Biosynthesis of (-)-(1*S*,2*R*)-allocoronamic acyl thioester by an Fe^{II}-dependent halogenase and a cyclopropane-forming flavoprotein.

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Supporting Information

Contents

1. General figures

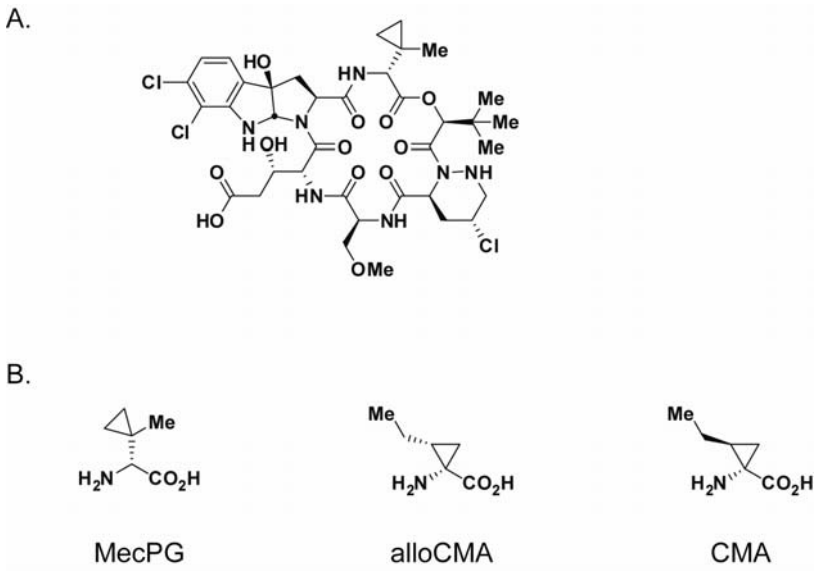
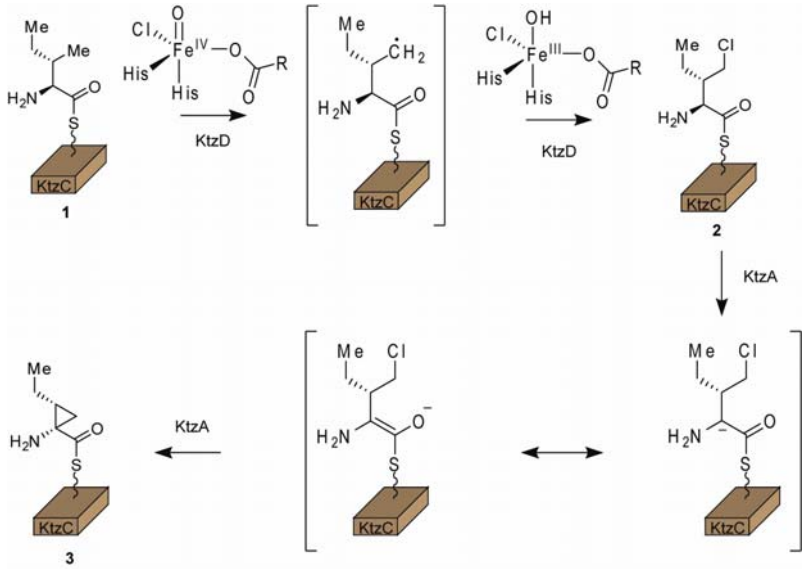


Figure S1: A. Kutzneride 2, a representative of the kutzneride family of natural products. B. Cyclopropane containing amino acids: D-2-(1-methylcyclopropyl)glycine (MecPG), (*1S*, *2R*)-allocoronamic acid (alloCMA), and (*1S*, *2S*)-coronamic acid (CMA).

Scheme S1: Proposed biosynthetic pathway for alloCMA–S–KtzC with mechanistic detail.



2. Materials and general methods: Standard recombinant DNA, molecular cloning, and microbiological procedures were performed according to the methods described by Sambrook *et al*.[1] *E. coli* Top10 (Invitrogen) was used for routine cloning and propagation of DNA vectors. *E. coli* BL21 (DE3) (Invitrogen) transformed with pET-derived vectors (Novagen) was used for overexpression in Luria-Bertani (LB) medium. Oligonucleotide primers were synthesized by Integrated DNA Technologies (Coralville, IA). Phusion DNA polymerase, restriction enzymes, and T4 DNA ligase were purchased from New England Biolabs. Gel extraction of DNA fragments and restriction endonuclease cleanup was performed with Illustra GFX Purification Kit from GE Healthcare. Recombinant plasmid DNA was purified with a Qiaprep kit from Qiagen. DNA sequencing was performed at the Molecular Biology Core Facilities of the Dana Farber Cancer Institute (Boston, MA). Nickel-nitrilotriacetic acid-agarose (Ni-NTA) superflow resin and SDS-page gels were purchased from Qiagen. Protein samples were concentrated using 10K mwco Amicon Ultra filters from Millipore. Microfiltration devices were also purchased from Millipore (10K mwco). The concentration of purified protein solutions was determined by the method of Bradford, with BSA as a standard. Anaerobic manipulations were performed under a nitrogen atmosphere using an Mbraun Labmaster glovebox (Stratham, NH) maintained at 2 ppm O_2 or less. Anaerobic buffers were prepared by sparging with argon for 30 minutes and equilibrating with the inert atmosphere overnight. All synthesis chemicals were purchased from Sigma-Aldrich and used without further purification unless noted otherwise. All 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. Reported yields are unoptimized. Except where noted, NMR spectra were obtained on a Varian 600 MHz spectrometer. Chemical shifts are reported in ppm downfield from tetramethylsilane with residual solvent protium serving as the internal standard.[2] MS analyses for synthetic intermediates were performed at the Harvard University Center for Systems Biology Mass Spectrometry and Proteomics Resource Laboratory.

3. Cloning, overexpression, and purification of Ktz proteins

3a. Composition of common buffers

Buffer A: 20 mM HEPPS, 300 mM NaCl, 5 mM imidazole, pH 8.0
Buffer B: 20 mM HEPPS, 300 mM NaCl, 30 mM imidazole, pH 8.0
Buffer C: 20 mM HEPPS, 100 mM NaCl, 200 mM imidazole, pH 8.0
Buffer D: 20 mM HEPPS, 80 mM NaCl, pH 8.0
Buffer E: 20 mM HEPES, 80 mM NaCl, pH 7.5

3b. KtzA PCR amplification of *ktzA* was carried out with purified fosmid DNA containing the upstream portion of the kutzernide gene cluster. The forward primer was 5'-ATT ATT CCA TAT GAT GAG CAC GGT CCA GGA GTT GTC C-3' and the reverse primer was 5'-ATT ATT CCT CGA GCA TCT CCC CTG AGA CCA GGT CA-3'. Purified PCR product was digested with NdeI and XhoI, ligated into analogously digested pET24b vector, and transformed into Top 10 cells. Purified vector was

sequenced to confirm the insert and then transformed into BL21 (DE3) cells. A saturated culture of the expression strain was prepared by growing overnight at 37 C. Six 2 L cultures containing kanamycin (50 μg/mL) were inoculated with 20 mL of the overnight culture and incubated at 35 °C for ~3 h, then 25 °C for ~30 min, and then cooled to 15 °C prior to the addition of isopropyl-β-D-galactopyranoside to a final concentration of 0.1 mM. The cultures were incubated for another 16-17 h, then harvested. The cell pellet was resuspended in 60 mL buffer A and lysed by two passes through a french press. The cell debris was removed by centrifugation at 27,000 g for 30 minutes. The supernatant was carefully removed and applied to Ni-NTA agarose resin. After 1-2 hours, the mixture was poured into a low pressure chromatography column. The resin was washed with 25 mL buffer A followed by 8 ml buffer B to remove weakly bound contaminants. The protein was eluted with five 10 mL portions of buffer C. Fractions containing pure protein as judged by SDS-PAGE were combined and concentrated. The protein was desalted using Bio-Gel P6-DG desalting gel (Biorad) equilibrated in buffer D and fractions positive for protein by Bradford analysis were combined and concentrated. The protein stocks were flash-frozen in liquid nitrogen in 15-25 μl aliquots and stored at -80 °C. FAD content was measured by boiling solutions of KtzA for 5 minutes and centrifuging to remove denatured protein. The released cofactor was quantitated by UV-Vis (l=441 nm and l=365 nm) and comparison to standard solutions of FAD.

