Published in final edited form as:
ACS Chem Biol. 2013 May 17; 8(5): 908–913. doi:10.1021/cb400102t.

Discovery of the antibiotic phosacetamycin via a new mass spectrometry-based method for phosphonic acid detection

Bradley S. Evans[1,*], **Changming Zhao**[1,†], **Jiangtao Gao**[1], **Courtney M. Evans**[1], **Kou-San Ju**[1], **James R. Doroghazi**[1], **Wilfred A. van der Donk**[1,2,4], **Neil L. Kelleher**[5], and **William W. Metcalf**[1,3,*]

[1]The Institute for Genomic Biology, University of Illinois, Urbana-Champaign, Urbana, IL

[2]Department of Chemistry, University of Illinois, Urbana-Champaign, Urbana, IL

[3]Department of Microbiology, University of Illinois, Urbana-Champaign, Urbana, IL

[4]Howard Hughes Medical Institute

[5]Northwestern University, Evanston IL

Abstract

Naturally occurring phosphonates such as phosphinothricin (Glufosinate, a commercially used herbicide) and fosfomycin (Monurol, 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 has 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.

Keywords

Secondary metabolism; natural products; phosphonates; antibiotics; mass spectrometry

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 p*K*a values for phosphonates (2–3 for pKa_1 and 8–9 for pKa_2) (3) relative to phosphate esters (<1–2 for pKa_1 and 6–7 for pKa_2) (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

*bsevans2@illinois.edu. *metcalf@life.illinois.edu.
†Current Address: Key Laboratory of Combinatorial Biosynthesis and Drug Discovery, School of Pharmaceutical Sciences, Wuhan University, Wuhan China

SUPPORTING INFORMATION Additional experimental procedures and data as noted in the text. This information is available free of charge via the internet at http://pubs.acs.org.

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-26 (13) is the phosphoenoylpyruvate 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 (^{31}P 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 range (14) in complex matrices, however the relatively low sensitivity, throughput (15) and limited structural information ^{31}P 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 presents 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 matrices 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 co-migration 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 phosphonate enrichment protocol based on iron-immobilized metal affinity chromatography (IMAC), patterned after phosphopeptide enrichment strategies (18) that includes 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.

RESULTS AND DISCUSSION

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 (Figures S1, S2 and 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 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 phosphorous containing compounds (20) 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 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 $C_7H_{11}O_6NP^-$ (Figure 2 and S4). LC peak 2 in Figure 2 was identified as a contaminant from the glaswash (Figure S5). The presence of the phosphonate moiety was further supported by ^{31}P NMR analysis; the chemical shift (17.90 ppm) and coupling constant (J = 21.2 Hz) are consistent with a C-P bond (Figure S6). Further investigation into the structure of this new compound using MS^2, MS^3 and MS^4 analysis (Figures S7, S8, and 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 Jg per liter. The ^1H NMR spectrum (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 ^{31}P NMR spectrum of compound 1 revealed a signal at 17.90 ppm. A direct P-CH_2 bond was evidenced by triplet splitting (J=21.2 Hz) in the ^1H-coupled ^{31}P NMR spectrum and by the large coupling constant of J=128.3 Hz in the ^{13}C NMR spectrum for a CH_2 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 ^{1}H-^{1}H COSY correlations of H-1 with H-2, H-3 with H-2 and H-4, ^{1}H-^{1}H TOCSY correlations of H-1, H-2, H-3, and H-4, and ^{1}H-^{13}C 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 ^{1}H-^{13}C HMBC correlations of C-1′ with H-4 and H-2′. NMR spectra are displayed in 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 hours. 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+ (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 observed (23) 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 respectively (21, 24), phosacetamycin was found to inhibit growth of both bacteria and fungi. The fungal and bacterial selectivity of the rhizocticins 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) mutant (26) 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 rhizocticin 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 homologs in the rhizocticin gene cluster (Fig. 3a). Based upon the shared similarity between the phosacetamycin and rhizocticin gene clusters, phosacetamycin biosynthesis is proposed to follow rhizocticin biosynthesis for the APPA moiety (Fig 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 hours. 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 (29). Detailed genome analysis procedures are described in the Supporting Information. The biosynthetic gene cluster for phosacetamycin is deposited under Genbank accession no. (to be added after acceptance).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Institutes of Health GM P01 077596 awarded to W.W.M. and W.A.V. and J.R.D a University of Illinois IGB postdoctoral fellowship. The 600 MHz NMR instrument was purchased with funds from the National Institutes of Health (S10 RR028833). We also thank Svetlana Borisova for preparation of the APPA standard from rhizocticin.

