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Supporting Information

Biosynthesis of Piperazic Acid via N^5-Hydroxy-Ornithine in *Kutzneria* spp. 744

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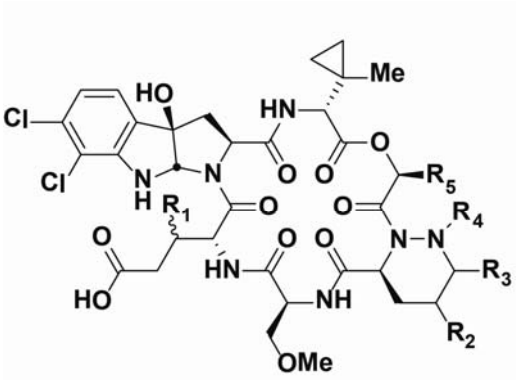
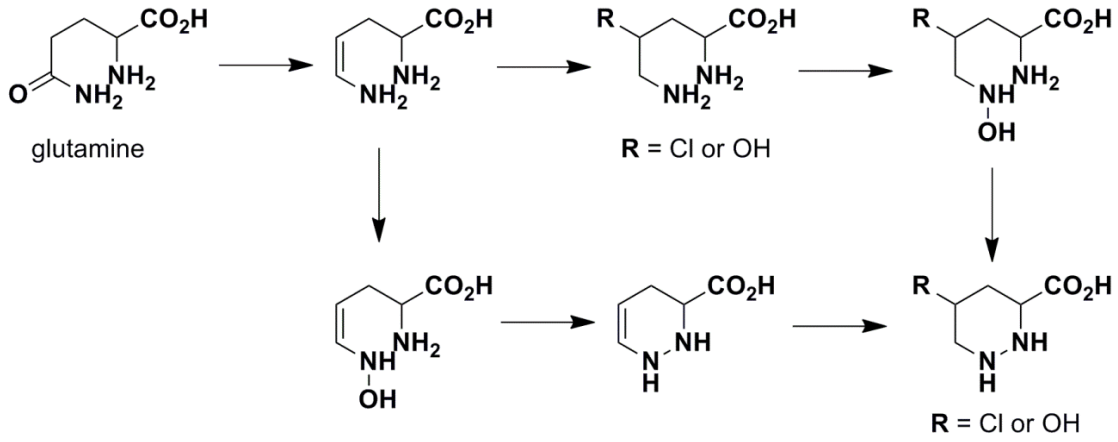


	R_1	R_2	R_3	R_4	R_5
Kutzneride 1	(S)-OH	H	H	H	*t*-Bu
Kutzneride 2	(S)-OH	Cl	H	H	*t*-Bu
Kutzneride 3	(R)-OH	H	H	H	*t*-Bu
Kutzneride 4	(R)-OH	H	π-bond		*t*-Bu
Kutzneride 5	(R)-OH	H	H	H	*i*-Pr
Kutzneride 6	(R)-OH	OH	π-bond		*t*-Bu
Kutzneride 7	(S)-OH	H	H	H	*i*-Pr
Kutzneride 8	(R)-OH	Cl	H	H	*t*-Bu
Kutzneride 9	(S)-OH	H	π-bond		*t*-Bu

Figure S1: Nine kutzneride derivatives have been isolated and characterized from the broth of Kutzneria spp. 744.[1] Kutznerides 1 and 3, kutznerides 4 and 9, and kutznerides 2 and 8 are each diastereomeric pairs varying in the stereochemistry of the R_1 hydroxyl group.



Scheme S1: Early hypotheses for biosynthesis of substituted piperazates begin with reduction and dehydration of glutamine to give an ornithine-like enamine intermediate.[2] The ordering of N^5-hydroxylation (Orn numbering) and tailoring at C5 (Cl or OH, Piz numbering) distinguish two possible pathways.

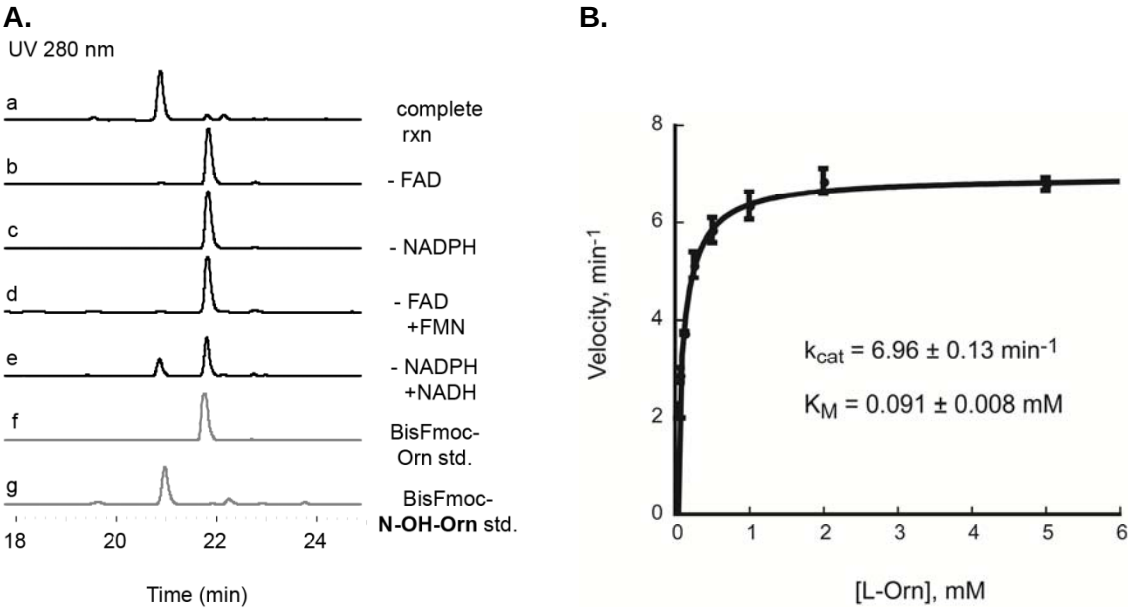


Figure S2: A) HPLC-UV_{280} analysis of in vitro KtzI assays performed as described below and derivatized with Fmoc chloroformate. A complete reaction (KtzI, NADPH, FAD, O_2, and ornithine) generates N^5-OH-Orn as the sole product (trace a). Removal of the FAD cofactor or the NADPH cosubstrate abrogates activity (traces b and c). Substitution of FMN for FAD is not tolerated (trace d), though substitution of NADH for NADPH results in a modest level of activity (trace e). Derivatized standards of Orn and N^5-OH-Orn are shown for reference (traces f and g). B) Pseudo-first order kinetic parameters were obtained with 0.5 μM KtzI and varying concentrations of L-Orn substrate as described in Biochemical Methods.