3c. KtzB The cloning, overexpression, and purification of KtzB have been described previously.[3]

3d. KtzC PCR amplification of *ktzC* was carried out with purified fosmid DNA containing the upstream portion of the kutzernide gene cluster. The forward primer was 5'-ATT ATT CCA TAT GAT GAG CGA CAC GGT CCG CTC C-3' and the reverse primer was 5'-ATT ATT CCT CGA GGA CGT CGA GTC CCT CAC CAC G-3'. Purified PCR product was digested with NdeI and XhoI, ligated into analogously digested pET24b vector, and transformed into Top 10 cells. Purified vector was sequenced to confirm the insert and the transformed into BL21 (DE3) cells. A saturated culture of the expression strain was prepared by growing overnight at 37 C. A 2 L culture containing kanamycin (50 μg/mL) was inoculated with 20 mL of the overnight culture and incubated at 35 °C for ~3 h, then 25 °C for ~30 min, and then cooled to 15 °C prior to the addition of isopropyl-β-D-galactopyranoside to a final concentration of 0.1 mM. The culture was incubated for another 16-17 h, then harvested. The cell pellet was resuspended in buffer A and lysed by two passes through a cell disruptor. The cell debris was removed by centrifugation at 27,000 g for 30 minutes. The supernatant was carefully removed and applied to Ni-NTA agarose resin. After 1-2 hours, the mixture was poured into a low pressure chromatography column. The resin was washed with 25 mL buffer A followed by 5 ml buffer B to remove weakly bound contaminants. The protein was eluted with 10 mL portions of buffer C. Fractions containing pure protein as judged by SDS-PAGE were combined and concentrated. The protein was desalted using Bio-Gel P6-DG desalting gel (Biorad) equilibrated in buffer D and fractions positive for protein by Bradford analysis were combined and concentrated. The protein stocks were flash-frozen in liquid nitrogen in 15-25 μl aliquots and stored at -80 °C.

3e. KtzD PCR amplification of *ktzD* was carried out with purified fosmid DNA containing the upstream portion of the kutzernide gene cluster. The forward primer was 5'-ATT ATT CCA TAT GGT GGT TTC GAA AAC ACT TGA TTT-3' and the reverse primer was 5'-ATT ATT CCT CGA GTC ATT ATG CCG GGC TTC CCG TCT CGC GCA-3'. Purified PCR product was digested with NdeI and XhoI, ligated into analogously digested pET28b vector, and transformed into Top 10 cells. Purified vector was sequenced to confirm the insert and the transformed into BL21 (DE3) cells. A saturated culture of the expression strain was prepared by growing overnight at 37 C. Six 2 L cultures containing kanamycin (50 μg/mL) were inoculated with 20 mL of the overnight culture and incubated at 35 °C for ~3 h, then 25 °C for ~30 min, and then cooled to 15 °C prior to the addition of isopropyl-β-D-galactopyranoside to a final concentration of 0.1 mM. The cultures were incubated for another 16-17 h, then harvested. The cell pellet was resuspended in 60 mL buffer A. $CaCl_2$ (1 mM), $MgCl_2$ (1 mM) and DNAse I (100 ug/mL) were added to the suspension. The cells were lysed by two passes through a French press. The cell debris was removed by centrifugation at 27,000 g for 30 minutes. The supernatant was carefully removed and applied to Ni-NTA agarose resin. After 1–2 hours, the mixture was poured into a low pressure chromatography column. The resin was washed with 40 mL buffer A followed by 13 ml buffer B to remove weakly bound contaminants. The protein was eluted with five 13 mL portions of buffer C. Fractions containing pure protein as judged by SDS-PAGE were combined and concentrated. The protein was desalted using Bio-Gel P6-DG desalting gel (Biorad) equilibrated in buffer D and fractions positive for protein by Bradford analysis were combined and concentrated. The N-terminal His tag was removed by cleavage with thrombin protease (Novagen) at room temperature for 4 hours according to the manufacturer's instructions. The protein was then purified by gel filtration on a Hi Load 26/60 Superdex 75 prep grade column (Amersham Biosciences) using anaerobic buffer E as the eluent. Fractions were collected in the glove box under a nitrogen atmosphere. Protein-containing fractions were concentrated using an Amicon Stirred Ultrafiltration Cell (Millipore). The resulting apo-KtzD protein was reconstituted by incubation with 2mM DTT, 2 mM α-ketoglutarate, and 1 mM $Fe(NH_4)_2(SO_4)_2$ for 3 minutes. The protein was again desalted using buffer E and protein-containing fractions concentrated. Aliquots of the holo-KtzD stock were prepared, removed from the inert atmosphere, and immediately flash-frozen in liquid nitrogen. The tubes were stored at -80 °C until use.

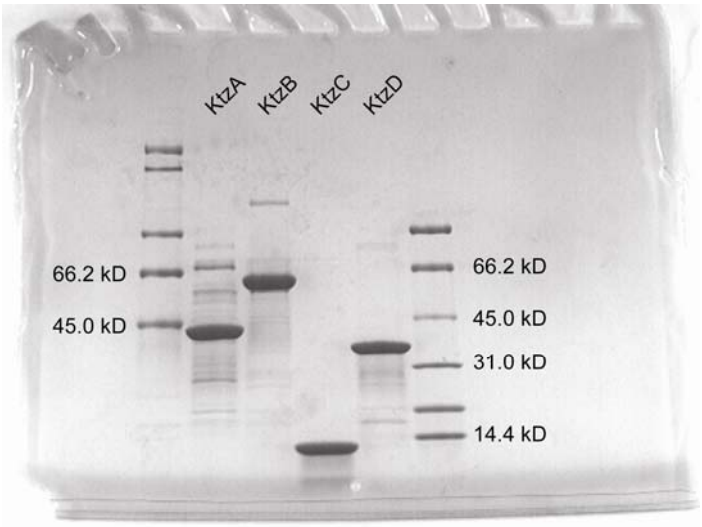
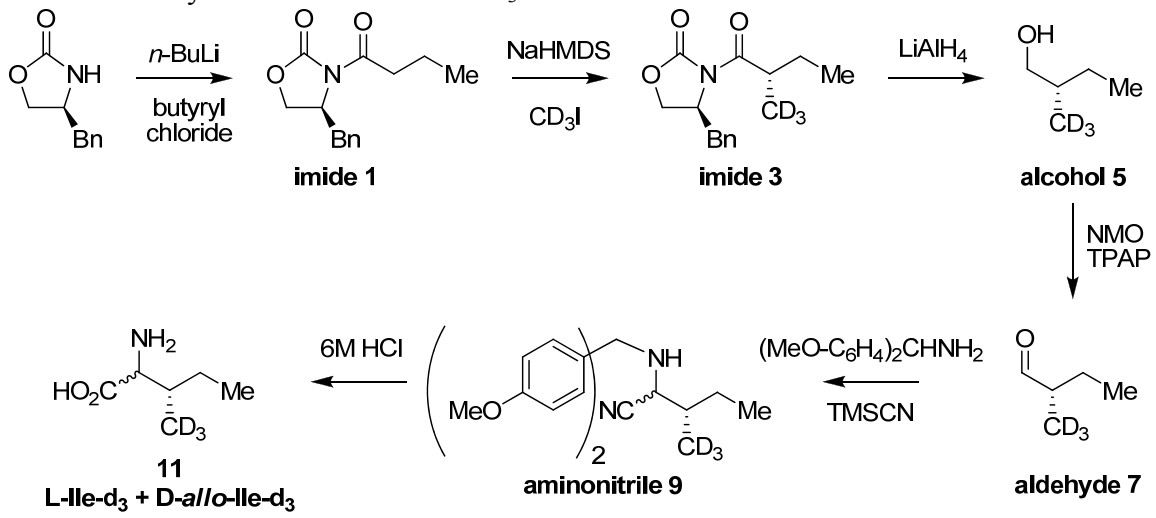


Figure S2: SDS-PAGE (4–15%) of purified Ktz proteins.