References

1. Metcalf WW, van der Donk WA. Biosynthesis of phosphonic and phosphinic acid natural products. Annu Rev Biochem. 2009; 78:65–94. [PubMed: 19489722]

2. Engel R. Phosphonates as Analogs of Natural Phosphates. Chem Rev. 1977; 77:349–367.
3. Freedman LD, Doak GO. The Preparation and Properties of Phosphonic Acids. Chem Rev. 1957; 57:479–523.
4. Kumler WD, Eiler JJ. The Acid Strength of Mono and Diesters of Phosphoric Acid. The n-Alkyl Esters from Methyl to Butyl, the Esters of Biological Importance, and the Natural Guanidine Phosphoric Acids. J Am Chem Soc. 1943; 65:2355–2361.
5. Sigel H. Metal ion-binding properties of the antiviral nucleotide analogue 9-[2-(phosphonomethoxy)ethyl]adenine (PMEA). Why is its diphosphorylated form, PMEApp(4-), initially a better substrate for nucleic acid polymerases than (2′-deoxy)adenosine 5′-triphosphate (dATP(4-)/ATP(4-))? Pure Appl Chem. 1999; 71:1727–1740.
6. Sigel H, Song B, Blindauer CA, Kapinos LE, Gregan F, Pronayova N. Why is the antiviral nucleotide analogue 9-[2-(phosphonomethoxy)ethyl]adenine in its diphosphorylated form (PMEApp(4-)) initially a better substrate for polymerases than (2′-deoxy)adenosine 5′-triphosphate (dATP(4-)/ATP(4-))? Considerations on the mechanism of nucleic acid polymerases. Chem Commun. 1999:743–744.
7. Bone HG, Hosking D, Devogelaer JP, Tucci JR, Emkey RD, Tonino RP, Rodriguez-Portales JA, Downs RW, Gupta J, Santora AC, Liberman UA. Ten years' experience with alendronate for osteoporosis in postmenopausal women. N Engl J Med. 2004; 350:1189–1199. [PubMed: 15028823]
8. Duke SO, Powles SB. Glyphosate: a once-in-a-century herbicide. Pest Manage Sci. 2008; 64:319–325.
9. Borrmann S, Lundgren I, Oyakhirome S, Impouma B, Matsiegui PB, Adegnika AA, Issifou S, Kun JF, Hutchinson D, Wiesner J, Jomaa H, Kremsner PG. Fosmidomycin plus clindamycin for treatment of pediatric patients aged 1 to 14 years with Plasmodium falciparum malaria. Antimicrob Agents Chemother. 2006; 50:2713–2718. [PubMed: 16870763]
10. Falagas ME, Kastoris AC, Kapaskelis AM, Karageorgopoulos DE. Fosfomycin for the treatment of multidrug-resistant, including extended-spectrum beta-lactamase producing, Enterobacteriaceae infections: a systematic review. Lancet Infect Dis. 2010; 10:43–50. [PubMed: 20129148]
11. Duke, Stephen O.; CLC; Meepagala, Kumudini M.; Wedge, David E.; Tabanca, Nurhayat; Schrader, Kevin K. Natural Toxins for Use in Pest Management. Toxins. 2010; 2:1943–1962. [PubMed: 22069667]
12. Wiesner J, Ortmann R, Jomaa H, Schlitzer M. Double ester prodrugs of FR900098 display enhanced in-vitro antimalarial activity. Arch Pharm (Weinheim, Ger). 2007; 340:667–669.
13. Ntai I, Manier ML, Hachey DL, Bachmann BO. Biosynthetic origins of C-P bond containing tripeptide K-26. Org Lett. 2005; 7:2763–2765. [PubMed: 15957941]
14. Nielsen ML, Pustinger JV, Strobel J. Phosphorus-31 Nuclear Magnetic Resonance Chemical Shifts of Phosphorus Compounds. J Chem Eng Data. 1964; 9:167–170.
15. Pan ZZ, Raftery D. Comparing and combining NMR spectroscopy and mass spectrometry in metabolomics. Anal Bioanal Chem. 2007; 387:525–527. [PubMed: 16955259]
16. Bennett BD, Kimball EH, Gao M, Osterhout R, Van Dien SJ, Rabinowitz JD. Absolute metabolite concentrations and implied enzyme active site occupancy in Escherichia coli. Nat Chem Biol. 2009; 5:593–599. [PubMed: 19561621]
17. Shoji J, Kato T, Hinoo H, Hattori T, Hirooka K, Matsumoto K, Tanimoto T, Kondo E. Production of fosfomycin (phosphonomycin) by Pseudomonas syringae. J Antibiot. 1986; 39:1011–1012. [PubMed: 3759643]
18. Andersson L, Porath J. Isolation of phosphoproteins by immobilized metal (Fe3+) affinity chromatography. Anal Biochem. 1986; 154:250–254. [PubMed: 3085541]
19. Alpert AJ. Hydrophilic-interaction chromatography for the separation of peptides, nucleic acids and other polar compounds. J Chromatogr A. 1990; 499:177–196.
20. Huddleston MJ, Annan RS, Bean MF, Carr SA. Selective Detection of Phosphopeptides in Complex Mixtures by Electrospray/Liquid Chromatography Mass Spectrometry. J Am Soc Mass Spectrom. 1993; 4:710–717.