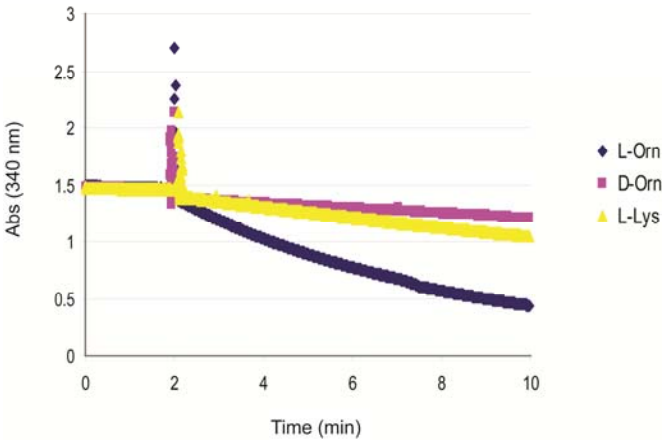


Figure S3: A. Levels of NADPH are measured during enzymatic reactions by absorbance at 340 nm. Background levels of NAPDH oxidation are determined from 0 to 2 minutes when amino acid substrates are not present. Addition of L-Orn at 2 min results in accelerated NADPH oxidation corresponding to formation of N^5-OH-Orn. D-Orn and L-Lys also increase NADPH oxidation rates, but do not serve as substrates for the enzyme (data not shown).

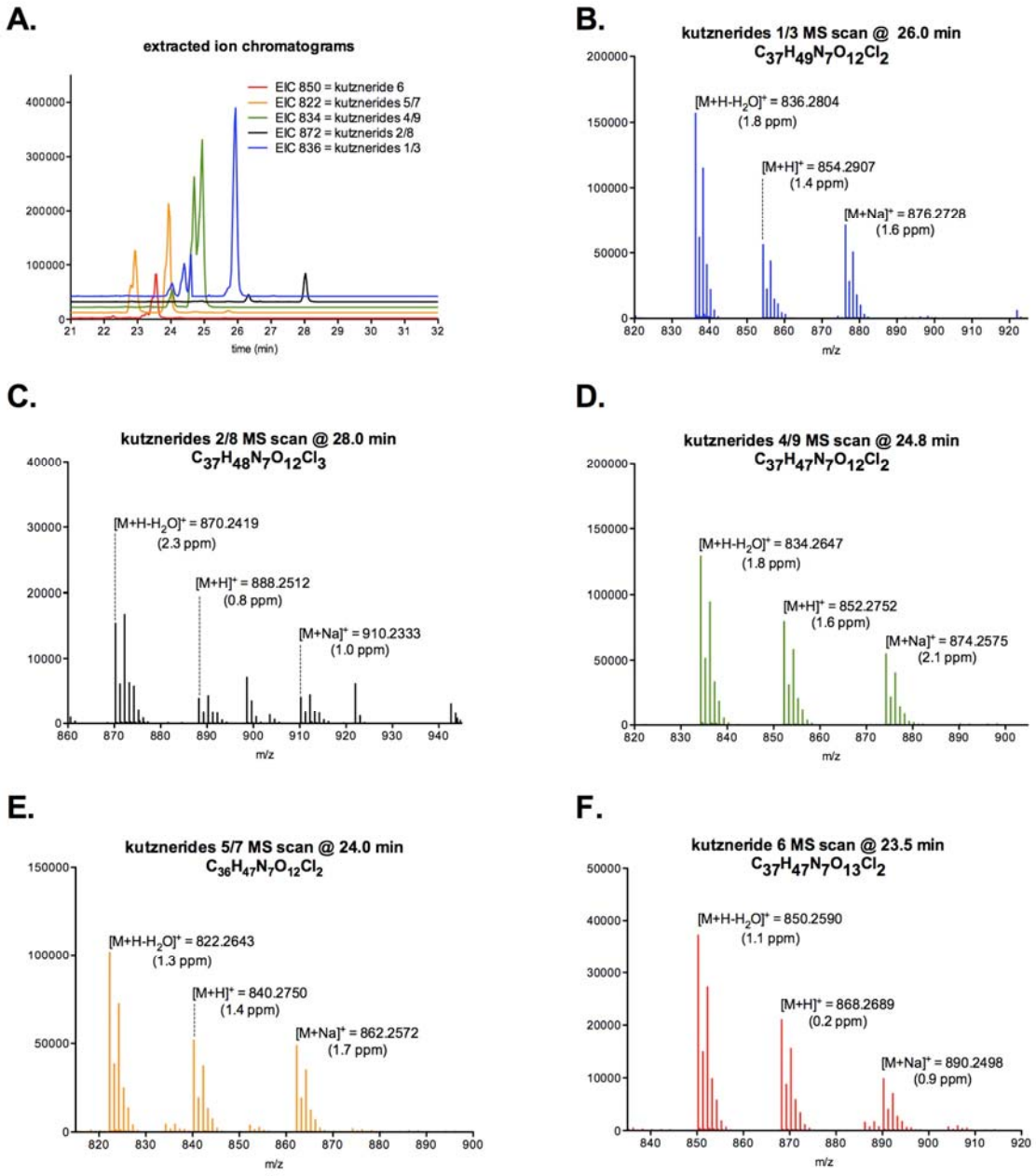


Figure S4: A) Extracted ion chromatograms for kutznerides. B-F) MS spectra for each set of isomers. The compounds predominantly ionize as the $[M+H-H_2O]^+$ species, though the $[M+H]^+$ and $[M+Na]^+$ species are also evident. Diastereomers (e.g., kutznerides 1 and 3) are not readily distinguished based on MS data alone and were processed as one species. For all panels, Y-axis is in units of spectral counts.