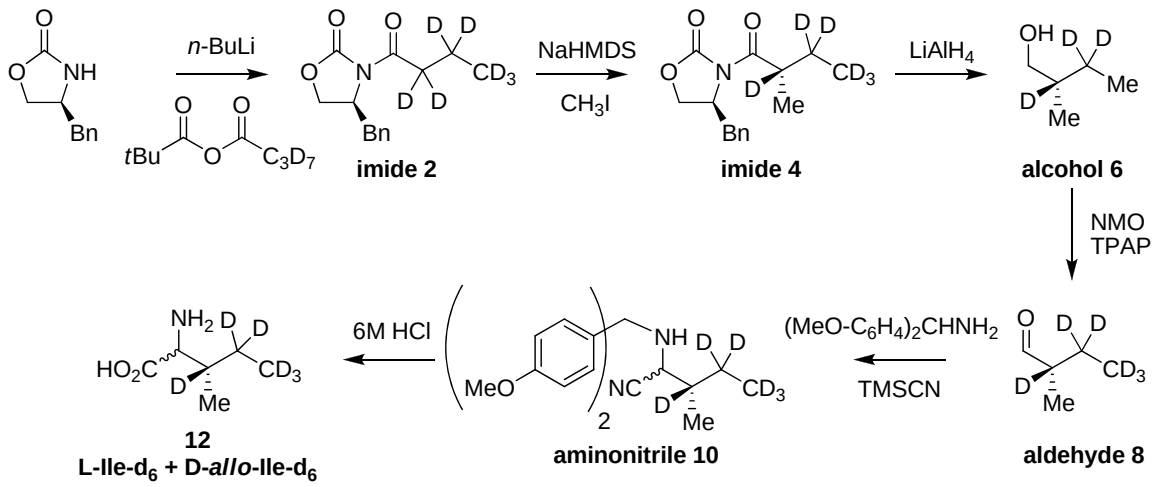
4. Synthesis of deuterated isoleucine isotopologues

4a. General Synthetic Schemes

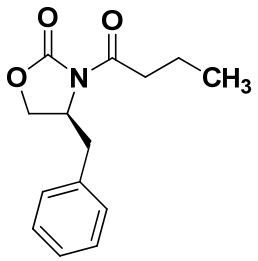
Scheme S2: Synthetic route for L-Ile-d_3



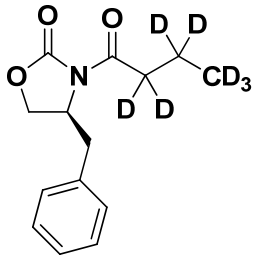
Scheme S3: Synthetic route for L-Ile-d_6



4. Synthetic methods and characterizations



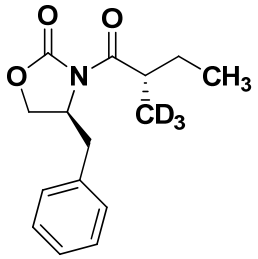
Imide 1: In a 250 mL flask with magnetic stir bar, (*S*)-4-benzyl-2-oxazolidinone (3.51 g, 19.8 mmol, 1.0 equiv) was dissolved in THF (25 mL). The solution was cooled to -78 C and 1.6M solution of *n*-butyl lithium (12.38 mL, 19.8 mmol, 1.0 equiv) was added. After stirring for 15 minutes, butyryl chloride (2.07 mL, 19.8 mmol, 1.0 equiv) was added dropwise. The reaction was stirred for 15 minutes at -78 °C and then warmed to room temperature for 2 hours. Saturated NH_4Cl was added and the product extracted 3X EtOAc. The combined organics were washed with brine and then dried over Na_2SO_4. The solvent was removed in vacuo to yield the desired compound as a slightly yellow oil (4.78 g, 98%). No further purification was performed. Spectra are consistent with those previously reported.[4] ^{1}H NMR (600 MHz, $CDCl_3$) δ ppm 7.33 (m, 2 H), 7.28 (m, 1 H), 7.21 (m, 2 H), 4.68 (m, 1 H), 4.20 (m, 1 H), 4.16 (dd, *J*=9.1, 2.9 Hz, 1 H), 3.30 (dd, *J*=13.5, 3.2 Hz, 1 H), 2.96 (ddd, *J*=17.0, 7.9, 6.7 Hz, 1 H), 2.88 (ddd, *J*=17.0, 7.9, 6.7 Hz, 1 H), 2.77 (dd, *J*=13.5, 9.7 Hz, 1 H), 1.73 (m, 2 H), 1.01 (t, *J*=7.3 Hz, 3 H). ^{13}C NMR (151 MHz, $CDCl_3$) δ ppm 173.1, 153.4, 135.3, 129.3, 128.8, 127.2, 66.1, 55.0, 37.8, 37.3, 17.6, 13.6. MS–ESI$^+$ (*m/z*): $[M+Na]^+$ calcd for $C_{14}H_{17}NO_3Na$, 248.1; found, 248.1.



Imide 2: In a 250 mL flask with magnetic stir bar, d_8-butyric acid (2.0 mL, 21.7 mmol, 1.0 equiv) was dissolved in anhydrous THF (50 mL). The solution was cooled to -78 °C at which point DIPEA (4.90 mL, 28.1 mmol, 1.3 equiv) was added followed by freshly distilled pivaloyl chloride (2.67 mL, 21.7 mmol, 1.0 equiv). The reaction was stirred for 30 minutes then warmed to room temperature. The resulting suspension continued stirring overnight.

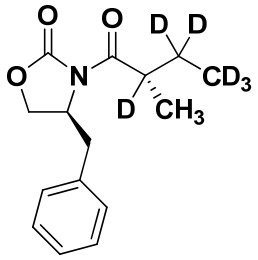
In a second flask, (S)-4-benzyloxazolidinone (4.61 g, 26.0 mmol, 1.2 equiv) was dissolved in anhydrous THF (50 mL). The solution was cooled to -78 °C and treated with a 1.6M solution of *n*-butyl lithium in THF (16.3 mL, 26.0 mmol, 1.2 equiv) and stirred for 20 minutes. The above suspension was added via cannula. After 15 minutes, the reaction was warmed to room temperature for an additional 2.5 hours. The reaction was quenched with saturated NH_4Cl and extracted 3X EtOAc. The combined organics were washed with brine then dried over Na_2SO_4. The solvent was removed in vacuo to yield a yellow oil. Flash chromatography (8:1->5:1 hex:EtOAc) provided the title compound (3.99 g, 72%).

^{1}H NMR (600 MHz, $CDCl_3$) δ ppm 7.33 (m, 2 H), 7.27 (m, 1 H), 7.21 (m, 2 H), 4.67 (m, 1 H), 4.20 (t, *J*=9.1, 8.5 Hz, 1 H), 4.16 (dd, *J*=9.1, 2.9 Hz, 1 H), 3.30 (dd, *J*=13.5, 3.5 Hz, 1 H), 2.77 (dd, *J*=13.2, 9.4 Hz, 1 H). ^{13}C NMR (151 MHz, $CDCl_3$) δ ppm 173.5, 153.6, 135.5, 129.5, 129.1, 127.5, 66.3, 55.3, 38.1. HRMS–ESI$^+$ (*m/z*): $[M+Na]^+$ calcd for $C_{14}H_{10}D_7NO_3Na$, 277.1648; found, 277.1634.

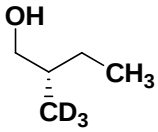


Imide 3: In a 250 mL flask with magnetic stir bar, anhydrous THF (20 mL) was cooled to -78 °C. A 1.0M solution of NaHMDS in THF (19.5 mL, 19.5 mmol, 1.4 equiv) was added followed by a solution of imide **1** (3.45 g, 13.95 mmol, 1.0 equiv) in anhydrous THF (20 ml). The reaction was stirred at -78 °C for 1 hour at which point CD_3I (10 g, 70 mmol, 5.0 equiv) was added. The reaction was stirred for 3 hours before quenching with 1.5 mL acetic acid and warming to room temperature. Water was added and the mixture extracted 3X EtOAc. The combined organics were washed with brine then dried over Na_2SO_4. The solvent was removed in vacuo to yield a yellow oil. Flash chromatography (9:1 hex:EtOAc) provided the title compound as a clear oil (2.75 g, 75%).