21. Kugler M, Loeffler W, Rapp C, Kern A, Jung G. Rhizocticin A, an antifungal phosphono-oligopeptide of Bacillus subtilis ATCC 6633: biological properties. Arch Microbiol. 1990; 153:276–281. [PubMed: 2110446]
22. Park BK, Hirota A, Sakai H. Structure of Plumbemycin A and B, Antagonists of L-Threonine from *Streptomyces plubeus*. Agric Biol Chem. 1977; 41:573–579.
23. Fredenhagen A, Angst C, Peter HH. Digestion of rhizocticins to (Z)-L-2-amino-5-phosphono-3-pentenoic acid: revision of the absolute configuration of plumbemycins A and B. J Antibiot (Tokyo). 1995; 48:1043–1045. [PubMed: 7592051]
24. Park BK, Hirota A, Sakai H. Studies on New Antimetabolite Produced by Microorganism .1. Studies on New Antimetabolite N-1409. Agric Biol Chem. 1977; 41:161–167.
25. Borisova SA, Circello BT, Zhang JK, van der Donk WA, Metcalf WW. Biosynthesis of rhizocticins, antifungal phosphonate oligopeptides produced by Bacillus subtilis ATCC6633. Chem Biol. 2010; 17:28–37. [PubMed: 20142038]
26. Evans BS, Chen Y, Metcalf WW, Zhao H, Kelleher NL. Directed evolution of the nonribosomal peptide synthetase AdmK generates new andrimid derivatives in vivo. Chem Biol. 2011; 18:601–607. [PubMed: 21609841]
27. Eliot AC, Griffin BM, Thomas PM, Johannes TW, Kelleher NL, Zhao H, Metcalf WW. Cloning, expression, and biochemical characterization of Streptomyces rubellomurinus genes required for biosynthesis of antimalarial compound FR900098. Chem Biol. 2008; 15:765–770. [PubMed: 18721747]
28. Evans BS, Ntai I, Chen Y, Robinson SJ, Kelleher NL. Proteomics-based discovery of koranimine, a cyclic imine natural product. J Am Chem Soc. 2011; 133:7316–7319. [PubMed: 21520944]
29. Doroghazi JR, Ju KS, Brown DW, Labeda DP, Deng Z, Metcalf WW, Chen W, Price NP. Genome sequences of three tunicamycin-producing Streptomyces Strains, S. chartreusis NRRL 12338, S. chartreusis NRRL 3882, and S. lysosuperificus ATCC 31396. J Bacteriol. 2011; 193:7021–7022. [PubMed: 22123769]

