Supporting information

NMR Spectroscopy of phosphonate samples

All NMR spectra were recorded at 25 ^{o}C on a DD2 600 MHz spectrometer (600 MHz for ^{1}H, 150 MHz for ^{13}C and 243 MHz for ^{31}P) equipped with an OneNMR probe (Agilent). Proton and carbon chemical shifts are reported in δ values relative to an external standard of 0.1% tetramethylsilane in D_2O. Phosphorus shifts are reported in δ values relative to an external standard of 85% phosphoric acid. ^{1}H-^{31}P gHMBC (gradient Heteronuclear Multiple-Bond Correlation) spectra were collected after optimization of long-range proton-phosphorus coupling at 18 Hz. An acquisition time of 1 s was used to obtain better spectral resolution. A total of 200 complex data points was collected in the F1 dimension. The spectrum was processed with a 90 degree shifted sine-bell square window function in MestReNova 7 software.

Mass spectrometry

Mass spectrometry (except FTMS) was performed at the University of Illinois Mass Spectrometry Center. EI mass spectra were recorded on a 70-VSE spectrometer. ESI mass spectra were obtained on a Quattro spectrometer. LC-FTMS analysis of the samples was performed on a custom-made 11T LTQ-FT Ultra (ThermoFisher Scientific) equipped with a 1200 HPLC (Agilent) using a 2.1 x 150 mm Zic-pHILIC column (SeQuant). The samples were diluted 10-fold in 10 mM NH_4HCO_3 (pH = 9.2) containing 90% acetonitrile. The injection volume was 100 μL. A gradient elution profile was used starting with 100% solvent B (10 mM NH_4HCO_3 containing 90% acetonitrile) for 2 min followed by a linear gradient to 50% solvent A (10 mM NH_4HCO_3 in water) over 13 min and then a return to initial conditions over 5 min and re-equilibration for 15 min before injection of the next sample. For HRMS analyses, data were acquired in the FT cell at a nominal resolution of 50,000 and were summed over 1 min. Collisionally-induced dissociation (CID) spectra (50) were acquired in the ion trap at 35% normalized collision energy and 30 ms activation time.

Preparation of phosphonates produced by *Streptomyces* sp. WM6372 for DhpI-labeling

Streptomyces sp. WM6372[1] was cultivated in ATCC172 seed medium (per liter: 20 g soluble starch, 10 g glucose, 5 g yeast extract, 5 g N-Z Amine type A, 1 g calcium carbonate) for 3 days at 30 °C on a platform shaker rotating at 200 rpm before inoculation onto 12 L of ISP medium 2 (Difco, Sparks, MD) plates solidified with Bacto agar (20 g/L). After incubating at 30 °C for 14 days, the agar-solidified medium was liquefied by repeated freezing and subsequent thawing. The resulting supernatant (8 L) was separated from the residual agar by filtration, before being concentrated twenty-fold via rotary evaporation. Methanol (3.6 L) was added to a final concentration of 90% and the precipitate formed was discarded. The supernatant was concentrated to 80 mL of aqueous solution and methanol (720 mL) was added to a final concentration of 90% and the precipitate formed was discarded. The supernatant was concentrated to 15 mL of aqueous solution, which was then placed onto a Sephadex LH-20 column (25 × 1500 mm gel bed). The sample was eluted with distilled water (flow rate 60 mL/h; 10 mL/fraction) and ^{31}P NMR analysis of the fractions showed that three among thirty fractions collected contained phosphonates; they were combined and lyophilized. The dry sample was dissolved in 1 mL of distilled water for DhpI-catalyzed methylation.

Preparation of phosphonates produced by *Streptomyces* sp. XY332 for DhpI-labeling

Streptomyces sp. XY332 (reference [1]) was cultured in Nutrient Broth (Difco, Sparks, MD). After incubating at 30 °C for 5 days, the culture was separated from the cells by centrifugation, and the filtrate was concentrated twenty-fold via rotary evaporation. The culture broth (20 L) was centrifuged and the supernatant was concentrated by rotary evaporation to 500 mL. Two liters of methanol were added. The supernatant was concentrated by rotary evaporation to 50 mL of aqueous solution, which was then chromatographed over a Sephadex LH-20 column (42 ×1400 mm gel bed) eluted with distilled water (flow rate 200 mL/h; 10 mL/fraction) to yield 65 fractions. ^{31}P NMR analysis showed that fractions 14 to

29 contained phosphonates and they were combined and lyophilized. The dry sample was dissolved in 1 mL of distilled water for LC-MS analysis.