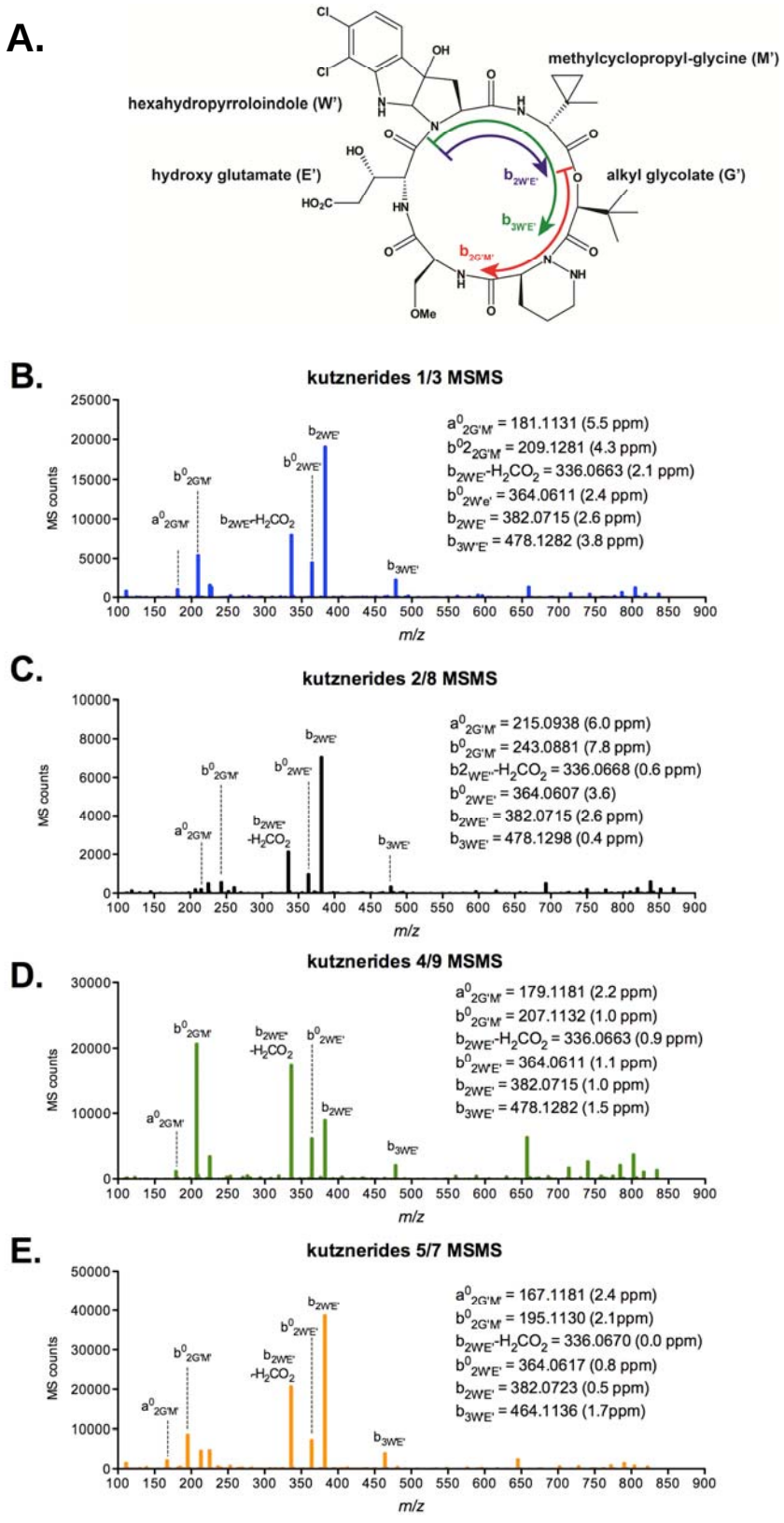
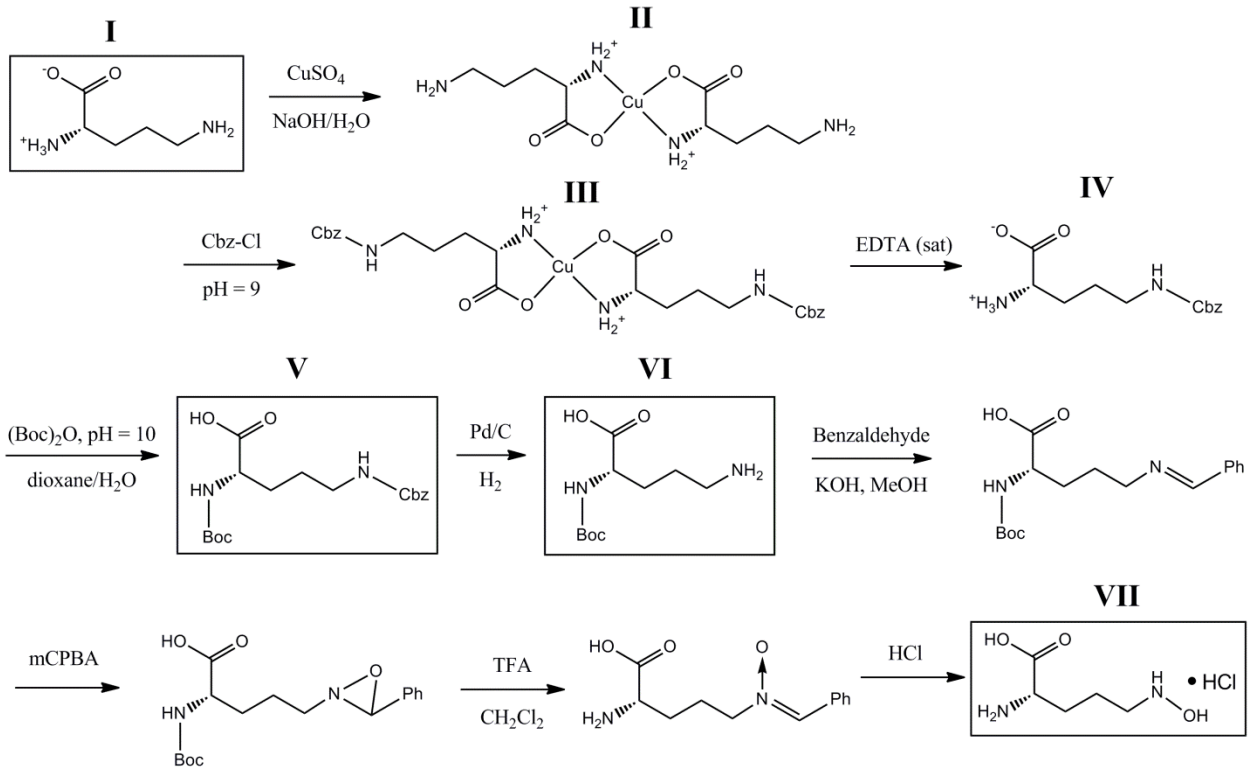


Figure S5: A) Key fragmentations of the kutzneride molecules annotated using nomenclature guidelines.[3] One-letter abbreviations of residues used for annotations are noted. B-E) MSMS spectra of kutznerides. Annotated peaks are ions described in panel A or neutral losses thereof: b^0 ions are neutral losses of H_2O from b ion, a ions are neutral losses of CO from b ion.



Scheme S2: Preparation of N^5-OH-Orn (**VII**) from Orn (**I**). Transformation of Orn to N^2-Boc-Orn (**VI**) follows the methods of Shao et al as applied to lysine.[4] Transformation of **VI** to **VII** follows the method of Heemstra et al.[5]

