Electronic Supporting Information

New insights into the biosynthesis of fosfazinomycin

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Table S1. List of strains and plasmids used in this study

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References
Chemicals
All chemicals were purchased from Sigma-Aldrich, Chem-Impex, and TCI-America unless otherwise specified. The isotopically labeled ammonium chloride and aspartic acid were purchased from Cambridge Isotope Laboratories. Compound 6, compound 10 and fosfazinomycin B were prepared following previously reported procedures.1-3

Procedures for the chemical synthesis of substrates and authentic standards

Scheme S1. Synthesis of Fmoc-N-hydroxy-aspartic acid (9)

Dibenzyl (benzylxoy)-l-aspartate (7)
Compound1 (446 mg, 1.42 mmol, 1.0 equiv.) was dissolved in dry CH2Cl2 (3 mL), and this solution was cooled to 0 °C. 2, 6-Lutidine (197 mg, 1.84 mmol, 1.3 equiv.) and triflic anhydride (1.7 mL, 1 M in CH2Cl2, 1.60 mmol, 1.2 equiv.) were added successively. The resulting mixture was stirred at the same temperature for 27 min. A solution of O-benzylhydroxylamine (349 mg, 2.84 mmol, 2.0 equiv.) in dry CH2Cl2 (2 mL) was added, and the resulting mixture was stirred for another 5 h. The solution was diluted with CH2Cl2 (60 mL) and then washed with 5% NaHCO3 (1 x 15 mL), water (1 x 15 mL), brine (1 x 15 mL), dried (Na2SO4), and evaporated to dryness. The product was purified by flash chromatography (silica gel, 8:1 hexanes : EtOAc) in 76% yield.1H NMR (CDCl3, 600 MHz): δ/ppm 7.50-7.18 (m, 15H, Ar-H), 6.25 (d, 1H, J = 6.0 Hz, NH), 5.23-5.06 (m, 4H, C(O)OCH2Ph), 4.71-4.62 (m, 2H, NOCH2Ph), 4.11 (m, 1H, Hα), 2.90 (dd, 1H, J = 12.0 Hz, J = 6.0 Hz, Hβ1), 2.55 (dd, 1H, J = 12.0 Hz, J = 6.0 Hz, Hβ2); 13C NMR (CDCl3, 150 MHz): δ/ppm 171.4, 170.5, 137.4, 135.6, 135.3, 128.5, 128.4, 128.3, 128.31, 128.30, 128.27, 128.21, 127.85, 76.6, 66.6, 60.3, 34.4; HRMS (ES) Calcd for C25H26NO5[M+H]+ 420.1811, found 420.1807.

Dibenzyl N-[(9H-fluoren-9-ylmethoxy)carbonyl]-N-(benzylxoy)-l-aspartate (8)
To a solution of compound 7 (361 mg, 0.86 mmol) and Fmoc-Cl (496 mg, 1.72 mmol, 2.0 equiv.) in dry CH2Cl2 (3 mL) was added triethylamine (174 mg, 1.72 mmol, 2.0 equiv.). After stirring at 20 °C for 6 h, more Fmoc-Cl (620 mg, 2.15 mmol, 2.5 equiv.) was added, and the resulting mixture was stirred for another 16 h. The solution was diluted with CH2Cl2 (50 mL) and was then washed with 3% NaHCO3 (1 x 15 mL), water (1 x 15 mL), brine (1 x 10 mL), dried (Na2SO4), filtered, and evaporated to dryness. The product was purified by flash chromatography (silica gel, CH2Cl2).1H NMR (CDCl3, 600 MHz): δ/ppm 7.74 (d, 2H, J = 6.0 Hz, Fmoc-H), 7.60 (d, 2H, J = 6.0 Hz, Fmoc-H), 7.38 (t, 2H, J = 6.0 Hz, Fmoc-H), 7.35-7.17 (m, 17H, Ar-H and Fmoc-H), 5.22 (t, 1H, J = 6.0 Hz, Hα), 5.15-5.05 (m, 4H, C(O)OCH2Ph), 4.81 (d, 1H, J = 6.0 Hz, NOCH2Ph), 4.70 (d, 1H, J = 6.0 Hz, NOCH2Ph), 4.55 (t, 1H, J = 6.0 Hz, Fmoc CHCHH), 4.51-4.46 (m, 1H,
Fmoc CHCH₂J, 4.16 (t, 1H, J = 6.0 Hz, Fmoc CHCH₂), 3.11 (dd, 1H, J = 18.0 Hz, J = 6.0 Hz, H₂), 2.80 (dd, 1H, J = 18.0 Hz, J = 6.0 Hz, H₁); ¹³C NMR (CDCl₃, 150 MHz): δ/ppm 170.1, 168.7, 157.9, 143.6, 143.4, 135.5, 135.0, 134.9, 129.3, 128.52, 128.41, 128.36, 128.33, 128.32, 128.24, 128.1, 127.76, 127.74, 127.1, 125.13, 125.06, 119.96, 119.95, 78.2, 68.2, 67.5, 66.8, 60.0, 47.0, 34.0; HRMS (ES) Calcd for C₄₀H₃₆NO₇[M+H]⁺ 642.2492, found 642.2505.

Fmoc-N-hydroxy-aspartic acid (9)

To a solution of compound 8 (22 mg, 0.034 mmol) in THF (6 mL) and water (2 mL) was added 4.6 mg palladium (black) and 5 drops of formic acid. This mixture was stirred at 20 °C under H₂ (1 atm) for 4 h. The suspension was filtered and the solvent evaporated to dryness. The product was purified by high-performance liquid chromatography (HPLC) using an Agilent Eclipse XDB-C18, 5µ C₁₈ column (9.4 mm x 250 mm). A gradient elution profile was used starting with 20% solvent B (acetonitrile with 0.1% trifluoroacetic acid) for 2 min, a linear gradient to 100% solvent B over 30 min, holding at 100% B for 2 min, a return to 20% solvent B over 5 min, and holding at 20% solvent B for 5 min. Solvent A was water with 0.1% trifluoroacetic acid, and the flow rate was 3 mL/min. The desired product was eluted with a retention time of 16.5 min. ¹H NMR (CD₂OD, 600 MHz): δ/ppm 7.79 (d, 2H, J = 6.0 Hz, Fmoc-H), 7.70 (t, 2H, J = 6.0 Hz, Fmoc-H), 7.38 (t, 2H, J = 6.0 Hz, Fmoc-H), 7.30 (t, 2H, J = 6.0 Hz, Fmoc-H), 5.17 (t, 1H, J = 6.0 Hz, H₂), 4.45-4.30 (m, 2H, Fmoc CHCH₂), 4.27 (t, 1H, J = 6.0 Hz, Fmoc CHCH₂), 3.01 (dd, 1H, J = 18.0 Hz, J = 6.0 Hz, H₁), 2.76 (dd, 1H, J = 18.0 Hz, J = 6.0 Hz, H₂); ¹³C NMR (CD₂OD, 150 MHz): δ/ppm 176.2, 171.0, 158.2, 143.64, 143.60, 127.4, 126.80, 126.77, 125.01, 124.95, 119.5, 68.3, 58.8, 46.8, 33.0; HRMS (ES) Calcd for C₁₉H₁₆NO₇[M-H]⁻ 370.09323, found 370.09412.