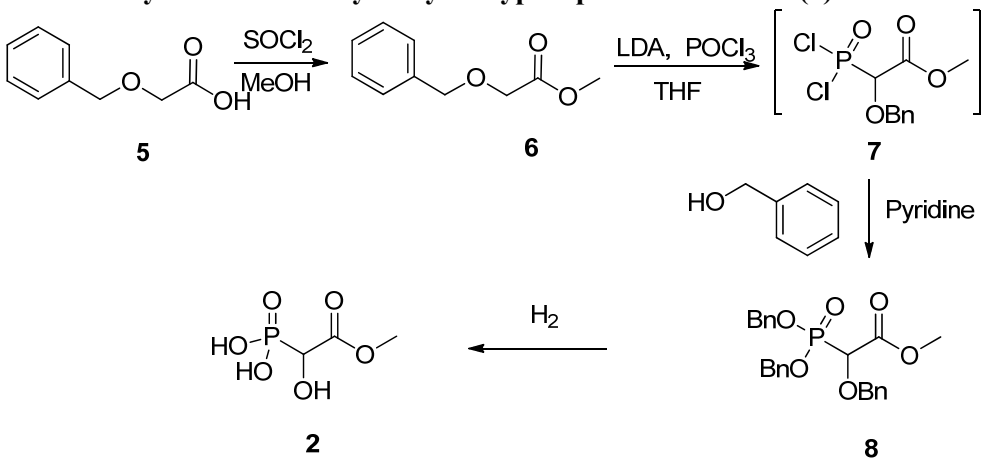
Purification of DhpI and SAH hydrolase

In a typical purification of the expressed proteins,[2] *E. coli* cells from a 3 L culture were harvested by centrifugation at 5,000×*g* for 15 min at 4 °C. The cell pellet was resuspended in 30 mL of ice cold buffer A (50 mM Tris·HCl, pH 7.5, 100 mM NaCl, 10 mM imidazole) supplemented with 0.4 mg/mL lysozyme and protease inhibitor cocktail tablets (Complete Mini, EDTA-free, Roche). Cells were disrupted in a chilled French press cell at 15,000 psi. The cell lysate was cleared by centrifugation at 25,000×*g* for 30 min at 4 °C, and the cleared soluble fraction was loaded on a column containing 8 mL of Ni-NTA affinity resin (Qiagen, Valencia, CA) equilibrated with Buffer A. After the loaded sample was passed through the column, unbound proteins were washed from the column with 10 column volumes of buffer B (50 mM Tris·HCl, pH 7.5, 100 mM NaCl, 20 mM imidazole) to remove any proteins nonspecifically bound to the column. Elution of the protein was achieved with buffer C (50 mM Tris·HCl, pH 7.5, 100 mM NaCl, 50-500 mM imidazole) with increasing imidazole concentrations in a step gradient from 50 mM to 500 mM with 30 mM increments with a total elution volume of 200 mL. The elution fractions containing the desired protein as detected by SDS-PAGE were concentrated by ultrafiltration using an Amicon Ultra 10 kDa membrane (Millipore). Imidazole and excess salt were removed by passage over a PD-10 column (GE Healthcare) pre-packed with Sephadex G-25. The protein was eluted from the column with buffer D (50 mM Tris·HCl, pH 7.5, 100 mM NaCl, 20% (w/v) glycerol), and the purified protein was flash frozen and stored at –80 °C. Protein concentration was determined by Bradford assay following the standard protocol using bovine serum albumin as a standard.

Methylation of phosphonates in cell extract by DhpI

Enzymatic reactions were carried out at 30 °C in 50 mM HEPES (pH 7.8) in 1.5 mL reaction vials for 2 days and analyzed by NMR spectroscopy and LC-FTMS. The reaction mixture contained 2 mM SAM, 2 mM d_3-SAM, 10 μM DhpI, 1 μM AdoHcy nucleosidase, and 100 μL of partially purified cell extract (see above) in a final volume of 300 μL. After adding DhpI, the reaction mixture was preincubated at 30 °C for 3 min before the reaction was initiated by addition of SAM.

Chemical synthesis of methyl 2-hydroxyphosphonoacetic acid (2)



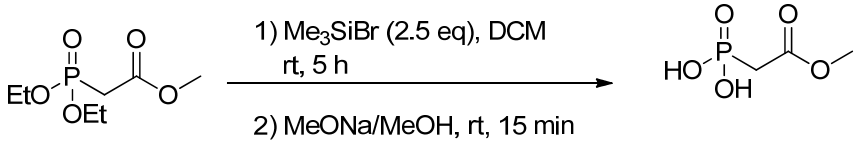
Methyl benzyloxyacetate (**6**): Thionyl chloride (1.64 mL, 22.6 mmol) was added dropwise to 10 mL of dry MeOH in a round bottom flask placed in an ice bath and the reaction was stirred for 5 min. To the reaction mixture, a solution of benzyloxyacetic acid **5** (3.13 g, 18.8 mmol) in 10 mL of dry MeOH was cannula transferred over a period of 15 min. The reaction was allowed to warm to room temperature and was stirred under N_2 for 22 h. The reaction mixture was concentrated on a rotary evaporator and dissolved in 30 mL of dichloromethane (DCM). The solution was then extracted with 2 × 30 mL of saturated

$NaHCO_3$ solution and 1 × 20 mL of brine. The organic layer was dried over Na_2SO_4 and concentrated on a rotary evaporator. The residue was purified by flash chromatography (SiO_2) using 100% DCM as eluent to generate product **6** as a colorless oil. TLC: R_f 0.61 (100% DCM). Yield: 3.1 g (91%). ^1H and ^{13}C NMR data matched the reported spectra.[3]

Methyl 2-(benzyloxy)-2-(bis(benzyloxy)phosphoryl)acetate (8): Lithium diisopropyl amide (LDA) was generated in-situ by adding *n*-BuLi (6.76 mL, 10.8 mmol, 1.3 equiv.) to a solution of dry diisopropyl amine (1.76 mL, 12.5 mmol, 1.5 equiv.) in 5 mL of dry THF at −78 ˚C. After stirring at −78 ˚C for 10 min, the solution was stirred for another 10 min in an ice bath and cooled back to −78 ˚C. To the generated LDA, a solution of methyl benzyloxyacetate **6** (1.49 g, 8.3 mmol) in 5 mL of dry THF was cannula-transferred over 10 min. The reaction was stirred at −78 ˚C for 10 min. This reaction mixture was cannula-transferred to a solution of phosphorus oxychloride (0.78 mL, 8.3 mmol, 1 equiv.) in 5 mL of dry THF at −78˚C over a period of 15 min and allowed to stir at −78 ˚C for 12 h. Then, a mixture of benzyl alcohol (1.72 mL, 16.6 mmol, 2 equiv.) and pyridine (1.34 mL, 16.6 mmol, 2 equiv.) was added to the reaction mixture, which was allowed to warm to room temperature overnight while stirring. The reaction was quenched by the addition of 5 mL of water after which the reaction mixture was immediately diluted with 60 mL of ethyl acetate and washed with 20 mL of brine. The organic layer was dried over Na_2SO_4 and concentrated. The residue was purified by flash chromatography (SiO_2) using a gradient from 20% to 50% EtOAc in hexanes. This procedure had to be used three times as a result of very closely eluting impurities to generate product **8**. TLC: R_f 0.38 (50% EtOAc in Hexanes). Yield = 251 mg (7%).
^1H NMR (600 MHz, $CDCl_3$) δ/ppm = 7.31-7.28 (m, 15H), 5.1 (d, *J* = 10 Hz, 4H), 4.79-4.77 (d, *J* = 10 Hz, 1H), 4.56-4.54 (d, *J* = 10 Hz, 1H), 4.45-4.41 (d, *J* = 20 Hz, 1H), 3.73 (s, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ/ppm=167.6, 136.0, 135.8, 128.0-128.6, 75.3 (d, *J*=158.5 Hz), 74.2 (d, *J*=12.1 Hz), 68.9 (d, *J*=10.6 Hz), 52.7. ^{31}P NMR (202 MHz, CDCl3) δ/ppm =15.96 (product). HRMS (ESI) m/z calculated for $C_{24}H_{26}O_6P$ 441.1467 ($M+H^+$), observed 441.1452.
^{31}P NMR analysis demonstrated the formation of product (δ = 15.96 ppm) along with a side product (δ = 1.36 ppm), which eluted with the same R_f value. The side product was identified as $PO(OBn)_2OMe$ by NMR and MS analysis.