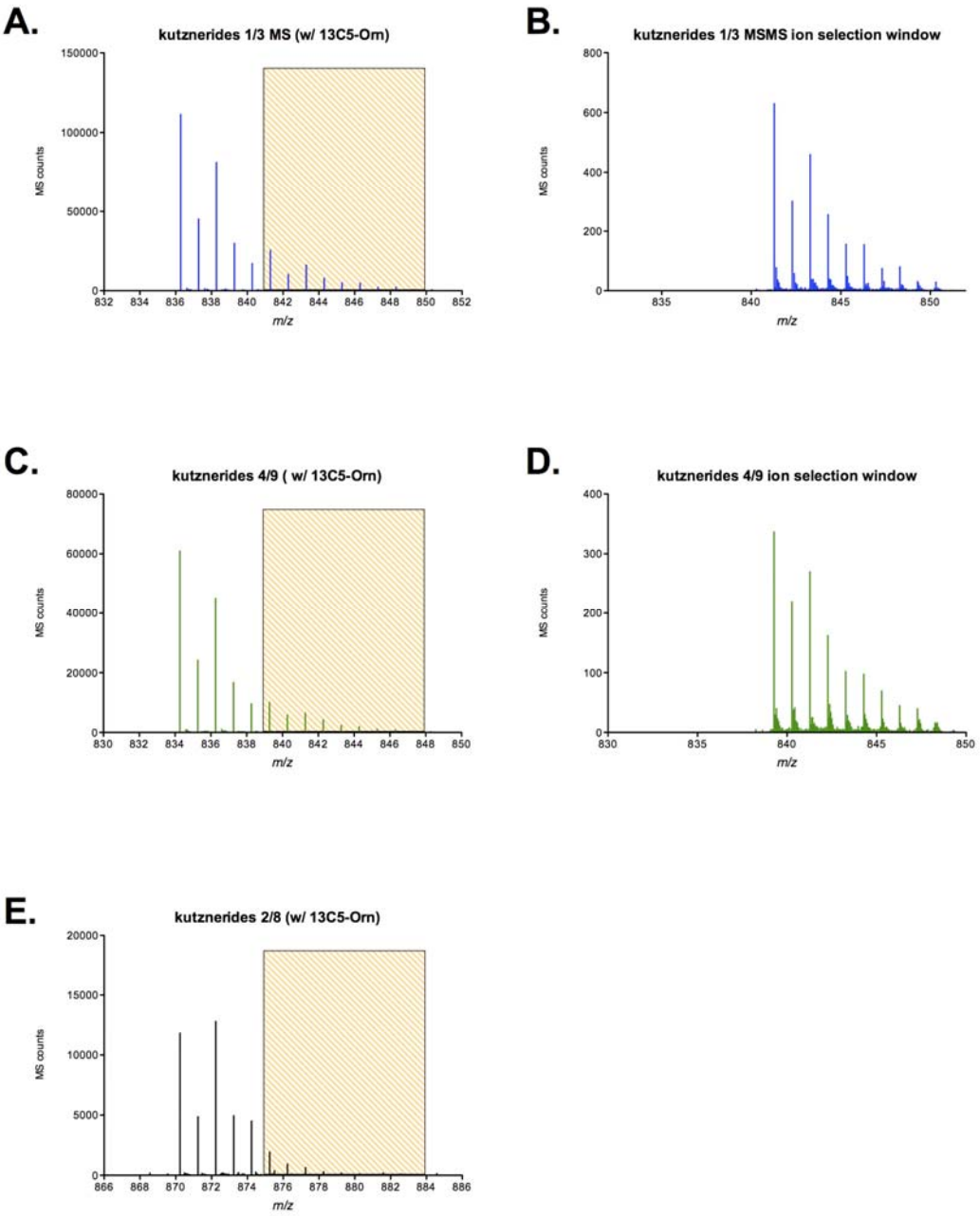


Figure S6: A) MS spectrum of kutznerides 1/3 following feeding of $^{13}C_5$-Orn (30 mg). Region of parent ion selection for subsequent MSMS analysis is indicated with shading (~9 *m/z* range). Label incorporation is estimated at 18%. B) MSMS spectrum with 0 V collision energy, demonstrating selection of only +5 labeled compounds and above. C) MS spectrum of kutznerides 4/9 under same experimental conditions. Label incorporation is estimated at 14%. D) MSMS spectrum with 0 V collision energy, demonstrating ion selection window. E) MS spectrum of kutznerides 2/8 under same experimental conditions. Labeling was not apparent in MS spectrum. No MSMS spectra were collected at 0 V to maximize collection of fragmented spectra.