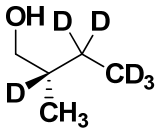
1H NMR (600 MHz, $CDCl_3$) δ ppm 7.33 (m, 2 H), 7.28 (m, 1 H), 7.22 (m, 2 H), 4.68 (m, 1 H), 4.20 (dd, *J*=9.1, 7.6 Hz, 1 H), 4.17 (dd, *J*=8.8, 2.6 Hz, 1 H), 3.62 (t, *J*=6.7 Hz, 1 H), 3.27 (dd, *J*=13.2, 3.2 Hz, 1 H), 2.77 (dd, *J*=13.2, 9.7 Hz, 1 H), 1.77 (m, 1 H), 1.47 (m, 1 H), 0.93 (t, *J*=7.6 Hz, 3 H). HRMS–ESI$^+$ (*m/z*): $[M+H]^+$ calcd for $C_{15}H_{17}D_3NO_3$, 265.1631; found, 265.1617.



Imide 4: The title compound was synthesized from imide **2** (2.83 g, 11.1 mmol, 1.0 equiv) and CH_3I using the above procedure. Product was obtained as a clear oil (2.34 g, 79%).
^{1}H NMR (600 MHz, $CDCl_3$) δ ppm 7.33 (m, 2 H), 7.27 (m, 1 H), 7.22 (m, 2 H), 4.68 (m, 1 H), 4.20 (dd, *J*=9.1, 7.6 Hz, 1 H), 4.17 (dd, *J*=9.1, 2.9 Hz, 1 H), 3.27 (dd, *J*=13.5, 3.5 Hz, 1 H), 2.77 (dd, *J*=13.2, 9.4 Hz, 1 H), 1.21 (s, 3 H). HRMS–ESI$^+$ (*m/z*): $[M+H]^+$ calcd for $C_{15}H_{14}D_6NO_3$, 268.1820; found, 268.1817.

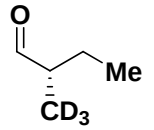


Alcohol 5: In a 250 mL flask with magnetic stir bar, diethyl ether (20 mL) was cooled to 0 °C. A 1.0M solution of $LiAlH_4$ in diethyl ether (16.4 mL, 16.4 mmol, 2.0 equiv) was added followed by a solution of imide **3** (2.17 g, 8.10 mmol, 1.0 equiv) in ether (20 mL). The reaction was stirred at 0 for 90 minutes, then quenched by the careful addition of 1 mL H_2O. The suspension was filtered through a short column of $MgSO_4$, silica, and celite which was then rinsed with additional ether. The solvent was carefully removed in vacuo to provide the crude alcohol. Flash chromatography (3:1 pentane:ether) provided the title compound as a clear liquid (270 mg, 34%).
^{1}H NMR (600 MHz, $CDCl_3$) δ ppm 3.52 (dd, *J*=10.6, 5.9 Hz, 1 H), 3.43 (dd, *J*=10.6, 6.5 Hz, 1 H), 1.53 (m, 1 H), 1.45 (m, 2 H), 1.15 (m, 1 H), 0.91 (t, *J*=7.6 Hz, 3 H). ^{13}C NMR (151 MHz, $CDCl_3$) δ ppm 68.2, 37.3, 25.8, 11.5.

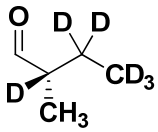


Alcohol 6: The title compound was synthesized from imide **4** (2.15 g, 8.03 mmol, 1.0 equiv) according to the above procedure. The product was obtained as a clear liquid (319 mg, 42%).

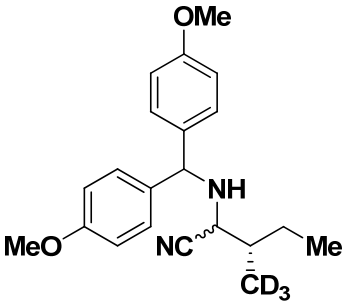
^{1}H NMR (600 MHz, $CDCl_3$) δ ppm 3.50 (d, *J*=10.3 Hz, 1 H), 3.42 (d, *J*=10.3 Hz, 1 H), 1.38 (br. s., 1 H), 0.90 (1:1:1 t, J_{HCCD}=0.9 Hz, 3 H). ^{13}C NMR (151 MHz, $CDCl_3$) δ ppm 68.1, 16.1.



Aldehyde 7: In a 50 mL flask with magnetic stir bar, molecular sieves (1 g) were suspended in dichloromethane. Alcohol **5** (265 mg, 2.9 mmol, 1.0 equiv) was added followed by *N*-methyl morpholine *N*-oxide (515 mg, 4.4 mmol, 1.5 equiv). The mixture was stirred for 20 minutes at which point tetrapropyl ammonium peruthenate (51 mg, 0.15 mmol, 0.05 equiv) was added and the resulting dark suspension was stirred for 2 hours. The reaction was filtered through a plug of silica topped with celite, which was washed with a small amount of additional solvent. The volume of the resulting aldehyde solution was reduced to ~7 mL by careful distillation in a 45 water bath. The solution was used without further purification; ^{1}H-NMR data for the aldehyde is provided to aid in analysis of the crude solution.
^{1}H NMR (600 MHz, $CDCl_3$) δ ppm 9.63 (d, *J*=2.1 Hz, 1 H), 2.26 (m, 1 H), 1.75 (m, 1 H), 1.44 (m, 1 H), 0.95 (t, *J*=7.6 Hz, 3 H).

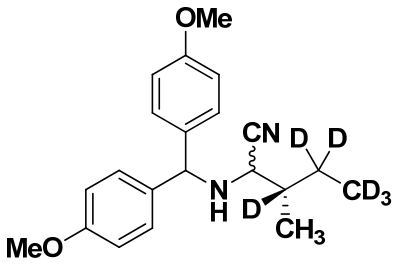


Aldehyde 8: A solution of aldehyde **8** was prepared from alcohol **6** (309 mg, 3.3 mmol, 1.0 equiv) according to the above procedure.
^{1}H NMR (600 MHz, $CDCl_3$) δ ppm 9.63 (s, 1 H), 1.08 (1:1:1 t, J_{HCCD}=0.9 Hz, 3 H).

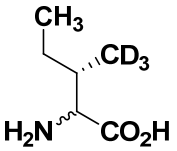


Aminonitrile 9: In a 50 mL flask with magnetic stir bar was added molecular sieves (1 g) and aldehyde **7** as a solution in dichloromethane. 1,1-di(*p*-anisyl)methylamine (TCI Chemicals, 779 mg, 3.2 mmol, 1.1 equiv relative to alcohol **5**) was added and the reaction stirred for 2 hours. Trimethylsilyl cyanide (426 µl, 3.2 mmol, 1.1 equiv) was added and the reaction stirred an additional 4 hours. The reaction was filtered through a plug of celite and concentrated to give a yellow oil. Flash chromatography (6:1 hex:EtOAc with

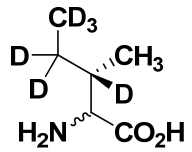
0.5% NEt_3) provided the title compound (1:1 mix of diastereomers) as a clear oil (42 mg, 4% from alcohol **5**).
^{1}H NMR (600 MHz, $CDCl_3$) δ ppm 7.37 (m, 4 H), 7.33 (m, 4 H), 6.85 (m, 8 H), 5.04 (d, *J*=11.2 Hz, 2 H), 3.78 (s, 6 H), 3.76 (s, 6 H), 3.33 (d, *J*=5.6 Hz, 1 H), 3.29 (d, *J*=5.6 Hz, 1 H), 1.64 (m, 2 H), 1.32 (m, 2 H), 0.89 (t, *J*=7.3 Hz, 3 H), 0.88 (t, *J*=7.3 Hz, 3 H).



