Electronic Supporting Information

Biosynthesis of fosfazinomycin follows a convergent strategy

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Table of Contents

Chemicals

All chemicals were purchased form Sigma-Aldrich and Chem-Impex unless otherwise specified. Phosphonoacetaldehyde (PnAA), methyl phosphonoacetate (Me-PnA), methyl 2-hydroxy-2-phosphono-acetate (Me-HPnA), 2-hydroxyethylphosphonate (2-HEP), 1, 2-dihydroxyethylphosphonate (1, 2-DHEP), NH_2NMeBoc, compound **15**, and fosfazinomycin B were prepared following literature procedures.[1,2,3,4,5,6]

Molecular Biology

Reagents

E. coli strains and plasmids used in this study are listed in Table S1. Restriction enzymes (*Nde*I and *Dpn*I), 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

Epicenter. 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.[7] Fosfazinomycin biosynthetic genes (Table S1) were PCR amplified from fosmids MMG 358 and 360[8] or genomic DNA WM6372 using the Fail Safe polymerase in buffer G. All cloning manipulations were based on the *in vitro* ligation isothermal assembly protocol described by Gibson et al.[9] Briefly, vector linearized with the appropriate restriction enzyme was PCR amplified using primers listed in Table S2, re-digested with *Dpn*I 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 mixture (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 ×), 6.8 µL Phusion Polymerase, 2 µL T5 exonuclease (10 × diluted in the T5 exonuclease buffer) and 50 µL Taq DNA ligase. Isothermal reaction buffer (6 mL final volume; 5×) 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 solution of each of the four dNTPs (800 µM) in the appropriate amount of distilled water.

Enzymology

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 EMB 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 and used for molecular biology procedures. Other reagents were purchased from Sigma-Aldrich. Lysis buffer consisted of 50 mM NaPi, 300 mM NaCl, 10 mM imidazole, 10% glycerol, pH 8.0. Wash buffer consisted of 50 mM NaPi, 300 mM NaCl, 20 mM imidazole, 10% glycerol, pH 8.0. Elution buffer consisted of 50 mM NaPi, 300 mM NaCl, 250 mM imidazole, 10% glycerol, pH 8.0. Storage buffer consisted of 50 mM NaPi, 300 mM NaCl, 10% glycerol, pH 8.0.

Analytical Methods

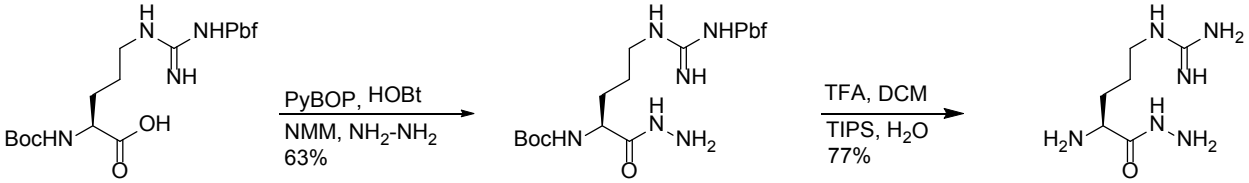
NMR experiments were carried out using an Agilent 600 MHz instrument equipped with a OneNMR probe. Mass spectrometry (except FTMS) was performed at the University of Illinois Mass Spectrometry Center. ESI mass spectra were obtained on a Quattro spectrometer. LC-FTMS analysis was performed on a custom-made 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 followed by a linear gradient to 95% solvent B over 10 min, elution at 95% B for 5 min, 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.

Expression and Purification of His_6-FzmB, His_6-FzmG and His_6-FzmH

An approximately 7 h culture of *E. coli* Rosetta 2 (DE3) pLysS cells freshly transformed with the appropriate plasmid and grown in $LB^{AMP, CAM}$ medium 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 ºC. All the following purification steps were carried out at 4 °C. The cells (5-7 g wet mass from 2 L 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 supplemented with 1 mg / mL lysozyme and 600 U DNase. The cells were lysed by passage twice 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 ca. 30 min, the flow-through was discarded and the resin was washed with 2 × 40 mL of wash buffer. Resin-bound protein was eluted with elution buffer. 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 was removed by passing the protein solution through 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 ºC. Typical yields: i) His_6-FzmB, 74 mg/L, ii) His_6-FzmG, 33 mg/L. iii) His_6-FzmH, 24 mg/L.

Synthetic Procedures for the Preparation of Substrates and Authentic Standards



Scheme S1. Synthesis of Arg-$NHNH_2$ (2)

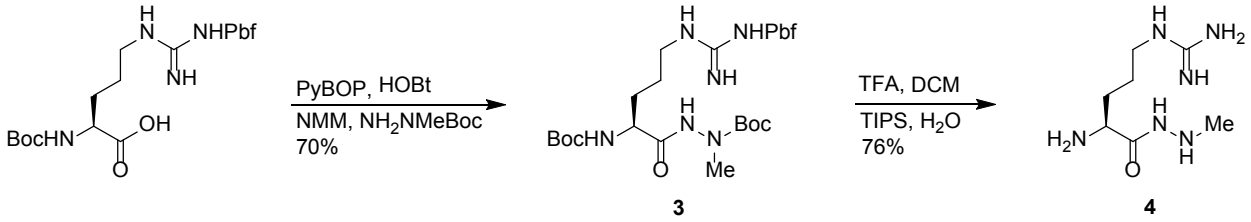
Boc-L-Arg(Pbf)-$NHNH_2$ (1)

Anhydrous hydrazine (0.078 mL, 2.5 mmol, 5.0 equiv.) was dissolved in dry CH_2Cl_2 (2 mL) and this solution was cooled to 0 °C. A mixture of Boc-L-Arg(Pbf)-OH (263.0 mg, 0.5 mmol), benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP; 260.0 mg, 0.5 mmol, 1.0 equiv.), hydroxybenzotriazole (HOBt) (67.5 mg, 0.5 mmol, 1.0 equiv.) and *N*-methylmorpholine (NMM) (0.055 mL, 0.5 mmol, 1.0 equiv.) in dry CH_2Cl_2 (3 mL) was added dropwise to the cooled solution of hydrazine. The resulting mixture was stirred at room temperature (rt) for 19 h. The solution was diluted with CH_2Cl_2 (25 mL) and was then washed with sat. $NaHCO_3$ (1 x 30 mL), water (1 x 15 mL), and brine (1 x 15 mL), dried (Na_2SO_4), and evaporated to dryness. The product was purified by flash chromatography (silica gel, 15:1 CH_2Cl_2 : MeOH), yielding 170 mg (63%) of white gum. ^1H NMR (CD_3OD, 600 MHz): δ/ppm 3.96 (t, 1H, *J* = 6.0 Hz, Arg-H_α), 3.14 (m, 2H, Arg-H_δ), 2.98 (s, 2H, Ar-CH_2), 2.55 (s, 3H, Ar-CH_3), 2.49 (s, 3H, Ar-CH_3),

2.07 (s, 3H, Ar-CH_3), 1.70-1.49 (m, 4H, Arg-H_β, Arg-H_γ), 1.44 (s, 6H, -OC($C\underline{H}_3$)$_2$$CH_2$), 1.43 (s, 9H, -C($CH_3$)$_3$); ^{13}C NMR ($CD_3OD$, 150 MHz): δ/ppm 172.5, 158.4, 156.6, 156.3, 137.9, 132.9, 132.1, 124.6, 117.0, 86.2, 79.2, 52.9, 42.5, 40.0, 29.3, 27.3, 25.5, 18.1, 16.9, 11.0; HRMS (ES) Calcd for $C_{24}H_{41}N_6O_6S[M+H]^+$ 541.2808, found 541.2811.

L-Arg-NHNH$_2$ (2)

A solution of Boc-L-Arg(Pbf)-$NHNH_2$ (**1**) (53.0 mg, 0.1 mmol) in trifluoroacetic acid (TFA; 2.5 mL), CH_2Cl_2 (2.25 mL), triisopropylsilane (TIPS; 0.125 mL) and water (0.125 mL) was stirred at rt for 65 min and then evaporated to dryness. The residue was dissolved in water (7 mL) and washed with EtOAc (3 x 5 mL). The aqueous layer was lyophilized to yield 32 mg product (77%) as a TFA salt. ^{1}H NMR (D_2O, 600 MHz): δ/ppm 3.98 (t, 1H, *J* = 6.0 Hz, Arg-H_α), 3.12 (t, 2H, *J* = 6.0 Hz, Arg-H_δ), 1.91-1.79 (m, 2H, Arg-H_β), 1.59-1.53 (m, 2H, Arg-H_γ); ^{13}C NMR (D_2O, 150 MHz): δ/ppm 167.8, 156.6, 51.5, 40.1, 27.7, 23.4; HRMS (ES) Calcd for $C_6H_{17}N_6O[M+H]^+$ 189.1464, found 189.1461.



Scheme S2. Synthesis of Arg-NHNHMe (4)

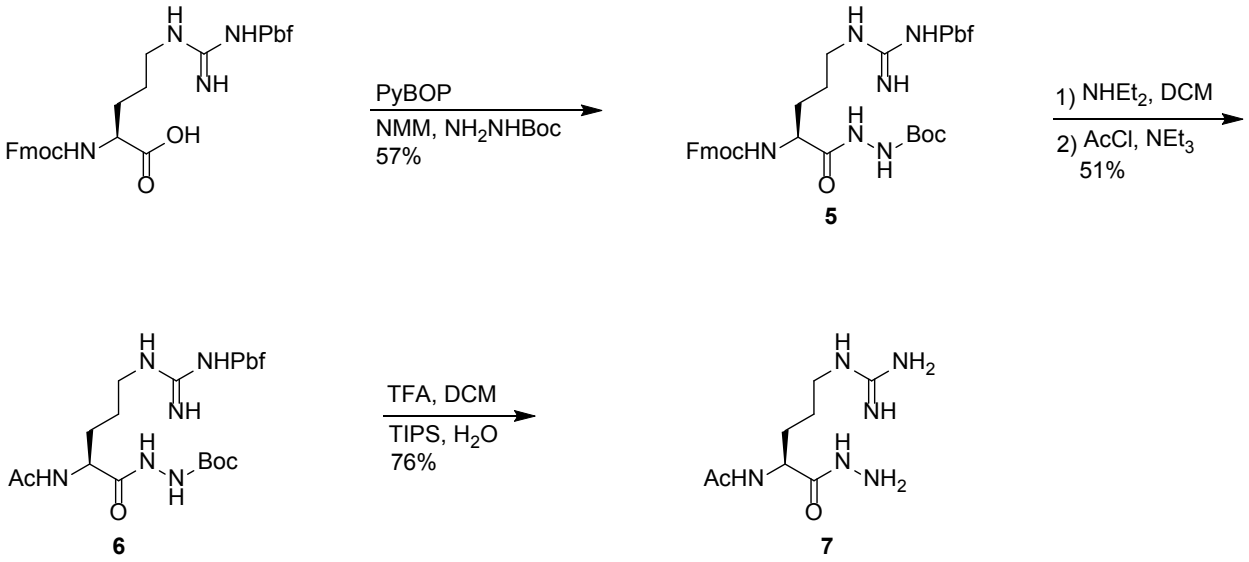
Boc-L-Arg(Pbf)-NHNMeBoc (3)

$NH_2NMeBoc$[5] (219 mg, 1.5 mmol, 3.0 equiv.) was dissolved in dry CH_2Cl_2 (2 mL) and the solution was cooled to 0 °C. A mixture of Boc-L-Arg(Pbf)-OH (263 mg, 0.5 mmol), PyBOP (260 mg, 0.5 mmol, 1.0 equiv.), HOBt (67.5 mg, 0.5 mmol, 1.0 equiv.) and NMM (0.055 mL, 0.5 mmol, 1.0 equiv.) in dry CH_2Cl_2 (3 mL) was added dropwise to the above cooled solution. The resulting mixture was stirred at rt for 17 h. The solution was diluted with CH_2Cl_2 (15 mL) and was then washed with sat. $NaHCO_3$ (1 x 10 mL), 10% citric acid (1 x 10 mL), water (1 x 10 mL), and brine (1 x 10 mL), dried (Na_2SO_4), and evaporated to dryness. The product was purified by flash chromatography (silica gel, 20:1 CH_2Cl_2 : MeOH), yielding 228 mg (70%) of white gum. ^{1}H NMR (CD_3OD, 600 MHz): δ/ppm 4.02 (t, 1H, *J* = 6.0 Hz, Arg-H_α), 3.25-3.10 (m, 2H, Arg-H_δ), 3.03 (s, 3H, N-CH_3), 2.98 (s, 2H, Ar-CH_2), 2.56 (s, 3H, Ar-CH_3), 2.50 (s, 3H, Ar-CH_3), 2.07 (s, 3H, Ar-CH_3), 1.82-1.68 (m, 1H, Arg-$H_{\beta1}$), 1.66-1.51 (m, 3H, Arg-$H_{\beta2}$, Arg-H_γ), 1.44 (s, 9H, -C(CH_3)$_3$), 1.42 (s, 9H, -C(CH_3)$_3$), 1.40 (s, 6H, -OC($C\underline{H}_3$)$_2$$CH_2$); ^{13}C NMR ($CD_3OD$, 150 MHz): δ/ppm 172.0, 158.4, 156.7, 156.2, 155.5, 138.0, 132.9, 132.0, 124.6, 117.0, 86.2, 80.9, 79.2, 52.6, 42.6, 39.8, 35.6, 29.1, 27.3, 27.1, 25.5, 18.2, 17.0, 11.1; HRMS (ES) Calcd for $C_{30}H_{51}N_6O_8S[M+H]^+$ 655.3489, found 655.3472.

L-Arg-NHNHMe (4)

A solution of Boc-L-Arg(Pbf)-NHNMeBoc (**3**) (46.0 mg, 0.07 mmol) in trifluoroacetic acid (TFA) (2.5 mL), CH_2Cl_2 (2.25 mL), triisopropylsilane (TIPS) (0.125 mL) and water (0.125 mL) was stirred at rt for 4 hand then evaporated to dryness. The residue was dissolved in water (15 mL) and washed with EtOAc (3 x 7 mL). The aqueous layer was lyophilized to yield 23 mg product (76%) as a TFA salt. ^{1}H NMR (D_2O, 600

MHz): δ/ppm 3.98 (t, 1H, *J* = 6.0 Hz, Arg-H_α), 3.12 (t, 2H, *J* = 6.0 Hz, Arg-H_δ), 2.83 (s, 3H, N-CH_3), 1.91-1.79 (m, 2H, Arg-H_β), 1.60-1.53 (m, 2H, Arg-H_γ); ^{13}C NMR (D_2O, 150 MHz): δ/ppm 167.6, 156.8, 52.0, 40.1, 36.3, 27.6, 23.4; HRMS (ES) Calcd for $C_7H_{19}N_6O[M+H]^+$ 203.1620, found 203.1612.



Scheme S3. Synthesis of Ac-Arg-$NHNH_2$ (7)

Fmoc-L-Arg(Pbf)-NHNHBoc (5)

tert-Butyl carbazate ($BocNHNH_2$) (0.29 g, 2.2 mmol, 1.1 equiv.) was dissolved in dry CH_2Cl_2 (2 mL) and the solution was cooled to 0 °C. A mixture of Fmoc-L-Arg(Pbf)-OH (1.29 g, 2.0 mmol), PyBOP (1.04 g, 2.0 mmol, 1.0 equiv.) and NMM (0.22 mL, 2.0 mmol, 1.0 equiv.) in dry CH_2Cl_2 (5 mL) was added dropwise to the cooled solution. The resulting mixture was stirred at rt for 19 h. The solution was diluted with CH_2Cl_2 (30 mL) and was then washed with 5% $NaHCO_3$ (1 x 10 mL), water (1 x 10 mL), and brine (1 x 10 mL), dried (Na_2SO_4), and evaporated to dryness. The product was purified by flash chromatography (silica gel, 2:1 EtOAc : hexanes), yielding 0.87 g (57%) of white gum. ^{1}H NMR (CD_3OD, 600 MHz): δ/ppm 7.73 (d, 2H, *J* = 12.0 Hz, Fmoc-H), 7.60 (t, 2H, *J* = 12.0 Hz, Fmoc-H), 7.33 (t, 2H, *J* = 6.0 Hz, Fmoc-H), 7.25 (t, 2H, *J* = 6.0 Hz, Fmoc-H), 4.36 (t, 1H, *J* = 12.0 Hz, Fmoc CHC<u>H</u>H), 4.30 (t, 1H, *J* = 12.0 Hz, Fmoc CHCH<u>H</u>), 4.17-4.09 (m, 2H, Fmoc C<u>H</u>CH_2, Arg-H_α), 3.25-3.08 (m, 2H, Arg-H_δ), 2.90 (s, 2H, Ar-CH_2), 2.56 (s, 3H, Ar-CH_3), 2.49 (s, 3H, Ar-CH_3), 2.03 (s, 3H, Ar-CH_3), 1.84-1.73 (m, 1H, Arg-$H_{\beta1}$), 1.68-1.49 (m, 3H, Arg-$H_{\beta2}$, Arg-H_γ), 1.42 (s, 9H, -$C(CH_3)_3$); 1.37 (s, 6H, -OC($C\underline{H}_3$)$_2CH_2$); ^{13}C NMR (CD_3OD, 150 MHz): δ/ppm 172.7, 158.4, 156.8, 156.7, 156.0, 143.9, 143.6, 141.1, 138.0, 133.0, 132.1, 127.4, 126.8, 124.8, 124.6, 119.5, 117.0, 86.2, 80.4, 66.9, 53.1, 47.0, 42.5, 40.0, 29.2, 27.3, 27.2, 25.3, 18.2, 17.0, 11.1; HRMS (ES) Calcd for $C_{39}H_{51}N_6O_8S[M+H]^+$ 763.3489, found 763.3478.

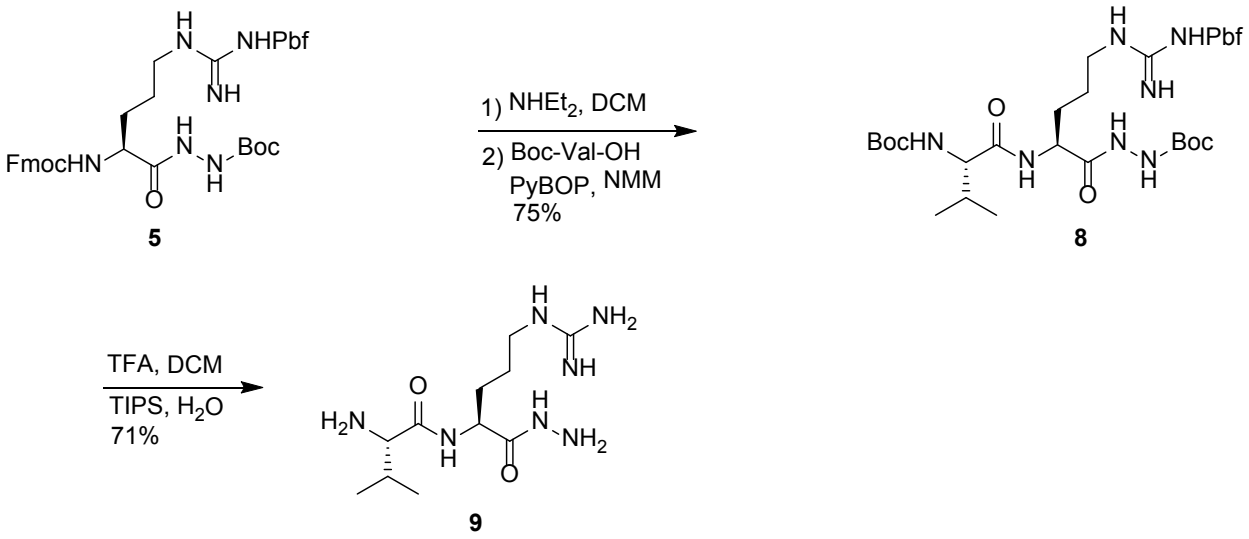
Ac-L-Arg(Pbf)-NHNHBoc (6)

A solution of Fmoc-L-Arg(Pbf)-NHNHBoc (**5**) (212 mg, 0.28 mmol) in dry CH_2Cl_2 (2.5 mL) and diethylamine (2.5 mL) was stirred at rt for 3 h. TLC indicated Fmoc was completely removed. This mixture was evaporated to dryness and re-dissolved in dry CH_2Cl_2 (3.5 mL), and cooled to 0 °C. Triethylamine (0.031 mL, 0.28 mmol, 1.0 equiv.) and acetyl chloride (AcCl) (0.020 mL, 0.28 mmol, 1.0 equiv.) were added sequentially and the resulting mixture was stirred at rt for 17 h. After evaporation to dryness, the product was purified by flash chromatography (silica gel, 15:1 CH_2Cl_2 : MeOH), yielding 82 mg (51%) of

white gum. ^{1}H NMR (CD_3OD, 600 MHz): δ/ppm 4.36 (t, 1H, *J* = 6.0 Hz, Arg-H_α), 3.24-3.11 (m, 2H, Arg-H_δ), 2.98 (s, 2H, Ar-CH_2), 2.56 (s, 3H, Ar-CH_3), 2.49 (s, 3H, Ar-CH_3), 2.06 (s, 3H, Ar-CH_3), 1.95 (s, 3H, $CH_3C(O)$), 1.85-1.76 (m, 1H, Arg-$H_{\beta1}$), 1.68-1.51 (m, 3H, Arg-$H_{\beta2}$, Arg-H_γ), 1.43 (s, 15H, -OC($C\underline{H}_3$)$_2CH_2$, -$C(CH_3)_3$); ^{13}C NMR (CD_3OD, 150 MHz): δ/ppm 172.4, 171.8, 158.4, 156.7, 156.1, 138.0, 132.9, 132.1, 124.9, 117.0, 86.2, 80.4, 51.3, 42.6, 40.0, 29.0, 27.3, 27.1, 25.2, 21.0, 18.1, 16.9, 11.0; HRMS (ES) Calcd for $C_{26}H_{43}N_6O_7S[M+H]^+$ 583.2914, found 583.2919.

Ac-L-Arg-NHNH2 (7)

A solution of Ac-L-Arg(Pbf)-NHNHBoc (**5**) (41.0 mg, 0.07 mmol) in trifluoroacetic acid (TFA) (2.5 mL), CH_2Cl_2 (2.25 mL), triisopropylsilane (0.125 mL) and water (0.125 mL) was stirred at rt for 4 h and then evaporated to dryness. The residue was dissolved in water (15 mL) and washed with EtOAc (3 x 7 mL). The aqueous layer was lyophilized to yield 24 mg product (76%) as a TFA salt. ^{1}H NMR (D_2O, 600 MHz): δ/ppm 4.23 (t, 1H, *J* = 6.0 Hz, Arg-H_α), 3.07 (t, 2H, *J* = 6.0 Hz, Arg-H_δ), 1.90 (s, 3H, $CH_3C(O)$), 1.80-1.70 (m, 1H, Arg-$H_{\beta1}$), 1.70-1.60 (m, 1H, Arg-$H_{\beta2}$), 1.60-1.45 (m, 2H, Arg-H_γ); ^{13}C NMR (D_2O, 150 MHz): δ/ppm 174.5, 171.9, 156.8, 52.0, 40.3, 27.8, 24.1, 21.6; HRMS (ES) Calcd for $C_8H_{19}N_6O_2[M+H]^+$ 231.1569, found 231.1570.