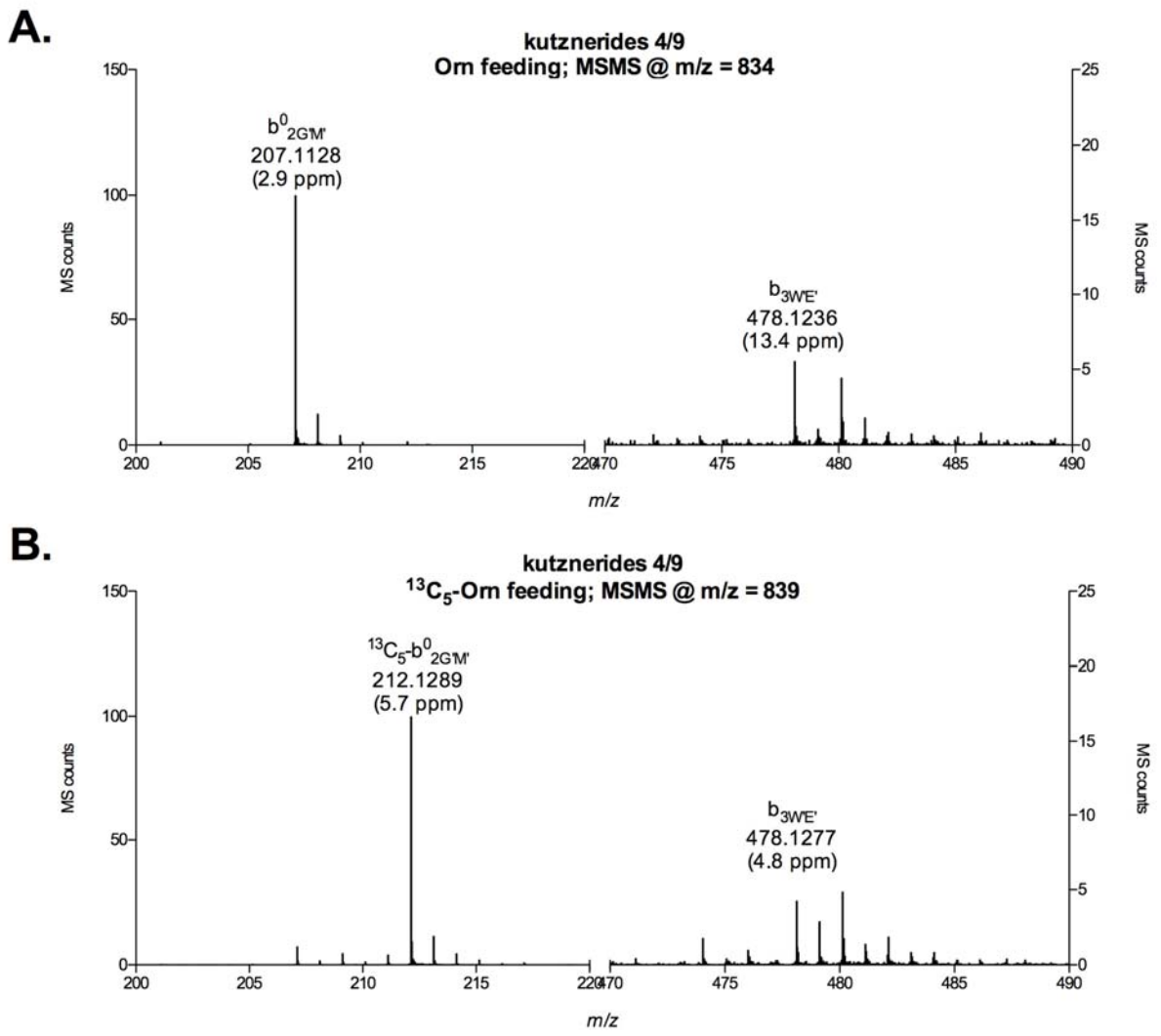


Figure S7: A) MSMS spectrum of kutznerides 4/9 with natural isotope abundance. B) MSMS spectrum of kutznerides 4/9 labeled by $^{13}C_5$-Orn. Label is incorporated into the $b^{o}_{2G'M'}$ daughter ion (*m*/*z* = 212.1289) but not the $b_{3W'E'}$ ion at *m*/*z* = 478.1277 (see Figure S5). Based on this observation, we conclude that the $^{13}C_5$-label is specifically located in the Piz residue.

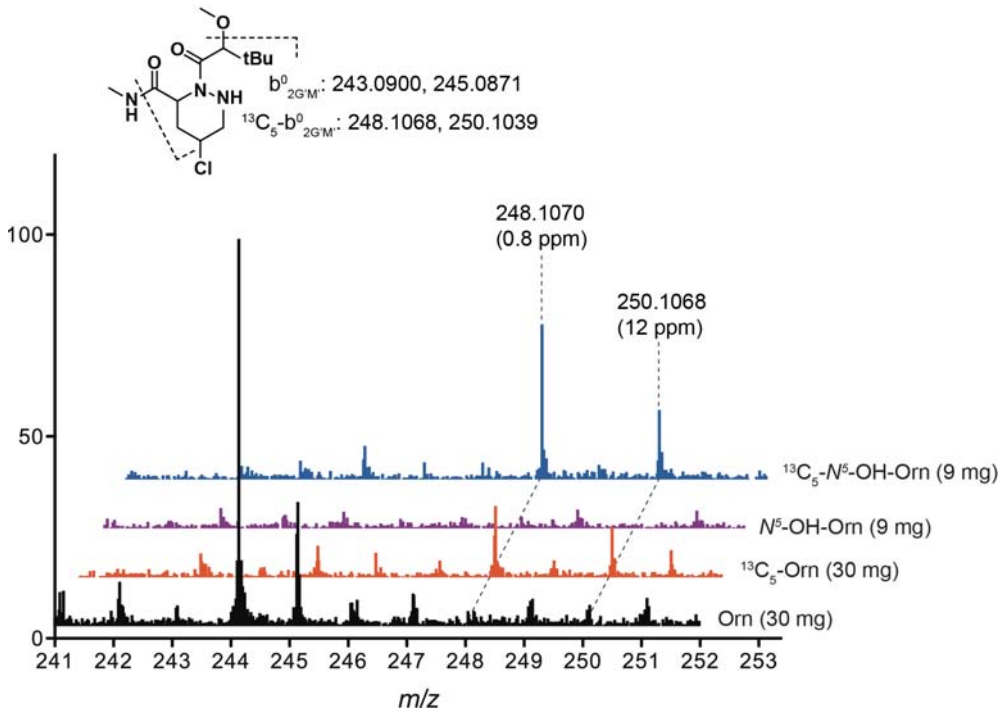


Figure S8: Labeling of Cl-Piz residue in kutznerides 2/8 was evident in the experiment with highest overall label incorporation. Both $^{13}C_5$-Orn and $^{13}C_5$-N^5-OH-Orn show incorporation into the $b^o_{G'M'}$ ion.

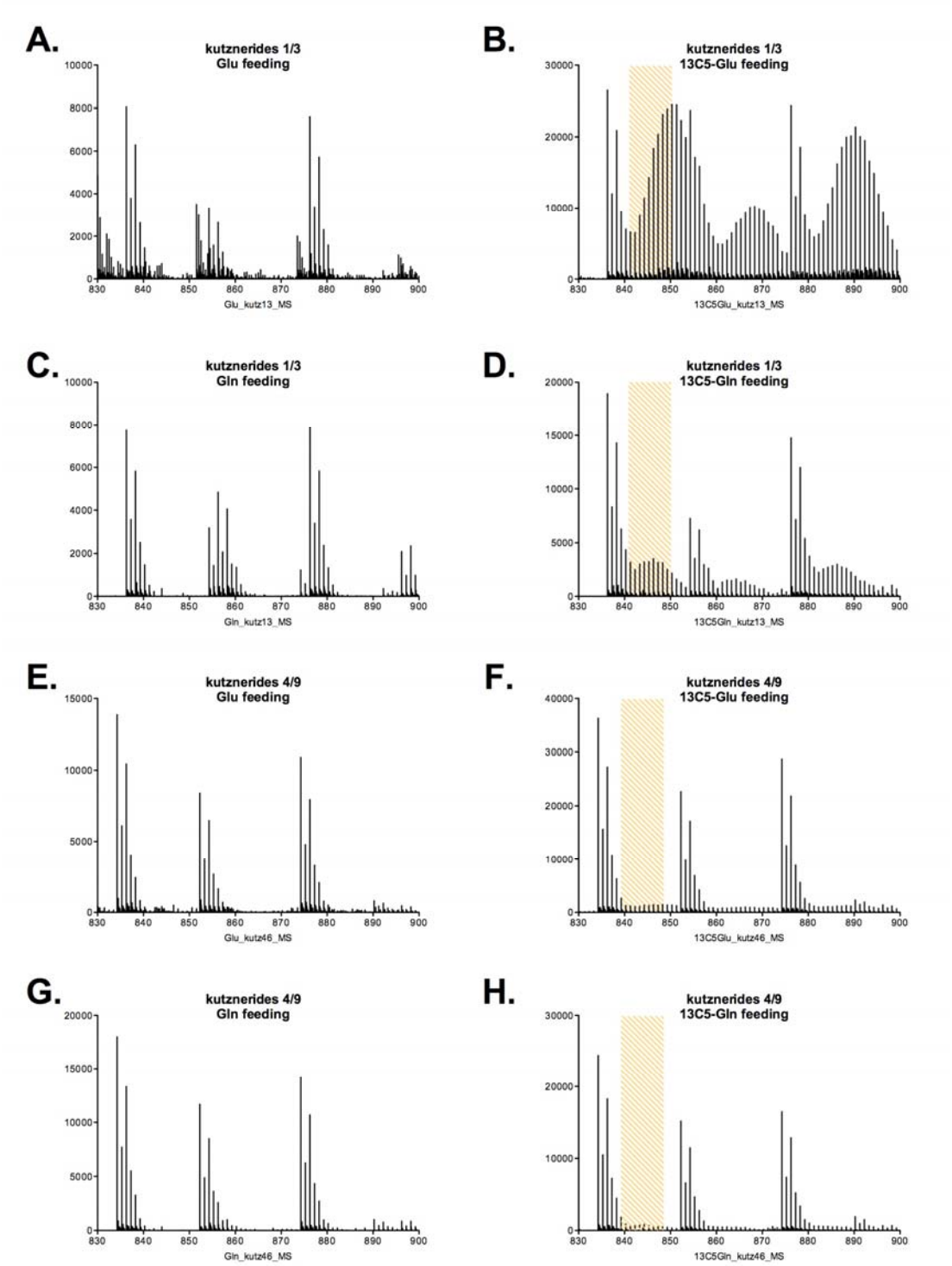


Figure S9: MS spectra of kutznerides 1/3 (panels **A**-**D**) and kutznerides 4/6 (panels **E**-**H**) following feeding of unlabeled and $^{13}C_5$-labeled Glu and Gln as indicated. Yellow shading area indicates zone of MSMS analysis (see Figure 4 in text and Figure S8 below).