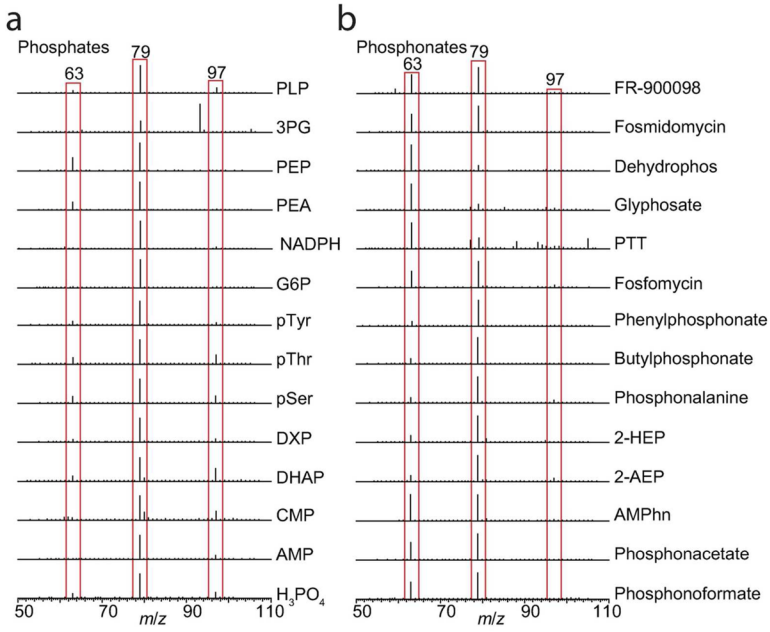


Figure 1. Differential fragmentation patterns of phosphonates and phosphorylated compounds

(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.

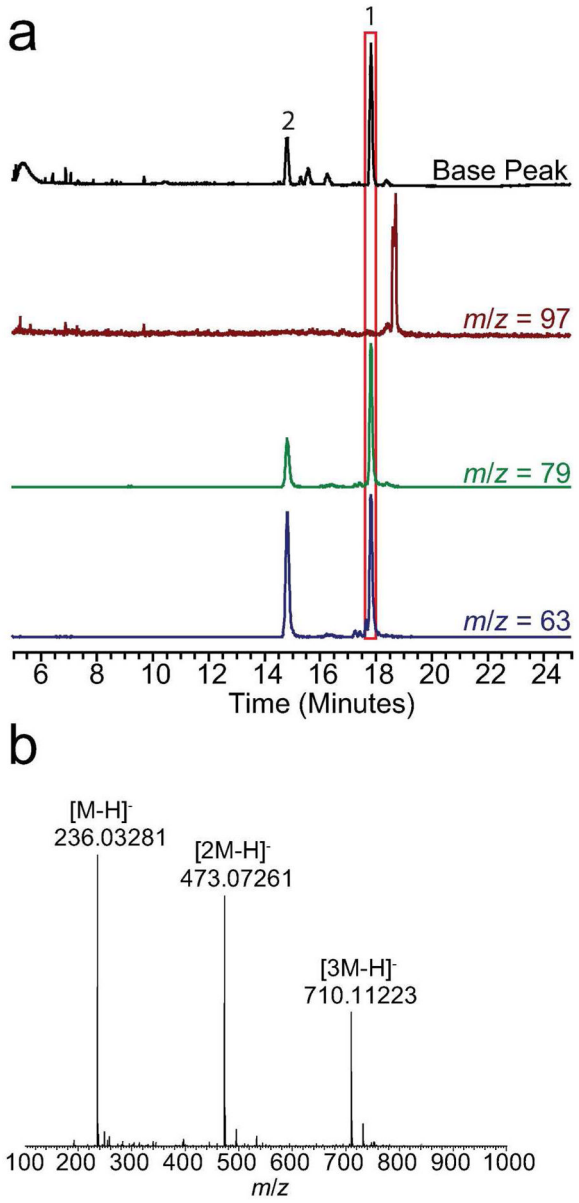
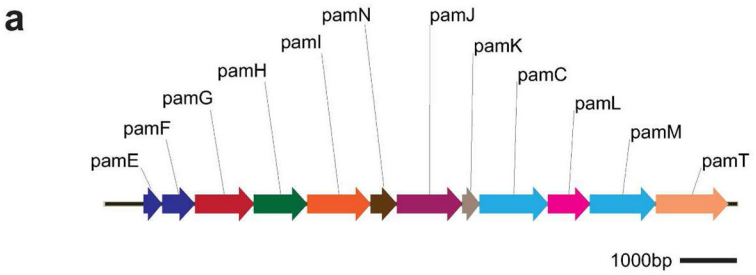


