Supplementary Information for:

Glutamic acid is a carrier for hydrazine during the biosyntheses of fosfazinomycin and kinamycin

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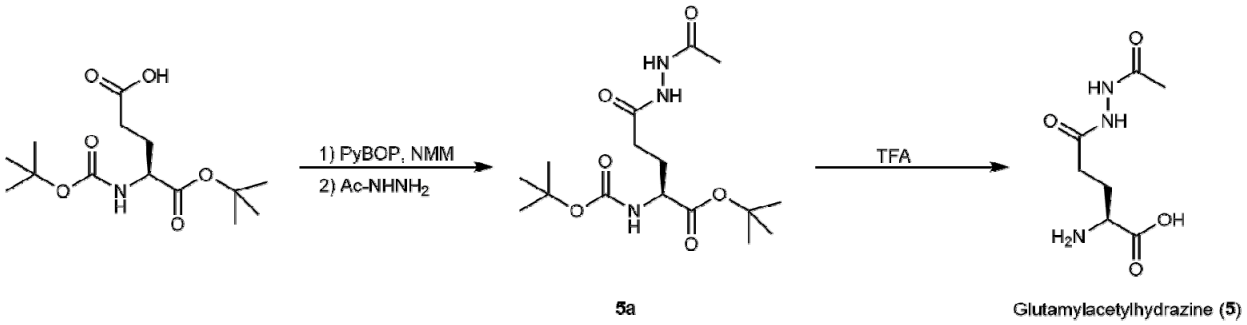
Synthesis of glutamylacetylhydrazine (5).



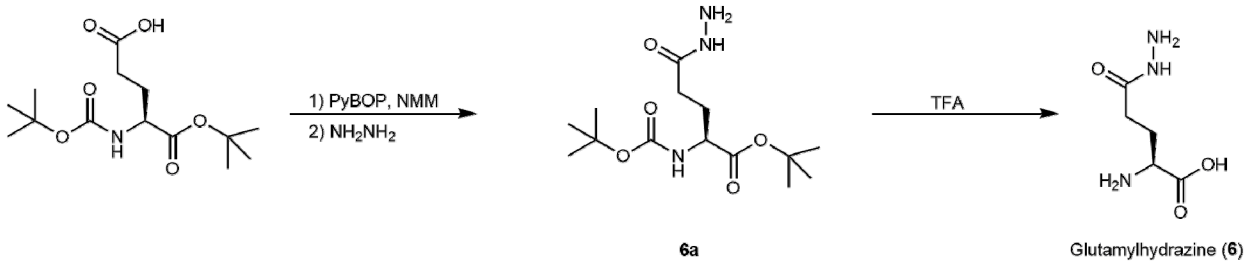
Synthesis of 5a

Boc-L-glutamic acid 1-*tert* butyl ester (606 mg, 2 mmol, 1.0 equiv.), (benzotriazol-1-xyloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP, 1.04 g, 2 mmol, 1.0 equiv.), and *N*-methylmorpholine (220 µL, 2 mmol, 1 equiv.) were dissolved in 8 mL of dry CH_2Cl_2. Acetylhydrazine (163 mg, 2.2 mmol, 1.1 equiv.) was then added, and the reaction mixture was stirred at ambient temperature for 16 h. The solution was diluted by the addition of 50 mL CH_2Cl_2 and washed successively with 15 mL of 5% $NaHCO_3$, 15 mL of 10% citric acid, water, and brine before being dried over Na_2SO_4. The product was then purified by flash chromatography (silica gel, 15:1 CH_2Cl_2 : MeOH).
^{1}H NMR (600 MHz, $CDCl_3$): δ (ppm) = 4.01 (m, 1H, CH), 2.02 (m, 2H, CH_2), 1.92 (s, 3H, CH_3), 1.92 (s, 3H, CH_3), 1.82 (m, 2H, CH_2), 1.34 (s, 9H, CH_3), 1.31 (s, 9H, CH_3). ^{13}C NMR (243 MHz, $CDCl_3$): δ (ppm) = 174.11, 173.12, 173.01, 157.59, 83.76, 81.39, 54.00, 29.57, 27.52, 27.05, 26.03, 19.61. HRMS: Calc'd for $C_{16}H_{29}N_3O_6$ $[M+H]^+$ = 360.2129, found = 360.2146

Synthesis of glutamylacetylhydrazine (5)

Compound **5a** (48 mg, 0.134 mmol) was dissolved in 2.25 mL of CH_2Cl_2 with 2.5 mL of trifluoroacetic acid, 0.125 mL of water, and 0.125 mL of triisopropylsilane cooled in an ice bath. The reaction mixture was stirred for 4 h. The mixture was concentrated under reduced pressure and then diluted with 20 mL of water before being washed three times with 8 mL of ethyl acetate. The solution was then dried by lyophilization.
^{1}H NMR (600 MHz, D_2O): δ (ppm) = 3.98 (t, J= 6.3 Hz, 1H, CH), 2.45 (octet, J=7.2 Hz, 2H, CH_2), 2.13 (m, 2H, CH_2), 1.92 (s, 3H, CH_3). ^{13}C NMR (243 MHz, D_2O): δ (ppm) = 173.17, 171.14, 162.94, 52.17, 28.80, 25.08, 19.60. HRMS: Calc'd for $C_7H_{13}N_3O_4$ $[M+H]^+$ = 204.0979, found = 204.0988

Synthesis of glutamylhydrazine (6).