Scheme S4. Synthesis of Val-Arg-NHNH$_2$ (9)

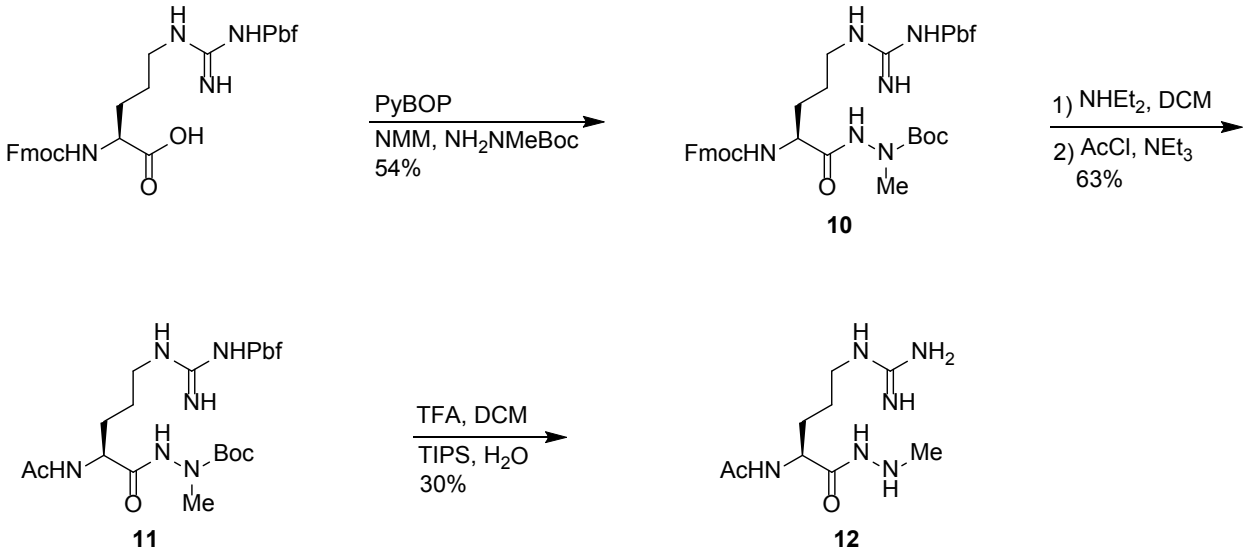
Boc-L-Val-L-Arg(Pbf)-NHNHBoc (8)

A solution of Fmoc-L-Arg(Pbf)-NHNHBoc (**5**) (434 mg, 0.57 mmol) in dry CH_2Cl_2 (2.0 mL) and diethylamine (2.0 mL) was stirred at rt for 3 h. TLC indicated Fmoc was completely removed. This mixture was evaporated to dryness and re-dissolved in dry CH_2Cl_2 (2.0 mL), and cooled to 0 °C. A mixture of Boc-L-Val-OH (154 mg, 0.71 mmol, 1.25 equiv.), PyBOP (370 mg, 0.71 mmol, 1.25 equiv.) and NMM (0.078 mL, 0.71 mmol, 1.25 equiv.) in dry CH_2Cl_2 (4.0 mL) was added dropwise to the cooled solution. The resulting mixture was stirred at rt for 18 h. The solution was diluted with CH_2Cl_2 (30 mL) and was then washed with sat. $NaHCO_3$ (1 x 10 mL), 10% citric acid (1 x 10 mL), water (1 x 10 mL), and brine (1 x 10 mL), dried (Na_2SO_4), and evaporated to dryness. The product was purified by flash chromatography (silica gel, 6:1 EtOAc : hexanes), yielding 317 mg (75%) of white gum. ^{1}H NMR (CD_3OD, 600 MHz): δ/ppm 4.41 (t, 1H, *J* = 6.0 Hz, Arg-H_α), 3.85 (t, 1H, *J* = 6.0 Hz, Val-H_α), 3.26-3.10 (m, 2H, Arg-H_δ), 2.98 (s, 2H, Ar-CH_2), 2.56 (s, 3H, Ar-CH_3), 2.49 (s, 3H, Ar-CH_3), 2.06 (s, 3H, Ar-CH_3), 2.04-1.94 (m, 1H, Val-H_β), 1.90-1.79 (m, 1H, Arg-

$H_{\beta 1}$), 1.73-1.54 (m, 3H, Arg-$H_{\beta 2}$, Arg-H_{γ}), 1.43 (s, 15H, -C(CH_3)$_3$, -OC($C\underline{H}_3$)$_2$$CH_2$), 1.41 (s, 9H, -C($CH_3$)$_3$), 0.93 (d, 3H, *J* = 6.0 Hz, -CHC$\underline{H}_3$$CH_3$), 0.91 (d, 3H, *J* = 6.0 Hz, -CHCH_3C$\underline{H}_3$); ^{13}C NMR (CD_3OD, 150 MHz): δ/ppm 173.0, 172.0, 158.4, 156.8, 156.7 156.0, 138.0, 133.0, 132.1, 124.5, 117.0, 86.2, 80.4, 79.3, 60.3, 51.0, 42.6, 40.0, 30.4, 29.0, 27.3, 27.2, 25.0, 18.4, 18.2, 17.2, 17.0, 11.1; HRMS (ES) Calcd for $C_{34}H_{58}N_7O_9S[M+H]^+$ 740.4017, found 740.4010.

L-Val-L-Arg-NHNH2 (9)

A solution of Boc-L-Val-L-Arg(Pbf)-NHNHBoc (**8**) (103 mg, 0.14 mmol) in trifluoroacetic acid (TFA) (2.5 mL), CH_2Cl_2 (2.25 mL), triisopropylsilane (0.25 mL) and water (0.25 mL) was stirred at rt for 2.5 h and then evaporated to dryness. The residue was dissolved in water (15 mL) and washed with EtOAc (3 x 7 mL). The aqueous layer was lyophilized to yield 51 mg product (71%) as a TFA salt. ^{1}H NMR (D_2O, 600 MHz): δ/ppm 4.28 (t, 1H, *J* = 6.0 Hz, Arg-H_{α}), 3.74 (d, 1H, *J* = 6.0 Hz, Val-H_{α}), 3.08 (t, 2H, *J* = 6.0 Hz, Arg-H_{δ}), 2.14-2.05 (m, 1H, Val-H_{β}), 1.80-1.66 (m, 2H, Arg-H_{β}), 1.60-1.45 (m, 2H, Arg-H_{γ}), 0.89 (d, 3H, *J* = 6.0 Hz, -CHC$\underline{H}_3$$CH_3$), 0.87 (d, 3H, *J* = 6.0 Hz, -CHCH_3C$\underline{H}_3$); ^{13}C NMR (D_2O, 150 MHz): δ/ppm 171.1, 169.5, 156.7, 58.2, 52.0, 40.3, 29.9, 27.6, 24.2, 17.5, 16.6; HRMS (ES) Calcd for $C_{11}H_{26}N_7O_2[M+H]^+$ 288.2148, found 288.2144.



Scheme S5. Synthesis of Ac-Arg-NHNHMe (12)

Fmoc-L-Arg(Pbf)-NHNMeBoc (10)

NH_2NMeBoc (443 mg, 3.0 mmol, 1.0 equiv.) was dissolved in dry CH_2Cl_2 (3 mL) and this solution was cooled to 0 °C. A mixture of Fmoc-L-Arg(Pbf)-OH (1.97 g, 3.0 mmol), PyBOP (1.58 g, 3.0 mmol, 1.0 equiv.) and NMM (0.333 mL, 3.0 mmol, 1.0 equiv.) in dry CH_2Cl_2 (7 mL) was added dropwise to the cooled solution. The resulting mixture was stirred at rt for 20 h. The solution was diluted with CH_2Cl_2 (30 mL) and was then washed with 5% $NaHCO_3$ (1 x 10 mL), water (1 x 10 mL), and brine (1 x 10 mL), dried (Na_2SO_4), and evaporated to dryness. The product was purified by flash chromatography (silica gel, 4:1 EtOAc : hexanes), yielding 1.28 g (54%) of white gum. ^{1}H NMR (CD_3OD, 600 MHz): δ/ppm 7.73 (d, 2H, *J* = 6.0 Hz, Fmoc-H), 7.60 (t, 2H, *J* = 6.0 Hz, Fmoc-H), 7.33 (t, 2H, *J* = 6.0 Hz, Fmoc-H), 7.24 (t, 2H, *J* = 6.0 Hz, Fmoc-H), 4.37 (t, 1H, *J* = 12.0 Hz, Fmoc CHC$\underline{H}$H), 4.31 (t, 1H, *J* = 12.0 Hz, Fmoc CHCH$\underline{H}$), 4.14 (t, 1H, *J* = 6.0 Hz, Fmoc C$\underline{H}$$CH_2$), 4.09 (m, 1H, Arg-$H_{\alpha}$), 3.26-3.07 (m, 2H, Arg-H_{δ}), 3.02 (s, 3H, N-CH_3), 2.90 (s, 2H, Ar-CH_2), 2.56 (s, 3H, Ar-CH_3), 2.49 (s, 3H, Ar-CH_3), 2.02 (s, 3H, Ar-CH_3), 1.82-1.49 (m, 4H, Arg-H_{β}, Arg-H_{γ}), 1.37 (s,

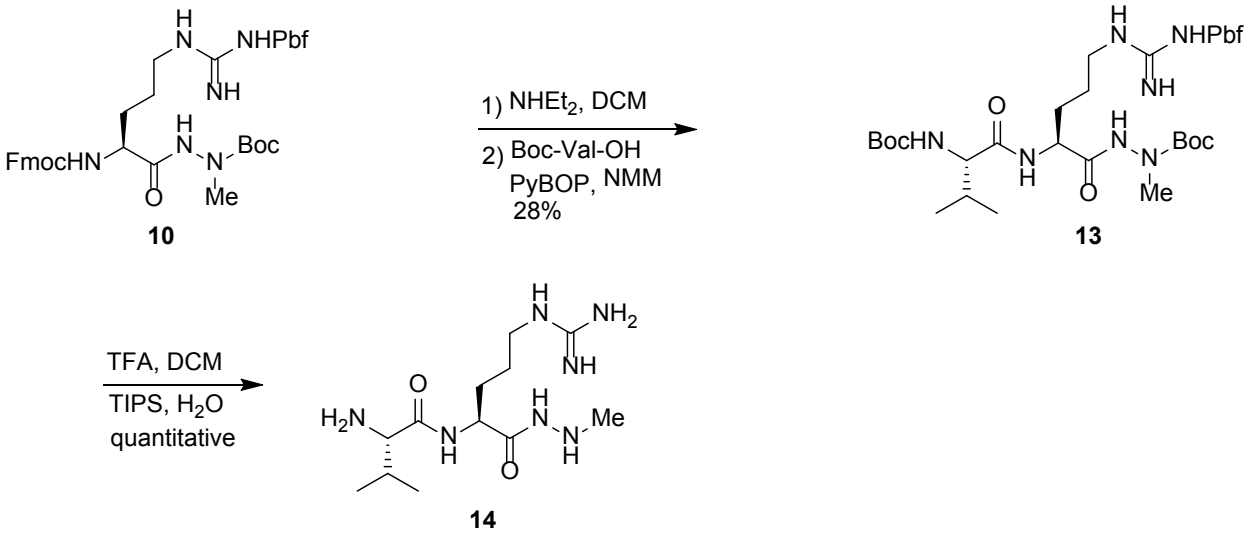
15H, -OC(C<u>H</u>$_3$)$_2$CH$_2$, -C(CH$_3$)$_3$); ^{13}C NMR (CD$_3$OD, 150 MHz): δ/ppm 171.7, 158.4, 156.9, 156.7, 155.5, 143.8, 143.6, 141.1, 138.0, 133.0, 132.1, 127.4, 126.8, 124.8, 124.6, 119.5, 117.0, 86.2, 81.0, 66.5, 53.0, 47.0, 42.5, 40.0, 36.0, 28.8, 27.3, 27.1, 25.5, 18.3, 17.1, 11.2; HRMS (ES) Calcd for $C_{40}H_{53}N_6O_8S[M+H]^+$ 777.3646, found 777.3646.

Ac-L-Arg(Pbf)-NHNMeBoc (11)

A solution of Fmoc-L-Arg(Pbf)-NHNMeBoc (**10**) (0.64 g, 0.82 mmol) in dry CH_2Cl_2 (4 mL) and diethylamine (4 mL) was stirred at rt for 3 h. TLC indicated Fmoc was completely removed. This mixture was evaporated to dryness, re-dissolved in dry CH_2Cl_2 (8 mL), and cooled to 0 °C. Triethylamine (0.091 mL, 0.82 mmol, 1.0 equiv.) and acetyl chloride (AcCl) (0.059 mL, 0.82 mmol, 1.0 equiv.) were added sequentially and the resulting mixture was stirred at rt for 15 h. After evaporation to dryness, the product was purified by flash chromatography (silica gel, 10:1 CH_2Cl_2 : MeOH), yielding 310 mg (63%) of white gum. ^{1}H NMR (CD$_3$OD, 600 MHz): δ/ppm 4.32 (t, 1H, Arg-H$_\alpha$), 3.27-3.10 (m, 2H, Arg-H$_\delta$), 3.02 (s, 3H, N-CH$_3$), 2.98 (s, 2H, Ar-CH$_2$), 2.56 (s, 3H, Ar-CH$_3$), 2.50 (s, 3H, Ar-CH$_3$), 2.06 (s, 3H, Ar-CH$_3$), 1.96 (s, 3H, CH$_3$C(O)), 1.84-1.75 (m, 1H, Arg-H$_{\beta1}$), 1.67-1.51 (m, 3H, Arg-H$_{\beta2}$, Arg-H$_\gamma$), 1.43 (s, 9H, -C(CH$_3$)$_3$), 1.39 (s, 6H, -OC(C<u>H</u>$_3$)$_2$CH$_2$); ^{13}C NMR (CD$_3$OD, 150 MHz): δ/ppm 171.8, 171.4, 158.4, 156.7, 155.4, 138.0, 132.9, 132.1, 124.6, 117.0, 86.2, 81.0, 51.2, 42.6, 39.8, 35.6, 28.7, 27.3, 27.1, 25.5, 21.0, 18.2, 17.0, 11.1; HRMS (ES) Calcd for $C_{27}H_{45}N_6O_7S[M+H]^+$ 597.3070, found 597.3064.

Ac-L-Arg-NHNHMe (12)

A solution of Ac-L-Arg(Pbf)-NHNMeBoc (**11**) (101 mg, 0.17 mmol) in trifluoroacetic acid (TFA) (5 mL), CH_2Cl_2 (4.5 mL), triisopropylsilane (0.25 mL), and water (0.25 mL) was stirred at rt for 4 h and then evaporated to dryness. The residue was dissolved in water (15 mL) and washed with EtOAc (3 x 7 mL). The aqueous layer was lyophilized to yield 24 mg product (30%) as a TFA salt. ^{1}H NMR (D$_2$O, 600 MHz): δ/ppm 4.18 (t, 1H, *J* = 6.0 Hz, Arg-H$_\alpha$), 3.07 (t, 2H, *J* = 6.0 Hz, Arg-H$_\delta$), 2.84 (s, 3H, N-CH$_3$), 1.90 (s, 3H, CH$_3$C(O)), 1.80-1.70 (m, 1H, Arg-H$_{\beta1}$), 1.69-1.61 (m, 1H, Arg-H$_{\beta2}$), 1.59-1.45 (m, 2H, Arg-H$_\gamma$); ^{13}C NMR (D$_2$O, 150 MHz): δ/ppm 174.6, 171.4, 156.7, 52.1, 40.3, 36.2, 27.6, 24.1, 21.5; HRMS (ES) Calcd for $C_9H_{21}N_6O_2[M+H]^+$ 245.1726, found 245.1725.



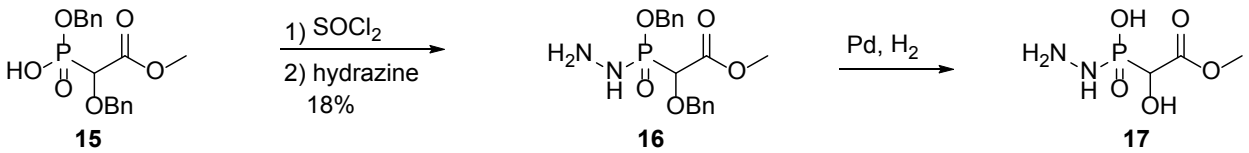
Scheme S6. Synthesis of Val-Arg-NHNHMe (14)

Boc-L-Val-L-Arg(Pbf)-NHNMeBoc (13)

A solution of Fmoc-L-Arg(Pbf)-NHNMeBoc (**10**) (94 mg, 0.12 mmol) in dry CH_2Cl_2 (1.5 mL) and diethylamine (1.5 mL) was stirred at rt for 3 h. TLC indicated Fmoc was completely removed. This mixture was evaporated to dryness, re-dissolved in dry CH_2Cl_2 (1.0 mL), and cooled to 0 °C. A mixture of Boc-L-Val-OH (39 mg, 0.18 mmol, 1.5 equiv.), PyBOP (93 mg, 0.18 mmol, 1.5 equiv.) and NMM (0.020 mL, 0.18 mmol, 1.5 equiv.) in dry CH_2Cl_2 (2.0 mL) was added dropwise to the cooled solution. The resulting mixture was stirred at rt for 19 h. The solution was diluted with CH_2Cl_2 (30 mL) and was then washed with 5% $NaHCO_3$ (1 x 10 mL), 10% citric acid (1 x 10 mL), water (1 x 10 mL), and brine (1 x 10 mL), dried (Na_2SO_4), and evaporated to dryness. The product was purified by flash chromatography (silica gel, 8:1 EtOAc : hexanes), yielding 26 mg (28%) of white gum. ^{1}H NMR (CD_3OD, 600 MHz): δ/ppm 4.39 (t, 1H, *J* = 6.0 Hz, Arg-H_α), 3.85 (t, 1H, *J* = 6.0 Hz, Val-H_α), 3.27-3.08 (m, 2H, Arg-H_δ), 3.02 (s, 3H, N-CH_3), 2.98 (s, 2H, Ar-CH_2), 2.56 (s, 3H, Ar-CH_3), 2.50 (s, 3H, Ar-CH_3), 2.06 (s, 3H, Ar-CH_3), 2.04-1.95 (m, 1H, Val-H_β), 1.90-1.77 (m, 1H, Arg-$H_{\beta1}$), 1.72-1.52 (m, 3H, Arg-$H_{\beta2}$, Arg-H_γ), 1.43 (s, 9H, -C(CH_3)$_3$), 1.41 (s, 9H, -C(CH_3)$_3$), 1.39 (s, 6H, -OC($\underline{CH_3}$)$_2$$CH_2$), 0.93 (d, 3H, *J* = 6.0 Hz, -CH$\underline{CH_3}$$CH_3$), 0.90 (d, 3H, *J* = 6.0 Hz, -$CHCH_3$$\underline{CH_3}$); ^{13}C NMR ($CD_3OD$, 150 MHz): δ/ppm 173.0, 170.9, 158.4, 156.7, 155.4, 138.0, 133.0, 132.1, 124.4, 117.0, 86.2, 81.0, 79.3, 60.3, 50.9, 42.6, 39.7, 35.6, 30.4, 29.0, 27.3, 27.1, 25.4, 18.4, 18.2, 17.2, 17.0, 11.1; HRMS (ES) Calcd for $C_{35}H_{60}N_7O_9S[M+H]^+$ 754.4173, found 754.4180.

L-Val-L-Arg-NHNHMe (14)

A solution of Boc-L-Val-L-Arg(Pbf)-NHNMeBoc (**13**) (12 mg, 0.016 mmol) in TFA (2.5 mL), CH_2Cl_2 (2.25 mL), triisopropylsilane (0.125 mL) and water (0.125 mL) was stirred at rt for 4 hours and then evaporated to dryness. The residue was dissolved in water (15 mL) and washed with EtOAc (3 x 10 mL). The aqueous layer was lyophilized to yield 10 mg product (quantitative) as a TFA salt. ^{1}H NMR (D_2O, 600 MHz): δ/ppm 4.23 (t, 1H, *J* = 6.0 Hz, Arg-H_α), 3.74 (d, 1H, *J* = 6.0 Hz, Val-H_α), 3.10 (t, 2H, *J* = 6.0 Hz, Arg-H_δ), 2.75 (s, 3H, N-CH_3), 2.15-2.08 (m, 1H, Val-H_β), 1.80-1.66 (m, 2H, Arg-H_β), 1.62-1.46 (m, 2H, Arg-H_γ), 0.91 (d, 3H, *J* = 6.0 Hz, -CH$\underline{CH_3}$$CH_3$), 0.89 (d, 3H, *J* = 6.0 Hz, -$CHCH_3$$\underline{CH_3}$); ^{13}C NMR ($D_2O$, 150 MHz): δ/ppm 170.6, 169.4, 156.8, 58.2, 52.1, 40.3, 36.5, 29.9, 27.6, 24.2, 17.5, 16.6; HRMS (ES) Calcd for $C_{12}H_{28}N_7O_2[M+H]^+$ 302.2304, found 302.2299.



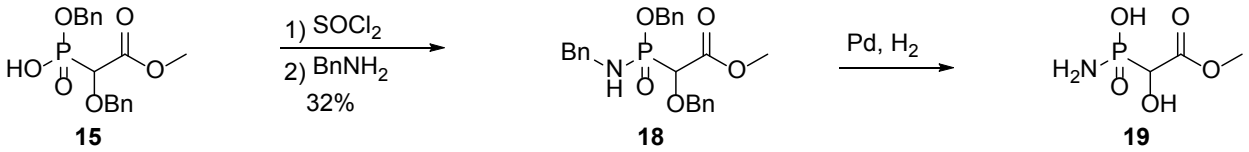
Scheme S7. Synthesis of compound 17

Methyl 2-(benzyloxy)-2-((benzyloxy)(hydrazinyl)phosphoryl)acetate (16)

To a solution of compound **15**[6] (413 mg, 1.18 mmol) in dry CH_2Cl_2 (3.5 mL), was added thionyl chloride (0.103 mL, 1.41 mmol, 1.2 equiv.). The mixture was refluxed for 8 h and cooled into an ice bath, followed by the addition of anhydrous hydrazine (0.074 mL, 2.36 mmol, 2.0 equiv.). After stirring for 30 min, the mixture was concentrated under reduced presssure. EtOAc (30 mL) was added to the residue, and the solution was washed with 0.1 N HCl (1 x 8 mL), water (1 x 8 mL), and brine (1 x 8 mL), dried (Na_2SO_4), and evaporated to dryness. The product (diastereomers) was purified by flash chromatography (silica gel, 20:1 CH_2Cl_2 : MeOH), yielding 78 mg (18%) of white gum. ^{1}H NMR ($CDCl_3$, 600 MHz) : δ/ppm 7.40-7.28 (m, 10H, Ar-H), 5.16-5.07 (m, 2H, P(O)OCH_2Ar), 4.83-4.76 (m, 1H, CHOC$\underline{H}$HAr), 4.62-4.46 (m, 3H, CHOCH$\underline{H}$Ar, C$\underline{H}$OCH_2Ar, P(O)NH), 3.81 (s, 1.35H, C(O)OCH_3), 3.74 (s, 1.62H, C(O)OCH_3), 3.41 (bs, 2H, P(O)NHN$\underline{H}_2$); ^{13}C NMR ($CDCl_3$, 150 MHz): δ/ppm 168.50 (d, *J* = 1.5 Hz), 168.46 (d, *J* = 1.5 Hz), 136.1, 136.0, 135.96 (d, *J* = 1.5 Hz), 135.92 (d, *J* = 1.5 Hz), 128.60, 128.58, 128.52, 128.50, 128.49, 128.44, 128.42, 127.94, 127.86, 76.1 (d, *J* = 58.5 Hz), 75.2 (d, *J* = 57.0 Hz), 74.4 (d, *J* = 12.0 Hz), 74.2 (d, *J* = 12.0 Hz), 67.06 (d, *J* = 7.5 Hz), 66.93 (d, *J* = 6.0 Hz), 52.8, 52.6; ^{31}P NMR ($CDCl_3$, 202 MHz): δ/ppm 21.57, 21.33; HRMS (ES) Calcd for $C_{17}H_{22}N_2O_5P[M+H]^+$ 365.1266, found 365.1275.