Aminonitrile 10: The aminonitrile (1:1 mix of diastereomers) was synthesized from a solution of aldehyde **8** according to the above procedure. The product was obtained as a clear oil (128 mg, 11% from alcohol **6**).
^{1}H NMR (600 MHz, $CDCl_3$) δ ppm 7.37 (m, 4 H), 7.32 (m, 4 H), 6.85 (m, 8 H), 5.03 (s, 1 H), 5.03 (s, 1 H), 3.78 (s, 6 H), 3.77 (s, 6 H), 3.29 (m, 2 H), 1.73 (m, 2 H), 1.08 (s, 3 H), 1.06 (s, 3 H). ^{13}C NMR (151 MHz, $CDCl_3$) δ ppm 159.16, 159.14, 159.01, 159.00, 136.15, 136.10, 133.80, 133.78, 128.55, 128.48, 128.18, 128.15, 120.19, 119.78, 114.28, 114.27, 114.17, 64.42, 64.40, 55.38, 55.36, 53.67, 53.33, 15.55, 15.32. HRMS–ESI$^+$ (*m/z*): $[M+H]^+$ calcd for $C_{21}H_{21}D_6N_2O_2$, 345.2449; found, 345.2437.



11 (L-Ile-d_3 + D-*allo*-Ile-d_3): Aminonitrile **9** (42 mg, 0.12 mmol, 1.0 equiv) was refluxed overnight in 6M HCl (10 mL). The organic impurities were removed by washing with 5 portions of dichloromethane. The acidic aqueous layer was applied to a small plug of Dowex 50WX8-200 ion exchange resin and washed with water. The amino acid was eluted with 0.25M NH_4OH. Ninhydrin positive fractions were combined and concentrated to yield the title compound as an off-white solid (1:1 ratio of diastereomers, 10 mg, 64%). HRMS–ESI$^+$ (*m/z*): $[M+H]^+$ calcd for $C_6H_{10}D_3NO_2$, 135.1213; found, 135.1203.



12 (L-Ile-d_6 + D-*allo*-Ile-d_6): Aminonitrile **10** (0.34 mmol, 1.0 equiv) was refluxed overnight in 6M HCl (10 mL). The organic impurities were removed by washing with 5 portions of dichloromethane. The aqueous layer was concentrated to provide the title compound in quantitative yield as an off-white HCl salt (1:1 ratio of diastereomers), and was used without further purification.
^{1}H NMR (600 MHz, D_2O) δ ppm 4.05 (s, 1 H), 4.01 (s, 1 H), 1.02 (s, 3 H), 0.98 (s, 3 H). HRMS–ESI$^+$ (*m/z*): $[M+H]^+$ calcd for $C_6H_8D_6NO_2$, 138.1401; found, 138.1400.

5. Enzymatic assays

5a. Preparation of aminoacyl thioesters of holo-KtzC. Apo-KtzC (120 uM, 10-40 nmol per reaction) was phosphopantetheinylated by incubation with coenzyme A (250 uM), $MgCl_2$ (2.5 mM), and Sfp[5] (3 uM) in 20 mM HEPES, pH 7.5 for 30 minutes at room temperature. Amino acid (2 mM) was added to the reaction followed by ATP (4 mM) and KtzB (19 uM). The reaction was incubated for a further 30 minutes at room temperature to provide aminoacyl–S–KtzC. When loading isotope-labeled amino acids, the concentration of amino acid was reduced to 0.2 mM and incubation time extended to 60 minutes.

5b. Cleavage of aminoacyl thioesters of KtzC; derivatization and HPLC analysis. Using Ultrafree centrifugal filters (Millipore), aminoacyl–S–KtzC preparations were washed five times with fresh buffer to remove untethered amino acid from the loading reaction. Fresh buffer was added to bring aminoacyl–S–KtzC to 100 uM and TycF[6] was added to a final concentration of 10 uM. The reaction was incubated at room temperature for 30–60 minutes. The released amino acid was collected by centrifugation of the filter apparatus and recovery of the flow-through. An additional portion of fresh buffer was added to the protein mix and collected again by centrifugation. The released amino acids were transformed to the isoindole derivative by reaction with *o*-pthalaldehyde and 3-mercaptopropionic acid.[7]

HPLC analyses were conducted on a Beckman Coulter System Gold HPLC equipped with a model 168 UV detector (λ_1=338 nm, λ_2=220 nm). HPLC conditions were as follows. Column: Phenomenex Luna 5u C18(2) 100A, 250 x 4.6 mm; Solvent A: 40 mM sodium phosphate, pH 7.4; Solvent B: 45:45:10 methanol:acetonitrile:water; Gradient: 20% B to 80% B over 25 minutes; Flow Rate: 1 mL/min.

Except where noted, LCMS analyses of enzymatic assays were performed with a Shimadzu LC-10AD HPLC system connected to a Shimadzu QP8000 MS with electrospray ionization in positive ion mode. LC conditions were as follows. Column: Phenomenex Luna 5u C18(2) 100A, 50 x 2.0 mm; Solvent A: 0.1% formic acid in water; Solvent B: 0.1% formic acid in acetonitrile; Gradient: 10% B to 100% B over 10 minutes; Flow Rate: 0.5 mL/min.

5c. Halogenase activity assay for KtzD. Preparations of aminoacyl–S–KtzC were transferred into the glove box and allowed to equilibrate with the nitrogen atmosphere. Frozen aliquots of reconstituted KtzD were transferred into the glove box in a cooling block and were allowed to equilibrate with the nitrogen atmosphere while frozen. An anaerobic solution of α-ketoglutarate was added to the reactions (2 mM). Aliquots of KtzD were thawed and immediately added to the reactions (17 uM). The reactions were removed from the glove box, aerated, and incubated for 60 minutes at room temperature.

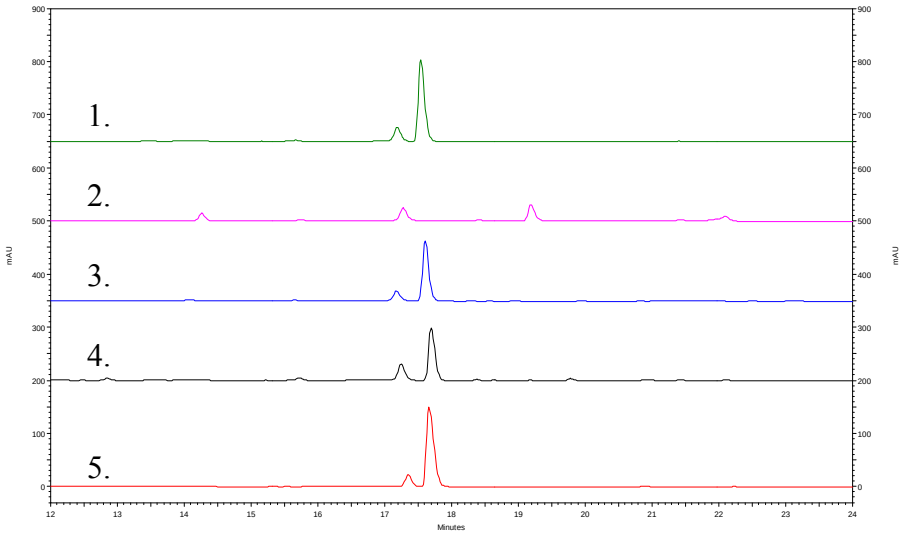


Figure S3: HPLC data for KtzD activity assay (λ=338 nm). 1) L-Ile–S–KtzC, –KtzD. 2) L-Ile–S–KtzC, +KtzD. 3) L-*allo*-Ile–S–KtzC, –KtzD. 4) L-*allo*-Ile–S–KtzC, +KtzD. 5) Ile standard.