Scheme S2. Synthesis of hydrazinosuccinic acid (12)

Hydrazinosuccinic acid (12)

Compound 10² (229 mg, 0.83 mmol, 1.0 equiv.) was dissolved in dry dichloroethane (DCE) (10 mL), and trimethyltin hydroxide (600 mg, 3.32 mmol, 4.0 equiv.) was added. This solution was refluxed for 14 h and concentrated in vacuo. EtOAc (120 mL) was added and the mixture was then washed with 10% citric acid (1 x 10 mL), brine (1 x 20 mL), dried (Na₂SO₄), filtered, and the solvent evaporated to yield compound 11. To this crude product was added CH₂Cl₂ (7 mL) and TFA (7 mL). The resulting solution was stirred at 20 °C for 3 h. The solvent was then evaporated, and the crude residue was purified by ion-exchange column chromatography on a cationic Dowex 50 resin (H⁺, 50 mesh, washed with water), eluting with 2.5% aqueous NH₃. The desired product 12 was obtained upon solvent evaporation and lyophilization. ¹H NMR (D₂O, 600 MHz): δ/ppm 3.71 (dd, 1H, J = 8.4 Hz, J = 3.6 Hz, H₄), 2.69 (dd, 1H, J = 17.4 Hz, J = 3.6 Hz, H₁), 2.55 (dd, 1H, J = 17.4 Hz, J = 8.4 Hz, H₂); ¹³C NMR (D₂O, 150 MHz): δ/ppm 177.8, 174.5, 61.9, 36.0; HRMS (ES) Calcd for C₄₆H₆₆NO₇[M+H]⁺ 149.0562, found 149.0569.
Dibenzyl acetamido-\(L\)-aspartate (13)
Compound 6 (419 mg, 1.33 mmol, 1.0 equiv.) was dissolved in dry \(\text{CH}_2\text{Cl}_2\) (3 mL), and this solution was cooled to 0 °C. 2, 6-Lutidine (185 mg, 1.73 mmol, 1.3 equiv.) and triflic anhydride (1.6 mL, 1 M in \(\text{CH}_2\text{Cl}_2\), 1.60 mmol, 1.2 equiv.) were added successively. The resulting mixture was stirred at the same temperature for 30 min. A solution of acetylhydrazide (196 mg, 2.66 mmol, 2.0 equiv.) in dry \(\text{CH}_2\text{Cl}_2\) (2 mL) was added, and the resulting mixture was stirred for another 19 h. The solution was diluted with \(\text{CH}_2\text{Cl}_2\) (60 mL) and was then washed with 10% citric acid (1 x 15 mL), 5% \(\text{NaHCO}_3\) (1 x 15 mL), water (1 x 15 mL), brine (1 x 15 mL), dried (Na2SO4), filtered and evaporated to dryness. The product was purified by flash chromatography (silica gel, 40:1 \(\text{CH}_2\text{Cl}_2\):MeOH).

**\(^1\text{H} NMR\) (CDCl\(_3\), 600 MHz) (rotational isomers):**

Major isomer: \(\delta/\text{ppm} 7.53\) (d, 0.74H, \(J = 6.0\) Hz, \(\text{CH}_3\text{C(O)NHNH}\)), 7.37-7.25 (m, 6.75H, Ar-H), 5.15 (bs, 0.71H, \(\text{CH}_3\text{C(O)NHNH}\)), 5.11 (s, 1.52H, \(\text{CH}_2\text{Ph}\)), 5.07 (s, 1.52H, \(\text{CH}_2\text{Ph}\)), 3.99 (m, 0.75H, \(H_\alpha\)), 2.88-2.80 (m, 1.53H, \(H_\beta\)), 1.86 (s, 2.26H, -\(\text{C(O)CH}_3\));

Minor isomer: \(\delta/\text{ppm} 7.37-7.25\) (m, 2.25H, Ar-H), 5.13 (s, 0.53H, \(\text{CH}_3\text{Ph}\)), 5.10-5.03 (m, 0.49H, \(\text{CH}_3\text{Ph}\)), 4.57 (d, 0.25H, \(J = 6.0\) Hz, \(\text{CH}_3\text{C(O)NHNH}\)), 3.86 (m, 0.25H, \(H_\alpha\)), 2.88-2.73 (m, 0.54H, \(H_\beta\)), 1.98 (s, 0.84H, -\(\text{C(O)CH}_3\));

**\(^{13}\text{C} NMR\) (CDCl\(_3\), 150 MHz) (rotational isomers):**

Major isomer: \(\delta/\text{ppm} 171.3, 170.4, 169.8, 135.4, 135.19, 128.58, 128.54, 128.44, 128.35, 128.32, 67.2, 66.8, 59.2, 35.4, 21.0;\)

Minor isomer: \(\delta/\text{ppm} 175.6, 171.9, 170.1, 135.18, 134.8, 128.70, 128.61, 128.46, 128.34, 67.6, 67.0, 60.4, 35.4, 19.5;\)

HRMS (ES) Calcd for \(\text{C}_{20}\text{H}_{23}\text{N}_2\text{O}_5\)[\(\text{M+H}^+\)]\(^+\) 371.1607, found 371.1607.

**\(\text{N-acetylhydrazinosuccinic acid}\) (14)**
To a solution of compound 13 (69 mg, 0.32 mmol) in THF (8 mL) and water (2 mL) was added 10 mg palladium (black) and 5 drops of formic acid. This mixture was stirred at 20 °C under \(\text{H}_2\) (1 atm) for 4 h.
The suspension was filtered and concentrated to yield the product. $^2$H NMR (D$_2$O, 600 MHz): δ/ppm 3.86 (t, 1H, $J$ = 6.0 Hz, $H_\alpha$), 2.78-2.66 (m, 2H, $H_\beta$), 1.83 (s, 3H, -C(O)CH$_3$); $^{13}$C NMR (D$_2$O, 150 MHz): δ/ppm 174.65, 174.62, 172.8, 58.8, 34.5, 19.8; HRMS (ES) Calcd for C$_6$H$_{11}$O$_2$[M+H]$^+$ 191.0668, found 191.0673.

Molecular Biology

Reagents

E. coli strains and plasmids used in this study are listed in Table S1. Restriction enzymes (NdeI and DpnI), Phusion polymerase, Taq DNA ligase and 100 mM stock solutions of dATP, dTTP, dGTP and dCTP were purchased from New England Biolabs. Fail Safe PCR system and T5 exonuclease were provided by Epicentre. DTT was provided by Promega. Oligonucleotides were purchased from IDT in standard desalted form and used without further purification (Table S2). All other reagents were purchased from Sigma-Aldrich unless otherwise specified. LB medium contained yeast extract (5 g/L), tryptone (10 g/L), NaCl (10 g/L). Antibiotics were used at the following concentration: ampicillin (AMP): 100 µg/mL, chloramphenicol: (CAM) 12.5 µg/mL.