Hydrazinyl(1-hydroxy-2-methoxy-2-oxoethyl)phosphinic acid (17)

To a solution of compound **16** (10.3 mg, 0.023 mmol) in THF (2 mL) and phosphate buffer (pH 7.0; 2 mL) was added 8 mg palladium (black). The mixture was stirred at rt under H_2 (1 atm) for 21 h, filtered, and concentrated under reduced pressure to yield the product. ^{1}H NMR (D_2O, 600 MHz) : δ/ppm 4.42 (d, 1H, *J* = 18.0 Hz, C$\underline{H}$C(O)OCH_3), 3.74 (s, 3H, CHC(O)OC$\underline{H}_3$); ^{31}P NMR (D_2O, 202 MHz): δ/ppm 16.12; HRMS (ES) Calcd for $C_3H_9NO_5P[M+H]^+$ 170.0213, found 170.0215.



Scheme S8. Synthesis of compound 19

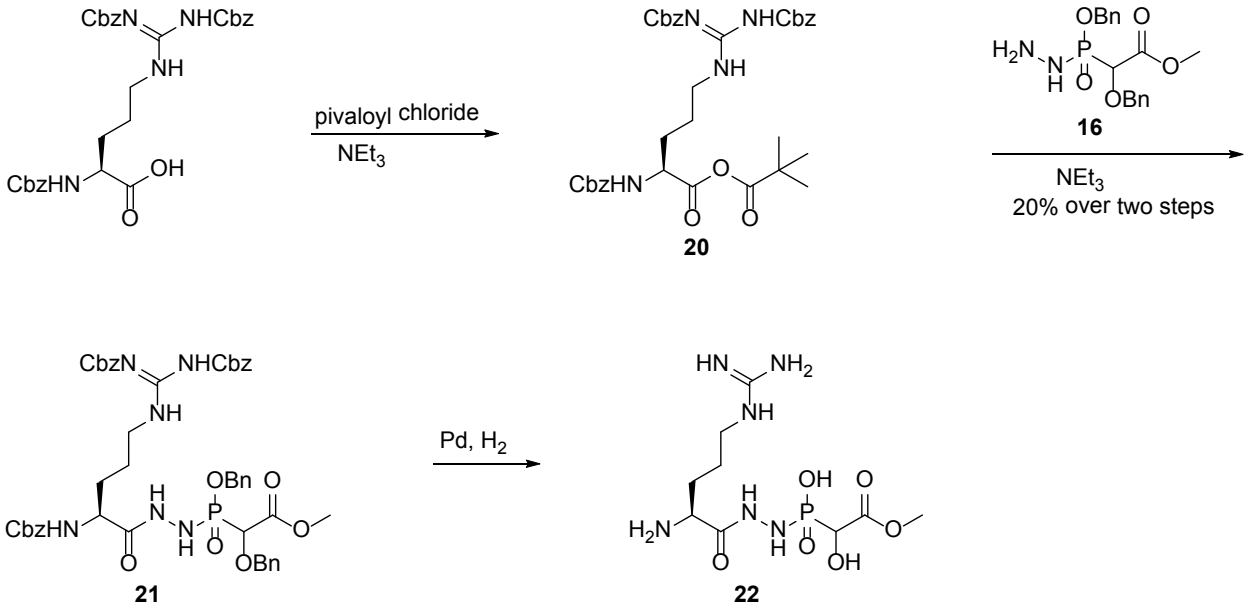
Methyl 2-((benzylamino)(benzyloxy)phosphoryl)-2-(benzyloxy)acetate (18)

To a solution of compound **15** (230 mg, 0.66 mmol) in dry CH_2Cl_2 (1.8 mL), was added thionyl chloride (0.057 mL, 0.79 mmol, 1.2 equiv.). The mixture was refluxed for 8 h and cooled into an ice bath, followed by the addition of benzylamine (0.143 mL, 1.32 mmol, 2.0 equiv.). After stirring for 30 min, the mixture was concentrated under reduced pressure. EtOAc (30 mL) was added to the residue, and the solution was washed with 0.1 N HCl (1 x 8 mL), water (1 x 8 mL), and brine (1 x 8 mL), dried (Na_2SO_4), and evaporated to dryness. The product (mixture of diastereomers) was purified by flash chromatography

(silica gel, 3:2 EtOAc : hexanes), yielding 94 mg (32%) of white gum. ^{1}H NMR ($CDCl_3$, 600 MHz) : δ/ppm 7.35-7.20 (m, 15H, Ar-H), 5.14-4.97 (m, 2H, P(O)OCH_2Ar), 4.78-4.74 (m, 1H, CHOC<u>H</u>HAr), 4.53-4.36 (m, 2H, CHOCH<u>H</u>Ar, C<u>H</u>OCH_2Ar), 4.24-4.09 (m, 2H, P(O)NHC<u>H</u>$_2$Ar), 3.78 (s, 1.64H, C(O)OCH_3), 3.70 (s, 1.40H, C(O)OCH_3), 3.30-3.16 (m, 1H, P(O)N<u>H</u>CH_2Ar); ^{13}C NMR ($CDCl_3$, 150 MHz): δ/ppm 168.53 (d, *J* = 3.0 Hz), 168.47, 139.66 (d, *J* = 3.0 Hz), 139.62 (d, *J* = 3.0 Hz), 136.22 (d, *J* = 3.0 Hz), 136.17 (d, *J* = 4.5 Hz), 128.56, 128.52, 128.51, 128.50, 128.46, 128.45, 128.41, 128.32, 128.29, 128.27, 127.73, 127.60, 127.43, 127.40, 127.35, 127.31, 76.9 (d, *J* = 58.5 Hz), 76.0 (d, *J* = 57.0 Hz), 74.3 (d, *J* = 13.5 Hz), 74.1 (d, *J* = 12.0 Hz), 66.76 (d, *J* = 6.0 Hz), 66.61 (d, *J* = 6.0 Hz), 52.7, 52.5, 45.1, 44.9; ^{31}P NMR ($CDCl_3$, 202 MHz): δ/ppm 20.31; HRMS (ES) Calcd for $C_{24}H_{27}NO_5P[M+H]^+$ 440.1627, found 440.1628.

P-(1-Hydroxy-2-methoxy-2-oxoethyl)phosphonamidic acid (19)

To a solution of compound **18** (16.5 mg, 0.045 mmol) in THF (3.5 mL) and phosphate buffer (pH 7.0; 3.5 mL) was added 10 mg palladium (black). The mixture was stirred at rt under H_2 (1 atm) for 9 h, filtered, and concentrated to yield the product. ^{1}H NMR (D_2O, 600 MHz) : δ/ppm 4.52 (d, 1H, *J* = 18.0 Hz, C<u>H</u>C(O)OCH_3), 3.68 (s, 3H, CHC(O)OC<u>H</u>$_3$); ^{31}P NMR (D_2O, 202 MHz): δ/ppm 16.19; HRMS (ES) Calcd for $C_3H_{10}N_2O_5P[M+H]^+$ 185.0322, found 185.0325.



Scheme S9. Synthesis of desmethyl fosfazinomycin B (22)

Protected desmethyl fosfazinomycin B (21)

To a solution of Z-Arg(Z^2)-OH (275 mg, 0.48 mmol) in dry CH_2Cl_2 (2.5 mL), was added triethylamine (0.064 mL, 0.48 mmol, 1.0 equiv.). The solution was cooled in an ice-brine bath, followed by the addition of pivaloyl chloride (0.057 mL, 0.48 mmol, 1.0 equiv.). After stirring for 1.5 h at the same temperature, the mixture was concentrated under reduced pressure, and EtOAc (20 mL) was added to the residue. The solution was then washed with water (1 x 8 mL) and brine (1 x 8 mL), dried (Na_2SO_4), and evaporated to dryness to yield compound **20** that was used without further purification.

To a solution of compound **16** (29 mg, 0.08 mmol) in dry THF (0.6 mL), was added triethylamine (0.011 mL, 0.08 mmol, 1.0 equiv.). The solution was cooled in a dry ice-acetone bath (-78 °C), followed by the addition of a solution of compound **20** (53 mg, 0.08 mmol, 1.0 equiv.) in dry THF (0.5 mL). After stirring for 2 h at the same temperature, the mixture was evaporated to dryness, and the product (mixture of diastereomers) was purified by flash chromatography (silica gel, 2:1 EtOAc : hexanes), yielding 15 mg (20% over two steps) of white solid. ^{1}H NMR ($CDCl_3$, 600 MHz) : δ/ppm 9.52-9.12 (m, 2H, NHC(N)NH), 8.46-8.12 (m, 1H, C(O)NH), 7.42-7.10 (m, 25H, Ar-H), 5.88-5.60 (m, 1H, NHCHC(O)), 5.24-4.96 (m, 9H, P(O)OCH_2Ar, 3 X NHC(O)OCH_2Ar, NHP(O)), 4.78-4.70 (m, 1H, CHOCHHAr), 4.66-4.52 (m, 2H, CHOCHHAr, CHOCH_2Ar), 4.32-4.20 (m, 1H, Arg-H_α), 4.15-3.75 (m, 2H, Arg-H_δ), 3.73-3.61 (m, 3H, C(O)OCH_3), 1.80-1.50 (m, 4H, Arg-H_β, Arg-H_γ); ^{31}P NMR ($CDCl_3$, 202 MHz): δ/ppm 18.84, 18.53, 18.20, 18.12; HRMS (ES) Calcd for $C_{47}H_{52}N_6O_{12}P[M+H]^+$ 923.3381, found 923.3400.

Desmethyl fosfazinomycin B (22)

To a solution of compound **21** (7.4 mg, 0.008 mmol) in THF (1.4 mL) and phosphate buffer (pH 7.0; 1.4 mL) was added 1.3 mg palladium (black). The mixture was stirred at rt under H_2 (1 atm) for 5 h, filtered, and concentrated to yield the product (misture of diastereomers). ^{1}H NMR (D_2O, 600 MHz) : δ/ppm 4.44-4.37 (m, 1H, CHC(O)OCH_3), 3.67 (s, 3H, CHC(O)OCH_3), 3.63 (t, 1H, *J* = 6.0 Hz, Arg-H_α), 3.11 (t, 2H, *J* = 6.0 Hz, Arg-H_δ), 1.77-1.65 (m, 2H, Arg-H_β), 1.58-1.51 (m, 2H, Arg-H_γ); ^{31}P NMR (D_2O, 202 MHz): δ/ppm 13.02, 12.90; HRMS (ES) Calcd for $C_9H_{22}N_6O_6P[M+H]^+$ 341.1333, found 341.1343.

Supplementary Figures

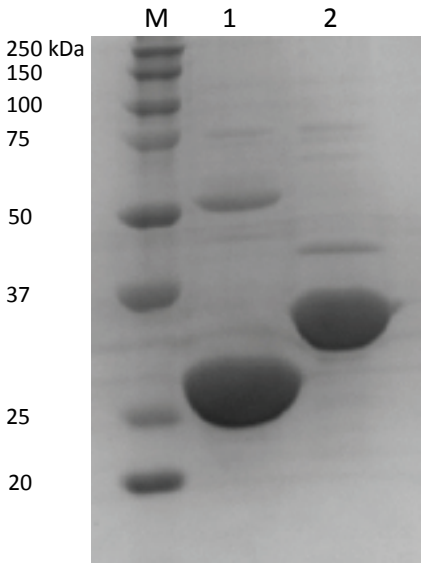


Figure S1. SDS-PAGE analysis of His_6-FzmB and His_6-FzmG after IMAC purification. Coomassie staining. M: Prestained precision Plus Protein Standards (Bio-Rad). Lane 1: His_6-FzmB. Lane 2: His_6-FzmG.

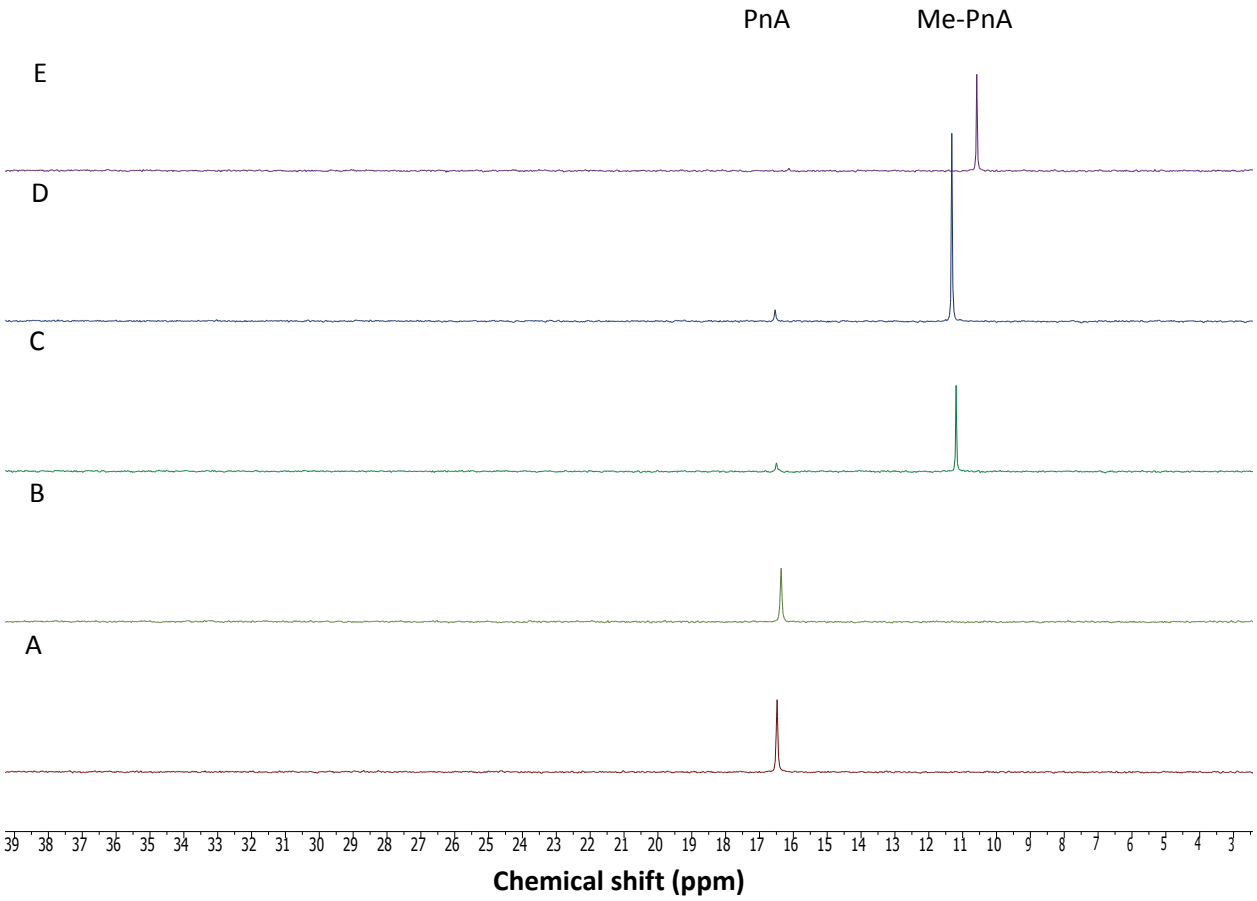


Figure S2. ^{31}P NMR analysis of the activity of His$_6$-FzmB on PnA. A. NMR spectrum of the reaction mixture in the absence of His$_6$-FzmB. **B.** NMR spectrum of the reaction mixture in the absence of SAM. **C.** NMR spectrum of the reaction mixture containing all the necessary components for catalysis (i.e. PnA, SAM, His$_6$-FzmB, AdoHcy nucleosidase). **D.** NMR spectrum of the enzymatic reaction mixture spiked with the authentic standard of Me-PnA. **E.** NMR spectrum of the authentic standard of Me-PnA.

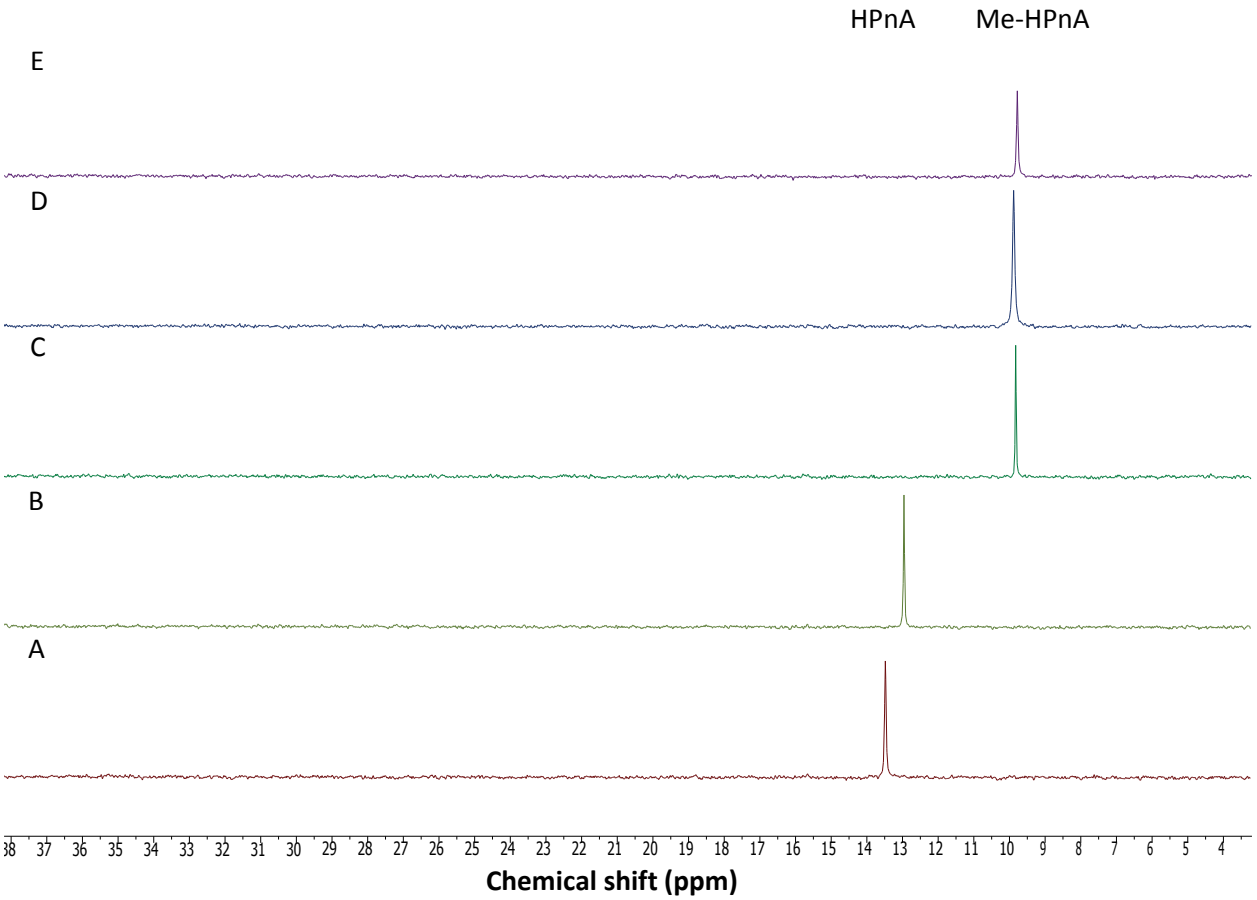


Figure S3. ^{31}P NMR analysis of the incubation of His$_6$-FzmB with HPnA. A. NMR spectrum of the reaction mixture in the absence of His$_6$-FzmB. **B.** NMR spectrum of the reaction mixture in the absence of SAM. **C.** NMR spectrum of the reaction mixture containing all the necessary components for catalysis (i.e. HPnA, SAM, His$_6$-FzmB, AdoHcy nucleosidase). **D.** NMR spectrum of the enzymatic assay mixture spiked with the authentic standard of Me-HPnA. **E.** NMR spectrum of the authentic standard of Me-HPnA.

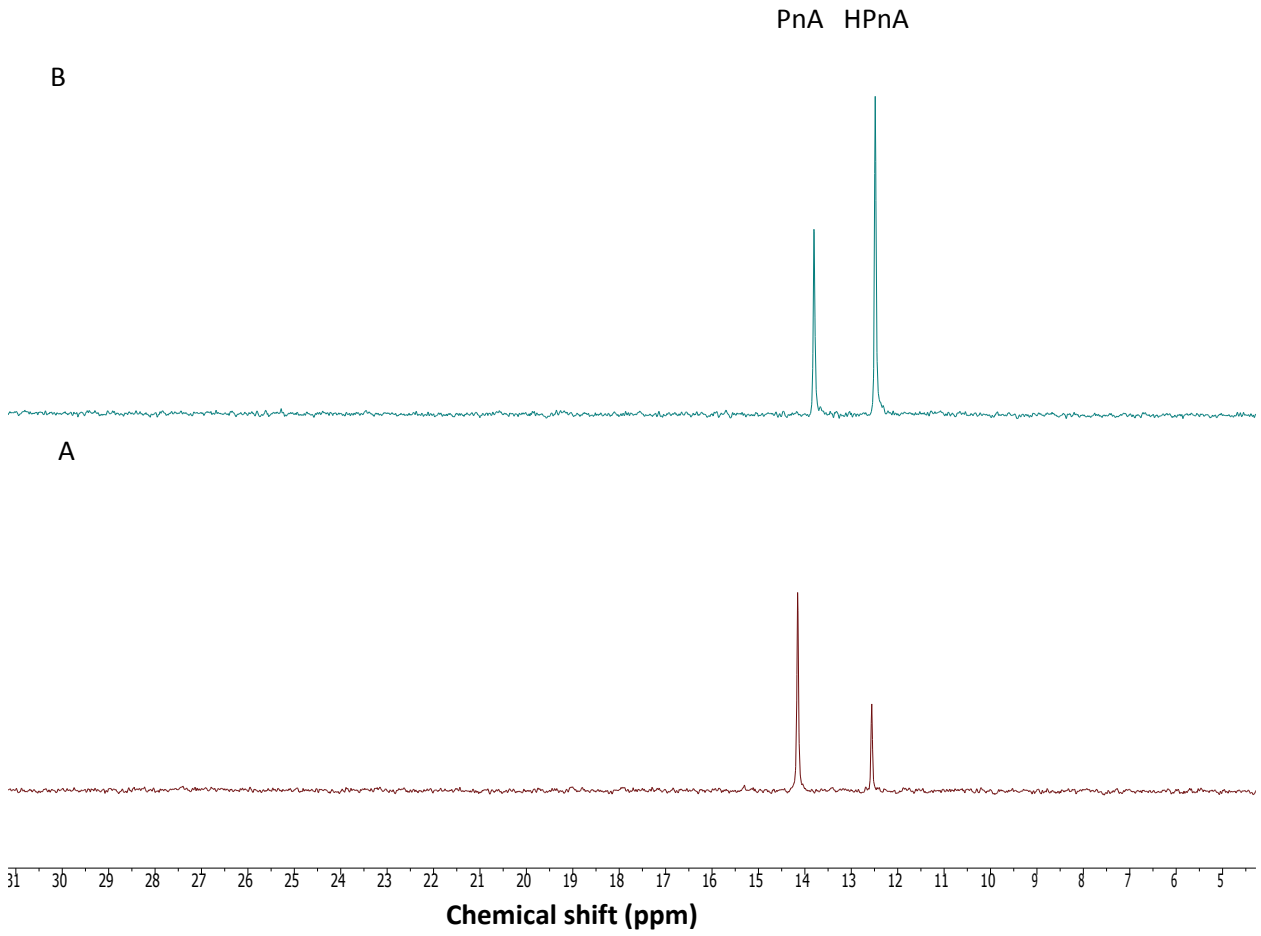


Figure S4. ^{31}P NMR analysis of the activity of His$_6$-FzmG on PnA. A. NMR spectrum of the reaction mixture containing all the necessary components for catalysis (i.e. PnA, O_2, α-KG, L-ascorbate, Fe(II), His$_6$-FzmG). **B.** NMR spectrum of the reaction mixture spiked with the authentic standard of HPnA.