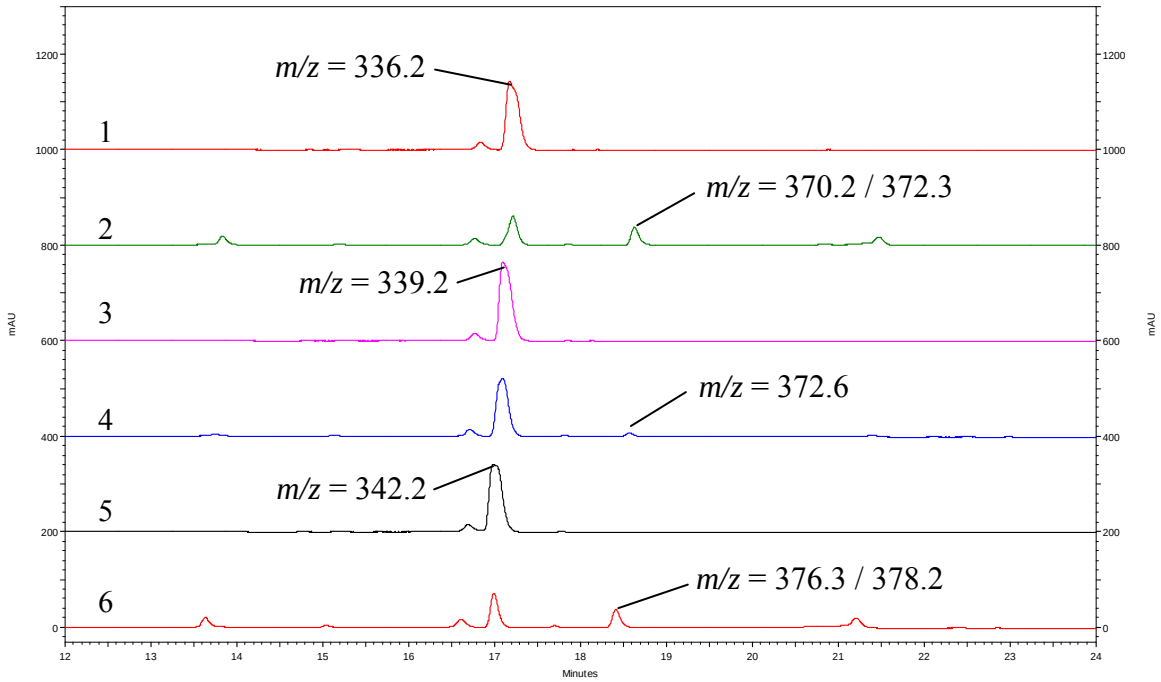


Figure S4: HPLC (λ=338 nm) and MS data for KtzD activity assay with deuterated Ile isotopologues. 1) Ile–S–KtzC, –KtzD. 2) Ile–S–KtzD, +KtzD. 3) Ile-d3–S–KtzC, –KtzD. 4) Ile-d3–S–KtzC, +KtzD (note slower conversion to chlorinated product). 5) Ile-d6–S–KtzC, –KtzD. 6) Ile-d6–S–KtzC, +KtzD.

5d. Cyclase activity assay for KtzA. To preparations of chlorinated aminoacyl–S–KtzC were added tris(2-carboxyethyl)phosphine (2 mM) and KtzA (15 uM). The reactions were incubated for 60 minutes at room temperature.

5e. H/D exchange assay for KtzA. Preparations of Ile–S–KtzC were first buffer exchanged into 40 mM sodium phosphate, pD 7.5 in D_2O using Ultrafree centrifugal filters (Millipore). Additional buffer was added to bring the concentration of Ile–S–KtzC to 100 uM. Tris(2-carboxyethyl)phosphine (2 mM) and KtzA (15 uM) were added and the reactions incubated for 30 minutes at room temperature. The tethered amino acids were released and derivatized as described above. LCMS analysis was performed on an Agilent 1200 series LC coupled to an Agilent 6210 TOF-MS with electrospray ionization collecting in the positive ion mode. LC conditions were as follows. Column: C18, 30 x 2.1 mm, 3.5 um; Solvent A: 0.1% formic acid in water; Solvent B: 0.1% formic acid in acetonitrile; Gradient: 5% B to 95% B over 10 minutes; Flow Rate: 0.5 mL/min.

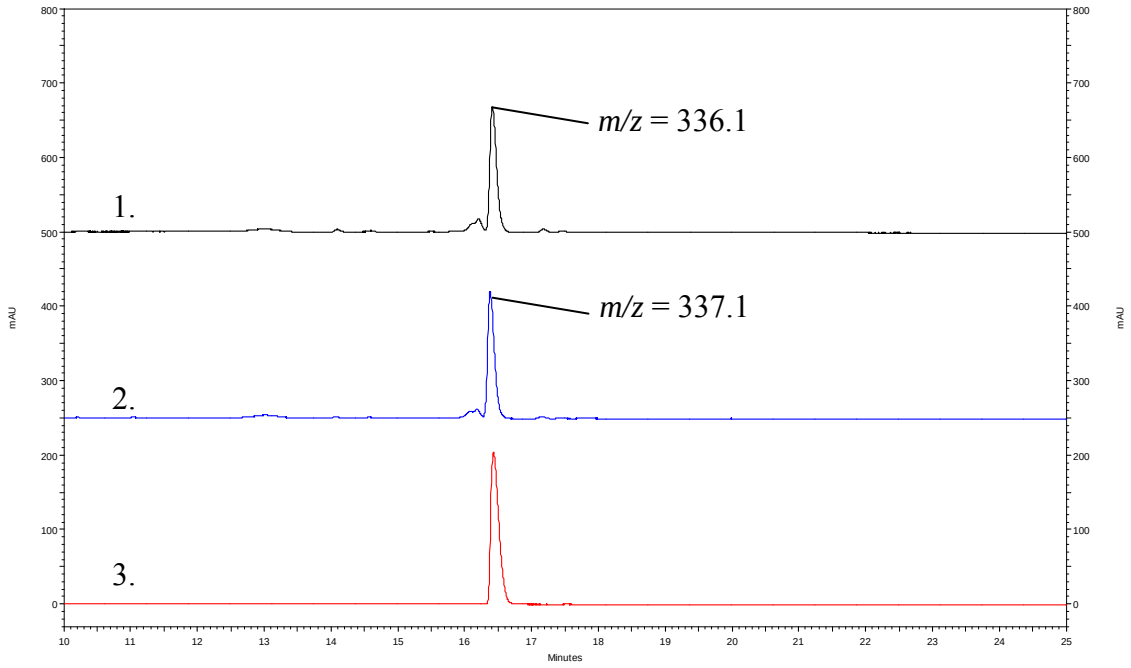


Figure S5: HPLC (λ=338 nm) and MS data for KtzA-mediated H/D exchange of Ile–S–KtzC. 1) Ile–S–KtzC, –KtzA. 2) Ile–S–KtzC, +KtzA. 3) Ile standard.

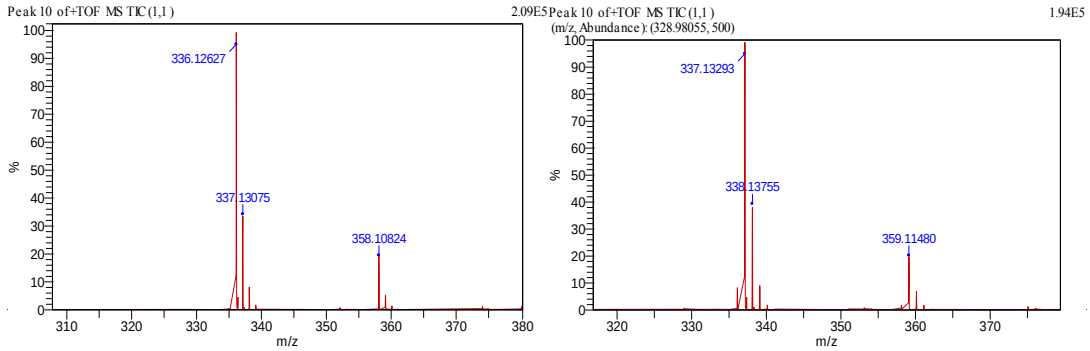


Figure S6: Raw MS data for KtzA-mediated H/D exchange of Ile–S–KtzC. Left: Ile–S–KtzC, –KtzA. Right: Ile–S–KtzC, +KtzA.