Cloning procedures

Plasmids were purified with Qiagen columns. PCR products were purified with Promega Wizard® PCR Clean-Up System. Electro-competent cells were prepared in-house according to standard protocols. Fosfazinomycin biosynthetic genes (Table S1) were PCR amplified from fosmids MMG 358 and 360 using the Fail Safe polymerase in buffer G (purchased from Epicentre). All cloning manipulations were based on the in vitro ligation isothermal assembly protocol described by Gibson et al. Briefly, vector linearized with the appropriate restriction enzyme was PCR amplified using primers listed in Table S2, digested with DpnI, and purified before being mixed (ca. 100 ng) with the appropriate insert (ca. 100 ng) in a final volume of 5 µL of distilled water. The solution of vector and insert was mixed with 15 µL of assembly master mix (ingredients see below) and incubated at 50 °C for 1 h in a PCR thermocycler. An aliquot (2 µL) from the ligation reaction mixture was used to transform electro-competent cells. The assembly master mixture was prepared by adding into 218 µL of distilled water the following reagents: 98.2 µL isothermal reaction buffer (5 x), 6.8 µL Phusion Polymerase, 2 µL T5 exonuclease (10 x diluted in the T5 exonuclease buffer) and 50 µL Taq DNA ligase. Isothermal reaction buffer (6 mL final volume; 5 x) was prepared by mixing the following reagents (final concentrations are indicated in parenthesis): 3 mL of 1 M Tris-HCl (500 mM; pH 7.5), 1.5 g PEG-4000 (25% (w/v)), 300 µL of 1 M DTT (50 mM), 300 µL of 1 M MgCl$_2$ (50 mM), 300 µL of 100 mM NAD$^+$ (5 mM), and 60 µL of 100 mM stock solutions of each of the four dNTPs (800 µM) in the appropriate amount of distilled water.

The gene encoding valyl-tRNA-synthetase in E. coli, valS, was amplified by colony-PCR using E. coli DH5α as a template and the primers listed in Table S2 before being cloned into a pET-15b vector as described above.

Enzymalogy

Reagents

Nickel(II)-nitrilotriacetic acid (Ni-NTA) agarose was purchased from Qiagen. Chelex® 100 resin, sodium form (50-100 mesh), was purchased from Sigma-Aldrich. Amicon ultracentrifugal filters were purchased from Millipore. Media components and salts were purchased from Fisher Scientific. Isopropylthio-β-D-galactoside (IPTG) was obtained from IBI Scientific. Nuclease-free water was purchased from...
Ambion. Other reagents were purchased from Sigma-Aldrich. Lysis buffer consisted of 50 mM NaP$_8$, 300 mM NaCl, 10 mM imidazole, 10% glycerol, pH 8.0. Wash buffer consisted of 50 mM NaP$_8$, 300 mM NaCl, 20 mM imidazole, 10% glycerol, pH 8.0. Elution buffer consisted of 50 mM NaP$_8$, 300 mM NaCl, 250 mM imidazole, 10% glycerol, pH 8.0. Storage buffer consisted of 50 mM NaP$_8$, 300 mM NaCl, 10% glycerol, pH 8.0.

**Expression and Purification of His$_6$-FzmR, His$_6$-FzmQ, His$_6$-FzmM, His$_6$-Fzml, His$_6$-Fzml and His$_6$-ValRS**

A culture of *E. coli* Rosetta 2 (DE3) pLysS cells freshly transformed with the appropriate plasmid (see Supporting Information) was grown in LB medium containing 100 mg/L ampicillin and 12.5 mg/L chloramphenicol was diluted 1:100 into 1 L of the same medium in a 4 L flask. The culture was shaken at 37 °C until the optical density at 600 nm reached 0.6-0.8. Then, the flask was placed in an ice/water bath for ca. 30 min before the addition of isopropylthio-β-D-galactoside (IPTG) to a final concentration of 100 μM. The culture was shaken for an additional 12-14 h at 18 °C. All the following purification steps were carried out at 4 °C. The cells (5-7 g wet mass from 2 L of culture) were collected by centrifugation, washed once with phosphate-buffered saline solution (PBS), pH 7.4, and then resuspended in 30 mL of lysis buffer [50 mM sodium phosphate (NaP$_8$), 300 mM NaCl, 10 mM imidazole, 10% glycerol, pH 8.0] supplemented with 1 mg/mL lysozyme and 600 U DNase. The cells were lysed by passage through a French pressure cell, and debris was removed by centrifugation at 37,000 x g for 60 min at 4 °C. The supernatant was loaded onto a column containing 5-7 mL of Ni-NTA resin previously equilibrated with lysis buffer. After equilibration of the resin with the lysate in a rocking platform for 30 min, the flow-through was discarded and the resin was washed with 2 x 40 mL of wash buffer (50 mM NaP$_8$, 300 mM NaCl, 20 mM imidazole, 10% glycerol, pH 8.0). Resin-bound protein was eluted with elution buffer (50 mM NaP$_8$, 300 mM NaCl, 250 mM imidazole, 10% glycerol, pH 8.0). Fractions of 3 mL were collected, and the absorbance at 280 nm was measured by a NanoDrop spectrophotometer. Fractions with strong absorbance at 280 nm were pooled and concentrated in an Amicon Ultra centrifugal filter unit with 10 kDa molecular weight cut off (MWCO) to a final volume of 2.5 mL. Imidazole and excess salt were removed with a PD10 desalting column previously equilibrated with storage buffer. Protein was eluted with 3.5 mL of storage buffer and stored in aliquots at -80 °C. Typical yields: i) His$_6$-FzmM, 11 mg/L, ii) His$_6$-FzmQ, 31 mg/L, iii) His$_6$-FzmR, 24 mg/L, iv) His$_6$-Fzml, 17 mg/L, v) His$_6$-ValRS, 36 mg/L, vi) His$_6$-Fzml, 48 mg/L. Protein concentration was measured using the Bradford method.

**Analytical Methods**

NMR experiments were carried out using an Agilent 600 MHz instrument equipped with a OneNMR probe. Direct infusion mass spectrometry (used to analyze synthetic samples) was performed at the University of Illinois Mass Spectrometry Center. ESI mass spectra were obtained on a Quattro spectrometer. LC-MS analysis was performed on an Agilent 1100 series LC system coupled to Agilent G1956B single quadrupole mass spectrometer. LC-FTMS analysis was performed on a custom-made 11T LTQ-FT Ultra (ThermoFisher Scientific) equipped with a 1200 HPLC (Agilent) using a 2.0 x 50 mm Onyx monolithic C18 column (Phenomenex). The injection volume was 2 μL. A gradient elution profile was used starting with 0% solvent B (acetonitrile with 0.1% formic acid) for 5 min, a linear gradient to 95% solvent B over 10 min, holding at 95% B for 5 min, and then a return to initial conditions over 5 min. Solvent A was water with 0.1% formic acid. For HRMS analyses, data were acquired in the FT cell at a nominal resolution of 50,000. DNA sequencing reactions were carried out at the W.M. Keck Center for Biotechnology at the University of Illinois at Urbana-Champaign. The purity of the isolated proteins was assessed by SDS-PAGE analysis. Coomassie (Bradford) protein assay (Thermo Scientific) was used for calculating protein concentrations. Sequencing data were analyzed by Vector NTI 9 (Invitrogen Co.).
NMR data were analyzed by MestReNova 8.0 (MestreLab Research). Kinetic data analyses were performed using Igor Pro version 6.1.