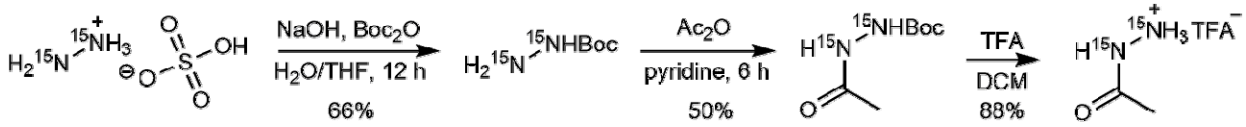
Synthesis of 6a

Boc-L-glutamic acid 1-*tert* butyl ester (0.909. g, 3 mmol, 1.0 equiv.) was dissolved in 7 mL of dry CH_2Cl_2 with PyBOP (1.56 g, 3 mmol, 1 equiv.) and *N*-methylmorpholine (330 µL, 3 mmol, 1 equiv.) before the addition of hydrazine (141 µL, 4.5 mmol, 1.5 equiv.), and the reaction mixture was stirred at ambient temperature for 15 h. The mixture was diluted by the addition of 30 mL of CH_2Cl_2 and washed sequentially with 12 mL of 5% $NaHCO_3$, 12 mL of water, and 10 mL of brine before being dried over Na_2SO_4. The product was then purified with flash chromatography (silica gel, 30:1 CH_2Cl_2 : MeOH). ^{1}H NMR (600 MHz, $CDCl_3$): δ (ppm) = 7.82 (br, 1H, NH), 5.38 (d, J=4.2 Hz, 1H, NH), 4.11 (m, 1H, CH), 3.93 (br, 2H, NH_2), 2.22 (t, J=7.3 Hz, 2H, CH_2), 2.12 (m, 1H, CH_2), 1.85 (m, 1H, CH_2), 1.42 (s, 9H, CH_3), 1.41 (s, 9H, CH_3). ^{13}C NMR (243 MHz, $CDCl_3$): δ (ppm) = 172.91, 171.29, 155.95, 82.32, 80.00, 53.40, 30.65, 29.35, 28.26, 27.93. HRMS: Calc'd for $C_{14}H_{27}N_3O_5$ $[M+H]^+$ = 318.2023, found = 318.2041

Synthesis of glutamylhydrazine (6)

Compound **6a** (0.67 g, 2.1 mmol) was dissolved in 21 mL of CH_2Cl_2, 21 mL of TFA, 1 mL of water, and 1 mL of triisopropylsilane cooled in an iced bath. The reaction was then stirred for 4.5 h before being concentrated under reduced pressure. The mixture was diluted with the addition of 30 mL of water and washed three times with 10 mL of ethyl acetate. The solution was then dried under reduced pressure.
^{1}H NMR (600 MHz, D_2O): δ (ppm) = 3.72 (t, J=6.3 Hz, 1H, CH), 2.43 (m, 2H, CH_2), 2.06 (q, J=7.24 Hz, CH_2). ^{13}C NMR (243 MHz, D_2O): δ (ppm) = 172.50, 162.97, 52.98, 28.68, 25.12. HRMS: Calc'd for $C_5H_{11}N_3O_7$ $[M+H]^+$ = 162.0873, found = 162.0875

Synthesis of $^{15}N_2$-acetylhydrazine

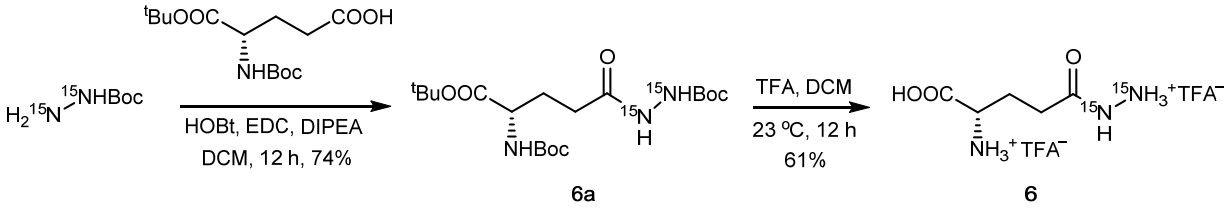


NaOH (187 mg, 4.68 mmol, 5.0 equiv.) was added to a solution of $^{15}N_2$-hydrazine sulfate (124 mg, 0.936 mmol, 1.0 equiv.) in H_2O (10 mL). Di-*tert*-butyl dicarbonate (Chem-Impex International, Inc.) (225 mg, 1.032 mmol, 1.1 equiv.) dissolved in dry THF (3 mL) was then added, and the reaction mixture allowed to stir vigorously for 12 h at room temperature. After 12 h, the reaction mixture was extracted with EtOAc (2 x 10 mL) and the combined organic layers were dried over Na_2SO_4, filtered, and concentrated to afford $^{15}N_2$-Boc hydrazide (83 mg, 66% yield) as a colorless oil. The crude product was used without further purification.

Acetic anhydride (65 µL, 0.681 mmol, 1.1 equiv.) was added to a solution of crude $^{15}N_2$-Boc hydrazide (83 mg, 0.619 mmol, 1 equiv.) dissolved in anhydrous pyridine (10 mL), and the reaction mixture stirred for 6 h at room temperature. After 6 h, the pyridine was removed under a stream of N_2 gas. The residue was then purified by silica gel chromatography (4:1 CH_2Cl_2 : MeOH) to afford $^{15}N_2$-Boc-acetylhydrazide (60 mg, 50% yield) as a colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm) = 2.00 (s, 3H, CH_3), 1.44 (s, 9H, CH_3). ^{13}C NMR (500 MHz, $CDCl_3$): δ (ppm) = 169.7, 155.8, 81.9, 28.0, 20.6. ^{15}N NMR (400 MHz, $CDCl_3$): δ (ppm) = 123.7, 96.2.