Methyl 2-hydroxyphosphonoacetic acid (2): Methyl 2-(benzyloxy)-2-(bis(benzyloxy)phosphoryl)acetate **8** (126.4 mg) was dissolved in 5 mL of dry MeOH in a 50 mL round-bottomed flask and 10% Pd/C (54 mg) was added. The heterogeneous mixture was sparged with H_2 (1 atmosphere) for 20 min, and allowed to stir for 20 h. The reaction mixture was filtered over Celite and concentrated. ESI MS indicated mainly the presence of mono-benzyl protected product. The product from this reaction was dissolved in 5 mL of dry THF and an additional 25 mg of 5% Pd/C was added, the vessel was kept under 1 atmosphere of H_2 and stirred for 23 h. The reaction mixture was filtered over Celite. The mixture was purified by size-exclusion chromatography Sephadex LH-20 to yield the desired product **2** as a white power (3 mg, 6% yield). ^1H and ^{13}C NMR data matched the reported spectra.[4]

Chemical synthesis of methyl phosphonoacetic acid (1)



9 **1**

Bromotrimethylsilane (10 mg, 60 µmol) was added to a solution of diethylphosphonoacetic acid methyl ester **9** (5.0 mg, 23.8 µmol) in dichloroethane (DCM; 1 mL). After stirring for 12 h, a 1 N solution of MeONa in MeOH (1 mL, 1 mmol) was added. The reaction mixture was stirred for 30 min. After evaporation of solvents, the residue was washed with MeOH. After evaporation of MeOH, the solid residue was dissolved in water and passed through a column filled with cationic exchange resin AG 50W-

X8, in the hydrogen form (100-200 mesh). The eluent was lyophilized to afford compound **1** as a white solid in 95% yield. ^{1}H and ^{13}C NMR data matched the reported spectra.[5]

Fosfazinomycin production by *Streptomyces* sp. WM6372

Strain WM6372 was cultivated in a variety of different growth media to identify conditions where fosfazinomycins are produced. The strain was first inoculated into glass culture tubes containing 5 mL of ATCC 172 medium and incubated at 30 °C on an angled roller drum rotating (80 rpm) for three days. This culture was used to seed starter cultures by transferring 2 mL into 250 mL Fernbach flasks containing 50 mL of fresh ATCC 172 medium. Flasks were incubated at 30 °C on a rotary platform shaker (200 rpm), yielding high-density cultures after five days. Fosfazinomycin production was screened in small-scale cultures by inoculating 500 mL Fernbach flasks containing 100 mL of either ATCC 172, Nutrient Broth, ISP2, ISP4, R2AS (per liter: 10.8 g sodium succinate hexahydrate, 0.5 g yeast extract, 0.5 g peptone, 0.5 g Casamino acids, 0.5 g glucose, 0.5 g potato starch, 0.3 g sodium pyruvate, 0.3 g monobasic potassium phosphate, 0.05 g magnesium sulfate heptahydrate) with 5 mL of the above starter culture. Additionally, production from growth in Nutrient Broth and R2AS amended with 25 mg of phosphonoacetate (per 100 mL of medium) was also tested. Flasks were incubated at 30 °C on a rotary platform shaker (200 rpm).

Production cultures were harvested by centrifugation (10 min at 10,000 rpm) after five (ATCC 172, Nutrient Broth, ISP2) or seven days (ISP4, R2AS). Clarified supernatants were concentrated by rotary evaporation to 25 mL and then lyophilized. After re-dissolving dried samples in 5 mL of deionized water, 25 mL of methanol was added and the vessel was stored at −80 °C for 30 min. Chilled samples were then centrifuged (10 min at 4,500 rpm) and methanol removed from the clarified supernatant by rotary evaporation prior to lyophilization. The samples were then dissolved in 1 mL of deionized water and prepared for LC-MS analysis by weak-anion exchange chromatography. Concentrated extracts (100 μL) were diluted in deionized water (900 μL) and treated with iron-charged IMAC resin as previously described.[8] Samples were stored at −20 °C prior to analysis.

Production of ^{15}N-Labeled fosfazinomycins from *Streptomyces* sp. XY332

Starter cultures for strain XY332 were grown as described for strain WM6372. Additional screening of strain XY332 in different growth media revealed that both fosfazinomycins were produced in R2AS. This medium recipe served as the starting point for modifications for ^{15}N-labelling without sacrificing fosfazinomycin production. Ultimately, four 2-L flasks containing 500 mL of R2AS-M2 medium (same as R2AS except yeast extract, peptone, and glucose were omitted, $(^{15}NH_4)_2SO_4$ [$^{15}N_2$ 99%; Cambridge Isotope Laboratories] was added as the sole nitrogen source [1 g/L], and Balch's vitamins [10 mL/L] were added) were each inoculated with 20 mL of XY332 starter cultures. Flasks were incubated at 30 °C on a rotary platform shaker (225 rpm) and cultures harvested by centrifugation (10 min at 10,000 rpm) after 7 days of growth. After clarified supernatants were concentrated to 50 mL by rotary evaporation, 200 mL of methanol was added, and the samples were incubated at −80 °C for 30 min. Chilled samples were centrifuged (10 min at 4,500 rpm) to remove precipitants, concentrated by rotary evaporation to 25 mL and then lyophilized. Phosphonates were enriched from the dried material by extracting twice with 100 mL of methanol, centrifuging in between extractions to remove insoluble materials. The methanol extracts were combined, dried by rotary evaporation, and re-dissolved in 10 mL of deionized water and re-lyophilized. The dried final material was dissolved in 1 mL of D_2O for NMR analysis.

Heterologous expression of the putative fosfazinomcyin gene cluster

Strains and plasmids used for heterologous expression are listed in Table S1. *Streptomyces* strains were grown at 30 °C on ISP2, Nutrient Broth or ISP4 agar or broth (Difco). *Escherichia coli* strains were grown at 37 °C on Luria-Bertani (LB) agar or broth supplemented with antibiotics where appropriate. Antibiotics were used at the following concentrations for plasmid maintenance: chloramphenicol 12.5 μg/mL and apramycin 50 μg/mL. 2,6-Diaminopimelic acid was added to the media (1 mM) for growth of *E. coli* WM6029.

To add functions necessary for transfer and integration into *S. lividans*, *pepM*$^+$ fosmids pMMG358 and pMMG364[1] from *Streptomyces* sp. XY332 were recombined *in vitro* with plasmid pAE4,[6] using the Gateway BP Clonase II enzyme mix (Invitrogen) following the manufacturer's instructions to yield pXY005 and pXY007. These were integrated into the ϕC31 *attB* of *S. lividans* TK24 by first transforming *E. coli* WM6029 and then conducting conjugative matings following standard methods.[7] Exconjugants MMG604 and MMG605 were selected on ISP4-apramycin plates and purified by repeated single colony isolation. Successful integration was verified by PCR amplification of a 406-bp *pepM* gene fragment from purified genomic DNA using primer pair pepMF-for (CGCCGGCGTCTGCNTNGARGAYAA) and pepMR-rev (GGCGCGCATCATGTGRTTNGCVYA).

To test phosphonate production, strains MMG604 and MMG605 were first inoculated to 3 mL of Nutrient Broth and grown at 30 ^{o}C for 3 days on a roller drum. The seed culture was then inoculated to 100 mL of Nutrient Broth and grown for additional 5 days in 500-mL Erlenmeyer flasks on a rotary shaker at 200 rpm and 30 ^{o}C. Cells were pelleted by centrifugation and the supernatant was concentrated for further NMR and MS analyses as described above. Compounds **1** and **2** were identified by both methods, but fosfazinomycin could not be detected.