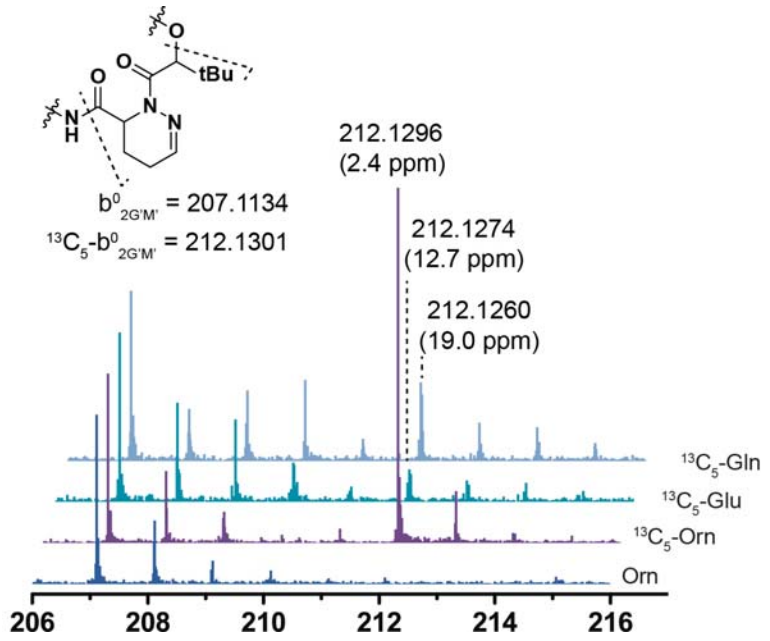


Figure S10: LC-MSMS analysis of kutznerides 4 and 9 following precursor feeding with Orn, $^{13}C_5$-Orn, $^{13}C_5$-Glu, and $^{13}C_5$-Gln. The $^{1\,3}C_5$-$b^0_{2P'S'}$ ion (*m*/*z* = 212.1296) is readily apparent with $^{13}C_5$-Orn feeding, and weakly visible with $^{13}C_5$-Glu and $^{13}C_5$-Gln feedings. Spectra are normalized to unlabeled $b^0_{2P'S'}$ ion at *m*/*z* = 207.1129.

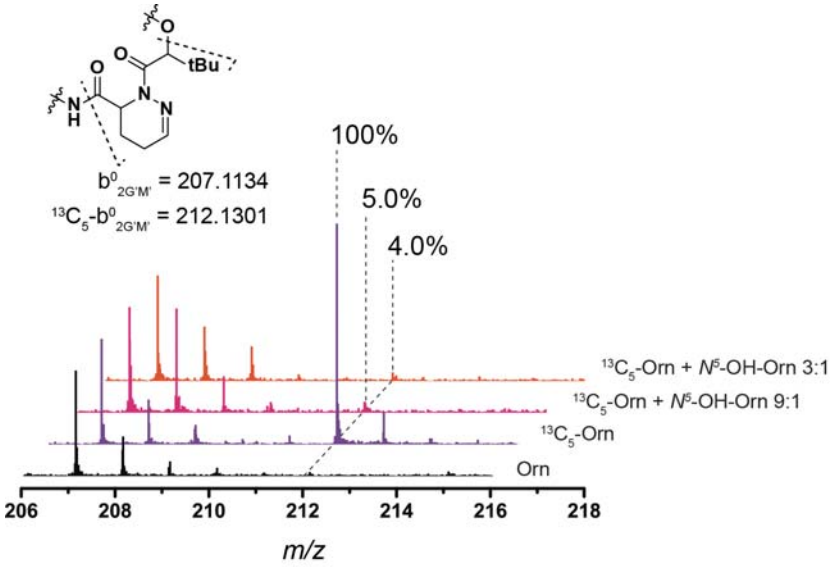


Figure S11: LC-MSMS analysis of kutznerides 4 and 9 following label suppression of $^{13}C_5$-Orn with increasing amounts of N^5-OH-Orn. At 9:1 (30 mg : 3.3 mg) and 3:1 (30 mg : 10 mg) ratios of $^{13}C_5$-Orn to N^5-OH-Orn, incorporation of a $^{13}C_5$-unit into the $b^0_{2G'M'}$ ion is almost completely suppressed. Spectra are normalized to signal at ~207.1128 (unlabeled $b^0_{2G'M'}$ ion). Percentages indicate signal intensity at ~212.1296 relative to $^{13}C_5$-Orn feeding.

2. Biochemical Methods

***2a. KtzI cloning and purification*:** The complete *ktzI* gene was PCR-amplified from a fosmid containing a portion of the kutzneride gene cluster.[6] Phusion DNA polymerase (New England Biolabs) was used according to manufacturer's protocol. Forward (aatcaatcatatggtggcgcacgccggtg) and reverse (gatcctcgagtcaactcttgcgtcgctc) primers (Integrated DNA Technologies, Coralville, IA) contained restriction sites for Nde and XhoI, respectively. The digested amplicon was ligated into similarly-digested pET28b vector (Novagen) to generate a gene fusion with the N-terminal hexahistidine tag. The construct was verified by bidirectional sequencing and then transformed into *E. coli* BL21 (DE3) cells (Invitrogen). Protein was overproduced by induction with IPTG at 16 °C. Purification from the soluble fraction of lysed cells was achieved by Ni-NTA affinity chromatography and gel filtration. KtzI-containing fractions were pooled and concentrated using Amicon Ultra centrifuge filters (Milliopore, Billerica, MA). Final yields were 7-8 mg per liter of bacterial culture with >95% purity.

After Ni-NTA chromatography, the enzyme purified with FAD as bound cofactor, as determined by boiling the protein and analyzing the supernatant by analytical HPLC using authentic standards. Using UV-Vis spectroscopy, the FAD occupancy was determined to be 22%. However, after gel filtration, the purified protein did not contain FAD as determined by the lack of an absorbance peak at 450 nm.