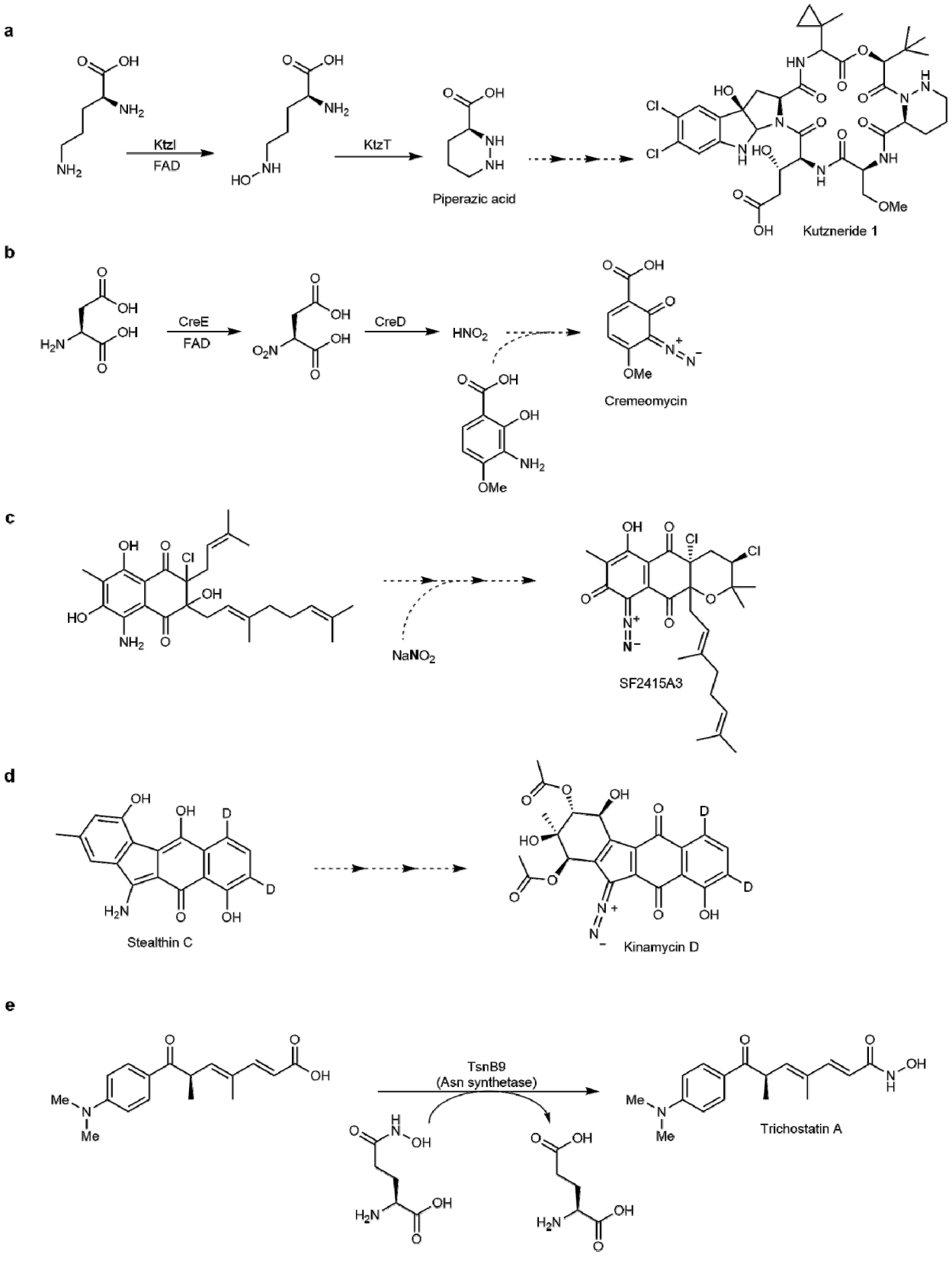
TFA (5 mL, 67.3 mmol, 198 equiv.) was added to $^{15}N_2$-Boc-acetylhydrazide (60 mg, 0.340 mmol, 1.0 equiv.) dissolved in anhydrous CH_2Cl_2 (5 mL). The reaction mixture was then stirred overnight at room temperature. After stirring overnight, the reaction was concentrated and the TFA removed *in vacuo.* The resulting residue was dried under vacuum to afford $^{15}N_2$-acetylhydrazine (57 mg, 0.300 mmol, 88% yield) as an oily solid. ^{1}H NMR (500 MHz, DMSO): δ (ppm) = 9.94 – 7.61 (br s, 3H), 1.93 (s, 3H. CH_3). ^{13}C NMR (500 MHz, DMSO): δ (ppm) = 169.2, 20.6. ^{15}N NMR (400 MHz, DMSO): δ (ppm) = 119.1, 44.5.