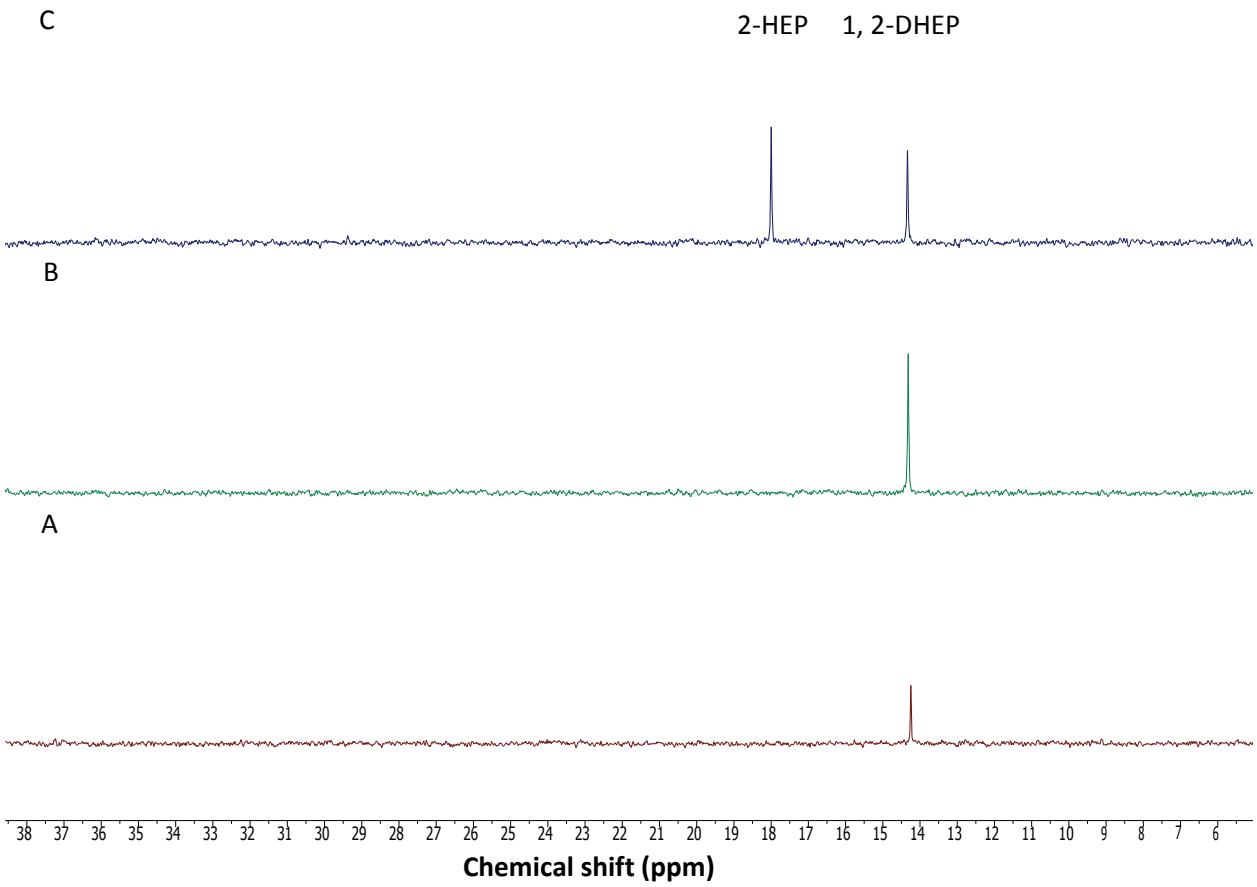


Figure S5. ^{31}P NMR analysis of the assay of His$_6$-FzmG incubated with 2-HEP. A. NMR spectrum of the reaction mixture containing all the necessary components for catalysis (i.e. 2-HEP, O_2, α-KG, L-ascorbate, Fe(II), His$_6$-FzmG). **B.** NMR spectrum of the reaction mixture spiked with the authentic standard of 1, 2-DHEP. **C.** NMR spectrum of the reaction mixture spiked with the authentic standard of 2-HEP.

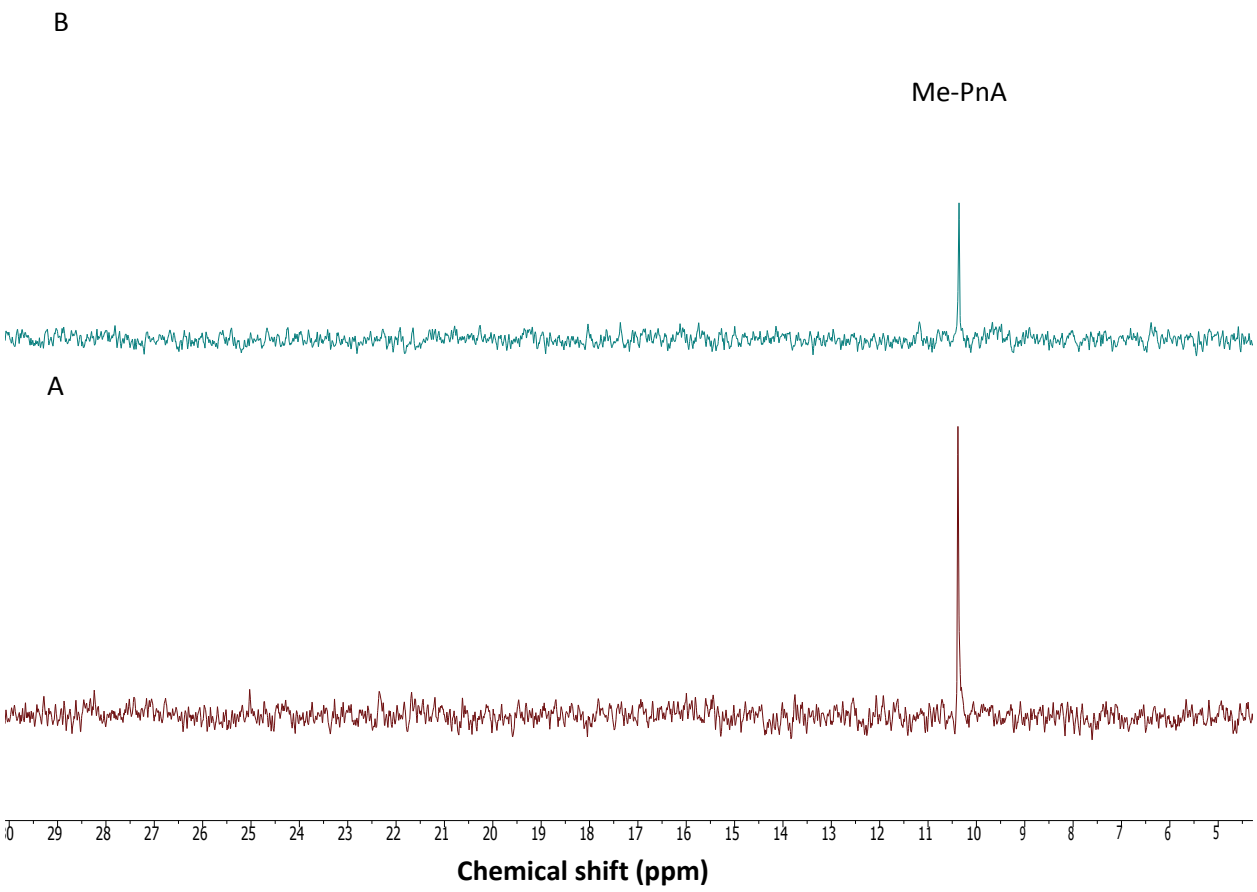
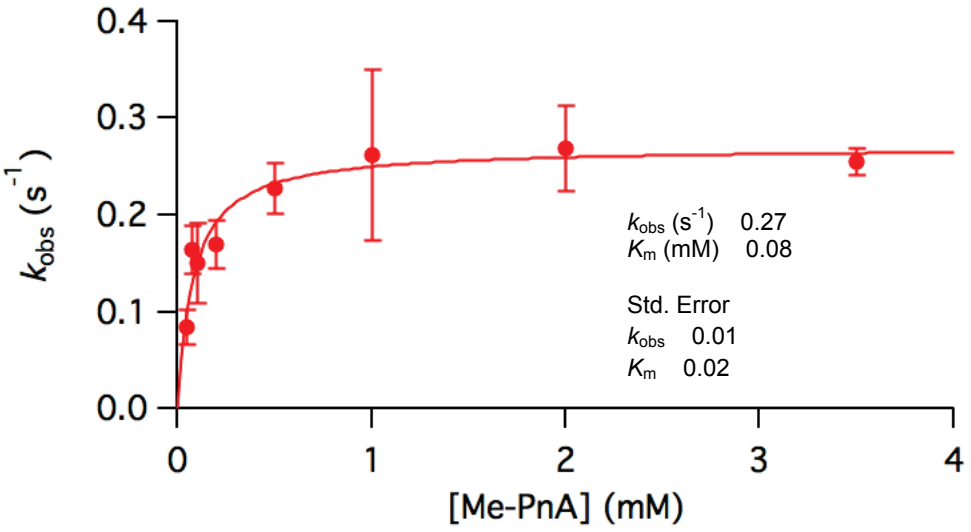
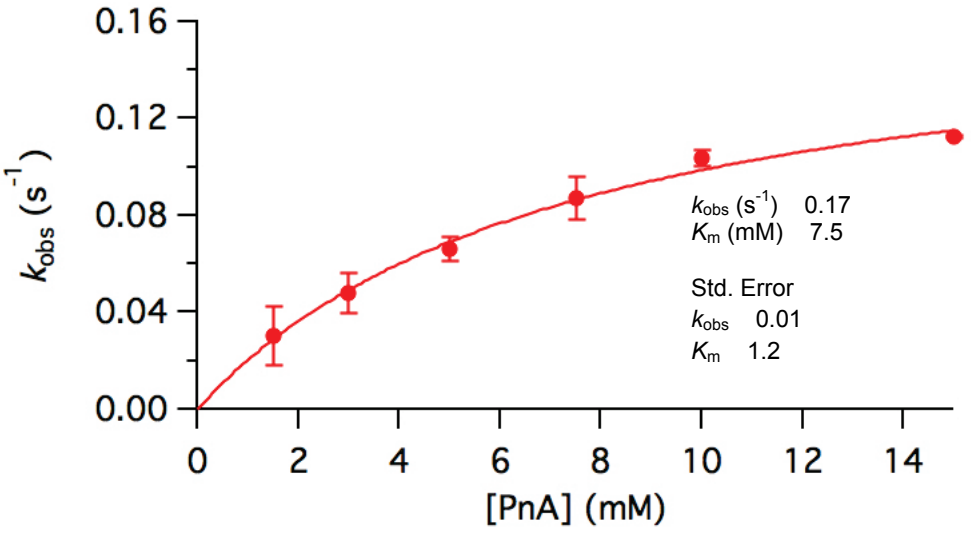


Figure S6. ^{31}P NMR analysis of the assay of His$_6$-DhpA with Me-PnA. A. NMR spectrum of the reaction mixture containing all the necessary components for catalysis (i.e. Me-PnA, O_2, α-KG, L-ascorbate, Fe(II), His$_6$-DhpA). **B.** NMR spectrum of the authentic standard of Me-PnA.

A


0.4
0.3
0.2
0.1
0.0
k_{obs} (s^{-1})
0
1
2
3
4
[Me-PnA] (mM)
k_{obs} (s^{-1}) 0.27
K_m (mM) 0.08
Std. Error
k_{obs} 0.01
K_m 0.02

B


0.16
0.12
0.08
0.04
0.00
k_{obs} (s^{-1})
0
2
4
6
8
10
12
14
[PnA] (mM)
k_{obs} (s^{-1}) 0.17
K_m (mM) 7.5
Std. Error
k_{obs} 0.01
K_m 1.2

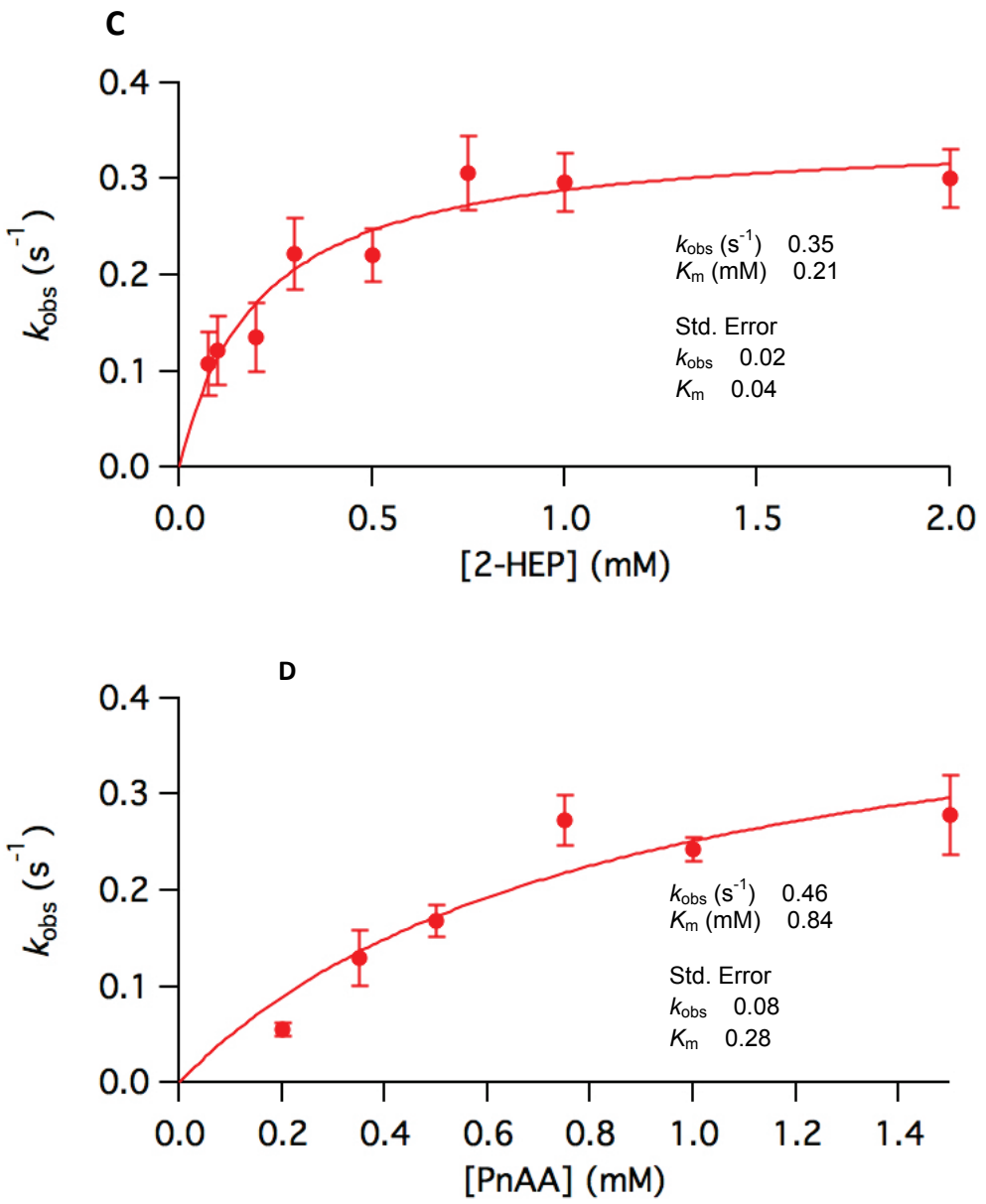


Figure S7. Dependence of the His_6-FzmG activity on the concentration of various substrates. A. Assay (1 mL final volume) was carried out at 20 ºC and contained: 0.05 – 3.5 mM Me-PnA, 1 mM α-KG, 0.2 mM ascorbate, and 3.7 µM His_6-FzmG reconstituted with 1 equivalent Fe(II), inf 50 mM NaPi, pH 7.7. **B**. Assay (1 mL final volume) was carried out at 20 ºC and contained: 1.5 – 15 mM PnA, 1 mM α-KG, 0.2 mM ascorbate, and 9.25 µM His_6-FzmG reconstituted with 1 equivalent Fe(II) in 50 mM NaPi, pH 7.7. **C**. Assay (1 mL final volume) was carried out at 20 ºC and contained: 0.075 – 2 mM 2-HEP, 1 mM α-KG, 0.2 mM ascorbate, and 3.7 µM His_6-FzmG reconstituted with 1 equivalent Fe(II), in 50 mM NaPi, pH 7.7. **D**. Assay (1 mL final volume) was carried out at 20 ºC and contained: 0.2 – 1.5 mM PnAA, 1 mM α-KG, 0.2 mM ascorbate, and 3.7 µM His_6-FzmG reconstituted with 1 equivalent Fe(II) in 50 mM NaPi, pH 7.7.

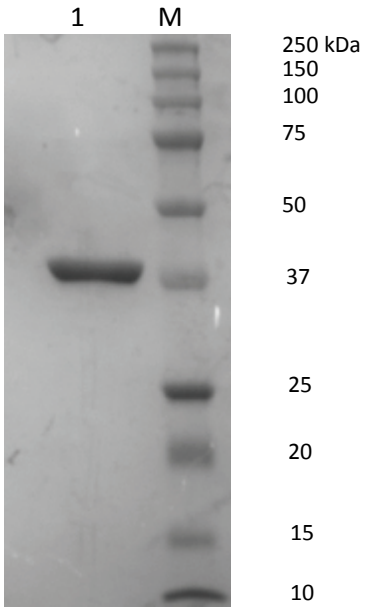


Figure S8. SDS-PAGE analysis of His_6-FzmH after IMAC purification. Coomassie staining. M: Prestained precision Plus Protein Standards (Bio-Rad). Lane 1: His_6-FzmH.

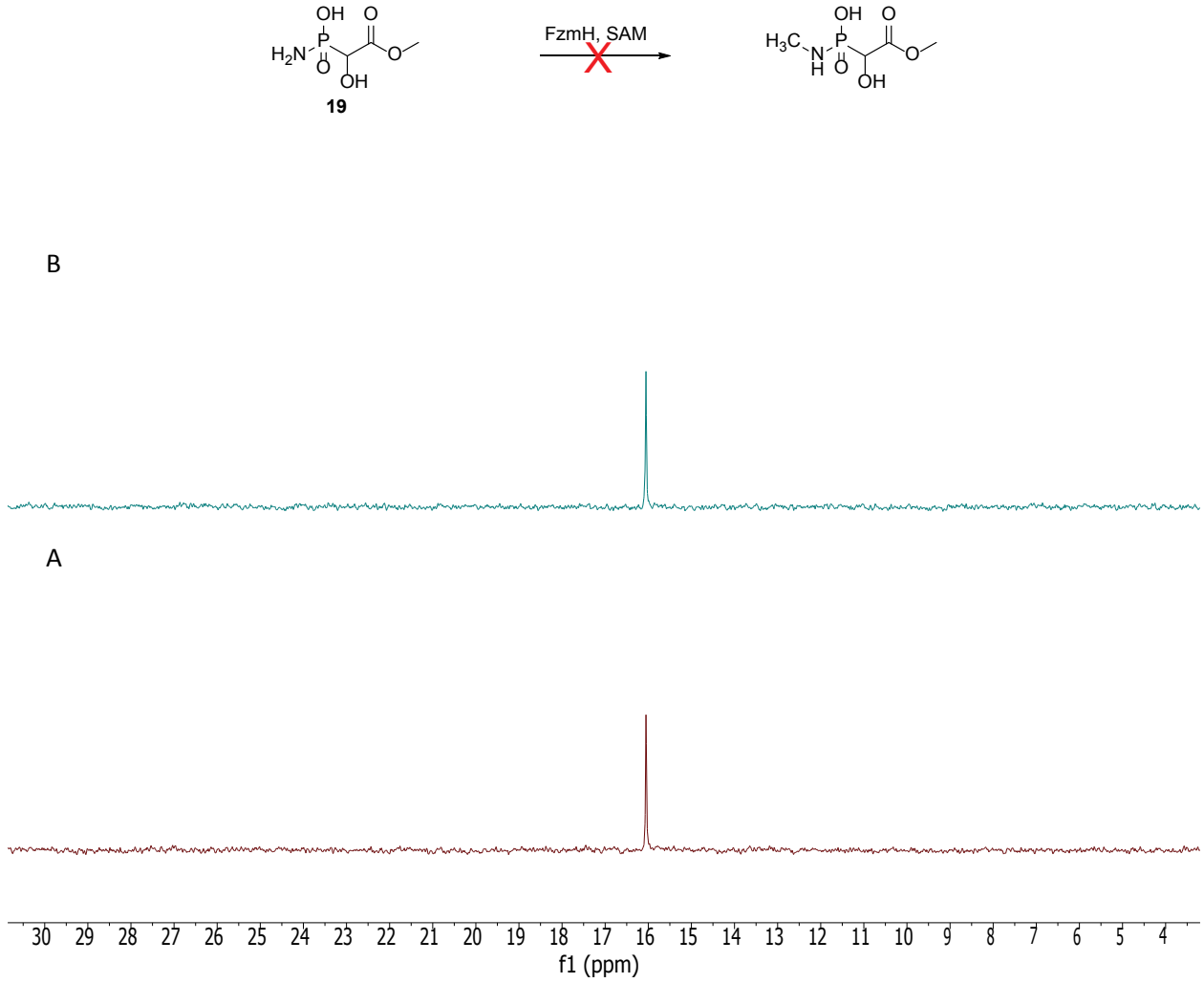


Figure S9. ^{31}P NMR analysis of the attempted His_6-FzmH reaction on compound 19. A. NMR spectrum of the reaction mixture in the absence of His_6-FzmH. **B.** NMR spectrum of the reaction mixture containing all the necessary components for catalysis (i.e. compound **19**, SAM, His_6-FzmH, AdoHcy nucleosidase).