Table S1. Chemical shift changes upon methylation of fosfomycin, fosmidomycin, compounds **1** and **2**.

Compounds	^{31}P chemical shift δ (ppm)[a]	Chemical shift change Δδ (ppm)
Fosfomycin	10.7	4.7
Monomethyl fosfomycin	15.4	
Fosmidomycin	24.2	4.3
Monomethyl fosmidomycin	28.5	
Compound **1**	10.7	5.4
Monomethyl ester of **1**	16.1	
Compound **2**	9.8	3.7
Monomethyl ester of **2**	13.5	

[a]pH was controlled at 7.8 (0.1 M HEPES).

Table S2. Characterization of methylated phosphonates. ^{31}P (242 MHz), ^{1}H (500 MHz) and ^{13}C (125 MHz) Spectroscopic Data for monomethyl fosfomycin and fosmidomycin in D_2O.

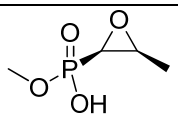
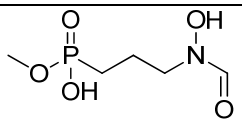
Compounds	Monomethyl fosfomycin	Monomethyl fosmidomycin
Structures		
^{31}P (Decoupled, pH 7.8)	15.4 ppm	28.5 ppm
^{1}H of methyl group (-OC**H$_3$**)	3.51 ppm (*d*, J=10.2 Hz)	3.43 ppm (*d*, J=10.2 Hz)
^{13}C of methyl group (-O**C**H$_3$)	51.79 ppm (*d*, J=5.6 Hz)	51.05 ppm (*d*, J=5.6 Hz)

Table S3. Bacterial strains and plasmids used for heterologous expression experiments

Strain or plasmid	Relevant characteristics	Source or reference
E. coli WM6029	*dam-3*, *dcm -9*, *metB1*, *galK2*, *galT27*, *lacY1*, *tsx-78*, *supE44*, *thi*, *mel-1*, *tonA31*, Δ*2* (*mcrC-mrr*)::*frt* Δ(*endA*)::*frt* *attλ*::pAE12-Δ*1*(*oriR6K-cat*::*frt5*), Δ*5816*(*dapA*)::*frt*, *uidA*(Δ*MluI*)::*pir*(wt), *attHK*::pJK1006:: Δ1/2(Δ *oriR6K-cat*, *trfA*); conjugal donor strain	This study
S. lividans TK24	Heterologous host for phosphonate production	USDA-ARS Strain Collection
S. lividans MMG604	Derivative of *S. lividans* TK24 containing putative fosfazinomycin gene cluster from pXY005 integrated at ϕC31 attB site; Apr^R	This study
S. lividans MMG605	Derivative of *S. lividans* TK24 containing putative fosfazinomycin gene cluster from pXY007 integrated at ϕC31 attB site; Apr^R	This study
pAE4	OriT, Apr^R, λ*attP,* ϕC31 *int*, ϕC31*attP*	[6]
pMMG364	Fosmid with putative fosfazinomycin gene cluster from XY332; Cm^R	[1]
pMMG358	Fosmid with putative fosfazinomycin gene cluster from XY332; Cm^R	[1]
pXY005	Fosmid pMMG364 recombined with pAE4 at λattB site; Cm^R; Apr^R	This study
pXY007	Fosmid pMMG358 recombined with pAE4 at λattB site Cm^R; Apr^R	This study

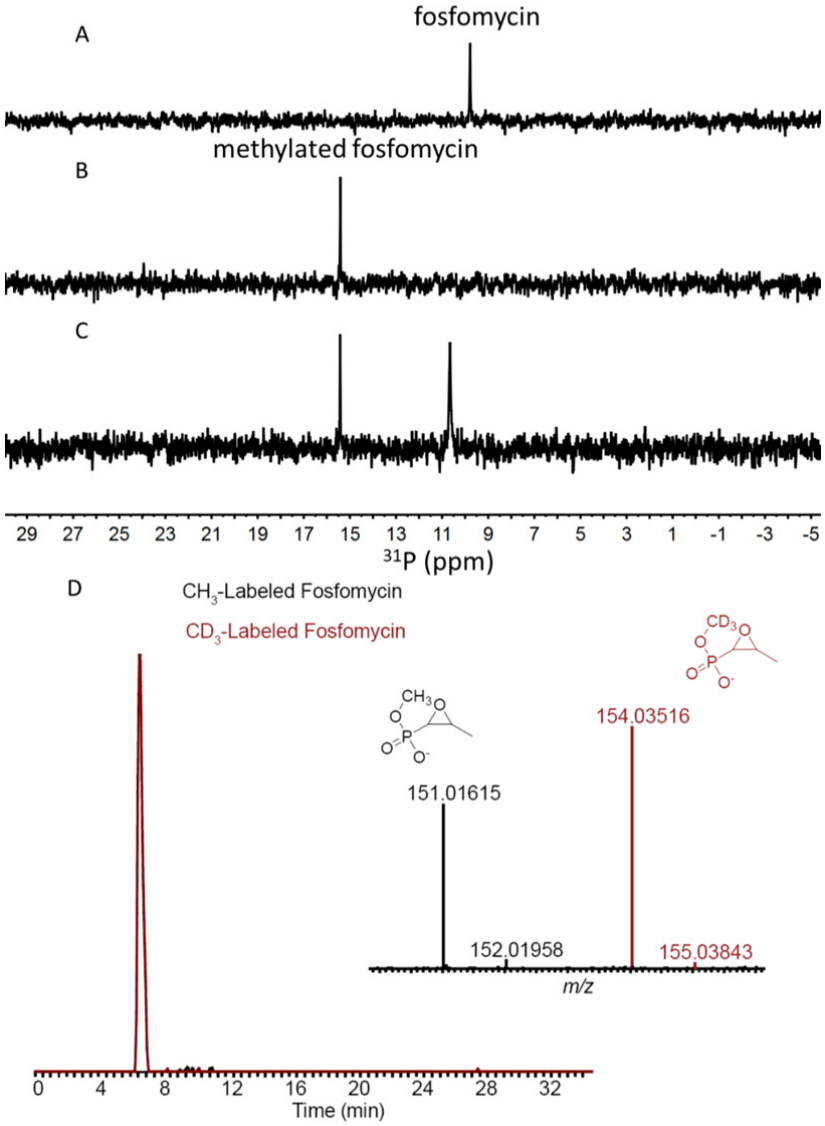


Figure S1. Identification of the antibiotic fosfomycin by SILPE. (A) ^{31}P NMR spectrum of fosfomycin. (B) ^{31}P NMR spectrum of a near equal mixture of fosfomycin methyl ester and its deuterated analog after DhpI labeling in crude spent medium. (C) ^{31}P NMR spectrum of methylated fosfomycin spiked with standard fosfomycin. (D) LC-FT-MS analysis of the spent medium containing the near-equal mixture of fosfomycin methyl ester and its deuterium labeled analog.