Synthesis of [$^{15}N_2$]-hydrazide 6

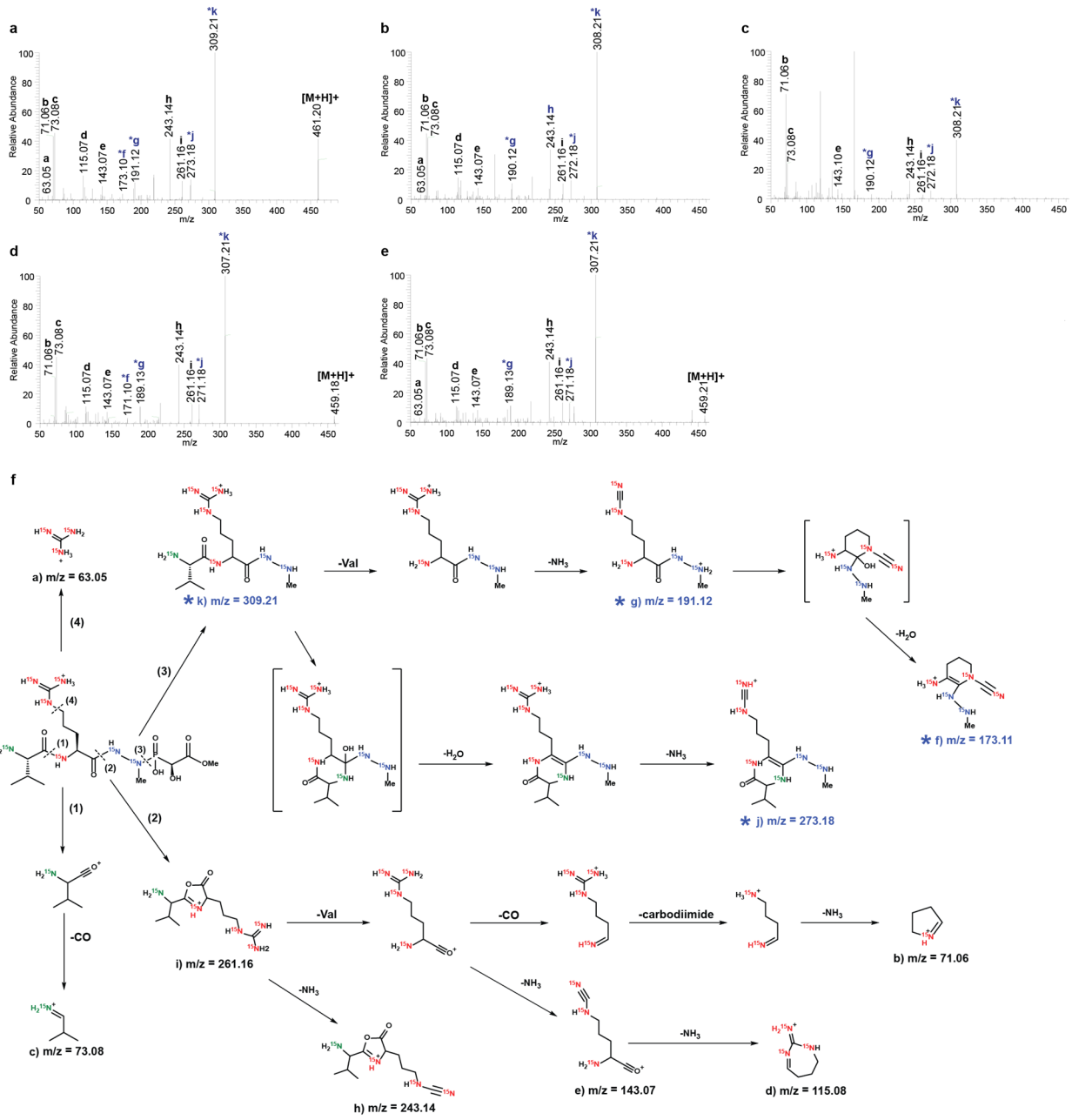


To a solution of $^{15}N_2$-Boc-hydrazide (87 mg, 0.649 mmol, 1.0 equiv.) in 10 mL of dry DCM was added Boc-L-glutamic acid 1-*tert* butyl ester (Ark Pharm, Inc.) (196 mg, 0.649 mmol, 1.0 equiv.), *N*,*N*-diisopropylethylamine (284 µL, 1.65 mmol, 2.5 equiv), and hydroxybenzotriazole (105 mg, 0.779 mmol, 1.2 equiv.). The reaction mixture was cooled on ice for 5 min and then 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (149 mg dissolved in 5 mL of DCM, 0.779 mmol, 1.2 equiv.) was added dropwise. The reaction mixture was then allowed to stir to room temperature overnight. After stirring overnight, the reaction mixture was concentrated *in vacuo*. The resulting residue was dissolved in EtOAc (20 mL) and then washed with 1 M HCl (10 mL), sat. aq. $NaHCO_3$ (10 mL), and brine (10 mL). The organic layer was dried over Na_2SO_4, filtered, and the filtrate was concentrated *in vacuo*. The resulting crude oil was then purified via silica gel chromatography (1:1 hexanes : EtOAc) to afford $^{15}N_2$-Boc-hydrazide **6a** (201 mg, 74% yield) as a colorless oil.