2b. N-hydroxylation assay: To a solution of L-ornithine (500 μM), FAD (50 μM), NADPH (2 mM) and pH 8.0 Tris buffer (50 mM) at 25 °C was added KtzI (5 μM) to initiate the reaction (total reaction volume of 50 μL). After 45 min the reactions were halted with MeCN (100 μL), chilled at −20 °C for 10 min and centrifuged at 13,000 rpm for 5 min. The supernatant was withdrawn and combined with 20 μL of 10 mM 9-fluorenylmethyl chloroformate (Fmoc-Cl) and the reaction was allowed to proceed at room temperature for 5 min. To remove any remaining derivatizing reagent, 20 mL of 0.1 M 1-aminoadamantane was added and the mixture was kept at room temperature for an additional 10 min. Conversion was determined by reverse phase HPLC analysis on a Supleco DiscoveryC18 column (250 × 4.6 mm) using a solvent gradient of 20 to 100% B over 40 min (solvent A, 0.1% TFA/H2O; solvent B, 0.1% TFA/MeCN). Product identity was confirmed by coelution with a N^5-hydroxy-L-ornithine synthetic standard Fmoc-derivatized in the same manner and by ESI-HRMS.

2c. NADPH oxidation assay: Enzyme reactions were reconstituted in 50 mM Tris pH 8.0 with KtzI (5 μM), NADPH (0.3 mM) and FAD (50 μM). NADPH levels were continuously monitored by UV spectroscopy (340 nm) using a Cary 50 Bio UV-visible spectrophotometer. During the first two minutes, no substrate was present to ascertain background rates of NADPH oxidation to $NADP^+$. After two minutes, amino acid substrate (500 μM) was added and NADPH levels monitored for an additional 8 minutes.

2d. KtzI kinetic characterization: KtzI N-hydroxylase activity was measured at 25 °C in a 300 μL reaction volume containing Tris-HCl (50 mM, pH 8.0), L-ornithine (0.065–5 mM), FAD (50 μM), and NADPH (300 μM). Assays were initiated by the addition of KtzI (500 nM). Activity was monitored continuously by following the decrease in absorbance at 340 nm. Initial velocities were calculated from the measured absorbance change over a 0.5–2 min time frame using the

extinction coefficient 6,300 $M^{-1}cm^{-1}$. Reactions were run in triplicate and the initial velocity data were fitted to the Michaelis-Menten equation in KaleidaGraph (Synergy Software) to obtain estimates for k_{cat} and K_m.

3. Synthetic Methods

3a. General methods and approach: All synthesis chemicals were purchased from Sigma-Aldrich and used without further purification unless noted otherwise. Organic solvents and amine bases were purchased from Sigma-Aldrich in anhydrous form and used without further purification. Glassware for chemical synthesis was dried overnight at 120 °C or alternatively flame-dried immediately before use. NMR spectra were obtained on Varian spectrometers at the indicated frequencies. Chemical shifts are reported in ppm downfield from tetramethylsilane with residual solvent protium serving as the internal standard.

Synthesis of N^2-Boc-ornithine (**VI**) from ornithine (**I**) (Scheme S2) was performed according to an analogous procedure described for preparation of Boc-Lys.[4] We report spectral data for *unlabeled* intermediates. Preparation of $^{13}C_5$-N^2-Boc-ornithine was performed with $^{13}C_5$-ornithine starting material (Cambridge Isotope Laboratories, Andover MA), and intermediates were verified by TLC and low res MS.

3b. N^2-Boc-N^5-Cbz-Ornithine (V): 350 mg Orn•HCl (2.08 mmol) was dissolved in 0.5M NaOH (4.3 ml) followed by the addition of a solution of $CuSO_4$ (196 mg, 1.23 mmol, 0.59 equiv) in water (18 ml). The reaction mixture was stirred at room temperature for 5 h. To the solution of Ornithine-copper complex (**II**), NaHCO3 (350 mg, 4.16 mmol, 2 equiv) was added followed by addition of benzyl chloroformate (399 μl, 2.81 mmol, 1.35 equiv) in ice bath. Then pH was adjusted to 9 by using 4 M NaOH. The solution was stirred at room temperature for 3 h. The blue precipitate was filtered and washed with small amount of water and ether to get cupric-ornithine complex (**III**). The complex was dissolved in 0.5 M EDTA solution (pH 8.0, 35 ml) and stirred overnight at room temperature. The white precipitate was collected by filtration and washed with small quantity of water and dried to give product (**IV**) as white solids.

The entire portion of **IV** (unpurified) was re-suspended in dioxane/water (1:1, 21 ml) and di-*tert*-butyl dicarbonate (778.8mg, 3.57 mmol) was added. 4M NaOH was used to adjust pH to 10. The reaction mixture was stirred at room temperature for 14 h followed by the addition of 4.4 ml MeOH. The reaction mixture was further stirred at room temperature for 12 h. The pH of reaction mixture was adjusted to 3 under ice bath by the addition of 1 M HCl. The mixture was extracted with ether (30 ml x 3). The ether layers were combined and washed with saturated NaCl and dried by dehydrated Na_2SO_4. The crude was purified by reversed-phased HPLC on preparative Phenomenex Luna C18 10 μm column eluting with a linear gradient of H_2O/MeCN (20 to 100% over 30 min, 0.1% TFA). The fractions containing product were combined and lyophilized to give compound **V** as white fluffy powder (402 mg, 53.7%). ^{1}H NMR (400 MHz, METHANOL-d_4) δ ppm 7.25 - 7.38 (m, 5 H), 5.06 (s, 2 H), 4.03 - 4.10 (m, 1 H), 3.13 (t, *J*=6.7 Hz, 2 H), 1.79 - 1.89 (m, 1 H), 1.53 - 1.68 (m, 3 H), 1.44 (s, 9 H).

3c. N^2-Boc-Ornithine (VI): 402 mg **V** (1.08 mmol) was dissolved in 25 ml methanol. 10% Pd/C (30 mg, 0.028 mmol) was added and the mixture was hydrogenated with a hydrogen balloon. After 14 h at room temperature, the catalyst was removed by filtration through a pad of celite.

The filtration solution was concentrated to give compound **VI**. (246 mg, 95.8%): ^{1}H NMR (400 MHz, METHANOL-d_4) δ ppm 3.94 - 3.99 (m, 1 H), 2.94 (t, J=7.0 Hz, 2 H), 1.81 - 1.86 (m, 1 H), 1.64 - 1.73 (m, 3 H), 1.44 (s, 9 H).