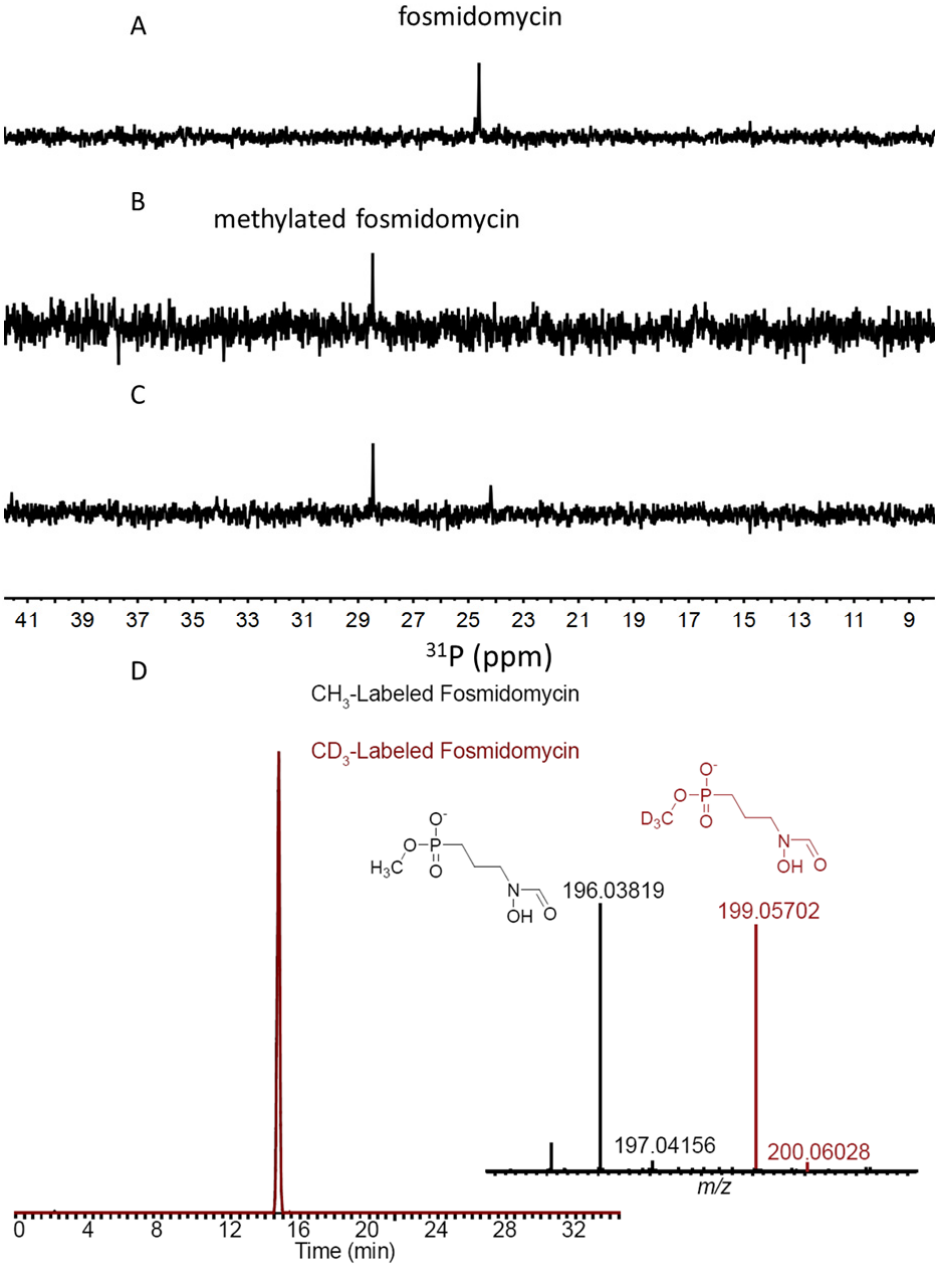


Figure S2. Identification of the antimalarial fosmidomycin by SILPE. (A) ^{31}P NMR spectrum of fosmidomycin. (B) ^{31}P NMR spectrum of a near-equal mixture of fosmidomycin methyl ester and its d_3-methyl analog after DhpI labeling in spent medium. (C) ^{31}P NMR spectrum of methylated fosmidomycin spiked with fosmidomycin standard. (D) LC-FT-MS analysis of the spent medium containing the near-equal mixture of fosmidomycin methyl ester and its d_3-methyl ester analog.

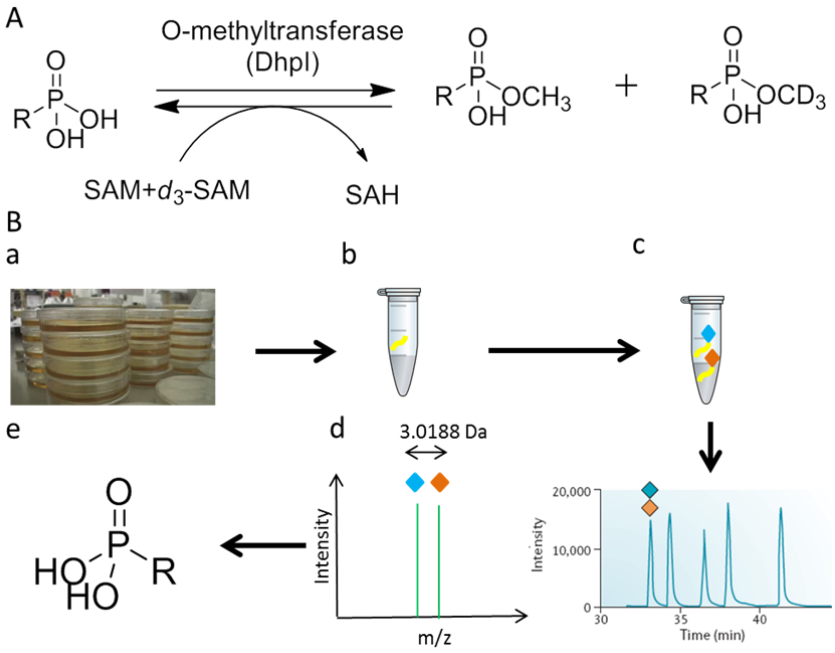


Figure S3. Workflow of SILPE. (A). Depiction of the SAM-dependent DhpI-catalyzed methyl transfer reaction during which a phosphonic acid is converted to a mixture of unlabeled and deuterium labeled phosphonate methyl ester. (B). (a) Organisms are grown in solid or liquid culture. (b) The extract is subjected to a simple purification step to reduce the salt level. (c) Phosphonates in the sample are converted to a mixture of phosphonate methyl ester and its deuterated analog. (d) The sample is subjected to LC-FT-MS and the elution profile is searched for peak pairs with a mass difference of 3.0188 Da with a tolerated error of 5 ppm. (e) After MS-guided purification, the chemical structure of the phosphonates is elucidated with MS and NMR spectroscopy.