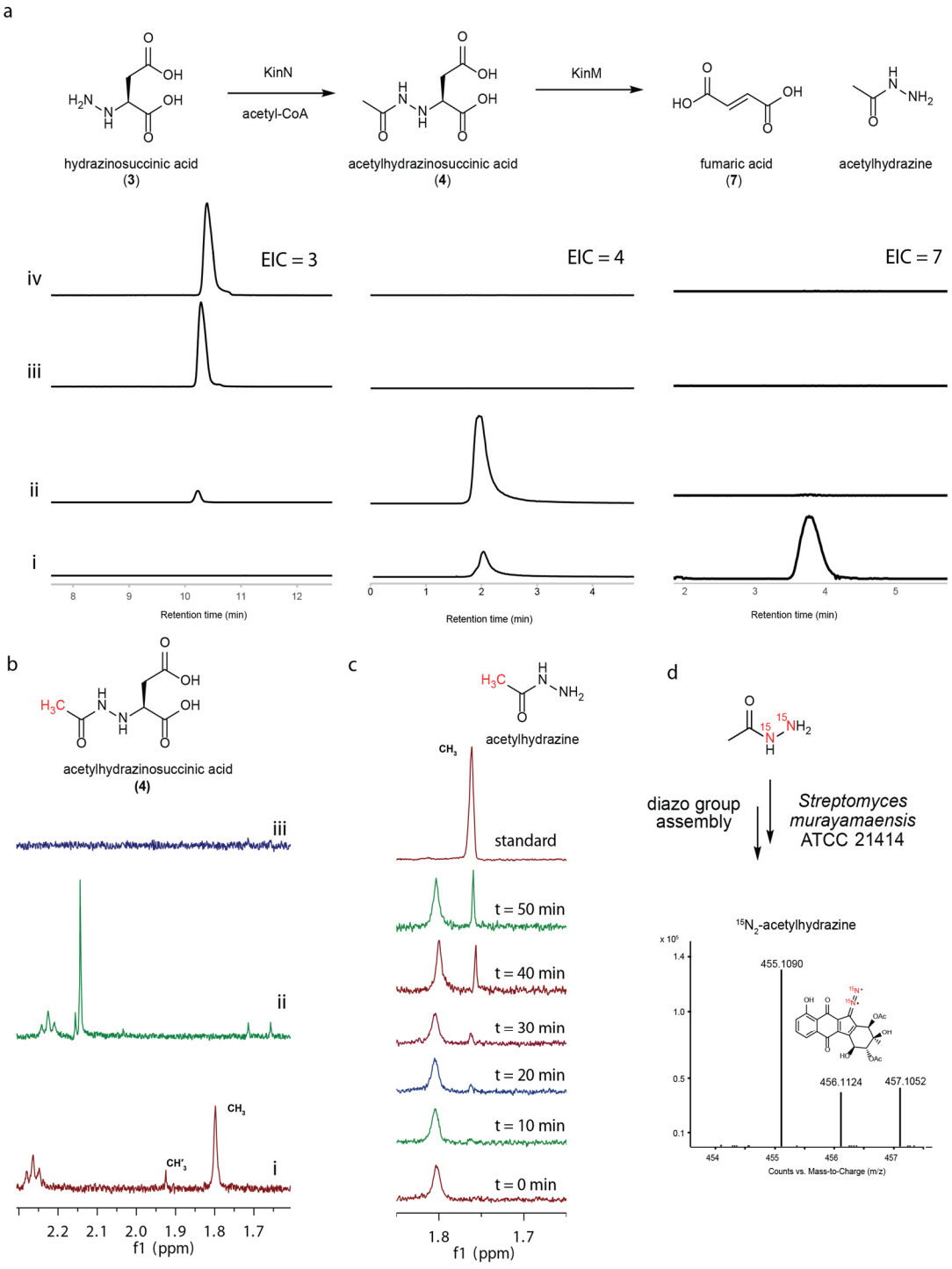
TFA (5 mL, 67.3 mmol, 140 equiv.) was added to a solution of hydrazide **6a** (201 mg, 0.480 mmol, 1.0 equiv.) in anhydrous DCM (5 mL), and the reaction mixture was allowed to stir overnight at room temperature. Once the reaction was deemed complete by TLC, the reaction was concentrated *in vacuo*. The resulting residue was then triturated with ether, filtered, and dried under vacuum to afford $^{15}N_2$-hydrazide **6** (116 mg, 61% yield) as a white solid. ^{1}H NMR (500 MHz, DMSO): δ (ppm) = 8.41 (br s, 2H), 3.95 (t, J=6.40 Hz, 1, 2.48 – 2.24, (m, 2H, CH_2), 2.08 – 1.92 (m, 2H, CH_2). ^{13}C (500 MHz, DMSO): δ (ppm) = 171.1, 159.0, 51.7, 28.8, 25.8. ^{15}N NMR (400 MHz, DMSO): δ (ppm) = 118.9, 51.7. HRMS: Calc'd for $C_5H_{11}{}^{15}NN_2O_3$ $[M+H]^+$ = 164.0814, found = 164.0835.


a
KtzI
FAD
KtzT
Piperazic acid
Kutzneride 1
b
CreE
FAD
CreD
HNO2
Cremeomycin
c
NaNO2
SF2415A3
d
Stealthin C
Kinamycin D
e
TsnB9
(Asn synthetase)
Trichostatin A

Supplementary Figure 1 Reconstituted or proposed biosynthetic pathways for the natural products discussed in the text. **(a)** Biosynthesis of the piperazic acid moiety in kutzneride. A flavin-dependent enzyme (KtzI) activates ornithine for an intramolecular attack catalyzed by a heme protein (KtzT) to form the N-N bond[1]. **(b)** Nitrous acid is formed from aspartic acid during cremeomycin biosynthesis before a proposed diazotization reaction to form the N-N bond[2]. **(c)** Labeling studies with ^{15}N-nitrite show incorporation of ^{15}N into the distal nitrogen in the diazo group of SF2415A3[3]. **(d)** Stealthin C was proposed to be an intermediate in kinamycin biosynthesis on the basis of a labeling study using deuterated stealthin C[4]. **(e)** TsnB9, an asparagine synthetase homolog, transfers hydroxylamine from the side chain of glutamic acid to complete the biosynthesis of trichostatin[5].