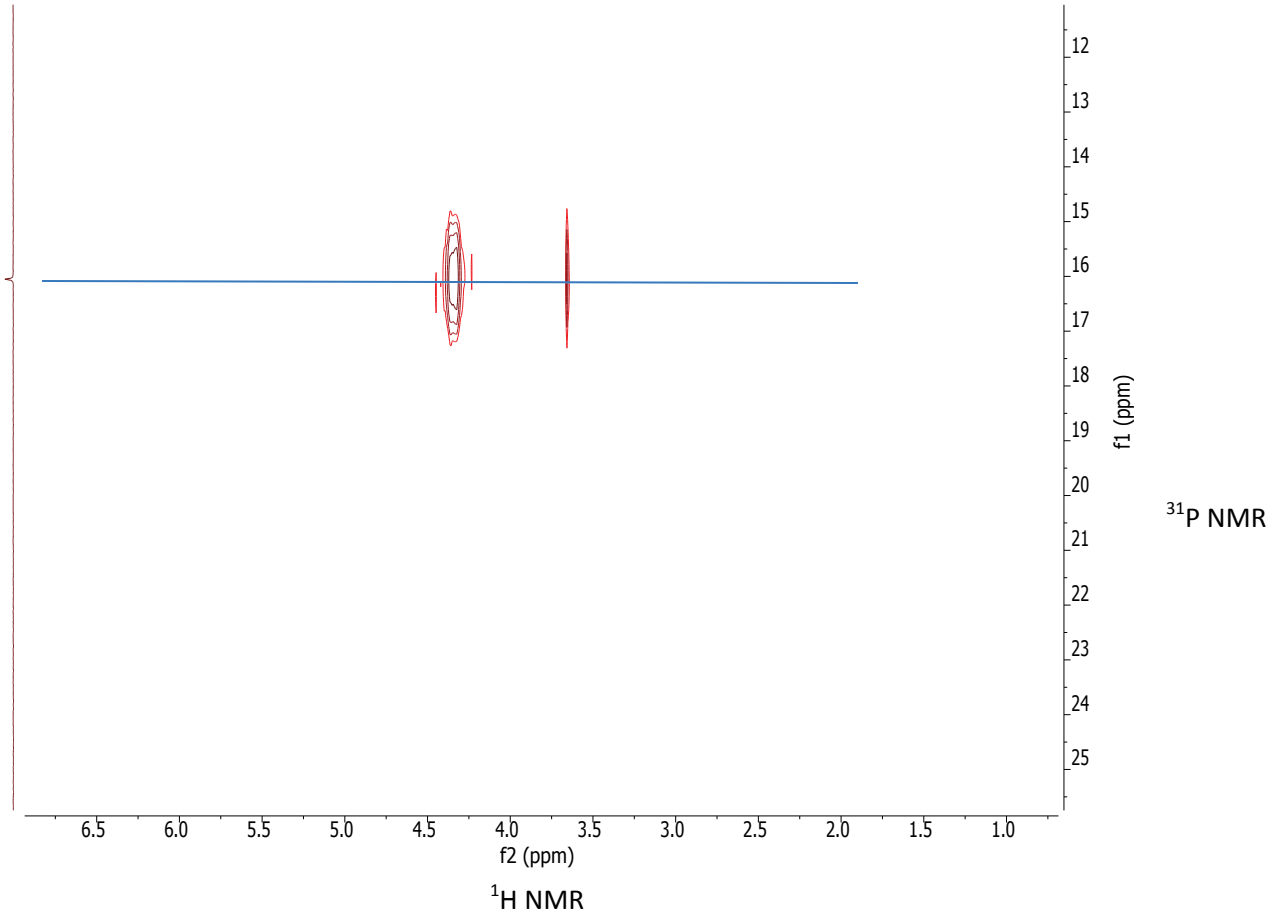


Figure S10. ^{1}H-^{31}P HMBC spectrum illustrating the incubation of His$_6$-FzmH reaction with compound 19 did not provide any new products. The assay contained all the necessary components for catalysis (i.e. compound 19, SAM, His$_6$-FzmH, AdoHcy nucleosidase).

Comparing spectrum B with spectrum A in Figure S9, no additional peak was seen, indicating no methylation occurred. This result was corroborated well with the ^{1}H-^{31}P HMBC study in Figure S10. If methylation had occurred, then the methylated product should have a signal around 2.8 ppm in the ^{1}H NMR dimension in Figure S10, which was not seen. Thus, based on these results, compound **19** is not a substrate of FzmH.

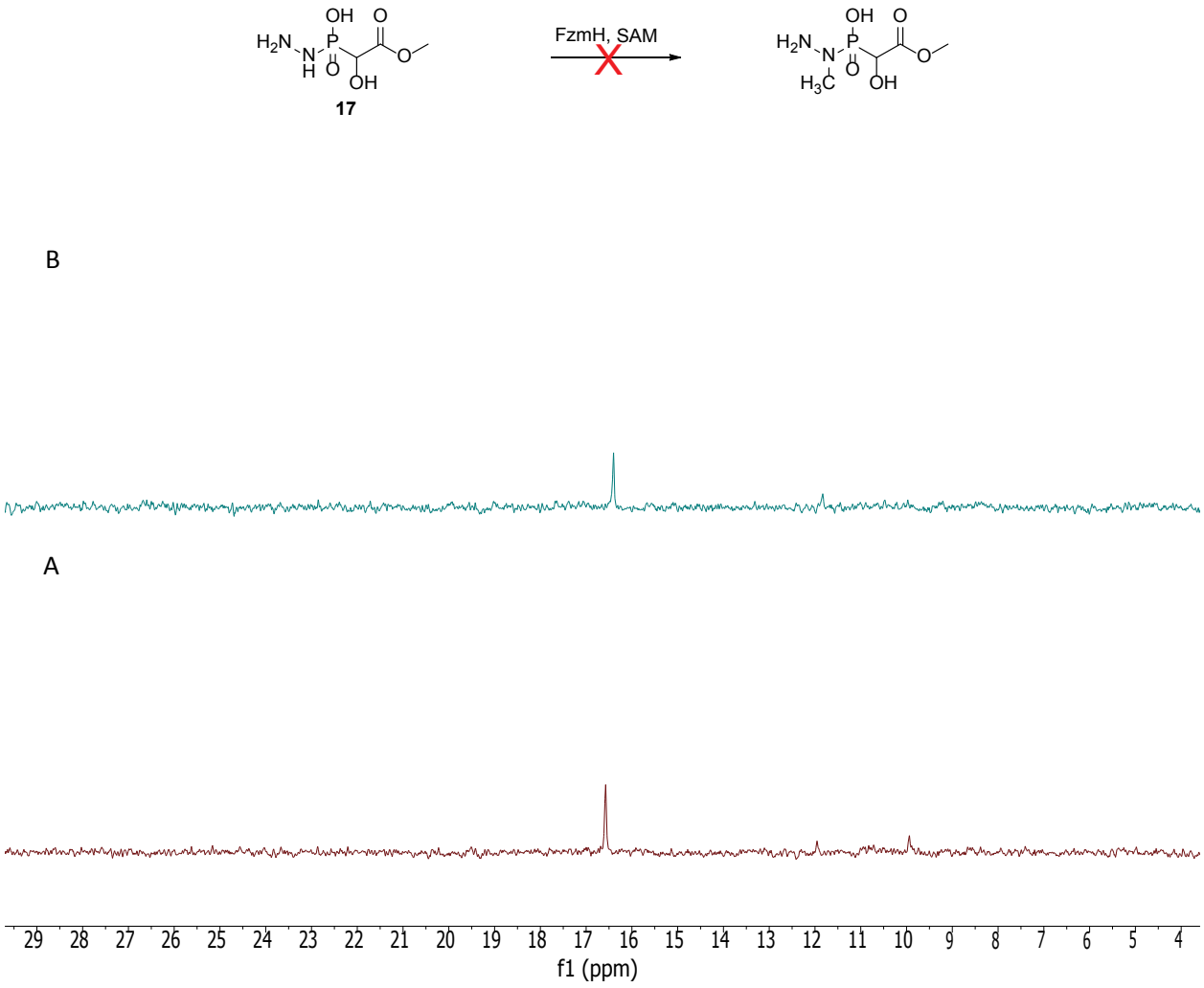


Figure S11. ^{31}P NMR analysis of the incubation of His$_6$-FzmH reaction with compound 17. A. NMR spectrum of the reaction mixture containing all the necessary components for catalysis (i.e. compound **17**, SAM, His$_6$-FzmH, AdoHcy nucleosidase). **B.** NMR spectrum of the authentic compound **17**.

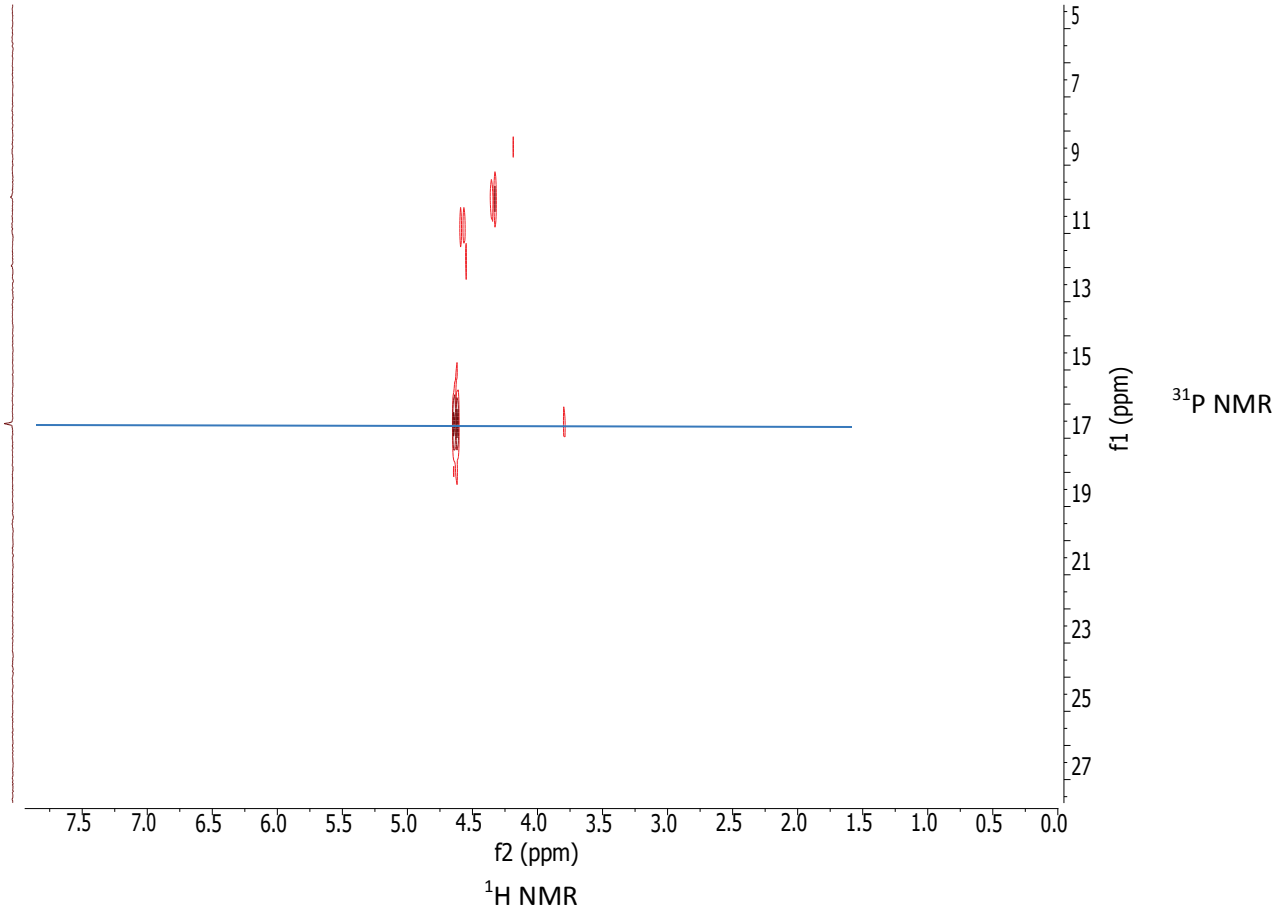


Figure S12. ^{1}H-^{31}P HMBC spectrum of compound 17 after incubation with His_6-FzmH. Tha assay contained all the necessary components for catalysis (i.e. compound 17, SAM, His_6-FzmH, AdoHcy nucleosidase).

Comparing spectrum A with spectrum B in Figure S11, no additional peak was seen, indicating no methylation occurred. This result was corroborated with the ^{1}H-^{31}P HMBC study in Figure S12. If methylation had occurred, then the methylated product should have a signal around 2.8 ppm in the ^{1}H NMR dimension in Figure S12, which was not observed. Thus, based on these results, compound **17** is not a substrate of FzmH.

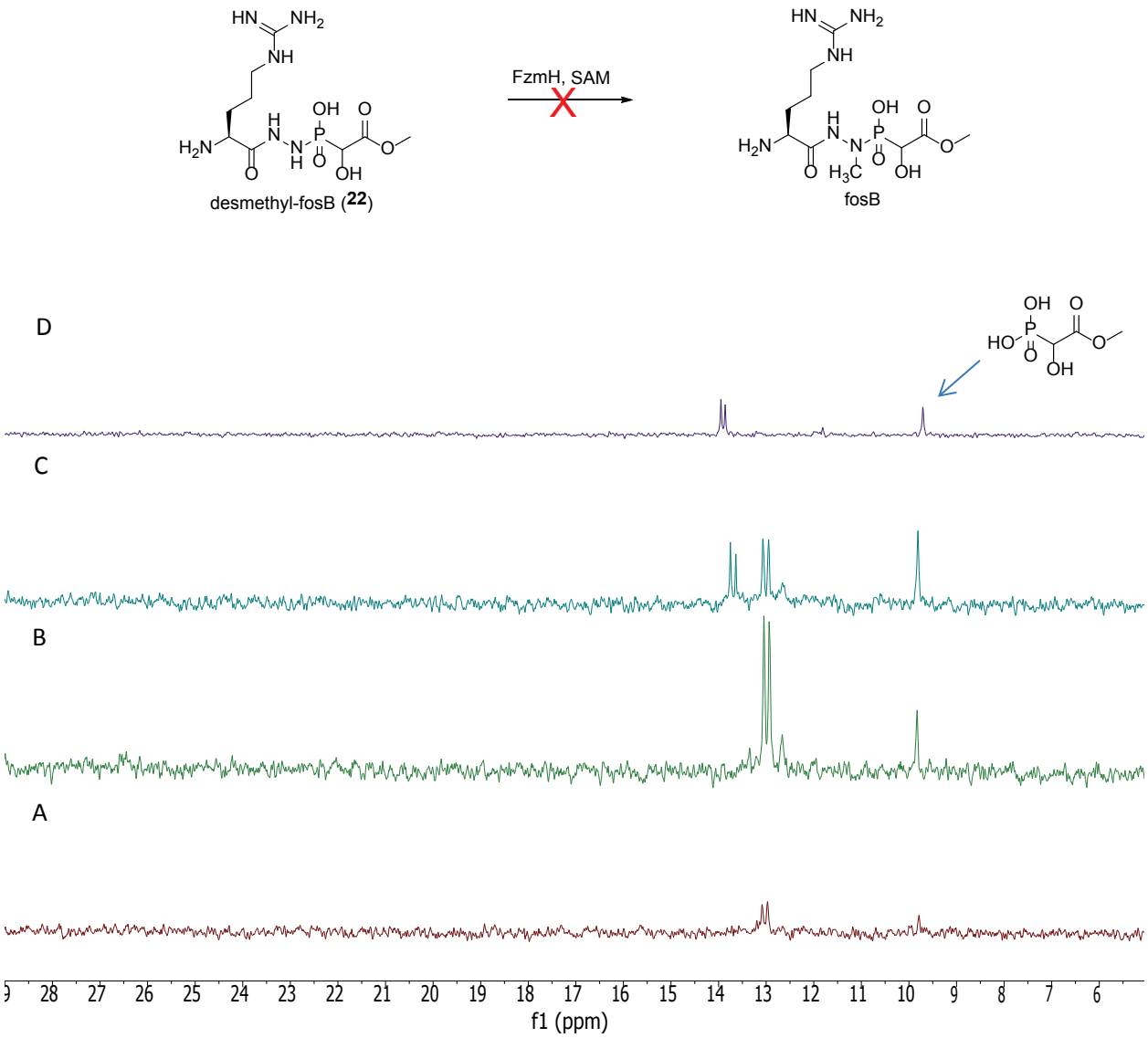


Figure S13. ^{31}P NMR analysis of the incubation of His$_6$-FzmH reaction with desmethyl fosfazinomycin B (22). **A.** NMR spectrum of the reaction mixture in the absence of His$_6$-FzmH. **B.** NMR spectrum of the reaction mixture containing all the necessary components for catalysis (i.e. desmethyl fosB (**22**), SAM, His$_6$-FzmH, AdoHcy nucleosidase). **C.** NMR spectrum of the reaction mixture in panel B spiked with a synthetic standard of fosfazinomycin B (2 diastereomers).[6] **D.** NMR spectrum of the synthetic standard of fosfazinomycin B (2 diastereomers).

Based on the above results, it is clear that FzmH could not methylate desmethyl fosfazinomycin B to make fosfazinomycin B.

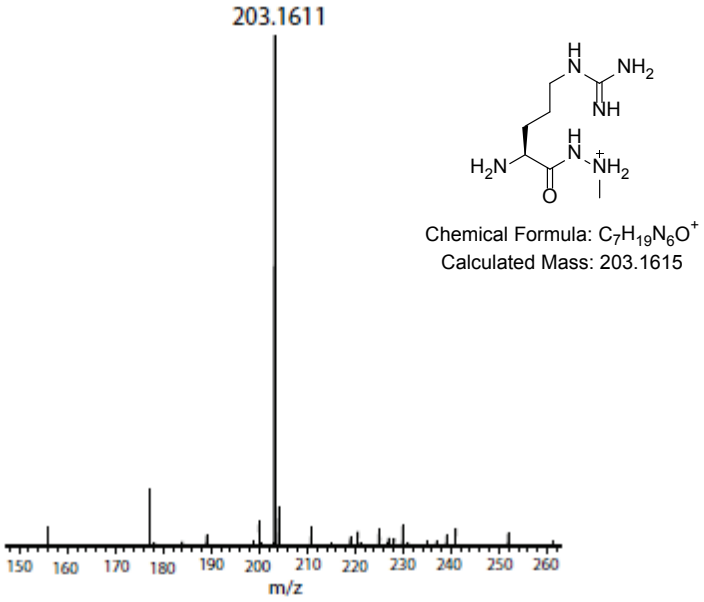


Figure S14. FT-ICR MS analysis of Arg-NHNHMe (4), the product of His_6-FzmH catalyzed methylation of Arg-$NHNH_2$ (2).

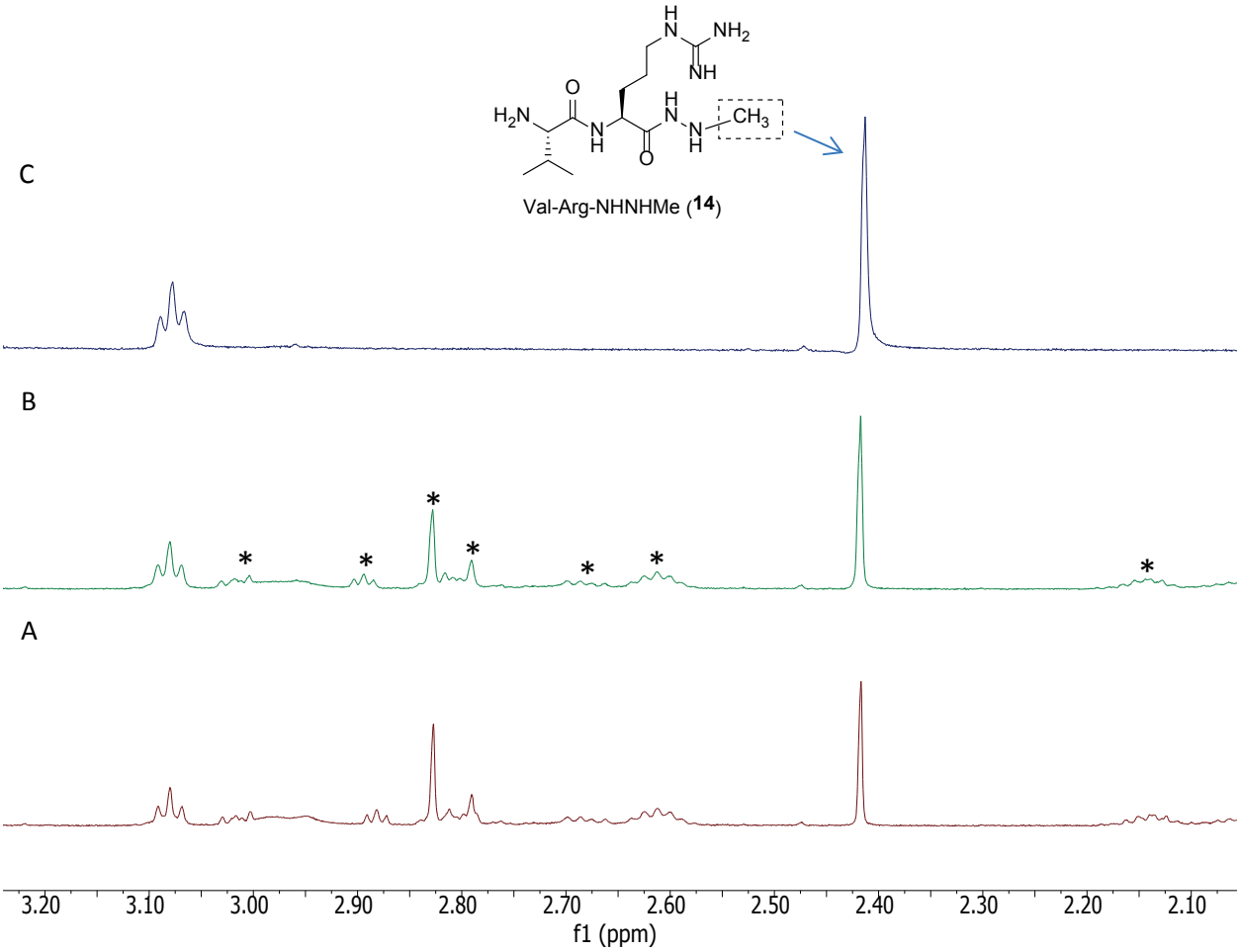


Figure S15. ^{1}**H NMR spectra of the assay of His$_6$-FzmH with Val-Arg-NHNH$_2$ (9). A.** NMR spectrum of the reaction mixture containing all the necessary components for catalysis (i.e. Val-Arg-NHNH$_2$, SAM, His$_6$-FzmH, AdoHcy nucleosidase). **B.** NMR spectrum of the assay in panel A spiked with an authentic standard of Val-Arg-NHNHMe **C.** NMR spectrum of the authentic standard of Val-Arg-NHNHMe . *These peaks are from SAM and its related products.