Figure 2. Identification of a new phosphonate from the culture of a *ppm*$^+$ actinomycete

(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 $C_7H_{11}O_6NP^-$. LC peak 2 is a contaminant peak from the glass wash, see Figure S5.



orf	Putative Function	Homolog	aa identity
pamE	Phosphonopyruvate decarboxylase α	ADB43070.1	50%
pamF	Phosphonopyruvate decarboxylase β	ADB43071.1	53%
pamG	2-keto-4-hydroxy-5-phosphonopentanoic acid aldolase	ADB43072.1	43%
pamH	Phosphoenoylpyruvate mutase	ADB43073.1	55%
pamI	Dehydratase	ADB43074.1	31%
pamN	Thiamin pyrophosphate synthase	ADB43075.1	51%
pamJ	Aminotransferase	ADB43076.1	60%
pamK	Glutaredoxin	ADB43077.1	55%
pamC	ATP-Grasp	ZP_06587016.1	40%
pamL	Metallophosphoesterase	ADB43078.1	32%
pamM	ATP-Grasp	ADB43079.1	32%
pamT	Major facilitator superfamily protein	YP_004081371	42%

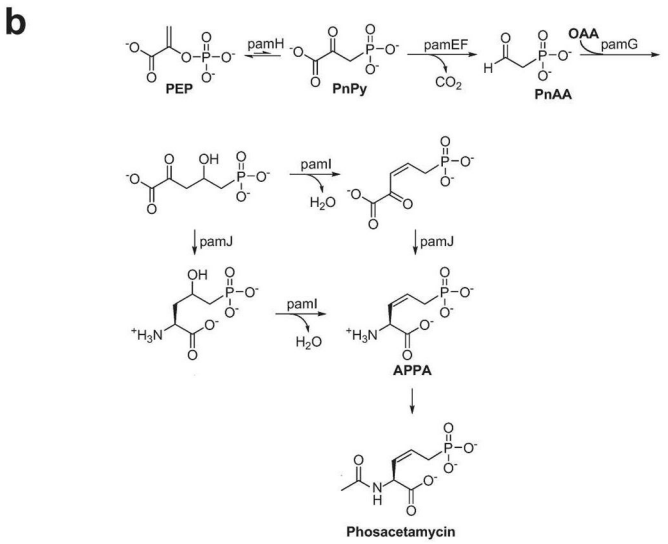


Figure 3. The phosacetamycin biosynthetic gene cluster

(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.

Table 1

Biological Activity Data for Phosacetamycin and Standard Antibiotics Against Representative Microorganisms

	Zone of Inhibition (diameter, mm)		
	Phosacetamycin	**Kanamycin**	**Nystatin**
Escherichia coli imp ASR	10.5	15.0	NT*
Escherichia coli MG1655	10.0	15.0	NT*
Escherichia coli WM6242 −IPTG	11.0	15.5	NT*
Escherichia coli WM6242 +IPTG	10.5	15.5	NT*
Serratia marcescens ATCC 274	0	17.0	NT*
Salmonella typhimurium LT2 ATCC 700720	0	13.5	NT*
Staphylococcus aureus ATCC 25923	10.0	18.0	NT*
Bacillus subtilis 168	9.5	17.0	NT*
Paecilomyces variotii	12	NT*	31
Saccharomyces cerevisiae	0	NT*	26

*Not Tested