6. Structure determination of alloCMA as the isoindole derivative (alloCMA–OPA)

6a. Preparation of material. To obtain sufficient material for NMR analysis, twenty reactions (40 nmol KtzC per reaction) were run according to the procedures described above. The products released after TycF cleavage were combined and lyophilized. The material was dissolved in 1 mL water, derivatized as described, and purified in batches by analytical HPLC using a slightly modified gradient (20 –> 60%B over 25 minutes). The peaks corresponding to the final product were collected, flash frozen, and lyophilized. Care was taken to protect the samples from prolonged exposure to light. The samples were redissolved in 600 µl D_2O for NMR analysis. ^{13}C-enriched samples were prepared in analogous fashion starting with $^{13}C_6$-L-Ile (95%+ labeling, Cambridge Isotope Labs). Preliminary NMR analysis showed the compound to be present as a 4:1 ratio of rotamers.

6b. NMR spectroscopy. ^{1}H and gCOSY spectra were obtained using standard pulse sequences. ^{13}C-decoupled ^{1}H NMR of the enriched sample was recorded using the WURST 40 decoupling sequence on the carbon channel during acquisition on the proton channel. For each scan, 4096 points were collected (0.426 sec acquisition time) and the recycle delay was increased to 3 sec. The data was processed using an exponential weighting function with 5 Hz line broadening. HMQC data was obtained on a Bruker 500 MHz instrument equipped with a TXO probe using a combined HMQC–ROESY pulse sequence. The basic acquisition parameters were as follows: 1024 points per scan, 16 scans per increment, 512 increments, 90 ms mixing time. Only HMQC correlations were analyzed for structure assignment.

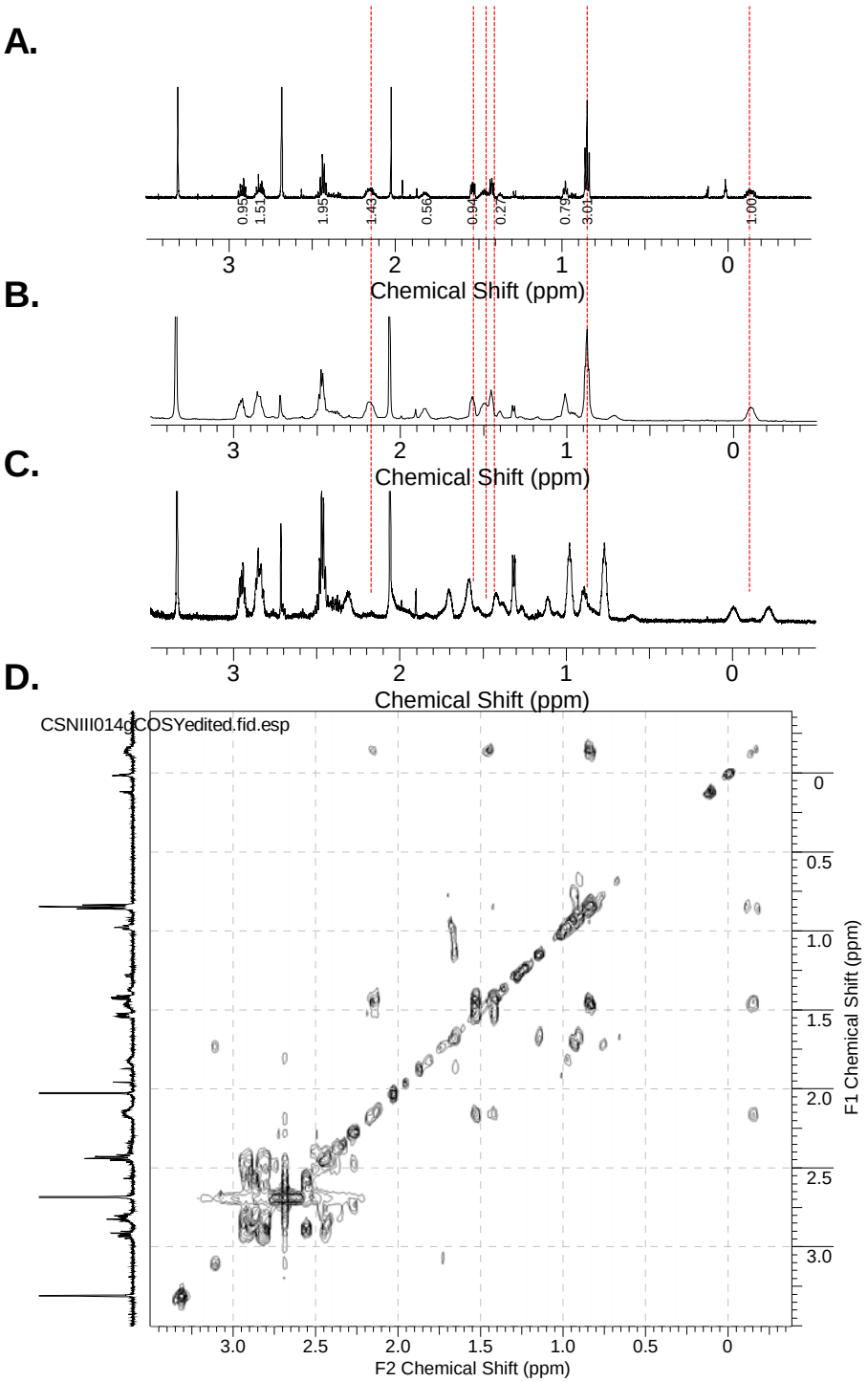


Figure S7: Expanded aliphatic regions of NMR spectra. Red lines indicate Ile-derived signals A) ^{1}H spectrum of alloCMA–OPA. B) ^{13}C-decoupled ^{1}H spectrum of ^{13}C-enriched alloCMA–OPA. C) ^{1}H spectrum of ^{13}C-enriched alloCMA–OPA. D) COSY spectrum of alloCMA–OPA.

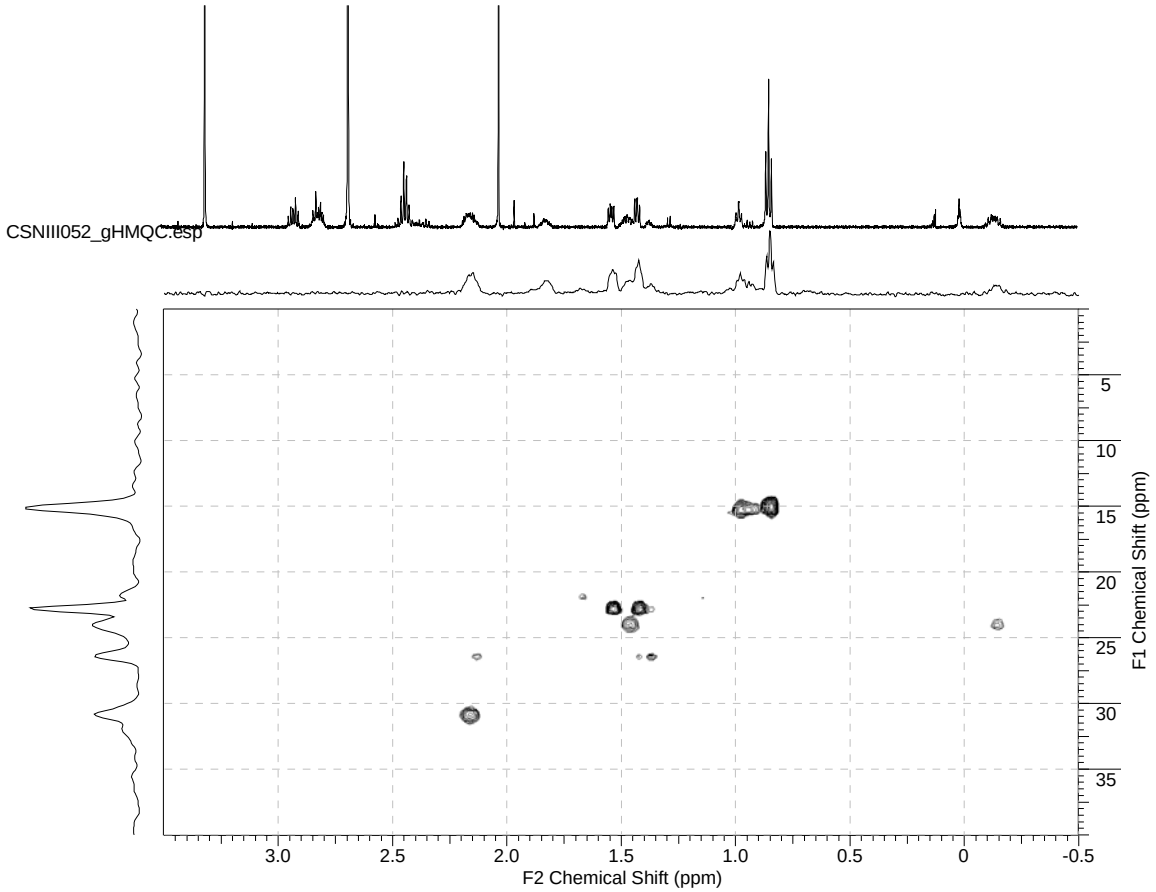
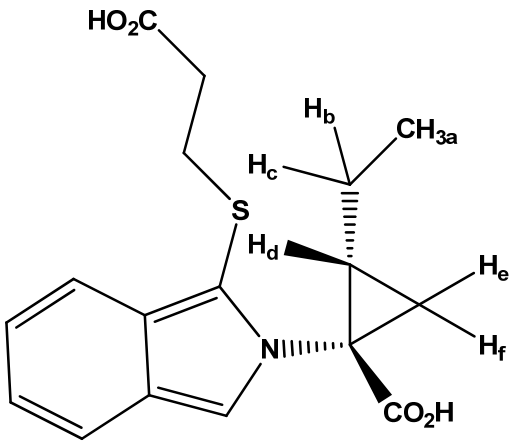