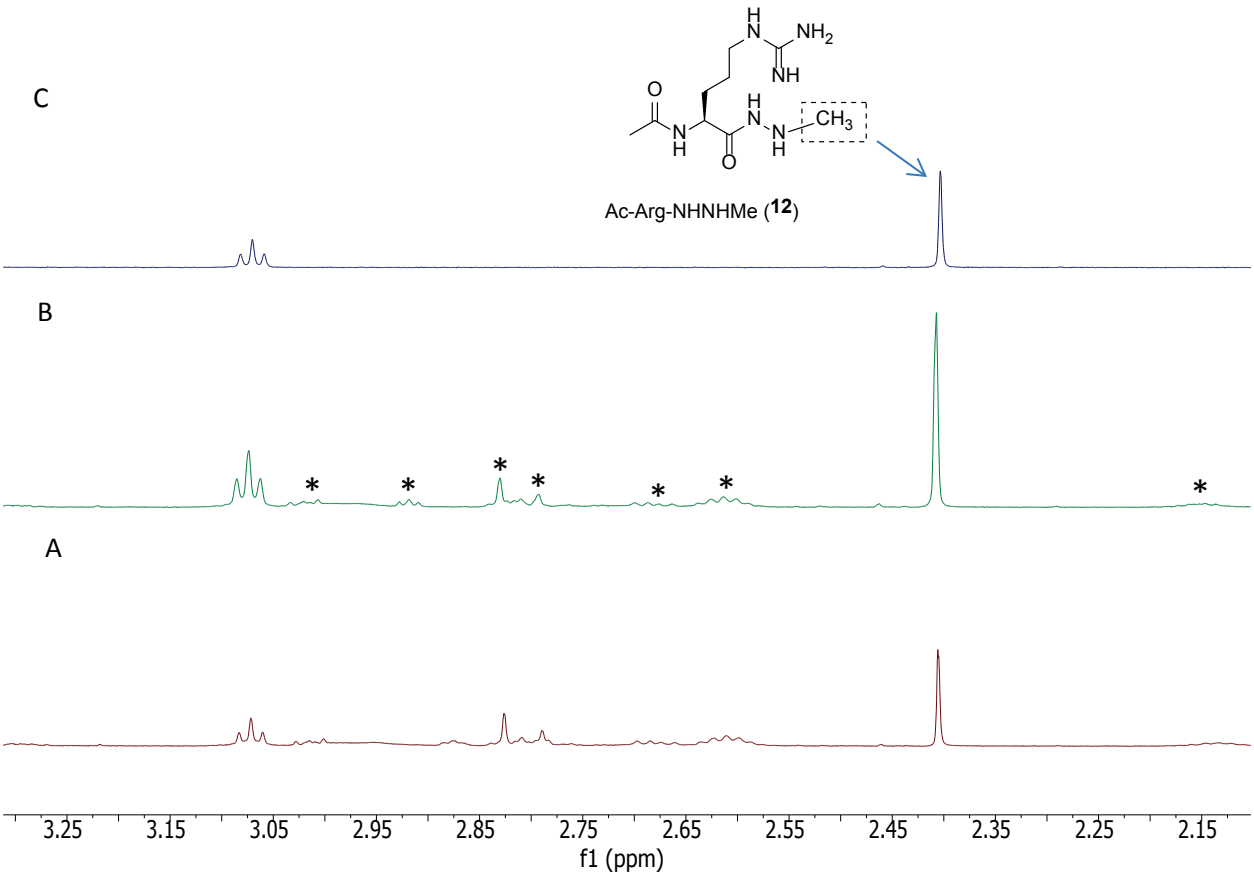


Figure S16. ^{1}H NMR analysis the reaction of His$_6$-FzmH with Ac-Arg-NHNH$_2$ (7). A. NMR spectrum of the reaction mixture containing all the necessary components for catalysis (i.e. Ac-Arg-NHNH$_2$, SAM, His$_6$-FzmH, AdoHcy nucleosidase). **B.** NMR spectrum of the real mixture spiked with the authentic standard of Ac-Arg-NHNHMe **C.** NMR spectrum of the authentic standard of Ac-Arg-NHNHMe. *These peaks are from SAM and its related products.

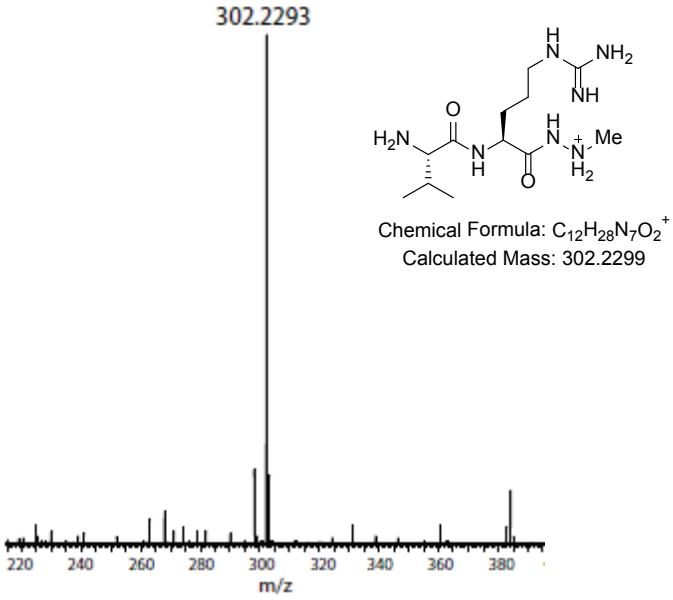


Figure S17. FT-ICR MS analysis of Val-Arg-NHNHMe (14), the product of His_6-FzmH catalyzed methylation of Val-Arg-$NHNH_2$ (9).

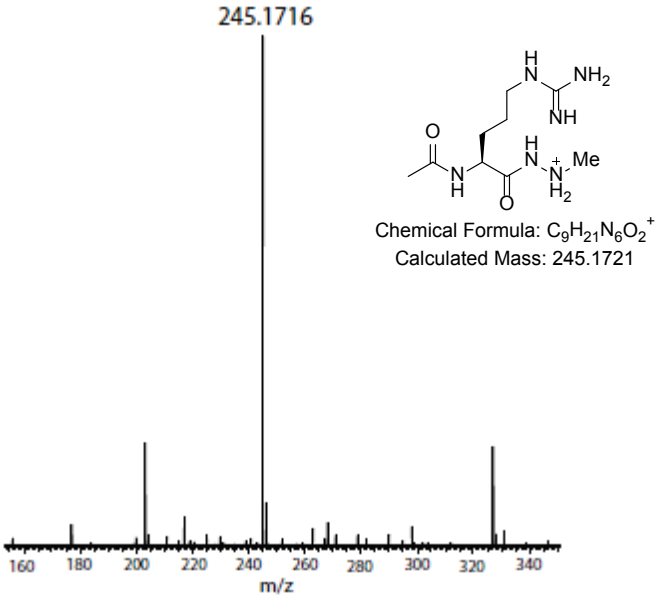


Figure S18. FT-ICR MS analysis of Ac-Arg-NHNHMe (12), the product of His_6-FzmH catalyzed methylation of Ac-Arg-$NHNH_2$ (7).

Supplementary Tables

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

Name	Features	Source
***E. coli* Rosetta 2(DE3)pLysS**	CAM^R; provides seven rare, in *E. coli*, tRNAs for the codons CGG, AUA, AGG, AGA, CUA, CCC, and GGA in the same plasmid that habors the T7 lysozyme	Novagen
***E. coli* DH5α λpir**	sup E44, ΔlacU169 (ΦlacZΔM15), recA1, endA1, hsdR17, thi-1, gyrA96, relA1, λpir phage lysogen.	in house
pET15b	AMP^R; encodes an N terminal 6xHis-tag® of the protein of interest, and a thrombin recognition sequence	Novagen
Fosmid MMG 358	CAM^R; carries the fosfazinomycin biosynthetic gene cluster from *Streptomyces* sp. XY332	in house[8]
Fosmid MMG 360	CAM^R; carries the fosfazinomycin biosynthetic gene cluster from *Streptomyces* sp. XY332	in house[8]
Genomic DNA WM6372	Carries the fosfazinomycin biosynthetic gene cluster from *Streptomyces* sp. WM6372	in house
pET15b-fzmB		this study
pET15b-fzmG		this study
pET15b-fzmH		this study

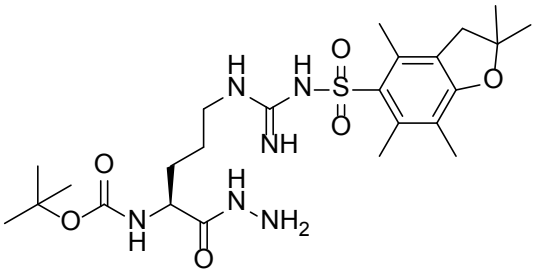
Table S2. List of oligonucleotides used in this study.

Name	Sequence (5'→3')[a]	Scope
pET15-XhoI_fw	ctcgaggatccggctgctaacaaagcccgaaagg	Amplification of pET15b vector during Gibson cloning
pET15-NdeI_rc	catatggctgccgcgcggcaccaggccgctg	Amplification of pET15b vector during Gibson cloning
NdeI-fzmB_fw	GcagcggcctggtgccgcgcggcagccatATGACAGCCCATCTCTACTGGGACGAG	Amplification of *fzmB* for Gibson cloning into pET15b vector
XhoI-fzmB_rc	CctttcgggctttgttagcagccggatcctcgagCTATTCCCCGGCCCTCTGCGCGAAGAGG	Amplification of *fzmB* for Gibson cloning into pET15b vector
NdeI-fzmG_fw	GcagcggcctggtgccgcgcggcagccatatgAACAGCAACGATCCGTTCTCGAC	Amplification of *fzmG* for Gibson cloning into pET15b vector
XhoI-fzmG_rc	CctttcgggctttgttagcagccggatcctcgagTCACAGGAGGGCCCCGGCCGCGGCGGCACCCT	Amplification of *fzmG* for Gibson cloning into pET15b vector
NdeI-fzmH_fw	GcagcggcctggtgccgcgcggcagccatatgGTGA	Amplification of *fzmH* for

	CACGGCTCGTCGCCGAGCTCGAACG	Gibson cloning into pET15b vector
XhoI-fzmH_rc	CctttcgggctttgttagcagccggatcctcgagTCAC CCGCCCGCGCGGTCGAGGACACGA	Amplification of *fzmH* for Gibson cloning into pET15b vector

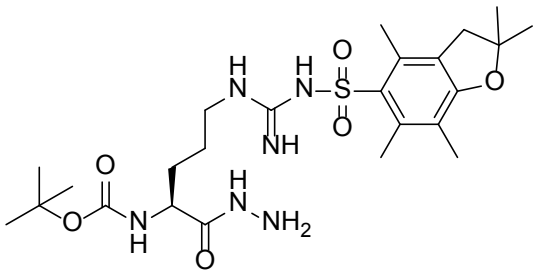
[a]Underlined sequence indicates homology to expression vector.

Boc-Arg(Pbf)-NHNH2, HNMR

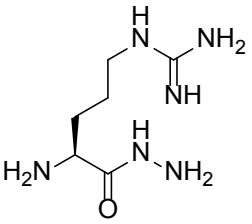


1

Boc-Arg(Pbf)-NHNH2, CNMR



1



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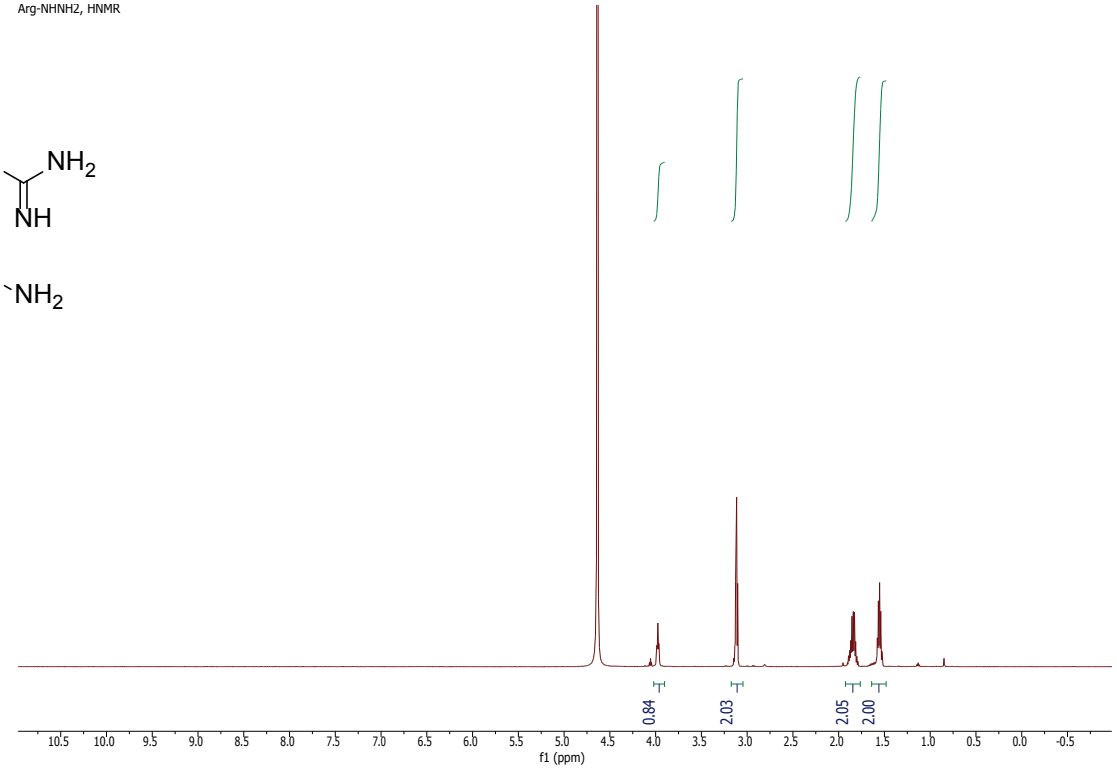
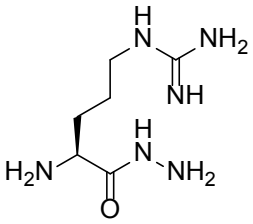
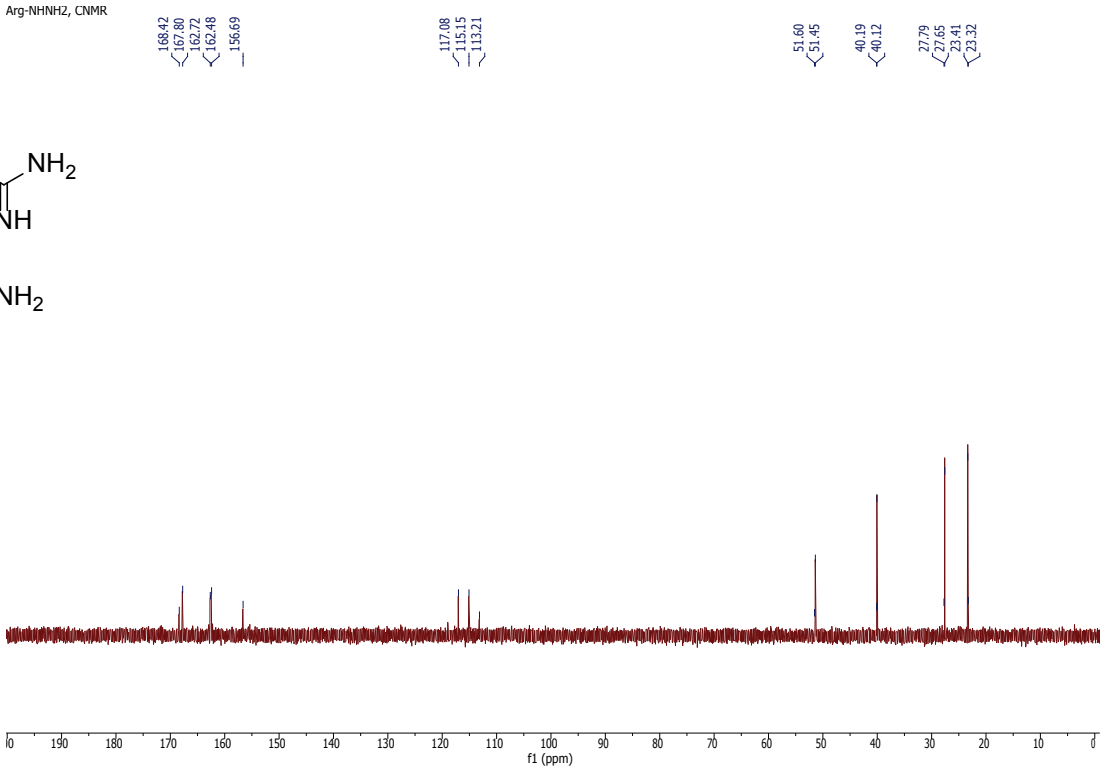
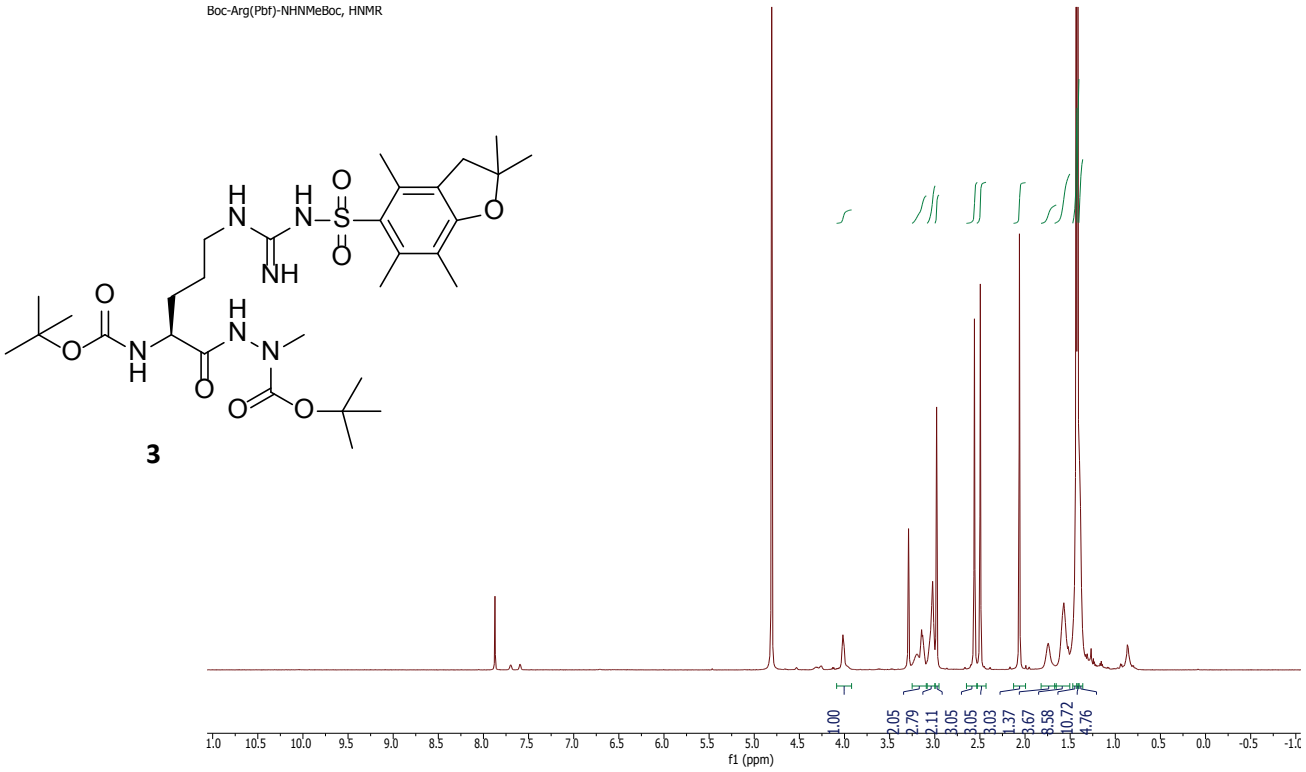

Arg-NHNH2, HNMR
0.84
2.03
2.05
2.00
f1 (ppm)

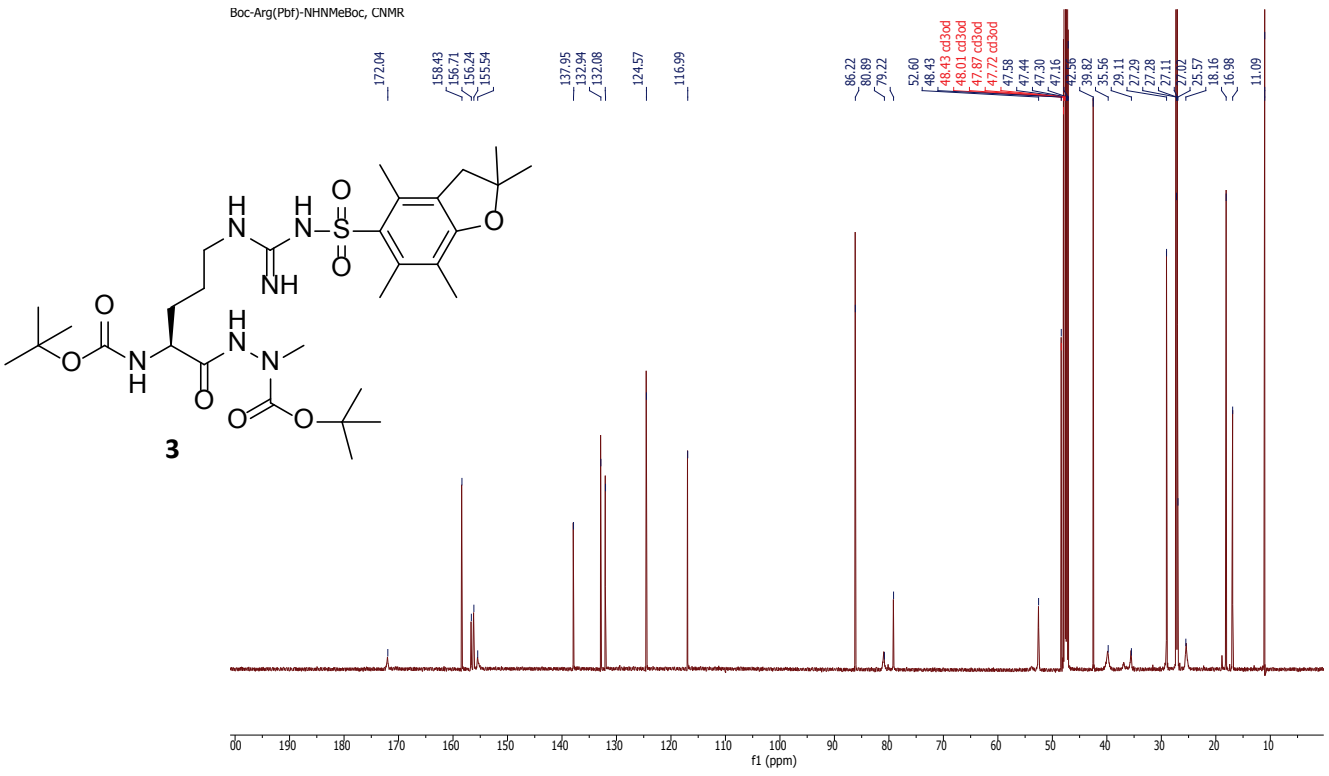


2


Arg-NHNH2, CNMR
168.42
167.80
162.72
162.48
156.69
117.08
115.15
113.21
51.60
51.45
40.19
40.12
27.79
27.65
23.41
23.32
f1 (ppm)

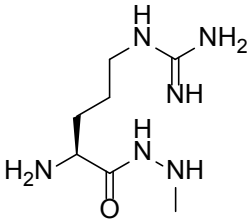

Boc-Arg(Pbf)-NHNMeBoc, HNMR
1.00
2.05
2.79
2.11
3.05
3.05
3.03
1.37
3.67
8.58
10.72
4.76
f1 (ppm)

3


Boc-Arg(Pbf)-NHNMeBoc, CNMR
172.04
158.43
156.71
156.24
155.54
137.95
132.94
132.08
124.57
116.99
86.22
80.89
79.22
52.60
48.43
48.43 cd3od
48.01 cd3od
47.87 cd3od
47.72 cd3od
47.58
47.44
47.30
47.16
39.82
35.56
29.11
27.29
27.28
27.11
27.02
25.57
18.16
16.98
11.09
f1 (ppm)