**Procedures for in vitro enzymatic assays**

**FzmR activity assays**
The reaction mixture (500 µL) contained 5 mM of N-acetylhydrazinosuccinic acid and 23 µM FzmR in 50 mM sodium phosphate buffer (NaPi), pH 7.7. After a 6 h incubation at ambient temperature, the proteins were removed by passing the reaction mixture through an Amicon spin column (30 kDa MWCO), and 150 µL D₂O was added into the sample before NMR analysis.

**FzmQ activity assays**
The reaction mixture (250 µL) contained 2 mM of hydrazinosuccinic acid (12), 2.5 mM acetyl-CoA, and 45 µM FzmQ in 50 mM NaPi, pH 7.7. After a 2 h incubation at ambient temperature, the proteins were removed by passing the reaction mixture through an Amicon spin column (10 kDa MWCO), and the filtrate was used for NMR and LC-MS analysis. Method 2 was used for the LC-MS analysis: A Grace Vydac protein & peptide 5µ C₁₈ column (4.6 mm x 250 mm) and a gradient elution profile was used starting with 5% solvent B (acetonitrile with 0.1% formic acid) for 2 min, a linear gradient to 25% solvent B over 30 min, a gradient to 100% solvent B over 5 min, holding at 100% B for 3 min, a return to 5% solvent B over 2 min, and holding at 5% solvent B for 5 min. Solvent A was water with 0.1% formic acid, and the flow rate was 0.4 mL/min.

**FzmM activity assays on L-Asp and L-Asp (2,3,3-D₃) using excess NADPH**
The reaction mixture (50 µL) contained 2.5 mM of L-Asp or L-Asp (2,3,3-D₃), 0.1 mM FAD, 5 mM NADPH and 10 µM FzmM in 50 mM NaPi, pH 7.7. After a 1 h incubation at 20 °C, 100 µL CH₃CN was added. This mixture was chilled at -20 °C for 10 min and centrifuged at 13000 rpm for 5 min. The supernatant was withdrawn and combined with 40 µL of 10 mM Fmoc-Cl, and the reaction was allowed to proceed at 20 °C for 20 min. To remove any remaining derivatizing reagent, 20 µL 100 mM 1-aminoadamantane was added, and the mixture was kept at 20 °C for an additional 10 min. The mixture was then filtered (0.22 µm) prior to LC-MS analysis (50 µL) injection. Method 1: A Grace Vydac protein & peptide 5µ C₁₈ column (4.6 mm x 250 mm) was used, and a gradient elution profile was used starting with 20% solvent B (acetonitrile with 0.1% formic acid) for 2 min, a linear gradient to 100% solvent B over 30 min, holding at 100% B for 2 min, a return to 20% solvent B over 5 min, and holding at 20% solvent B for 5 min. Solvent A was water with 0.1% formic acid, and the flow rate was 0.4 mL/min. The desired products 9 and 15 were eluted with a retention time of 20.0 min and 20.2 min, respectively.

**FzmM activity assays on L-3-¹³C-Asp using excess NADPH**
The reaction mixture (400 µL) contained 2 mM of L-3-¹³C-Asp, 0.1 mM FAD, 2.5 mM NADPH, 60 µL D₂O and 10 µM FzmM in 50 mM NaPi, pH 7.7. ¹³C NMR spectra were recorded at various time points.

**FzmM activity assays with L-3-¹³C-Asp and L-2-¹³C-Asp using an NADPH regeneration system**
The reaction mixture (400 µL) contained 2 mM of L-3-¹³C-Asp, 0.1 mM FAD, 0.05 mM NADP, 6 mM phosphite, 60 µL D₂O, 23 µM phosphite dehydrogenase, and 10 µM FzmM in 50 mM NaPi, pH 7.7. ¹³C NMR spectra were recorded after incubation at 25 °C for 24 h.

**¹⁸O-labeling experiments with FzmM**
The experiments were conducted in an anaerobic glove box (Coy, Grass Lake, MI). The reaction mixture (300 µL) contained 2 mM L-Asp, 0.1 mM FAD, 0.05 mM NADP, 6 mM phosphite, 23 µM phosphite.
The dehydrogenase and 10 µM FzmM in 50 mM NaP, pH 7.7. The reaction was initiated in a vessel stoppered with a septum by injection of 5 mL of 10 psi by 18O2 gas (Sigma-Aldrich, 97% 18O). For comparison, a parallel experiment was setup in the same manner and the reaction vessel was then moved outside the anaerobic box and exposed to air to initiate the reaction.

After a 24 h incubation at 25 °C, the proteins were removed by passing the reaction mixture through an Amicon spin column (10 kDa MWCO), and the filtrate was taken for LC-MS analysis. Method: A Grace Vydac peptide C18 column (5µ; 4.6 mm x 250 mm) was used and a gradient elution profile was used starting with 5% solvent B (acetonitrile with 0.1% formic acid) for 2 min, a linear gradient to 25% solvent B over 30 min, a gradient to 100% solvent B over 5 min, holding at 100% B for 3 min, return to 5% solvent B over 2 min, and holding at 5% solvent B for 5 min. Solvent A was water with 0.1% formic acid, and the flow rate was 0.4 mL/min.

Coincubation of L-3-13C-Asp with FzmM and either FzML or FzMR using an NADPH regeneration system

The reaction mixture (400 µL) contained 2 mM of L-3-13C-Asp, 0.1 mM FAD, 0.05 mM NADP, 6 mM phosphite, 60 µL D2O, 23 µM phosphite dehydrogenase, 10 µM FzmM and 45 µM FzML or FzMR in 50 mM NaP, pH 7.7. The 13C NMR spectra were recorded after incubation at 20 °C for about 20 h.

Detection of nitrous acid during the coincubation of FzmM with FzML using Saltzman’s reagent8

The reaction mixture (100 µL) contained 1 mM of L-Asp, 0.02 mM FAD, 2 mM NADPH, 2 mM FzmM and 2 µM FzML in 10 mM Tris-buffer, pH 7.5. After incubation at 30 °C for 1 h, the reaction was stopped by the addition of 50 µL of methanol. To this mixture was added 10 µL of 100 mM sulphanilamide in 1 M HCl. After incubation at 20 °C for 5 min, 1 µL of 10 mM N-(1-naphthyl)ethylenediamine in 60 mM HCl was added and the nitrous acid containing solution turned red-purple.

FzML activity assays with fosB

The reaction mixture (500 µL) contained about 0.25 mM of synthetic fosB, 2 mg of total tRNA from E. coli MRE 600 (Roche), 6 mM L-Val, 6 mM ATP, 12 µM valyl-tRNA synthetase from E. coli (ValRS), 2 Units of thermostable inorganic pyrophosphatase (TIPP), 40 µM FzML in 100 mM HEPES, 10 mM KCl, and 20 mM MgCl2, pH 7.5. After 7 h incubation at ambient temperature ca. 100 µL of Chelex resin was added to the reaction tube. The resin was allowed to chelate any divalent cations to minimize line broadening of 31P NMR signals. Prior to NMR analysis, the protein and the nucleic acids were removed by passing the reaction mixture through an Amicon spin column (10 kDa MWCO).