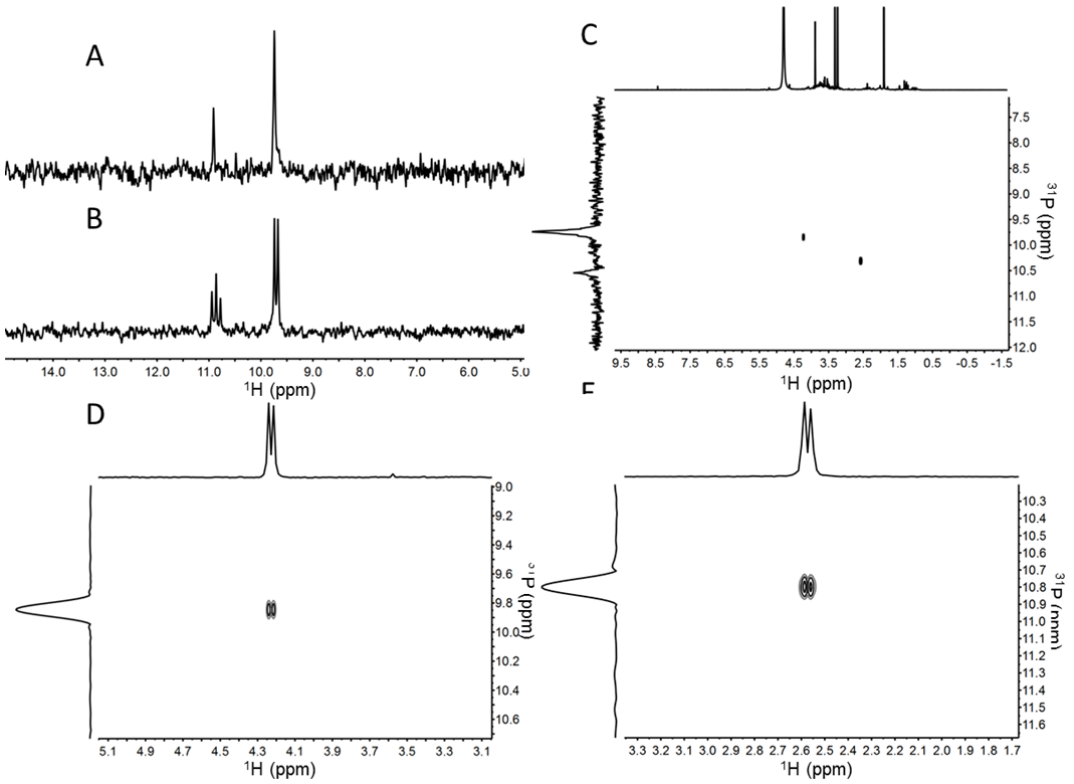


Figure S4. NMR spectra of phosphonates in crude cell extract of *Streptomyces* sp. WM6372. (A) ^{1}H-decoupled ^{31}P NMR spectrum of two phosphonates (9.74 and 10.90 ppm). (B) ^{1}H-coupled ^{31}P NMR spectrum of the same two phosphonates, 9.74 (d, J=16.5 Hz) and 10.90 (t, J=19.9 Hz). (C) ^{1}H-^{31}P HMBC spectrum showing the correlations in the ^{1}H dimension with the two ^{31}P resonances from panels A and B. (D) and (E) Expanded view of the ^{1}H-^{31}P HMBC spectrum of the two phosphonates in extracts.

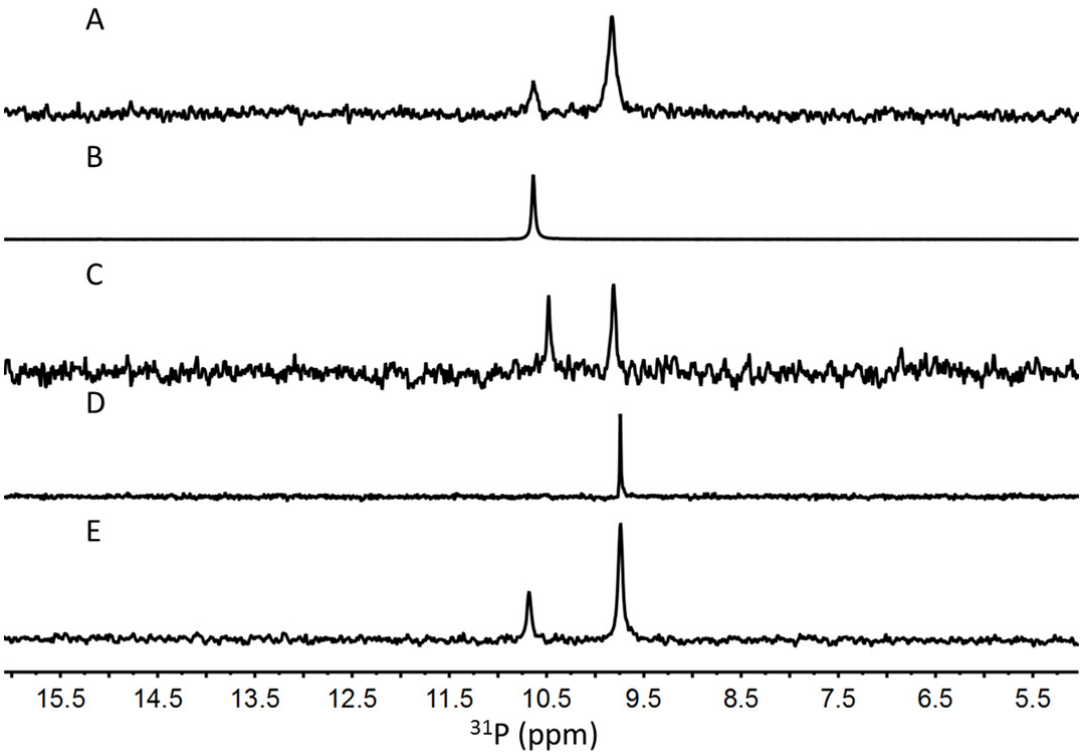


Figure S5. The two phosphonates produced by *Streptomyces* sp. WM6372 spiked with two standards that were chemically synthesized. (A) ^{31}P NMR spectrum of the original extract of *Streptomyces* sp. WM6372. (B) ^{31}P NMR spectrum of synthetic compound **2** (C) ^{31}P NMR spectrum of the extract spiked with synthetic compound **2** (D) ^{31}P NMR spectrum of synthetic compound **1**. (E) ^{31}P NMR spectrum of the extract in C spiked with synthetic compound **1**.