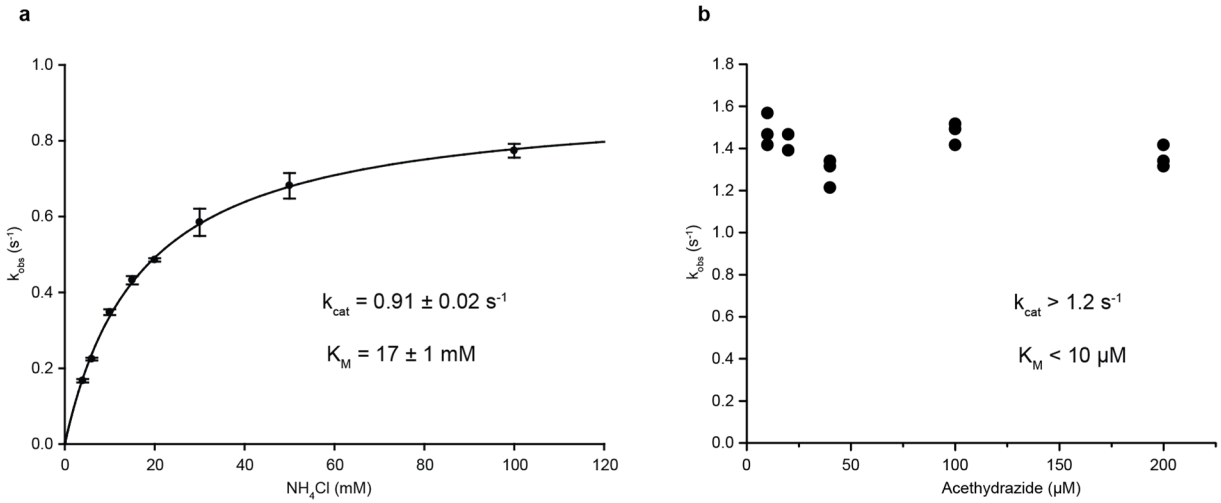


Supplementary Figure 2 MS/MS analysis of fosfazinomycin A from *Streptomyces* sp. NRRL S-149. **(a)** MS/MS spectrum of uniformly ^{15}N-labeled fosfazinomycin A (precursor ion m/z = 461.20). **(b, c)** MS/MS spectra of fosfazinomycin with one ^{14}N-incorporation (precursor ion m/z = 460.20) from ^{15}N-labeled media with added $NaNO_2$ **(b)** or N-hydroxyaspartic acid **(c)** at natural abundance. **(d, e)** MS/MS spectra of fosfazinomycin A with two ^{14}N-incorporations (precursor ion m/z = 459.20) from ^{15}N-labeled media with added hydrazinosuccinic acid **(d)** or acetylhydrazine **(e)**, at natural abundance. **(f)** The proposed fragmentation pathway of uniformly ^{15}N-labeled fosfazinomycin with assigned ions. Product and fragment ions containing the N-N bond are indicated by asterisks and blue lettering.

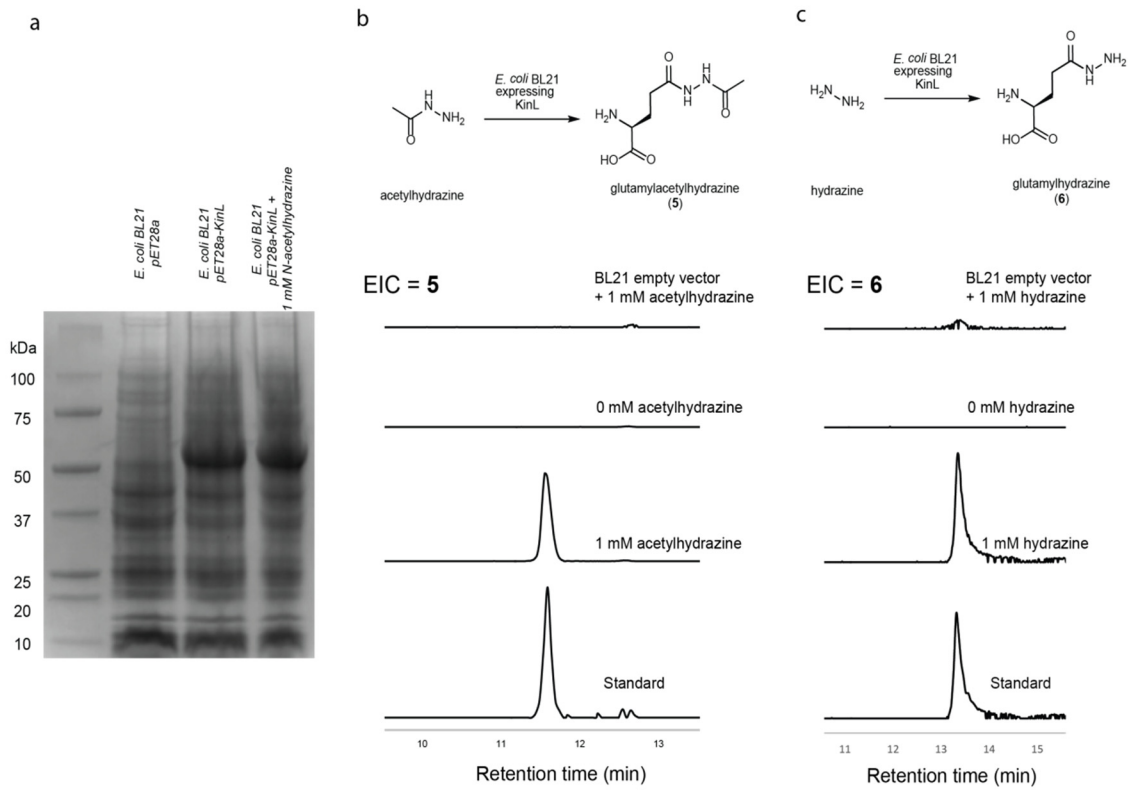


Supplementary Figure 3 *in vitro* generation of acetylhydrazine by KinNM and *in vivo* feeding experiment with acetylhydrazine. **(a)** EIC for *N*-hydrazinosuccinic acid (**3**, $[M-H]^-$ = 147.0411), acetylhydrazinosuccinic acid (**4**, $[M-H]^-$ = 189.0517), and fumaric acid (**7**, $[M-H]^-$ = 115.0037). i. **3** + acetyl-CoA + KinNM, ii. **3** + acetyl-CoA + KinN, iii. **3** + acetyl-CoA, iv. **3** + KinNM. **(b)** 1D ^{1}H-NMR spectrum of KinM assay mixtures. i. **3** + acetyl-CoA + KinN, ii. **3** + acetyl-CoA, iii. **3** + KinN. **(c)** 1D ^{1}H-NMR spectrum of acetylhydrazine