FzML activity assays with Arg-OH, Arg-NHNH2 and Arg-NHNHMe

The reaction mixture (50 µL) contained 3 mM Arg-OH, Arg-NHNH2 or Arg-NHNHMe,9 0.2 mg of total tRNA from E. coli MRE 600, 6 mM L-Val, 6 mM ATP, 12 µM valyl-tRNA synthetase from E. coli (ValRS), 1 Unit of thermostable inorganic pyrophosphatase (TIPP), 31 µM FzML in 100 mM HEPES, 10 mM KCl, and 20 mM MgCl2, pH 7.5. After a 5 h incubation at ambient temperature, 100 µL CH3CN was added. This mixture was chilled at -20 °C for 10 min and centrifuged at 13000 rpm for 5 min. The supernatant was withdrawn and combined with 80 µL of 10 mM Fmoc-Cl, and the reaction was allowed to proceed at 20 °C for 20 min. To remove any remaining derivatizing reagent, 20 µL of 100 mM 1-aminoadamantane was added and the mixture was kept at 20 °C for an additional 10 min. The mixture was then filtered (0.22 µm) before LC-MS analysis. Method 1: A Grace Vydac protein & peptide 5µ C18 column (4.6 mm x 250 mm) was used and a gradient elution profile was used starting with 20% solvent B (acetonitrile with 0.1% formic acid) for 2 min followed by a linear gradient to 100% solvent B over 30 min, holding at 100% B for
2 min, return to 20% solvent B over 5 min and holding at 20% solvent B for 5 min. Solvent A was water with 0.1% formic acid, and the flow rate was 0.4 mL/min.

Production of uniformly $^{15}$N-labeled fosfazinomycin A from *Streptomycetes* sp. NRRL S-149
A 10% glycerol stock of *Streptomycetes* sp. NRRL S-149 was used to streak onto a R2A agar plate (0.05% yeast extract, 0.05% peptone, 0.05% casamino acids, 0.05% soluble potato starch, 2.8 mM glucose, 2.7 mM sodium pyruvate, 0.9 mM potassium phosphate dibasic, 0.2 mM magnesium sulfate heptahydrate, 1.5% agar). After an incubation period of three days at 30°C, a single colony was picked and used to inoculate 5 mL of liquid ATCC172 media (55.5 mM glucose, 10.0 mM calcium carbonate, 2% soluble starch, 0.5% yeast extract, 0.5% N-Z Amine Type A). The ATCC172 starter culture was then incubated for 3 days at 30°C on a rolling drum. An aliquot of 1 mL of the starter culture was then used to inoculate a secondary seed culture of 25 mL of modified R2A media (3.8 mM ammonium chloride, 0.05% soluble potato starch, 2.8 mM glucose, 2.7 mM sodium pyruvate, 0.9 mM potassium phosphate dibasic, 0.2 mM magnesium sulfate heptahydrate) in a 125 mL baffled flask. The modified R2A seed culture was then incubated at 30°C on a shaker at 200 rpm for 3 days. A sample of 20 mL of the modified R2A starter culture was then used to inoculate 500 mL of uniformly $^{15}$N-labeled modified R2AS production media [3.8 mM ammonium chloride (15N, 99%, Cambridge Isotope Laboratories), 100 μM L-aspartic acid ($^{15}$N, 98%), 0.05% soluble potato starch, 2.8 mM glucose, 2.7 mM sodium pyruvate, 0.9 mM potassium phosphate dibasic, 0.2 mM magnesium sulfate heptahydrate, 40 mM sodium succinate, 0.5% Balch’s vitamins] in a 2 L baffled flask. After 3 days at 30°C on a shaker at 200 rpm, the spent media was separated from the cell mass by centrifugation. The spent media was then concentrated to 10 mL under reduced pressure, 40 mL of methanol was then added, and the precipitant was removed via centrifugation. The supernatant was again dried under reduced pressure before being reconstituted in 5 mL of distilled water. Solid phase extraction was then preformed with an Oasis HLB resin cartridge; the sample was applied onto the resin and washed with 20 mL of distilled water. Fosfazinomycin A was then eluted from the resin with 15 mL of 20% methanol (in water). The eluate was then dried under reduced pressure and reconstituted in 600 μL of D$_2$O for NMR and LC-MS analysis.

Production of unlabeled fosfazinomycin A from *Streptomycetes* sp. NRRL S-149
The procedure used was the same as that for producing the uniformly $^{15}$N-labeled sample described above except using unlabeled ammonium chloride and aspartic acid.

Biotransformation experiments with ammonium chloride and $^{15}$N-labeled aspartic acid using *Streptomycetes* sp. NRRL S-149
The initial starter and seed cultures for the biotransformation experiments were cultivated in the same manner as described above. An aliquot of 1 mL of the seed culture in R2A medium was used to inoculate production cultures in 50 mL of modified R2AS media (3.8 mM ammonium chloride, 100 μM L-aspartic acid, 0.05% soluble potato starch, 2.8 mM glucose, 2.7 mM sodium pyruvate, 0.9 mM potassium phosphate dibasic, 0.2 mM magnesium sulfate heptahydrate, 40 mM sodium succinate, 0.5% Balch’s vitamins) in 250 mL baffled flasks. After 40 h shaking at 200 rpm at 30°C, aspartic acid ($^{15}$N, 98%, Cambridge Isotope Laboratories) was added to a final concentration of 1 mM. After an additional 4 h shaking at 200 rpm at 30°C, the spent media was separated from the cell mass by centrifugation. The supernatant was then dried under reduced pressure. The dried spent media was extracted three times with 1 mL of methanol. The methanol extract was then dried under reduced pressure and the residue reconstituted in 600 μL of deuterium oxide for NMR and LC-MS analysis.
Biotransformation experiments with $^{15}$N-ammonium chloride and aspartic acid for labeled fosfazinomycin A in Streptomyces sp. NRRL S-149

The initial starter and seed cultures for the biotransformation experiments were cultivated in the same manner as described above. An aliquot of 1 mL of the seed culture in R2A medium was used to inoculate production cultures in 50 mL of modified R2AS media containing uniformly $^{15}$N-labeled ammonium chloride (3.8 mM; $^{15}$N, 99%, Cambridge Isotope Laboratories), 100 µM L-aspartic acid ($^{15}$N, 98%), 0.05% soluble potato starch, 2.8 mM glucose, 2.7 mM sodium pyruvate, 0.9 mM potassium phosphate dibasic, 0.2 mM magnesium sulfate heptahydrate, 40 mM sodium succinate, and 0.5% Balch's vitamins in 250 mL baffled flasks. After 40 h shaking at 200 rpm at 30°C, aspartic acid (with nitrogen at natural abundance) was added to a final concentration of 1 mM. The spent media was then processed as described above before NMR and LC-MS analysis.