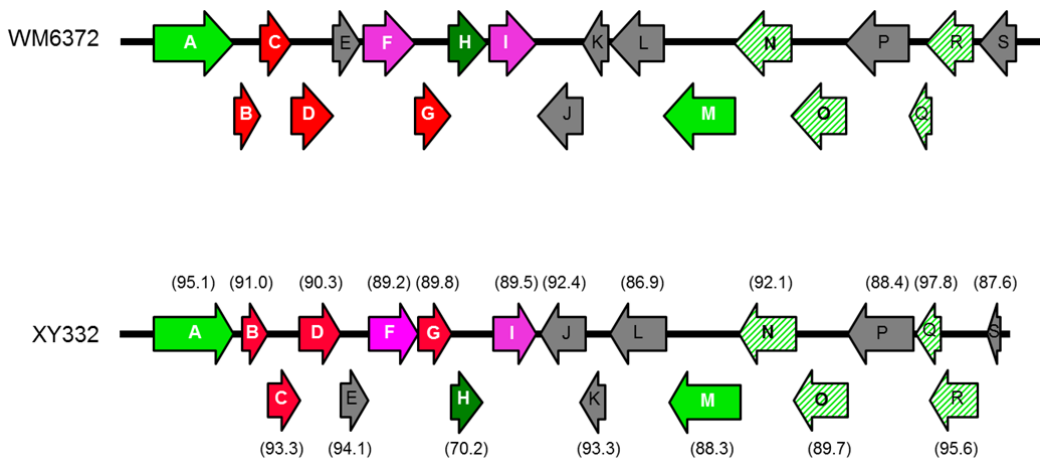


Figure S6. Comparison of the putative fosfazinomycin gene clusters in WM6372 and XY332 shows complete conservation of gene content and synteny, and a high degree of shared sequence between the Fzm homologs. Putative genes encoding key biosynthetic enzymes are shaded as follows: formation of Me-HPnA (red), nitrogen methylation (dark green), amide ligation (pink), installation of the N-N bond (light green; the four genes shared with the clusters for biosynthesis of diazo-containing natural products are in striped light green). Percent amino acid identities between Fzm homologs are indicated above or below the genes from XY332. (Note – gene clusters are not drawn to scale; the *fzmS* sequence from XY332 was recovered as a truncated gene due to its location at the end of the fosmid clone. The *fzmH* sequence from XY332 may also be incomplete).

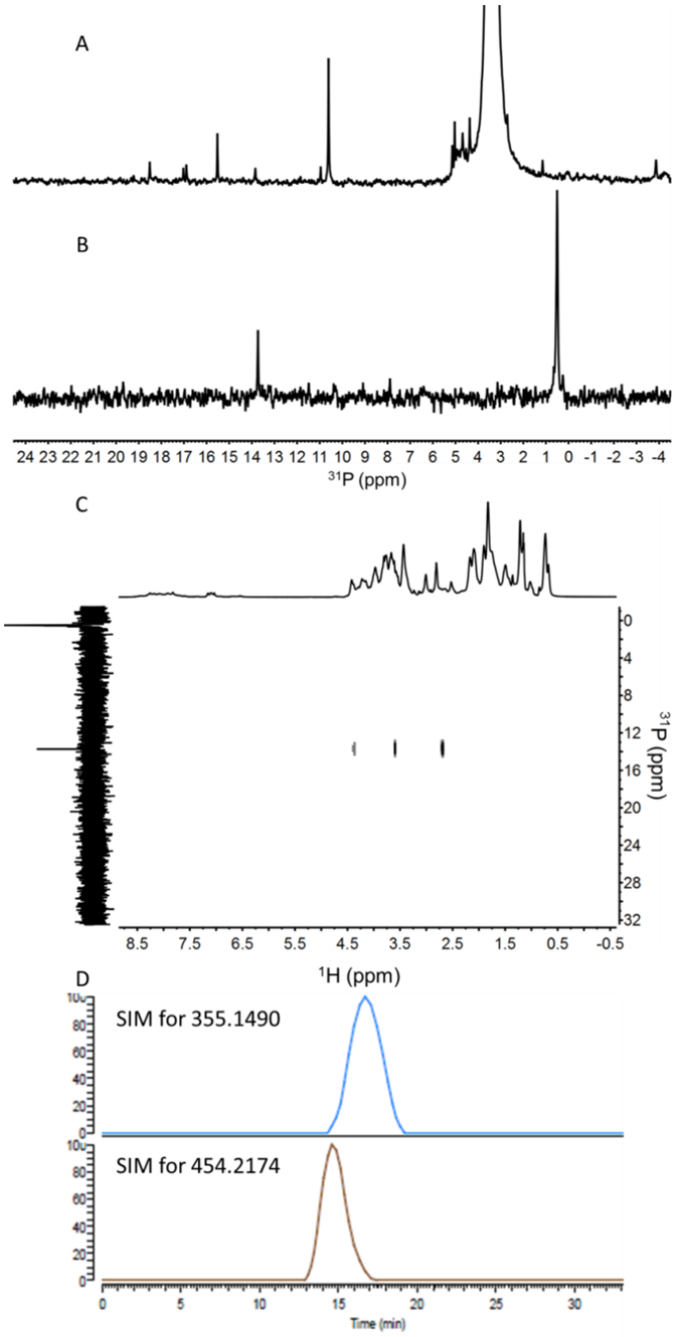


Figure S7. (A) ^{31}P NMR spectrum of phosphonates in the extract of *Streptomyces* sp. XY332. (B) ^{31}P NMR spectrum of a fraction of *Streptomyces* sp. XY332 after Sephadex LH-20 chromatography that contains phosphonates **3** and **4**. (C) ^{1}H-^{31}P HMBC NMR spectrum of the fraction in panel B. (D) LC-FT-MS analysis of the fraction in panel B. Selected ion monitoring (SIM) data are shown for fosfazinomycin A (**3**) and B (**4**).