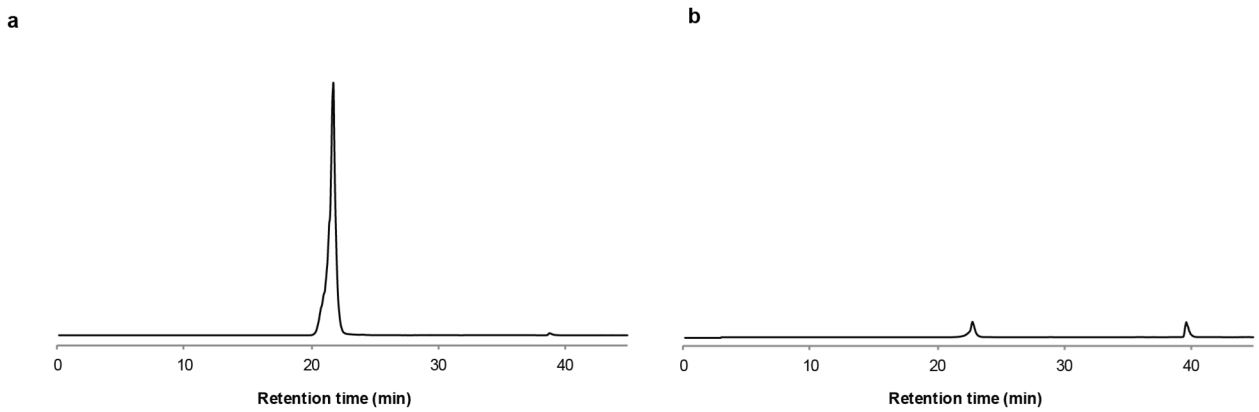
generation from **4** with KinM. (**d**) HRMS of kinamycin D when *S. murayamaensis* is fed $^{15}N_2$-acetylhydrazine.



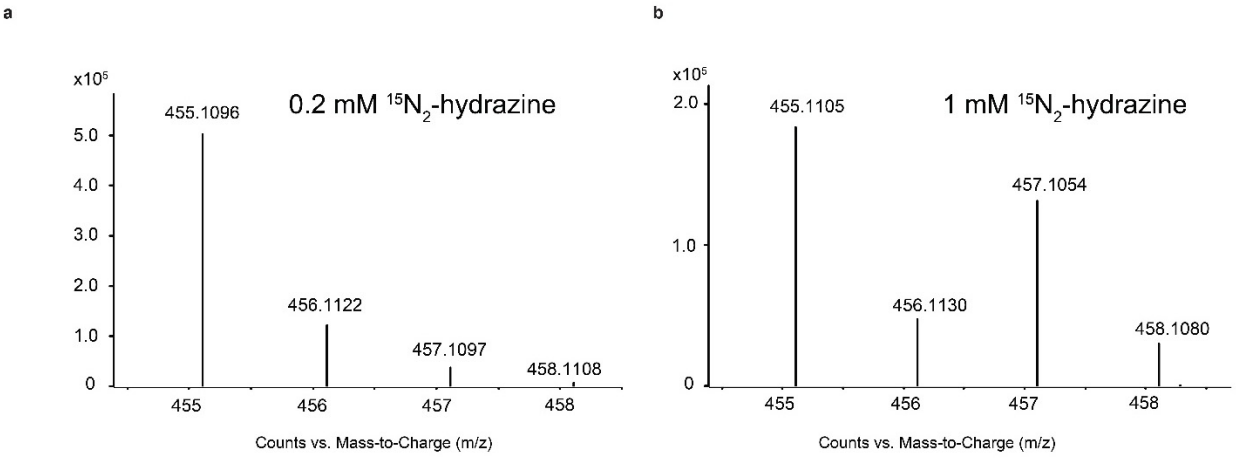
Supplementary Figure 4 Kinetic analysis of FzmN-catalyzed reactions. **(a)** Michaelis-Menten plot of FzmN catalyzed Gln-formation from Glu, NH_4Cl, ATP, and $MgCl_2$. The data points represent the average values obtained from triplicate experiments, and the error bars indicate the standard deviation. **(b)** Attempted kinetic analysis of the FzmN-catalyzed formation of glutamylacetylhydrazine from Glu, acetylhydrazine, NH_4Cl, ATP, and $MgCl_2$. Triplicate experiments are plotted.



Supplementary Figure 5 *in vivo* activity assay of KinL. **(a)** SDS-PAGE of cell lysate demonstrating heterologous expression of KinL in *E. coli* BL21 (DE3) in the presence and absence of acetylhydrazine. The molecular weight for *N*-terminal His_6-tag KinL is 55.8 kDa. **(b)** KinL ligates acetylhydrazine to glutamic acid *in vivo* to afford **5**. EIC for **5** ($[M-H]^-$ = 202.0833). (**c**) KinL ligates hydrazine to glutamic acid *in vivo* to afford **6**. EIC for **6** ($[M-H]^-$ = 160.0728).



Supplementary Figure 6 LC-MS analysis of the FzmA-catalyzed reaction. The mass of Fmoc-derivatized glutamic acid ([M-H]$^-$ = 368) was monitored. **(a)** Chromatogram (selected ion monitoring for m/z = 368) of the reaction after Fmoc-derivatization. **(b)** Chromatogram (selected ion monitoring for m/z = 368) of the reaction mixture with FzmA omitted.



Supplementary Figure 7 HRMS analysis of $^{15}N_2$-hydrazine-labeled kinamycin D from *Streptomyces murayamaensis* ATCC 21414. HRMS analysis of ^{15}N incorporation into kinamycin D from feeding either (**a**) 0.2 mM or (**b**) 1 mM $^{15}N_2$-hydrazine to fermentation cultures of *S. murayamaensis*. $^{15}N_2$-kinamycin D ([M+H]$^+$ = 457.1026).