Analysis of fosfazinomycin A labeling by LC-MS

LC-MS analysis was performed on an Agilent 1100 series LC system coupled to an Agilent G1956B single quadrupole mass spectrometer with a Waters XBridge BEH 5µ C18 column (4.6 mm x 250 mm) using a mobile phase of water (solvent A) and acetonitrile (solvent B). The following gradient was used for analysis of fosfazinomycin A: 5% B for 5 min, from 5% B to 35% B for 30 min, from 35% B to 95% B for 5 min, holding at 95% B for 15 min, and from 95% B to 5% B for 10 min. The spectra were processed with Agilent ChemStation software before being replotted in Microsoft Excel. The percent incorporations of the nitrogen from aspartic acid in samples derived from the producing organism grown in $^{15}$N-labeled media were calculated after subtracting the mass spectrum of fosfazinomycin A at natural abundance (Fig. S29). Thus, the values were already adjusted for the presence of natural abundance $^{13}$C, which is indicated as normalized abundance in Figure S30.

With the current labeling data, it is not possible to conclusively determine whether both of the nitrogens in the hydrazine moiety derive from aspartic acid. We examined the MS data for fosfazinomycin A produced in media containing $^{15}$NH$_4$Cl and aspartic acid (natural abundance) (Fig. S5C), because it provides the highest level of incorporation of nitrogen from Asp. The relative abundance of analyte with zero, one, two, and three $^{14}$N-incorporations from Asp was determined from the mass spectra (Fig. S30A). If only one of the nitrogens in the hydrazine moiety comes from aspartic acid, then the relative abundance of doubly incorporated material would be zero (assuming nonspecific labeling does not take place). However, if it is assumed that aspartic acid is the biosynthetic origin of both nitrogens (and again ignoring nonspecific incorporation), then the predicted relative abundance of doubly $^{14}$N-incorporated material (8.6, see below) matches well with the experimental value (8.3).

The prediction of 8.6 was made as follows. The experimental mass spectrum of fosfazinomycin shows that the relative abundances of zero $^{14}$N incorporations and one $^{14}$N incorporation are 100 and 58.7, respectively (Fig. S30B). However, the mass spectrometer cannot distinguish between nitrogen incorporation at the two different sites of the hydrazine. If both sites are assumed to originate from aspartic acid, the abundance of fosfazinomycin labeled at each site would be 29.35 ($^{58.7/2} = 29.35$). The partitioning of the aspartic acid nitrogen compared to nitrogen arising from $^{15}$NH$_4$Cl into fosfazinomycin can then be calculated as: $^{58.7/2}_{100} = 22.7%$ (i.e. 22.7% of the hydrazine nitrogens come from unlabeled exogenous Asp and 77.3% from endogenously biosynthesized $^{15}$N-Asp).

Since this partitioning is assumed to arise from the relative concentrations of labeled and unlabeled aspartic acid within the cell at the time of fosfazinomycin biosynthesis, it is assumed to be the same for both nitrogens. Hence, the partitioning scheme shown in Figure S30B would apply, and fosfazinomycin containing two $^{14}$N atoms would be expected to have a relative abundance of 8.6.
However, throughout this prediction, it is assumed that the $^{14}\text{N}$ from Asp does not scramble into any other positions. There is almost certainly some degree of nonspecific labeling as the exogenously provided aspartic acid is assimilated into primary metabolism. Indeed, a small amount of material containing three $^{14}\text{N}$ atoms is observed (Fig. S29A). Thus, currently, it is not possible to unambiguously conclude that both nitrogens derive from Asp. The NMR data also does not have sufficient resolution to unambiguously settle the question.
Supplementary Figures

Figure S1. SDS-PAGE analysis of His6-FzmM and His6-FzmQ after IMAC purification. Coomassie staining. M: Prestained precision Plus Protein Standards (Bio-Rad). Lane 1: His6-FzmM. Lane 2: His6-FzmQ.
Figure S2. SDS-PAGE analysis of His$_6$-FzmL after IMAC purification. Coomassie staining. M: Prestained precision Plus Protein Standards (Bio-Rad). Lane 1: His$_6$-FzmL.

Figure S3. SDS-PAGE analysis of His$_6$-FzmR after IMAC purification. Coomassie staining. M: Prestained precision Plus Protein Standards (Bio-Rad). Lane 1: His$_6$-FzmR.
Figure S4. SDS-PAGE analysis of His$_6$-FzmI after IMAC purification. Coomassie staining. M: Prestained precision Plus Protein Standards (Bio-Rad). Lane 1: His$_6$-FzmI.

Figure S5. SDS-PAGE analysis of His$_6$-ValRS after IMAC purification. Coomassie staining. M: Prestained precision Plus Protein Standards (Bio-Rad). Lane 1: His$_6$-ValRS.
Figure S6. $^1$H NMR analysis of His$_6$-FzmR incubated with N-acetylhydrazinosuccinate: zoom-in of the fumarate region. 

a) $^1$H NMR spectrum of the assay in panel b spiked with an authentic standard of fumarate. b) NMR spectrum of the reaction mixture containing all the necessary components for catalysis (i.e. N-acetylhydrazinosuccinate, His$_6$-FzmR). c) NMR spectrum of the reaction mixture (N-acetylhydrazinosuccinate in NaPi, pH 7.7) in the absence of His$_6$-FzmR.
Figure S7. $^1$H NMR analysis of His$_6$-FzmR incubated with N-acetylhydrazinosuccinate, zoom-in of the upfield region. a) $^1$H NMR spectrum of the assay in panel b spiked with an authentic standard of AcNH$_2$H$_2$ b) NMR spectrum of the reaction mixture containing N-acetylhydrazinosuccinate and His$_6$-FzmR. c) NMR spectrum of the reaction mixture in the absence of His$_6$-FzmR.

Note: N-acetylhydrazinosuccinate exists as a pair of rotational isomers. The existence of rotational isomers was also observed for the authentic synthetic standard of N-acetylhydrazinosuccinate (see Figure S8).
Figure S8. $^1$H NMR analysis of an authentic synthetic standard of $N$-acetylhydrazinosuccinate in different solvents. a) $^1$H NMR spectrum of the authentic synthetic standard of $N$-acetylhydrazinosuccinate acquired in D$_6$-acetone. b) NMR spectrum of the authentic synthetic standard of $N$-acetylhydrazinosuccinate acquired in 50 mM phosphate buffer with 15% D$_2$O, pH 7.7.

The above results suggest $N$-acetylhydrazinosuccinate exists as multiple rotational isomers, with the ratio of the isomers depending on the solvents used. For example, three main rotational isomers exist in acetone, while two main isomers exist in phosphate buffer.
Figure S9. Overlaid selected ion monitoring (SIM) LC-MS chromatograms (negative mode) of N-acetylhydrazinosuccinate (14) incubated with FzmQ. 