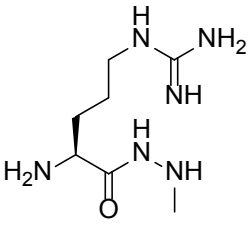
3

Arg-NHNHMe, HNMR

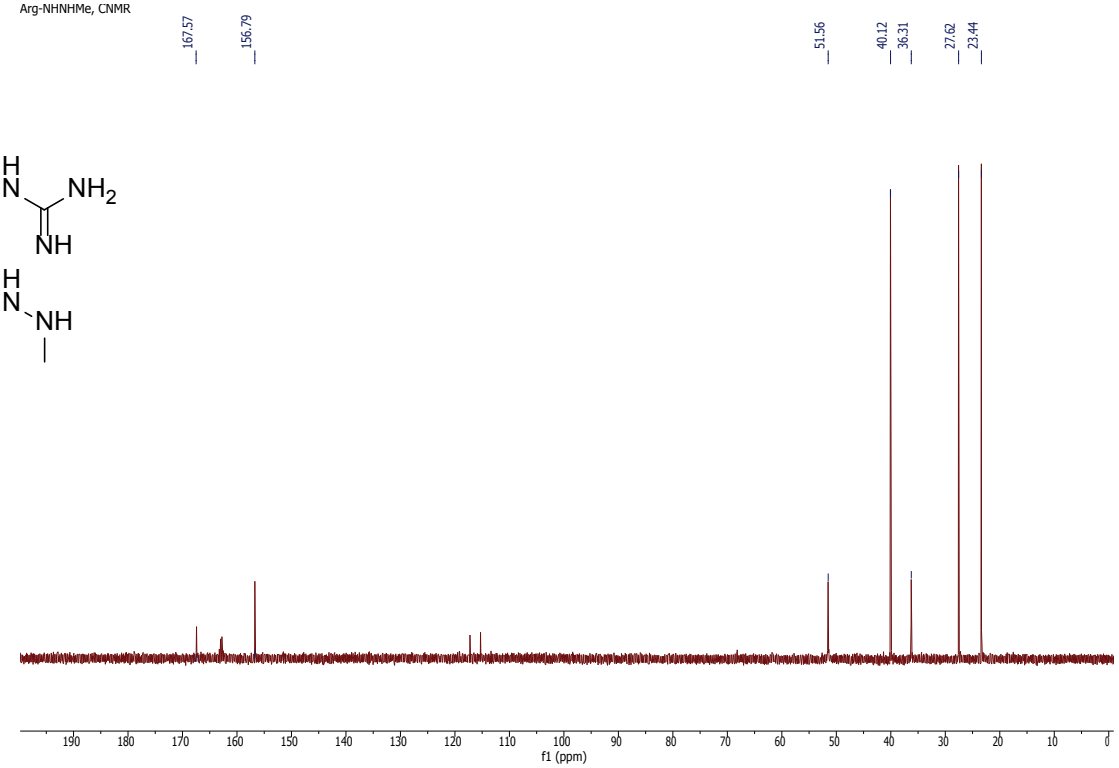


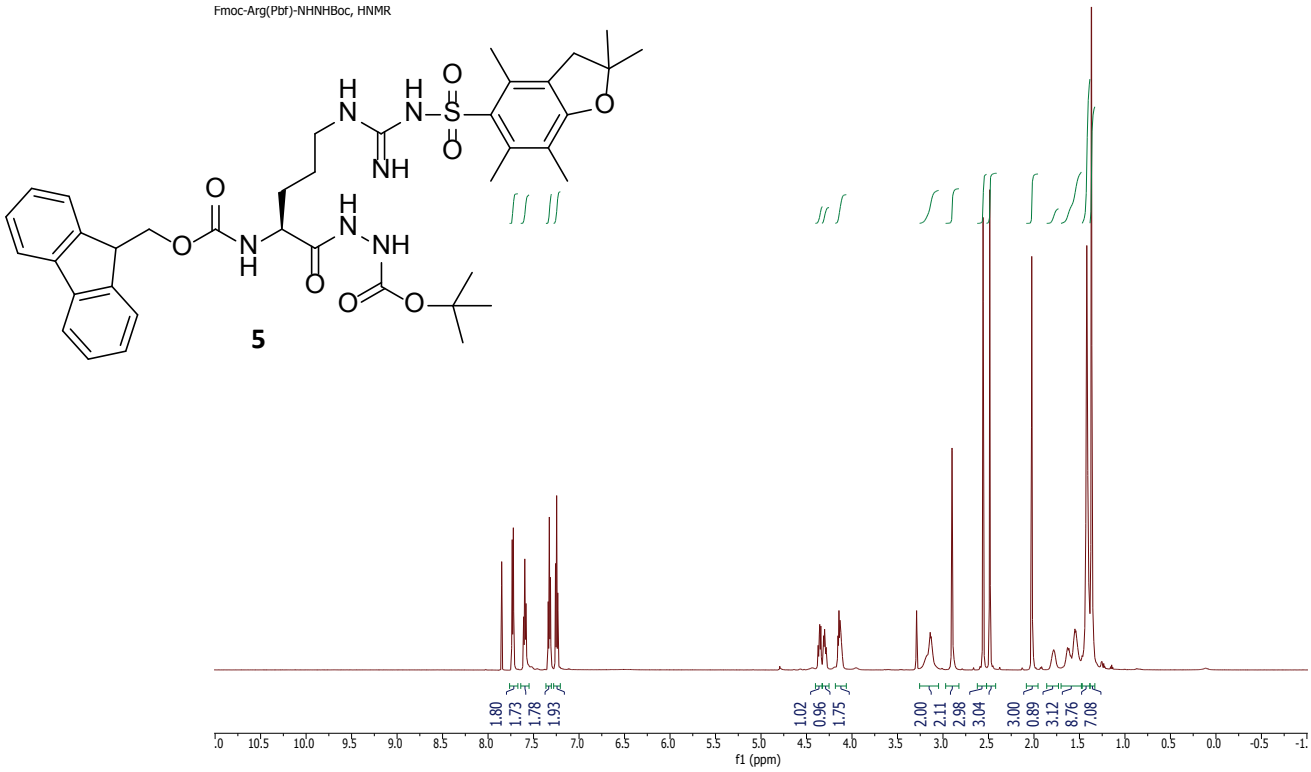
4

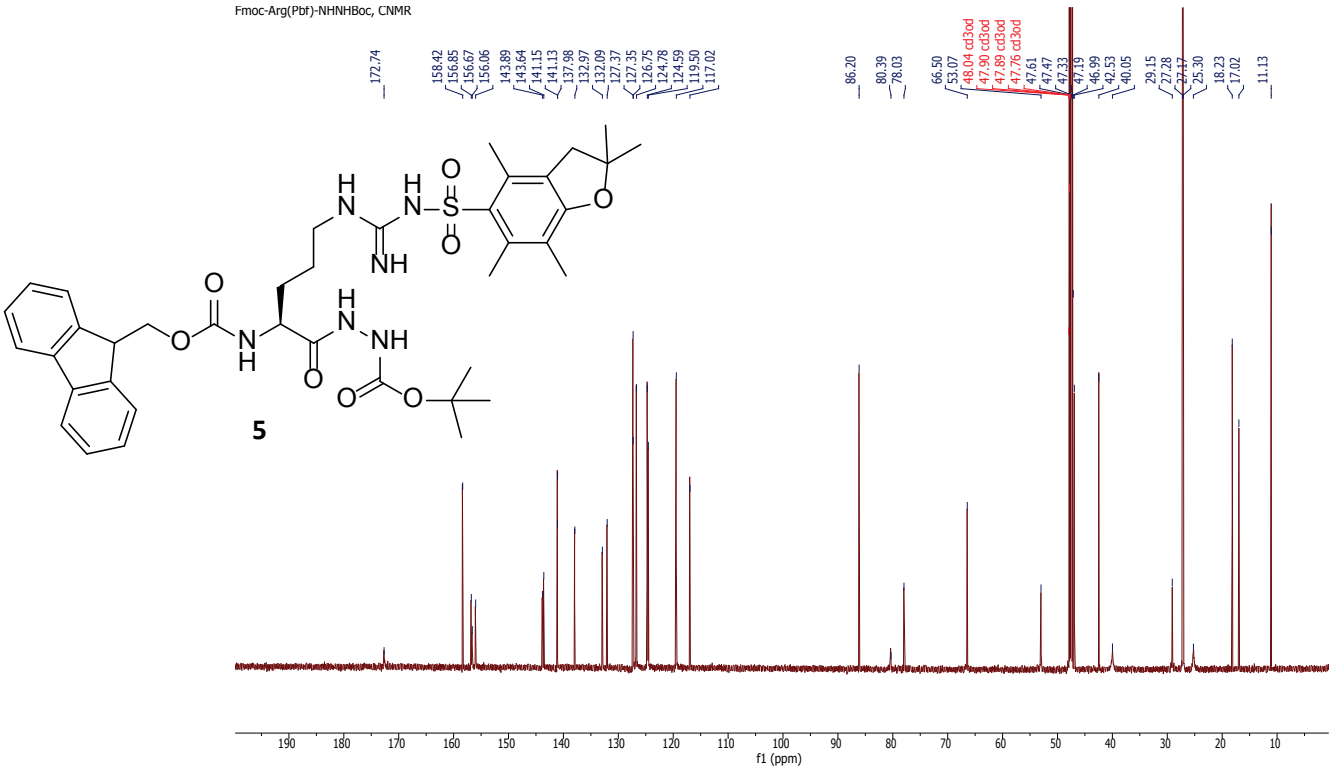
Arg-NHNHMe, CNMR



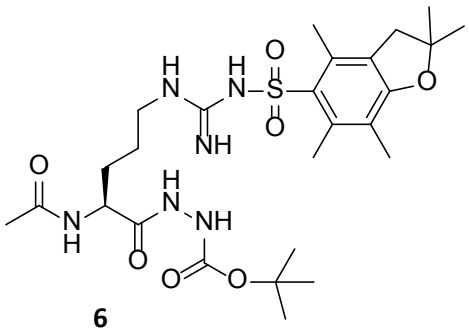
4




Fmoc-Arg(Pbf)-NHNHBoc, HNMR
5
1.80
1.73
1.78
1.93
1.02
0.96
1.75
2.00
2.11
2.98
3.04
3.00
0.89
3.12
8.76
7.08
f1 (ppm)

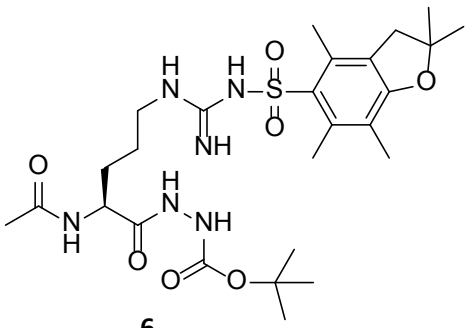

Fmoc-Arg(Pbf)-NHNHBoc, CNMR
5
172.74
158.42
156.85
156.67
156.06
143.89
143.64
141.15
141.13
137.98
132.97
132.09
127.37
127.35
126.75
124.78
124.59
119.50
117.02
86.20
80.39
78.03
66.50
53.07
48.04 cd3od
47.90 cd3od
47.89 cd3od
47.76 cd3od
47.61
47.47
47.33
47.19
46.99
42.53
40.05
29.15
27.28
27.17
25.30
18.23
17.02
11.13
f1 (ppm)

Ac-Arg(Pbf)-NHNHBoc, HNMR



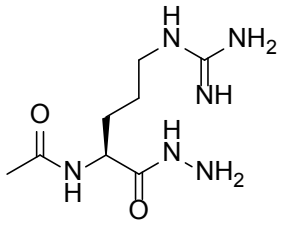
6

Ac-Arg(Pbf)-NHNHBoc, CNMR

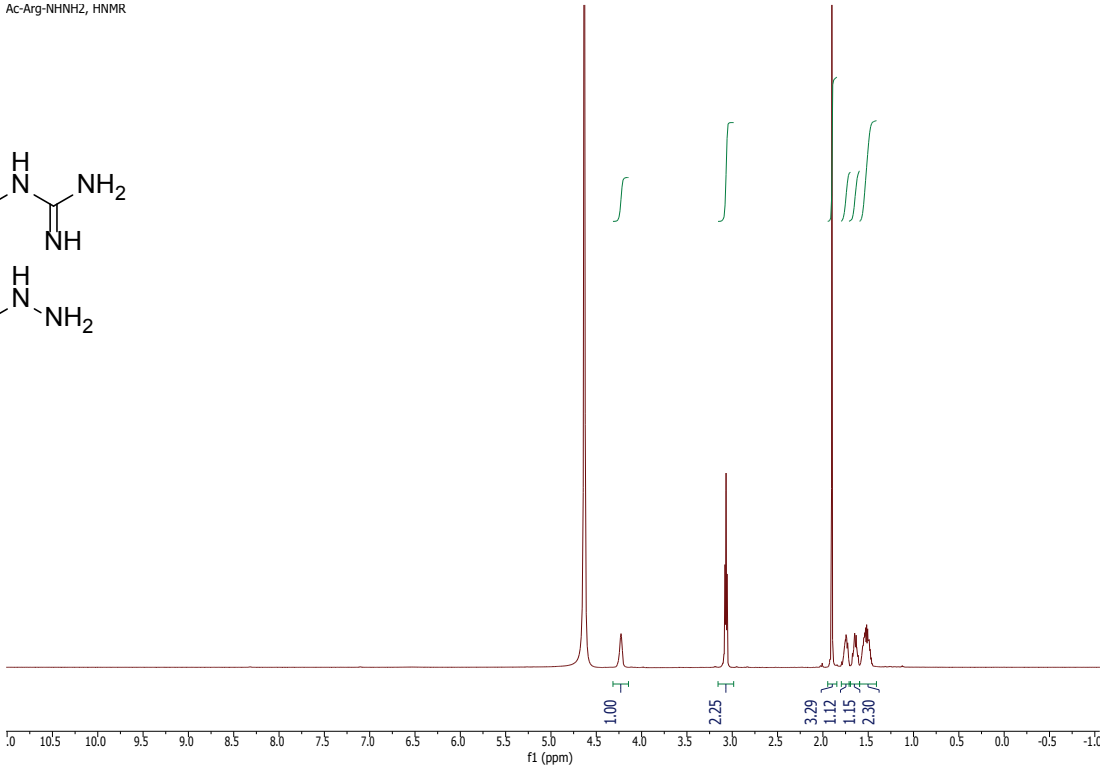


6

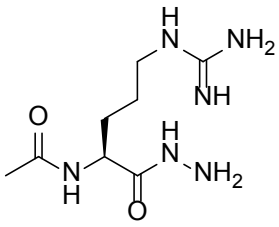
Ac-Arg-NHNH2, HNMR



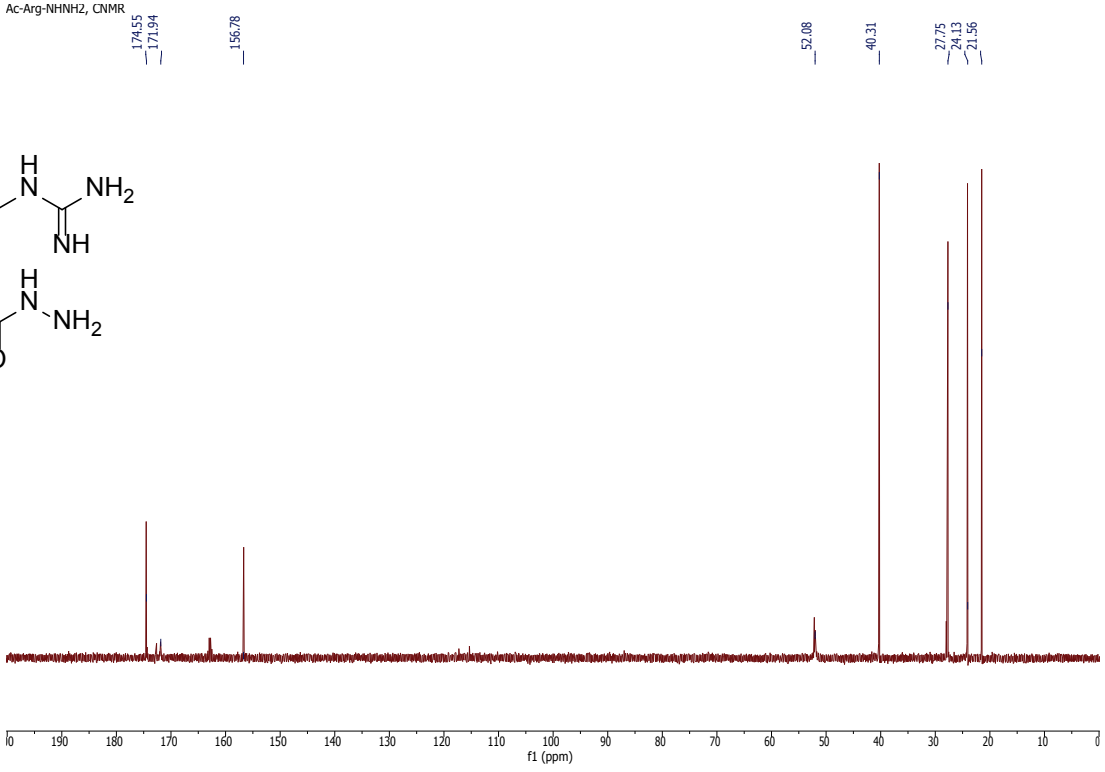
7



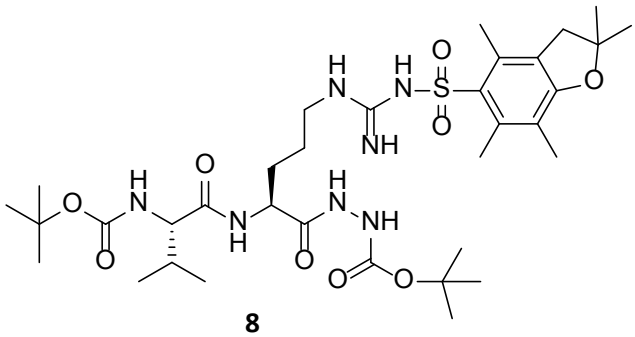
Ac-Arg-NHNH2, CNMR



7



Boc-Val-Arg(Pbf)-NHNHBoc, HNMR



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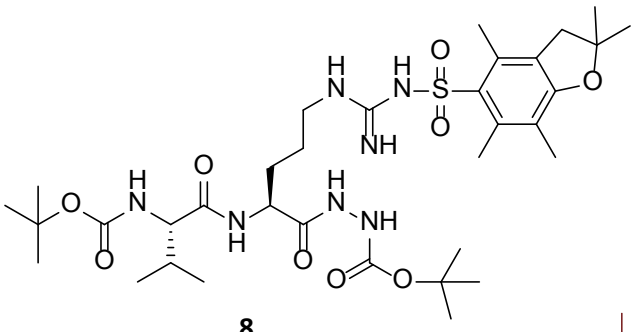
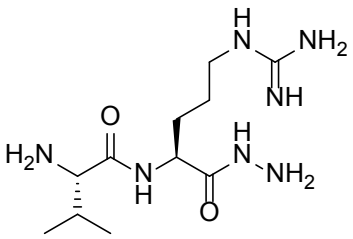
Boc-Val-Arg(Pbf)-NHNHBoc, CNMR



8

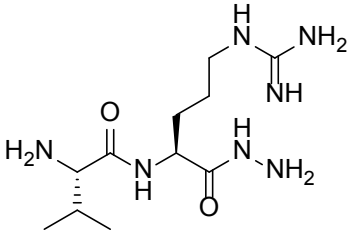
Val-Arg-NHNH2, HNMR



9

Val-Arg-NHNH2, CNMR

171.77
171.09
169.50
169.27
163.18
162.95
162.71
162.48
156.70
117.25
115.31
58.18
52.11
52.00
40.40
40.32
29.97
29.92
27.86
27.61
24.19
24.16
17.53
17.45
16.65
16.62



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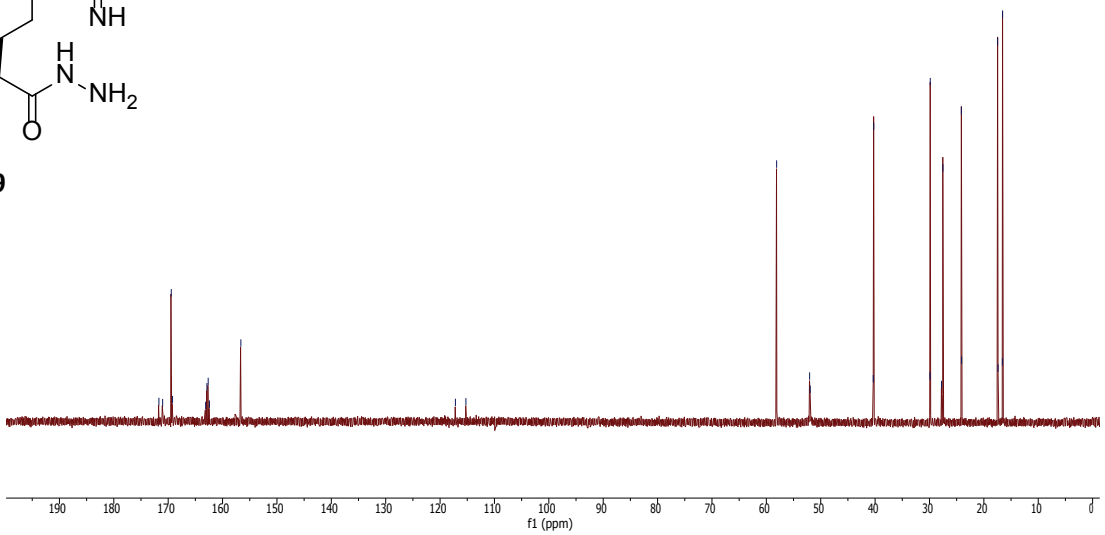
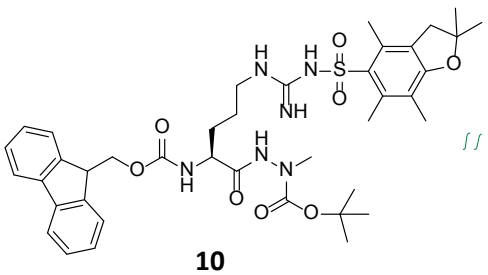
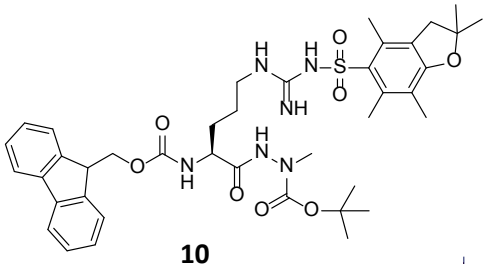


Fmoc-Arg(Pbf)-NHNMeBoc, HNMR



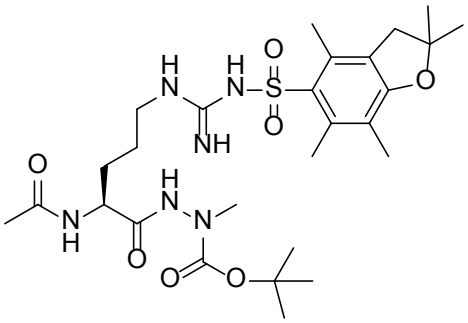
10

Fmoc-Arg(Pbf)-NHNMeBoc, CNMR



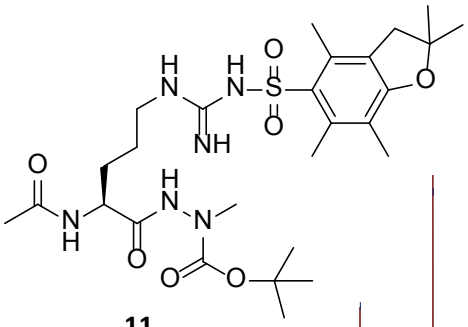
10

Ac-Arg-NHNMeBoc, HNMR



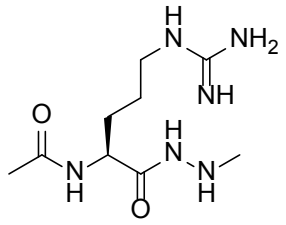
11

Ac-Arg-NHNMeBoc, CNMR



11

Ac-Arg-NHNHMe, HNMR



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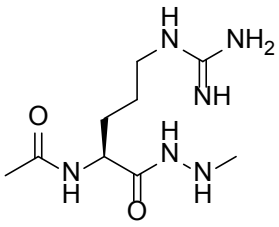
Ac-Arg-NHNHMe, CNMR