Figure S8: Aliphatic region of gHMQC spectrum. ^{1}H reference spectrum (top) is unenriched alloCMA–OPA.

6c. Assignment of proton resonances.



alloCMA–OPA

^{1}H NMR (600 MHz, D_2O) δ ppm 7.68 (d, *J*=8.5 Hz, 1 H), 7.59 (d, *J*=8.5 Hz, 1 H), 7.08 (m, 1 H), 6.98 (m, 1 H), 2.92 (ddd, *J*=12.9, 7.0, 6.5 Hz, 1 H), 2.82 (m, 1 H), 2.44 (m, 2 H),

2.15 (m, 1 H), 1.54 (dd, *J*=9.4, 5.3 Hz, 1 H), 1.47 (m, 1 H), 1.42 (dd, *J*=7.0, 5.9 Hz, 1 H), 0.85 (t, *J*=7.6 Hz, 3 H), -0.14 (m, 1 H).

Proton	Chemical Shift (ppm)	COSY correlations	HMQC correlation (ppm)
H_a (3)	0.85	H_b, H_c	15.1
H_b	-0.14	H_a, H_c, H_d	24.0
H_c	1.47	H_a, H_b, H_d	24.0
H_d	2.15	H_b, H_c, H_e, H_f	30.7
H_e	1.54 or 1.42	H_d, H_f	22.6
H_f	1.54 or 1.42	H_d, H_e	22.6

Table S1: Correlation data for 2D NMR experiments.

6d. Comparison with coronamic acid; deduction of stereochemistry: An authentic sample of coronamic acid was derivatized to the isoindole compound as described and compared to purified alloCMA–OPA by HPLC. The differential retention times of the two compounds confirmed the diastereomeric relationship.

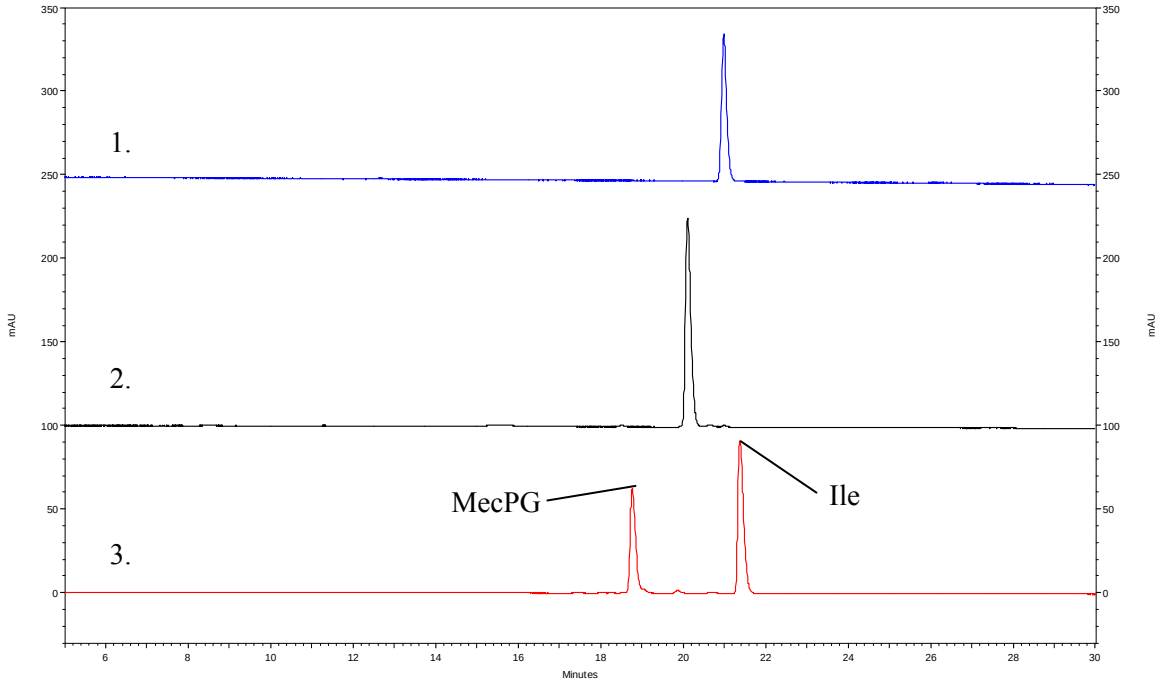


Figure S9: HPLC analysis (λ=338 nm) of isoindole derivatives of 1) experimentally-derived allocoronamic acid, 2) authentic coronamic acid, and 3) Ile and 2-(1-methylcycolpropyl)glycine (MecPG).

7. References

(1) Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning: A Laboratory Manual.* 3rd ed, Cold Spring Harbor Laboratory Press, Cold Spring Habor, NY, 2001.

(2) Gottlieb, H. E.; Kotlyar, V.; Nudelman, A. *J. Org. Chem.* **1997**, *62*, 7512–7515.

(3) Fujimori, D. G.; Hrvatin, S.; Neumann, C. S.; Strieker, M.; Marahiel, M. A.; Walsh, C. T. *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104*, 16498–16503.
(4) Evans, D. A.; Rieger, D. L.; Jones, T. K.; Kaldor, S. W. *J. Org. Chem.* **1990**, *55*, 6260–6268.
(5) (a) Yin, J.; Lin, A. J.; Golan, D. E.; Walsh, C. T. *Nat. Protoc.* **2006**, *1*, 280-285. (b) Quadri, L. E. N.; Weinreb, P. H.; Lei, M.; Nakano, M. M.; Zuber, P.; Walsh, C. T. *Biochemistry* **1998**, *37*, 1585–1595.
(6) Yeh, E.; Kohli, R. M.; Bruner, S. D.; Walsh, C. T. *Chembiochem* **2004,** *5,* 1290–1293.
(7) (a) Morineau, G.; Azoulay, M.; Frappier, F. *J. Chrom., A* **1989**, *467*, 209–216. (b) Vaillancourt, F. H.; Yeh, E.; Vosburg, D. A.; O'Connor, S. E.; Walsh, C. T. *Nature* **2005**, *436*, 1191–1194.