- a) Chromatogram of the assay in panel c spiked with an authentic synthetic standard of N-acetylhydrazinosuccinate (14).
- b) Chromatogram of an authentic synthetic standard of N-acetylhydrazinosuccinate (14).
- c) Chromatogram of the reaction mixture containing hydrazinosuccinate (12), Ac-CoA, and His$_6$-FzmQ.
- d) Chromatogram of the reaction mixture in the absence of His$_6$-FzmQ.
Figure S10. Michaelis-Menten plot of the dependence of the rate of hydrazinosuccinate conversion by FzmQ on concentration. The assay (0.2 mL final volume) was carried out at 23 °C and contained: 1 – 50 µM hydrazinosuccinate, 0.48 mM Ac-CoA, 0.4 mM DTNB, and 0.05 µM His6-FzmQ in 50 mM Tris, pH 7.7.
Figure S11. Overlaid selected ion monitoring (SIM) chromatograms (negative ion mode LC-MS) showing the formation of compound 9 upon incubation of L-Asp with FzmM. a) Chromatogram of the assay in panel c spiked with an authentic synthetic standard of 9. b) Chromatogram of an authentic synthetic standard of 9. c) Chromatogram of the reaction mixture containing L-Asp, FAD, NADPH, O₂, and His₆-FzmM. d) Chromatogram of the reaction mixture in the absence of His₆-FzmM.
Figure S12. FT-ICR MS analysis of compound 9, the product of His$_6$-FzmM-catalyzed N-hydroxylation of l-Asp followed by Fmoc derivatization.
Figure S13. Overlaid selected ion monitoring (SIM) chromatograms (negative ion mode LC-MS) demonstrating the formation of compound 15 upon incubation of $d_3$-L-Asp with FzmM. a) Chromatogram of the reaction mixture containing $L$-Asp (2,3,3-D$_3$), FAD, NADPH, $O_2$, and His$_6$-FzmM. b) Chromatogram of the reaction mixture in the absence of His$_6$-FzmM.
Figure S14. FT-ICR MS analysis of compound 15, the product of His$_6$-FzmM-catalyzed $N$-hydroxylation of L-Asp (2,3,3-D3) followed by Fmoc derivatization.
Figure S15. $^{13}$C NMR analysis of incubation of L-3-$^{13}$C-Asp with His$_6$-FzmM using a NADPH regeneration system. 

a) $^{13}$C NMR spectrum of the reaction mixture containing 2 mM of L-3-$^{13}$C-Asp, 0.1 mM FAD, 0.05 mM NADP, 6 mM phosphite, 60 µL D$_2$O, 23 µM phosphite dehydrogenase, 10 µM FzmM in 50 mM NaPi, pH 7.7. 

b) NMR spectrum of the sample of panel a spiked with an authentic standard of NPA.

c) NMR spectrum of the authentic standard of NPA.

Note: The authentic NPA was at natural abundance for $^{13}$C, and a much higher concentration (50 mM) was required compared to the labeled material in the assay (2 mM), which changes the pH. This accounts for the changes in the chemical shifts between experiments, and thus spiking experiments were required for the assignments.
Figure S16. $^1$H-$^{13}$C HMBC analysis of the product formed upon incubation of His$_6$-FzmM with L-3-$^{13}$C-Asp using a NADPH regeneration system.
Figure S17. $^{13}$C NMR analysis of the products formed upon incubation of L-2-$^{13}$C-Asp with His$_6$-FzmM using an NADPH regeneration system. 

a) $^{13}$C NMR spectrum of an authentic standard of 50 mM NPA in NaPi buffer (pH 7.7) with 15% D$_2$O. 

b) NMR spectrum of the sample in panel c spiked with the authentic standard of NPA. 

c) NMR spectrum of the reaction mixture containing 2 mM of L-2-$^{13}$C Asp, 0.1 mM FAD, 0.05 mM NADP, 6 mM phosphite, 60 µL D$_2$O, 23 µM phosphite dehydrogenase, and 10 µM FzmM in 50 mM NaPi, pH 7.7. 

d) NMR spectrum of the reaction mixture in the absence of His$_6$-FzmM.

Note: The authentic NPA in panel a was at natural abundance for $^{13}$C, and thus a much higher concentration was required compared to the labeled material. These much higher concentrations likely lead to changes in pH which accounts for the differences in the chemical shifts between panels a and c. For that reason, a spiking experiment was employed (panel b) for the assignment.

# glycerol peak from the protein storage buffer.
Figure S18. LC-MS analysis of NPA generated in FzmM assays performed with $^{18}$O$_2$. Overlaid selected ion monitoring (SIM) chromatograms of NPA in negative mode in LC-MS produced in FzmM conversion of L-Asp performed under $^{18}$O$_2$. a) Chromatogram monitored at m/z 122. b) Chromatogram monitored at m/z 120. c) Chromatogram monitored at m/z 118.
Figure S19. LC-MS analysis of NPA generated in FzmM assays performed with $^{16}$O$_2$. Overlaid selected ion monitoring (SIM) chromatograms of NPA in negative mode in LC-MS in FzmM assays performed under $^{16}$O$_2$. a) Chromatogram monitored at m/z 118. b) Chromatogram monitored at m/z 120. c) Chromatogram monitored at m/z 122.
Figure S20. Michaelis-Menten plot of the rates of $\text{O}_2$ consumption during FzmM oxidation of L-Asp. Dependence of the His$_5$-FzmM activity on the concentration of L-Asp. Assay (0.2 mL final volume) was carried out at 23 °C and contained: 0.05 – 15 mM L-Asp, 0.02 mM FAD, 0.25 mM NADPH, and 0.3 µM His$_5$-FzmM in 50 mM Hepes containing 100 mM NaCl, pH 7.8. 

$\begin{align*}
    k_{\text{cat}} &\quad 2.48 \\
    K_m &\quad 0.79 \\
    \text{Std. Error} &\quad k_{\text{cat}} \quad 0.06 \\
    &\quad K_m \quad 0.07
\end{align*}$
Figure S21. $^{13}$C NMR analysis of coincubation of L-3-$^{13}$C-Asp with His$_6$-FzmM and either His$_6$-FzmL or His$_6$-FzmR using an NADPH regeneration system. a) NMR spectrum of an authentic standard of fumarate. b) NMR spectrum of an authentic standard of malate. c) NMR spectrum of the coincubation of His$_6$-FzmM with His$_6$-FzmL and L-Asp. d) NMR spectrum of the coincubation of His$_6$-FzmM with His$_6$-FzmR.

*glycerol signals from the protein storage buffer.
Figure S22. Detection of nitrous acid in the coincubation of L-Asp with His$_6$-FzmM and His$_6$-FzmL using Saltzman’s reagent.\(^8\)

1) Solution containing authentic nitrous acid. 2) Coincubation of His$_6$-FzmM with His$_6$-FzmL in the presence of L-Asp and NADPH. 3) Negative control: same as tube 2 except omitting His$_6$-FzmM. 4) Negative control: same as tube 2 except omitting His$_6$-FzmL.
Figure S23. $^{31}$P NMR analysis of incubation of His$_6$-FzmI with synthetic fosB. a) NMR spectrum of an authentic standard of synthetic fosB. b) NMR spectrum in panel c spiked with an authentic standard of synthetic fosB. c) NMR spectrum of the assay in panel d spiked with an authentic standard of natural fosA. d) NMR spectrum of the reaction mixture containing synthetic fosB, His$_6$-FzmI, Val, ValRS, ATP, and tRNA. e) NMR spectrum of an authentic standard of natural fosA.