Supplementary Table 1 Oligonucelotides and synthetic gene used for cloning. The primers are named for the amplicon and direction of amplification: forward (fwd or F) or reverse (rev or R); uppercase letters indicate portions that anneal to the amplicon while lowercase letters indicate the overhang regions for Gibson assembly

Cloning primer	**Sequence**
FzmN_fwd	gcagcggcctggtgccgcgcggcagccatATGACGAAGCCCACCACCGCGCGGGAAGCC
FzmN_rev	cctttcgggctttgttagcagccggatcctcgagTCAGTAGGCGCCGAAGTACTCACGGTG
FzmO_fwd	gcagcggcctggtgccgcgcggcagccatATGGCGCCTGACCTGCCCGCCCCACCT
FzmO_rev	cctttcgggctttgttagcagccggatcctcgagTCATGTCGATTCCTCTTCCGGTTCCGGCGAT
FzmA_fwd	tttaagaaggagatatacatATGTGCGGAATTGCAGGATTCG
FzmA_rev	gatctcagtggtggtggtggtgctcgagTCCGGCGCTGTCGACCGT
pET15b_fwd	CTCGAGGATCCGGCTGCTAACAAAGCCCGAAAGG
pET15b_rev	CATATGGCTGCCGCGCGGCACCAGGCCGCTG
AspB_fwd	agcggcctggtgccgcgcggcagccatatgATGAATACGGATGTGCGTATTGAAA
AspB_rev	cctttcgggctttgttagcagccggatcctcgagTCACTTACGGCCTGCAATG
Kin1K-F	ctggtgccgcgcggcagccatGTGCAGTCGGTCCTCACCGC
Kin1K-R	tcgagtgcggccgcaagcttTTACAGGATCCTTTCGGTTA
Kin1L-F	ctggtgccgcgcggcagccatATGTACTCCCGGTCGTGGT
Kin1L-R	ctcgagtgcggccgcaagcttTCAGAAGGCCTCGAAGTATT
KinM-F	ctggtgccgcgcggcagccatATGATTCCCCGCTATACCC
KinM-R	cgagtgcggccgcaagcttTCAGTCGTCCAGGTCACGAAG
KinN-F	ctggtgccgcgcggcagccatGTGACCTGGACGACTGAGC
Kin1N-R	cgagtgcggccgcaagcttCTACGGGTTGAGCACCCAGGC
Kin1J-F	ctggtgccgcgcggcagccatATGACAAGTTCCGCCGAGC
Kin1J-R	ctcgagtgcggccgcaagcttTCACGAGACCTGCTGCGGC
pET28a-F	AAGCTTGCGGCCGCACTCGAG
pET28a-R	ATGGCTGCCGCGCGGCACCAG
aspB	atgaatacggatgtgcgtattgaaaaagattttctgggcgaaaaagaaattccgaaagatgcatattatggcgtgcaaacgat tcgtgcaacggaaaattttccgattacgggctatcgtattcatccggaactgattaaaagcctgggcattgtgaaaaaaagcgc agcactggcaaatatggaagtgggcctgctggataaagaagtgggccaatatattgtgaaagcagcagatgaagtgattga aggcaaatggaatgatcaatttattgtggatccgattcaaggcggcgcaggcacgagcattaatatgaatgcaaatgaagtg attgcaaatcgtgcactggaactgatgggcgaagaaaaaggcaattatagcaaaattagcccgaatagccatgtgaatatg agccaaagcacgaatgatgcatttccgacggcaacgcatattgcagtgctgagcctgctgaatcaactgattgaaacgacg aaatatatgcaacaagaatttatgaaaaaagcagatgaatttgcaggcgtgattaaaatgggccgtacgcatctgcaagatg cagtgccgattctgctgggccaagaatttgaagcatatgcacgtgtgattgcacgtgatattgaacgtattgcaaatacgcgta ataatctgtatgatattaatatgggcgcaacggcagtgggcacgggcctgaatgcagatccggaatatattagcattgtgacg gaacatctggcaaaatttagcggccatccgctgcgtagcgcacaacatctggtggatgcaacgcaaaatacggattgctata cggaagtgagcagcgcactgaaagtgtgcatgattaatatgagcaaaattgcaaatgatctgcgtctgatggcaagcggcc cgcgtgcaggcctgagcgaaattgtgctgccggcacgtcaaccgggcagcagcattatgccgggcaaagtgaatccggtg atgccggaagtgatgaatcaagtggcatttcaagtgtttggcaatgatctgacgattacgagcgcaagcgaagcaggccaat ttgaactgaatgtgatggaaccggtgctgttttttaatctgattcaaagcattagcattatgacgaatgtgtttaaaagctttacgga aaattgcctgaaaggcattaaagcaaatgaagaacgtatgaaagaatatgtggaaaaaagcattggcattattacggcaatt aatccgcatgtgggctatgaaacggcagcaaaactggcacgtgaagcatatctgacgggcgaaagcattcgtgaactgtgc attaaatatggcgtgctgacggaagaacaactgaatgaaattctgaatccgtatgaaatgacgcatccgggcattgcaggcc gtaagtga

Supplementary Table 2 Buffer conditions used for refolding FzmO. The 216 solutions were made by combinatorially combining each element in a column with one other element from each of the other columns. Conditions were adapted from another study[7].

Buffer (50 mM)	Glycerol	NDSB 256	Salt	Arginine	Glucose
HEPES, pH=7.0	20%	100 mM	300 mM NaCl	800 mM	250 mM
HEPES, pH=8.0	0%	0 mM	100 mM NaCl	400 mM	0 mM
CHES, pH=9.0			100 mM KCl	0 mM	

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