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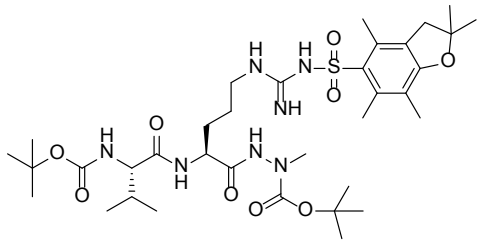
Boc-Val-Arg(Pbf)-NHNMeBoc, HNMR



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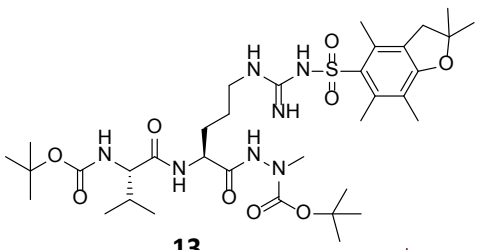
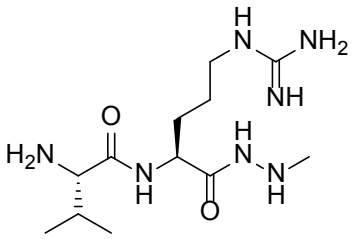
Boc-Val-Arg(Pbf)-NHNMeBoc, CNMR



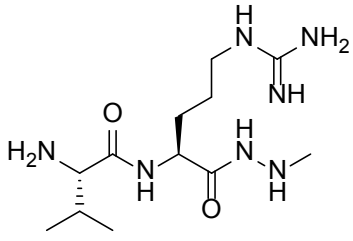
13

Val-Arg-NHNHMe, HNMR



14

Val-Arg-NHNHMe, CNMR



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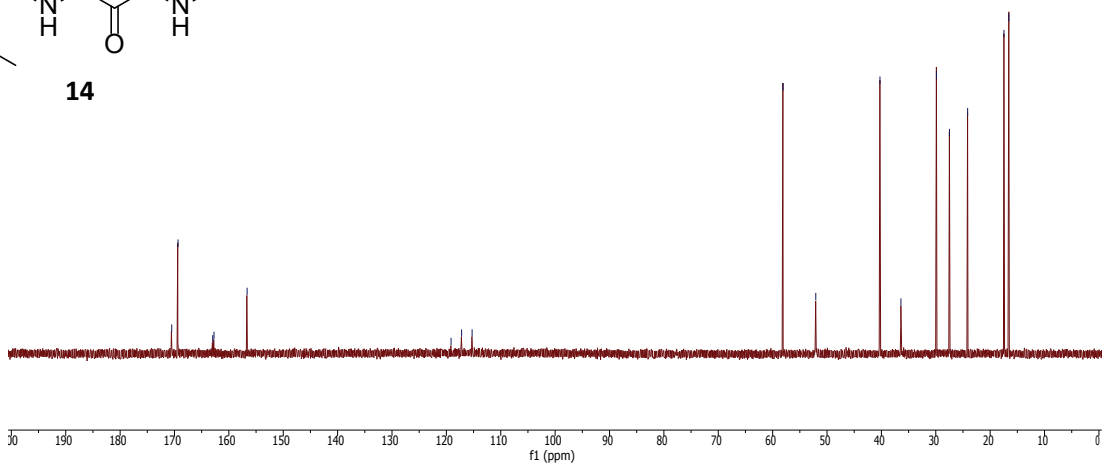
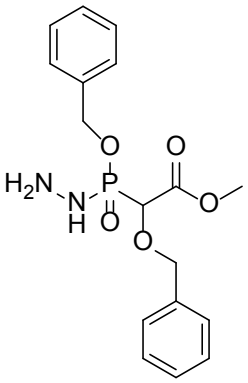
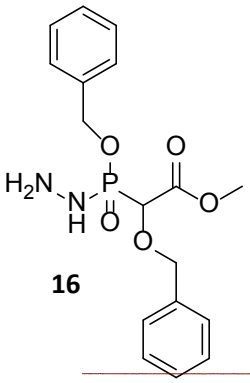


compound 16, PNMR

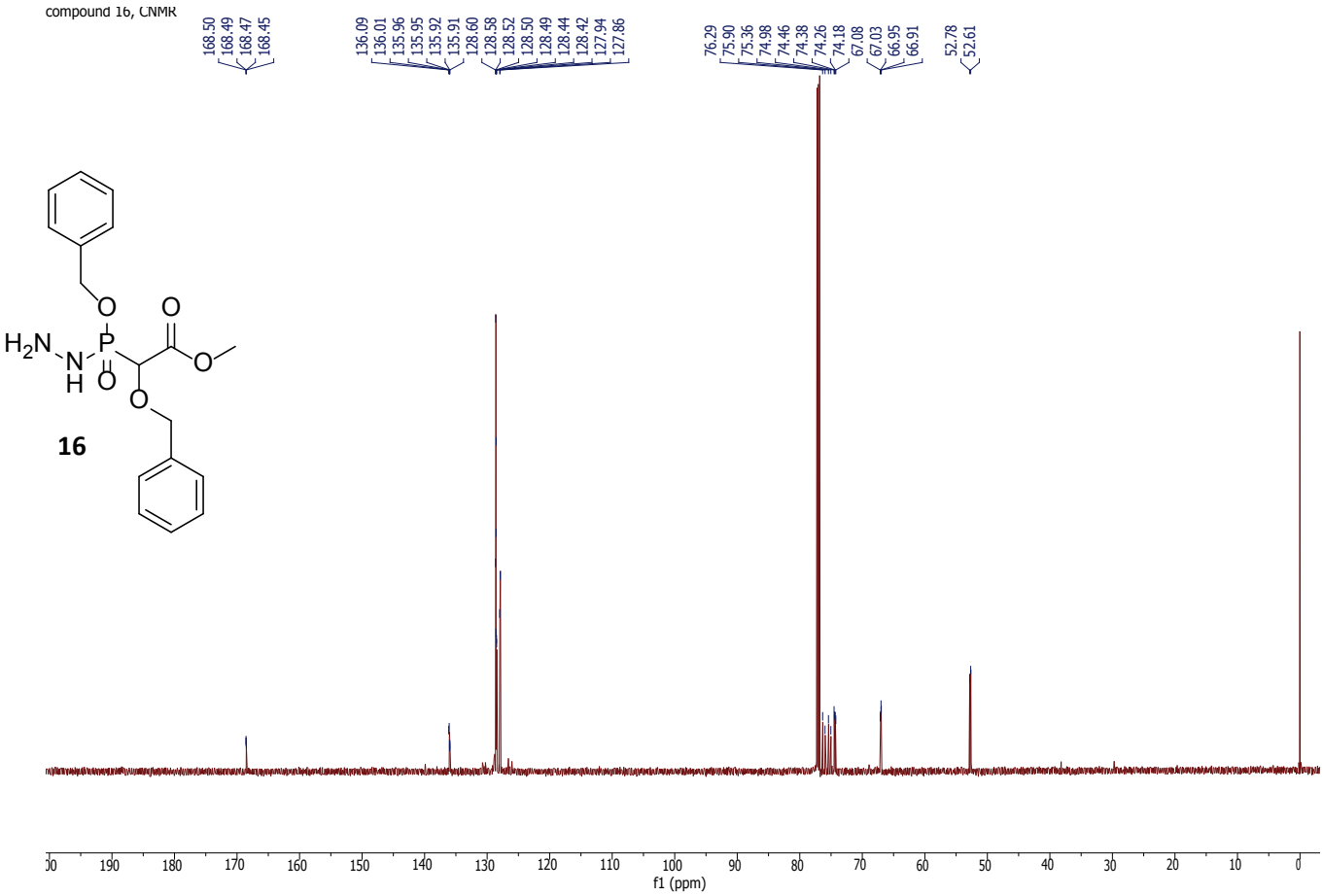


16

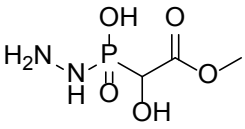
compound 16, HNMR



16

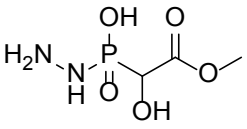

compound 16, CNMR
168.50
168.49
168.47
168.45
136.09
136.01
135.96
135.95
135.92
135.91
128.60
128.58
128.52
128.50
128.49
128.44
128.42
127.94
127.86
76.29
75.90
75.36
74.98
74.46
74.38
74.26
74.18
67.08
67.03
66.95
66.91
52.78
52.61
16
f1 (ppm)

compound 17, PNMR

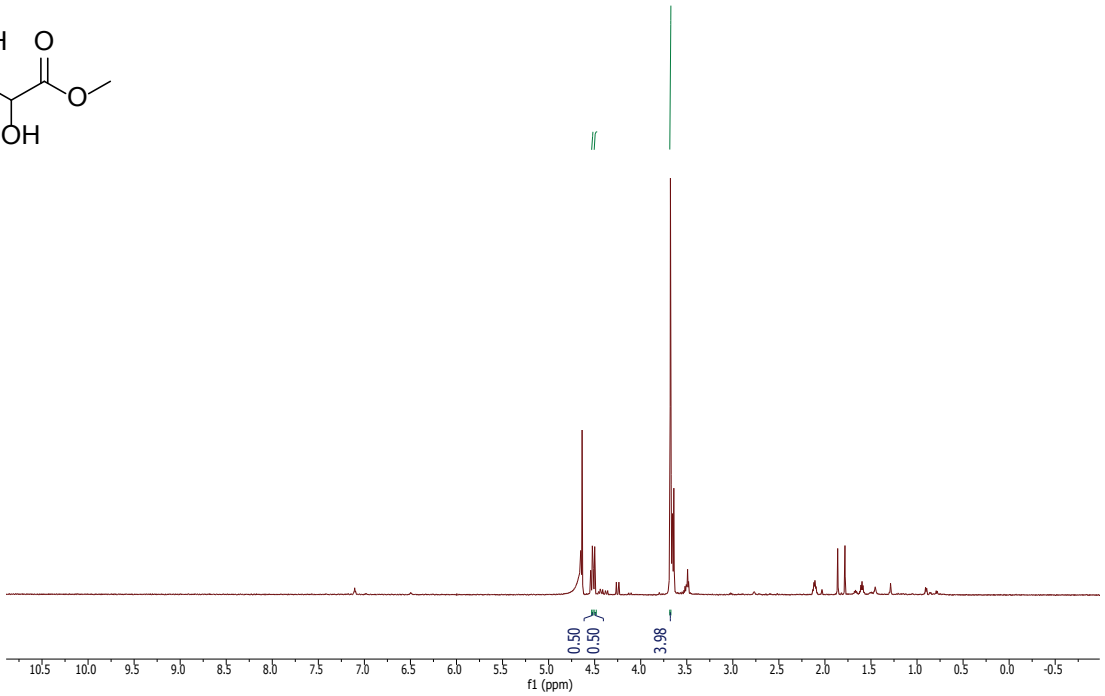


17

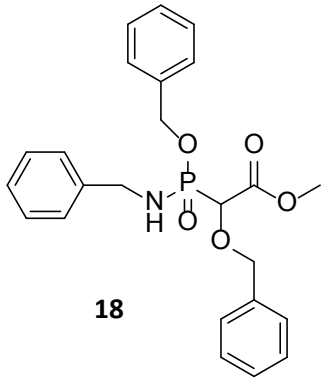
compound 17, HNMR



17

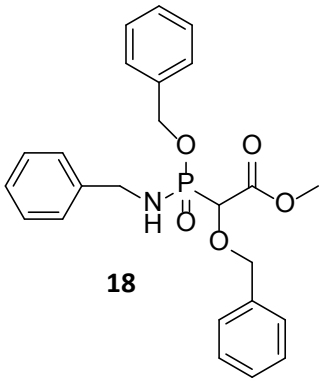


compound 18, PNMR

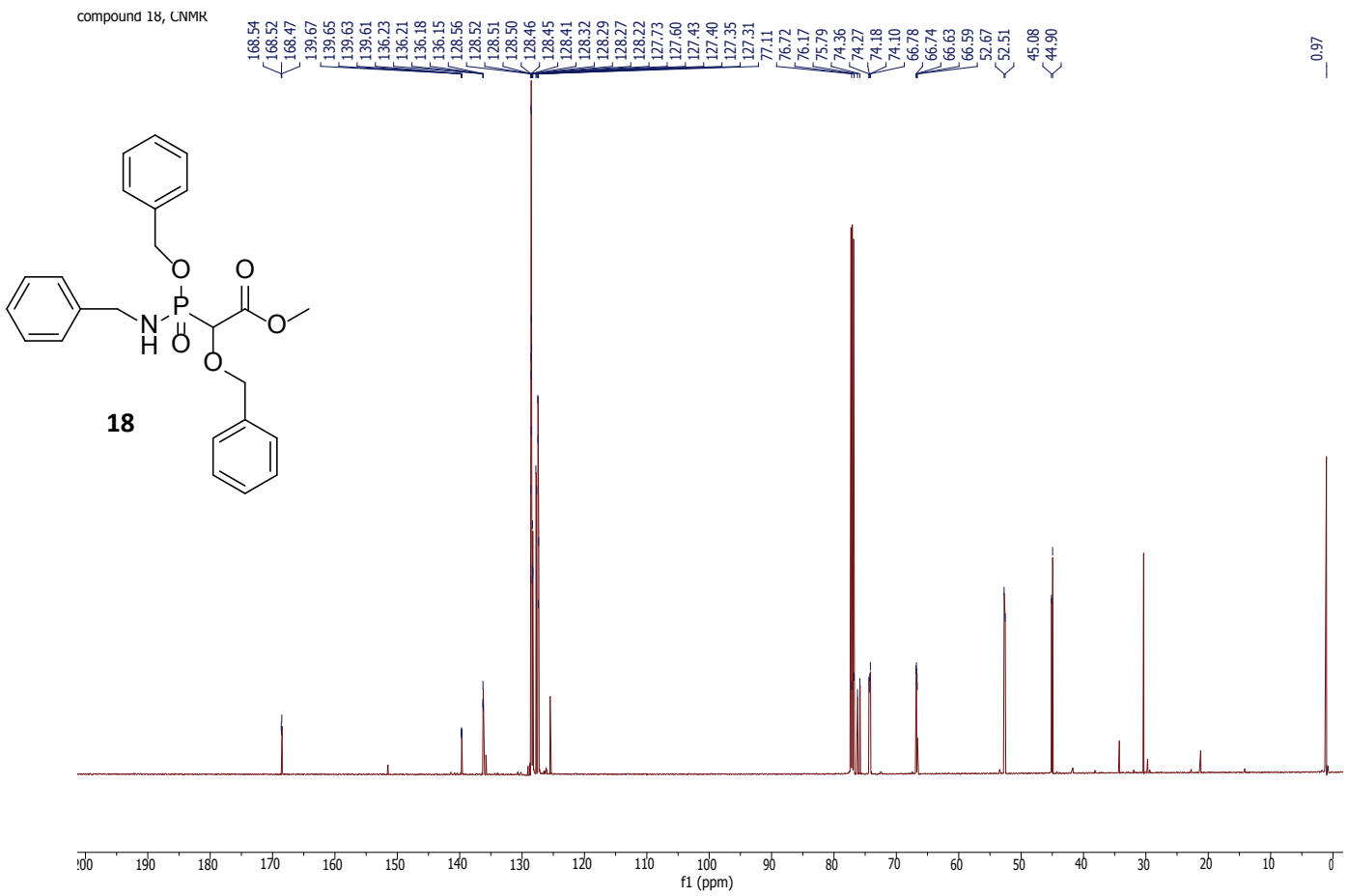


18

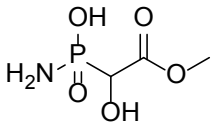
compound 18, HNMR



18

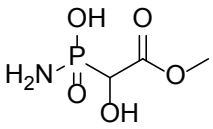

compound 18, CNMR
168.54
168.52
168.47
139.67
139.65
139.63
139.61
136.23
136.21
136.18
136.15
128.56
128.52
128.51
128.50
128.46
128.45
128.41
128.32
128.29
128.27
128.22
127.73
127.60
127.43
127.40
127.35
127.31
77.11
76.72
76.17
75.79
74.36
74.27
74.18
74.10
66.78
66.74
66.63
66.59
52.67
52.51
45.08
44.90
0.97
18
f1 (ppm)

compound 19, PNMR



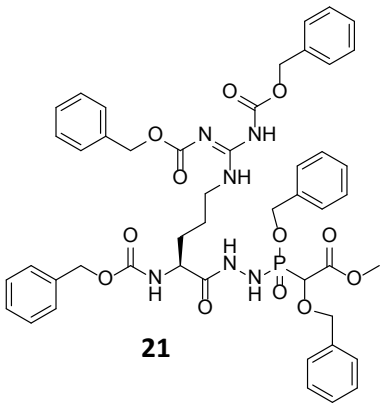
19

compound 19, HNMR



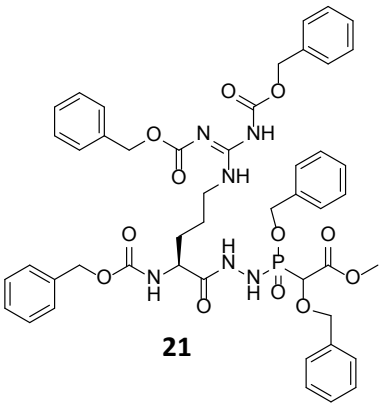
19

protected desmethyl fosB, PNMR

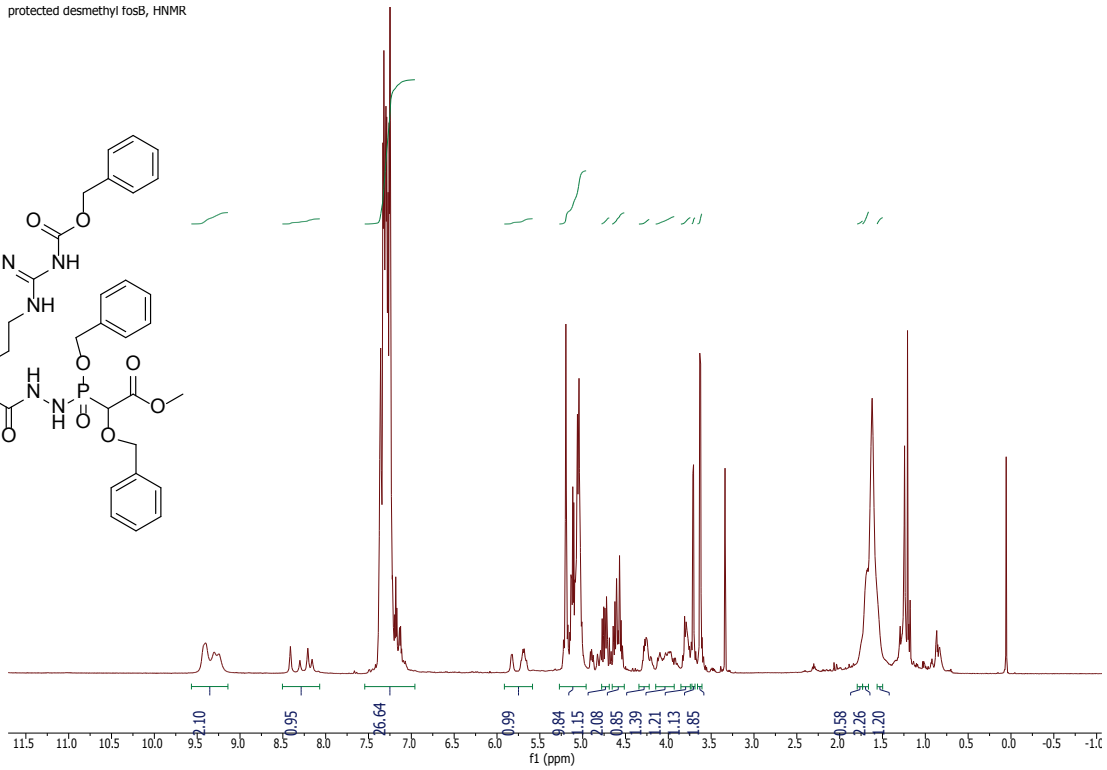


21

protected desmethyl fosB, HNMR

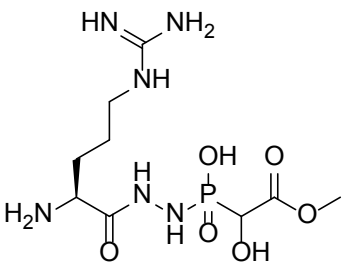


21



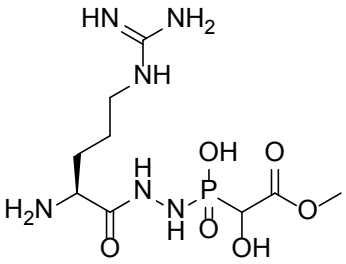
phosphate

desmethyl fosB, PNMR



22

desmethyl fosB, HNMR



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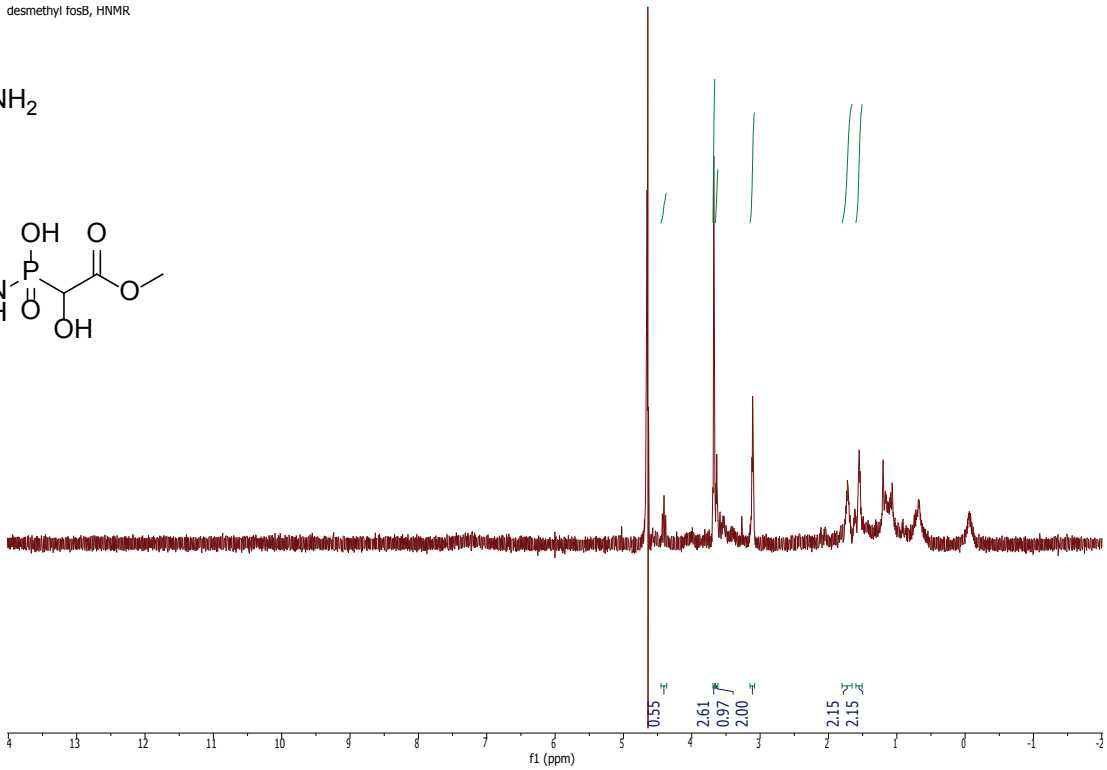

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