3d. N^5-OH-Ornithine (VII): Conversion of **VI** to **VII** was performed as previously described.[5] $^{13}C_5$-N^5-OH-Orn was prepared by initiating the synthetic sequence with $^{13}C_5$-Orn. Comparison of ^{1}H-NMR spectra for labeled and unlabeled N^5-OH-Orn are shown in Figure S10. For tabulation of ^{1}H NMR data, only the $^{1}J_{CH}$ coupling constants are noted. Further deconvolution of multiplets arising from $^{3}J_{HH}$, $^{2}J_{CH}$, and/or $^{3}J_{CH}$ couplings was not performed. ^{1}H NMR (400 MHz, *DEUTERIUM OXIDE*) δ ppm 3.74 (d-m, $^{1}J_{CH}$=148 Hz, 1H) 3.22 (d-m, $^{1}J_{CH}$=144 Hz, 2H) 1.78 (d-br m, $^{1}J_{CH}$≈128 Hz, 4H). ^{13}C NMR (100 MHz, *DEUTERIUM OXIDE*) δ ppm 173.29 (d, $^{1}J_{cc}$=55 Hz), 53.59 (ddd, $^{1}J_{cc}$=54, 34.3, $^{3}J_{cc}$=5.4 Hz), 49.89 (dd, $^{1}J_{cc}$ =34, $^{3}J_{cc}$=5.4 Hz), 27.19 (t, $^{1}J_{cc}$=34 Hz), 19.01 (t, $^{1}J_{cc}$=34 Hz).

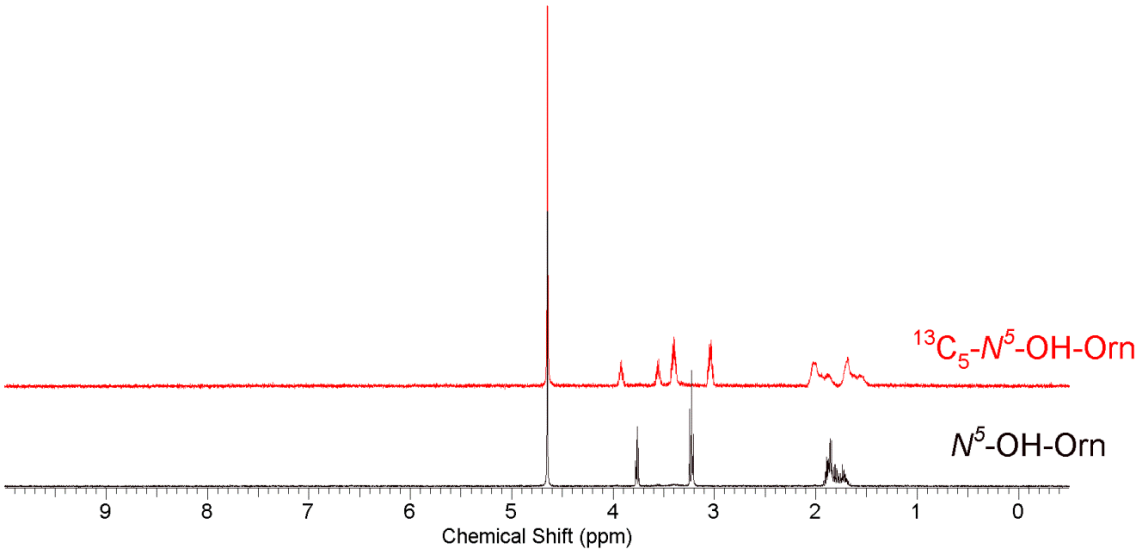


Figure S12: ^{1}H-NMR spectra of synthetic N^5-OH-Orn (black) and $^{13}C_5$-N^5-OH-Orn (red). Proton resonances of $^{13}C_5$-labeled compound are split from unlabeled compound by $^{1}J_{CH}$ couplings of 128-144 Hz.

4. Feeding and Analytical Methods

4a. Culturing, feeding, and extraction of Kutzneria: Routine culturing of Kutzneria sp. 744 was carried out on YEME7 agar plates (10 g malt extract, 4 g yeast extract, 4 g glucose, 900 ml deionized [DI] water, 100 ml 10x MOPS buffer [20.93 g MOPS, 0.436 g K_2HPO_4, 0.34 g KH_2PO_4], 100 ml DI water). Frozen stocks were prepared in YEME7 media supplemented with 15% glycerol. For feeding experiments, starter cultures were prepared by adding a 1 ml frozen stock into 50 ml of YEME7. The culture was incubated at 30°C with shaking at 200 rpm for 3 days and then 15 ml of the culture was spun at 7,000 rpm for 5 minutes to generate a loose pellet. After removing the supernatant, production cultures were prepared by transferring ~20

mg of cell material to 125 ml flasks containing 10 ml of medium MMN (10 g glucose, 3 g, malt extract, 0.5 g KH_2PO_4, 0.25 g $(NH_4)_2HPO_4$, 0.067 g $CaCl_2 \cdot 2H_2O$, 0.025 g NaCl, 0.15 g $MgSO_4 \cdot 7H_2O$, 0.1 mg thiamine, 1.2 μg $FeCl_3 \cdot 6H_2O$, 1 L DI water, pH 5.6). Adjusting the pH of medium MMN to 5.6 is essential for kutzneride production. The 10 ml cultures were incubated at 30°C with shaking at 200 rpm.

Amino acids for feeding studies were prepared as neutral aqueous solutions and sterile filtered. $^{13}C_5$-amino acids were obtained from Cambridge Isotope Laboratories (Andover, MA). 500 μL of compound solutions were added at 18-48 hours after inoculation of production cultures, with final label amounts varying from 9 to 30 mg. Total label incorporation was seen to depend on amount of labeled compound added to the cultures and the extent to which kutzneride compounds accumulated in the media before addition of the labeled compounds (as determined by analysis of control cultures at time of feeding). For compound extraction, an equal volume of ethyl acetate was added to the culture broth and the bilayer mixture was mixed on a rotary shaker at 4 °C for 1-2 hours. The organic layer was separated, dried with anhydrous Na_2SO_4, and filtered. The crude extract was dried *in vacuo* and stored at -20 °C until analysis.

4b. LCMS methods: LCMS analysis was carried out on an Agilent 1200 Series HPLC coupled to an Agilent 6520 QTOF spectrometer. The crude material was redissolved in 200 μl MeOH and filtered through a 0.45 μM membrane. 10 μl were injected for each analytical run. Separation was achieved on a 50 x 2.1 mm Phenomenex Kinetix C18 column (part # 00B-4462-AN) equipped with a guard column. Solvent A was composed of 0.1% formic acid in water; solvent B was composed of 0.1% formic acid in acetonitrile. The LC gradient was as follows:

Time range (min)	Flow rate (ml/min)	Solvent (%B)
0-5	0.1	5
5-45	0.4	5-100
45-53	0.5	100
53-60	0.5	5

MS data collection was performed between from 5 to 53 min of the separation. Ionization source temperature was 350 °C to optimize yields of $[M+H-H_2O]^+$ ions. MSMS spectra for labeling analyses were generated by collision-induced dissociation at 44 V, targeted to the predicted exact mass of the $^{13}C_5$-analyte, and using the wide ion selection window (~9 *m*/*z*, extending from target *m*/*z* to +9). MSMS spectra for each analyte were summed over the range in which the parent molecule was detected in MS mode.

5. References for Supporting Information

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