Note: Synthetic fosB has two peaks in the $^{31}$P NMR spectrum because the chemical synthesis yielded a pair of diastereomers.

Based on the results, both diastereomers of the synthetic fosB were accepted by FzmI and converted to natural fosA and its diastereomer. These observations are consistent with FzmI also accepting Arg and Arg-NHNH$_2$ (Figures S26 & S27).
Figure S24. FT-ICR MS analysis of fosfazinomycin A, the product of His₆-FzmI-catalyzed reaction between L-Val and synthetic fosfazinomycin B in the presence of ATP, ValRS and tRNA.
**Figure S25.** $^{31}$P NMR spectra analysis of the negative control of His$_6$-FzmI on synthetic fosB (in the absence of His$_6$-FzmI). 

a) NMR spectrum of an authentic standard of synthetic fosB. 

b) NMR spectrum of the assay in panel c spiked with an authentic standard of synthetic fosB. 

c) NMR spectrum of the reaction mixture (see legend Figure S23) in the absence of His$_6$-FzmI.

**Note:** Synthetic fosB has two peaks in the $^{31}$P NMR spectrum because the chemical synthesis yielded a pair of diastereomers.

Based on these results, synthetic fosB could not be converted to fosA in the absence of FzmI.
Figure S26. LC-MS analysis of incubation of His<sub>6</sub>-FzmI with Arg-OH. Overlaid selected ion monitoring (SIM) chromatograms (positive ion mode LC-MS) of Fmoc-Val-Arg-OH upon incubation of FzmI with Arg-OH, Val, ATP, ValRS, tRNA, and ATP. a) Chromatogram of Fmoc-derivatized authentic standard of Val-Arg-OH. b) Chromatogram of the reaction mixture containing Arg-OH, His<sub>6</sub>-FzmI, Val, ATP, ValRS, and tRNA.
Figure S27. LC-MS analysis of incubation of His$_6$-FzmI with Arg-NHNH$_2$. Overlaid selected ion monitoring (SIM) chromatograms (positive ion mode LC-MS) of Fmoc-Val-Arg-NHNH$_2$ upon incubation of FzmI with Arg-NHNH$_2$, ATP, Val, ValRS, and tRNA. a) Chromatogram of Fmoc-derivatized authentic synthetic standard of Val-Arg-NHNH$_2$. b) Chromatogram of the reaction mixture containing Arg-NHNH$_2$, His$_6$-FzmI, ATP, Val, ValRS, and tRNA.
Figure S28. LC-MS analysis of incubation of His<sub>6</sub>-FzmI with Arg-NHNHMe. Overlaid selected ion monitoring (SIM) chromatograms of Fmoc-Val-Arg-NHNHMe under positive mode in LC-MS in FzmI assays. a) Chromatogram of Fmoc-derivated authentic synthetic standard of Val-Arg-NHNHMe. b) Chromatogram of the reaction mixture containing Arg-NHNHMe, His<sub>6</sub>-FzmI, ATP, ValRS, Val, and tRNA.
Figure S29. Mass spectra of fosfazinomycin A at natural abundance.
Figure S30. Analysis of the isotope distribution for fosfazinomycin A produced in media containing $^{15}$NH$_4$Cl and unlabeled aspartic acid. a) The experimental relative abundances of fosfazinomycin A with 0, 1, 2, or 3 incorporations of nitrogen from aspartic acid compared to predicted abundances assuming either one or two incorporations of nitrogen from aspartic acid into the hydrazine moiety. b) The theoretical partitioning of the nitrogen from aspartic acid into the hydrazine moiety of fosfazinomycin A. The asterisk denotes a nitrogen originating from aspartic acid.
One obvious question that is raised by our proposed pathway is how it would fit in the lomaiviticin and kinamycin pathways, the clusters of which have the same set of five genes. One possibility is that these pathways also involve formation of hydrazine, but that would not be consistent with labelling studies that suggest stepwise incorporation of both nitrogens. Since it appears unlikely that the hydrazide functionality in fosfazinomycin originates from an acyl diazonium salt (diazoization product of an amide or phosphoramidate), we suggest that whereas the genes are conserved, the chemistry may have diverged, consistent with the considerable difference in the structures of the final products. This may also explain why all seven genes are present in only the fosfazinomycin cluster.
### Table S1. List of strains and plasmids used in this study.

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<td>CAM&lt;sup&gt;+&lt;/sup&gt;; provides seven rare, in <em>E. coli</em>, tRNAs for the codons CGG, AUA, AGG, AGA, CUA, CCC, and GGA in the same plasmid that harbors the T7 lysozyme</td>
<td>Novagen</td>
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<tr>
<td><em>E. coli</em> DH5α λpir</td>
<td>sup E44, ΔlacU169 (ΦlacZΔM15), recA1, endA1, hsdR17, thi-1, gyrA96, relA1, λpir phage lysogen.</td>
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<td>pET15b</td>
<td>AMP&lt;sup&gt;+&lt;/sup&gt;; encodes an N terminal 6xHis-tag&lt;sup&gt;®&lt;/sup&gt; of the protein of interest, contains a thrombin recognition sequence</td>
<td>Novagen</td>
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<td>Fosmid MMG 358</td>
<td>CAM&lt;sup&gt;+&lt;/sup&gt;; carries the fosfazinomycin biosynthetic cluster from <em>Streptomyces</em> sp. XY332</td>
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<td>Fosmid MMG 360</td>
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### Table S2. List of oligonucleotides used in this study.

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<td>Ndel-fzmM_fw</td>
<td>gcagcgcgctgtccggccgggacgagctatagTGAGTACTTCGGCGCTACTGAGCG</td>
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<td>gcagcgcgctgtccggccgggacgagctatagTGAGTACTTCGGCGCTACTGAGCG</td>
<td>Amplification of fzmR for Gibson cloning into pET15b vector</td>
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<tr>
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<td>Sequence</td>
<td>Amplification</td>
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<td>------------</td>
<td>--------------------------------------------------------------------------</td>
<td>------------------------------------------------------------------------------</td>
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<td>XhoI-fzmR_rc</td>
<td>cctttcgggctttttagcagccggatctccgagTCACACTTC CACATGGTCTCTGACGCTCTT</td>
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<tr>
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<td>Amplification of <em>fzmI</em> for Gibson cloning into pET15b vector</td>
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<tr>
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<td>gcagcggcctgtgccgctggccagccatcagAAAAGACA TATAACCCACAAGATCGAAGCC</td>
<td>Amplification of <em>valS</em> for Gibson cloning into pET15b vector</td>
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<td>cctttcgggctttttagcagccggatctccgagTTACACGG CGCGGATACGCGCTGC</td>
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<td>gcagcggcctgtgccgctggccagccatcagATGGACGGCT GAAGACGACCCCGGAC</td>
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<td>cctttcgggctttttagcagccggatctccgagTCATGGCT TGATCCGCGTGCGGTGCGGCGTGTCT</td>
<td>Amplification of <em>fzmL</em> for Gibson cloning into pET15b vector</td>
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*a* Underlined sequence indicates homology to expression vector.
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References