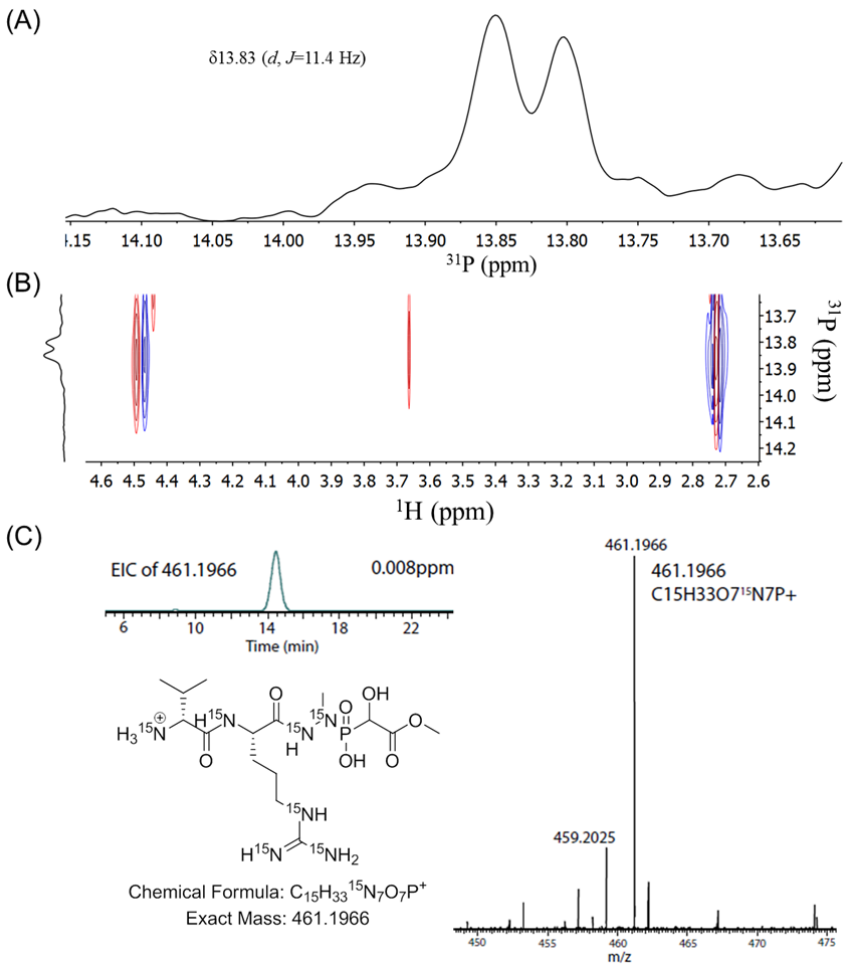


Figure S8. (A) Zoom-in of the ^{31}P NMR spectrum of crude ^{15}N-labeled fosfazinomycin. (B) ^{1}H-^{31}P HMBC spectrum of ^{15}N-labeled fosfazinomycin present in extracts. (C) FT-LC-MS data of fosfazinomycin A.

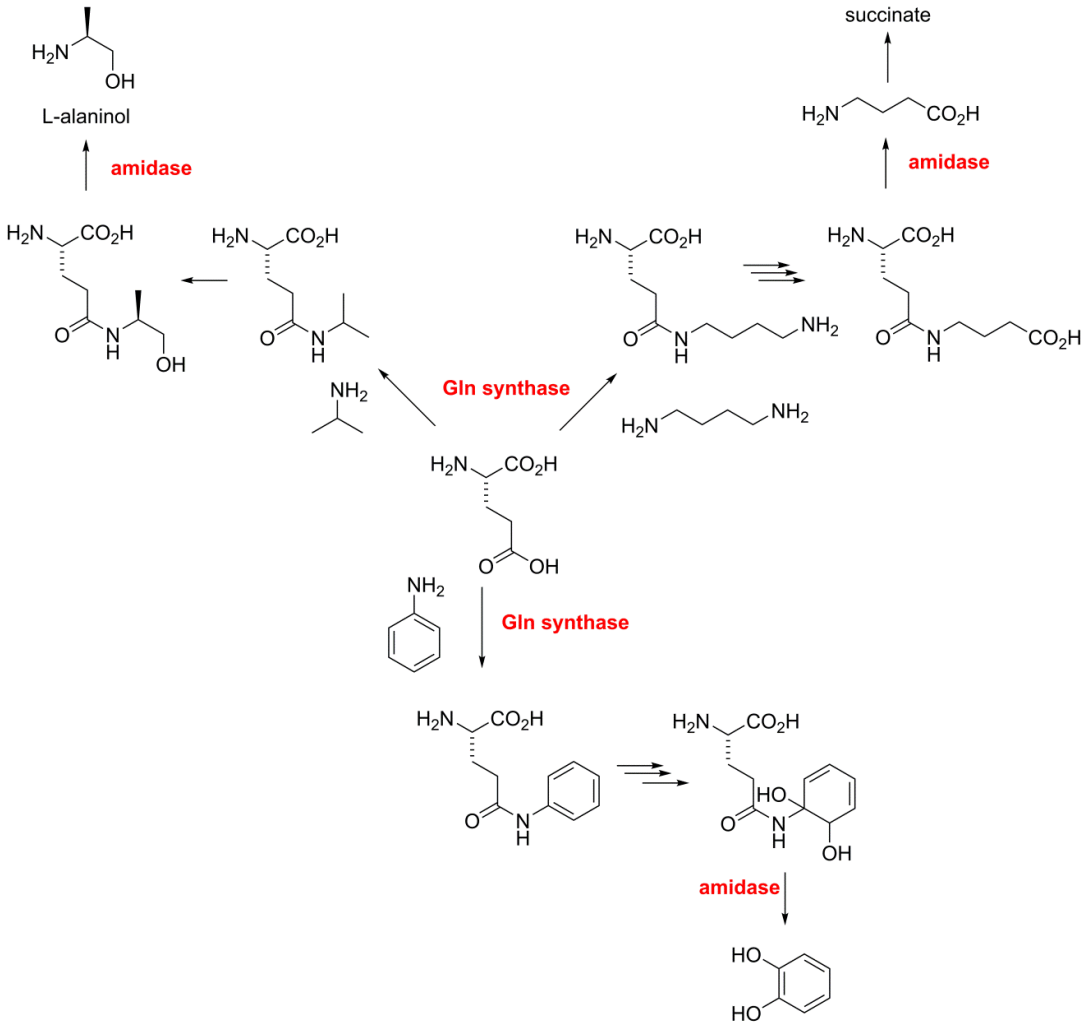


Figure S9. Three pathways that have been characterized in bacteria in which a glutamine synthetase and an amidase are involved in first linking a substrate to the Glu side chain carboxylate, followed by chemical elaboration of the substrate, and finally hydrolysis from Glu by an amidase. The frequent combination of a Gln synthetase and amidase in gene clusters, including those encoding natural products, has been noted before.[8] The catabolic pathways for isopropylamine,[9] putrescine,[10] and aniline[11] all involve a general strategy of using Glu as a scaffold. The recent use of Glu to dehydrate Ser and Thr residues in peptide natural product biosynthesis can be considered another example.[12]

The presence of a Gln synthetase and amidase in various clusters that encode natural products that contain N-N bonds (two of the green striped genes in Figure S6) suggests that perhaps Glu is also used as a scaffold in N-N bond formation. The other green-striped genes may involve the actual N-N bond formation step as well as a nitrogen atom delivery step. The observation of an adenylosuccinate lyase type gene is intriguing since this enzyme supplies a nitrogen atom in *de novo* purine biosynthesis.[13]

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