Discovery and biosynthesis of azabicyclene, a conserved nonribosomal peptide in *Pseudomonas aeruginosa*

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Experimental Procedures:

Chemicals were purchased from commercial suppliers and used without further purification. *N*-(3-Oxododecanoyl)-L-homoserine lactone (3-*O*-C_{12}-AHL), butyryl chloride, anhydrous dichloromethane (DCM), L-proline, L-serine, crotonyl-coenzyme A, L-azetidine-2-carboxylic acid (AZC), and coenzyme-A were purchased from Millipore-Sigma. Luria broth, 3-(N-morpholino) propane sulfonic acid (MOPS), dichloromethane (DCM), ethyl acetate (EtOAc), acetonitrile (ACN), agar, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), glycerol, sodium chloride (NaCl), imidazole, sodium bicarbonate, hexanes, and ampicillin were purchased from Fisher Scientific. Dimethyl sulfoxide (DMSO) molecular biology grade was purchased from Acros Organics. L-homoserine lactone hydrochloride was purchased from Frontier Scientific. Triethylamine was purchased from Alfa Aesar. Isopropylthio-D-galactoside (IPTG) was purchased from Goldbio. $CDCl_3$ and DMSO-d_6 were purchased from Cambridge Isotopes Laboratories.

NMR spectra were recorded either on a Bruker 500 MHz, 600 MHz, or 700 MHz NMR spectrometer, as specified in the experimental methods. NMR experiments are reported in δ units, parts per million (ppm), and were referenced to DMSO (δ 2.50 ppm) or $CDCl_3$ (δ 7.26 ppm) as internal standards.

Synthesis of L-C_4-acyl-homoserine lactone (C_4-AHL)

The procedure was adapted from Chhabra *et al*.[1] Dry DCM (15 mL), L-homoserine lactone hydrochloride (0.727 mmol) and triethylamine (1.74 mmol) were added to an oven dried round bottom flask under N_2 on ice. The mixture was stirred for 30 minutes at 0 °C, and butyryl chloride (0.727 mmol) was subsequently added dropwise over 5 minutes. This reaction was gradually warmed to room temperature and then stirred for 4 hours at room temperature. The reaction was extracted with 20 mL EtOAc, washed twice with saturated sodium bicarbonate and once with brine. The mixture was treated with sodium sulfate and dried under reduced pressure. The mixture was then resuspended in 50/50 EtOAc/hexanes, applied to a silica column and separated using a gradient of 0−100% hexanes/EtOAc. To fully elute the product, C_4-AHL, 20 column volumes of 100% EtOAc was used. The product was dried under reduced vacuum as a white powder (79 mg, 63% yield).

^{1}H NMR (500 MHz, $CDCl_3$) δ: 6.11 (br s, 1H), 4.58 (ddd, J = 11.6, 8.6, 5.9 Hz, 1H), 4.49 (td, J = 9.0, 1.2 Hz, 1H), 4.31 (ddd, J = 11.3, 9.3, 5.8 Hz, 1H), 2.88 (dddd, J = 12.6, 8.6, 5.9, 1.2 Hz, 1H), 2.25 (td, J = 7.3, 2.1 Hz, 1H), 2.15 (dtd, J = 12.6, 11.5, 8.8 Hz, 1H), 1.70 (h, J = 7.4 Hz, 1H), 0.98 (t, J = 7.4 Hz, 2H).
HRMS (ESI) m/z $[M + H]^+$ calc'd for $C_8H_{14}NO_3$ 172.2035, observed 172.2029.

Verification of *Pseudomonas aeruginosa* deletion mutants from the Manoil collection[2]

LacZ insertional mutants of *Pseudomonas aeruginosa* PAO1 were obtained in genes *azeB*, *C*, *D*, *E*, *F*, *H*, *I*, and *J* (PA3327, 3328, 3329, 3330, 3331, 3333, 3334, 3335) and PhoA insertional mutant of *azeG* (PA3332). Insertional mutants were verified by colony PCR of WT

PAO1 in comparison to the insertional mutant. The primers were designed to amplify the genomic region disrupted by the insertional mutant (Table S4). Fragments of ~1 kb size are amplified for WT PAO1, while the insertional mutants have a 3 kb *lacZ* insertion (*azeG* instead has a 1.5 kb *phoA* insertion) and are not amplified under the PCR conditions that use a short extension time.

Production of 1 and 2 by *P. aeruginosa* PAO1 wildtype and insertional mutants

P. aeruginosa PAO1 wildtype and insertional mutants were freshly streaked on LB plates and grown for 16 hours at 37 °C. Single colonies were picked and used to inoculate 2 mL liquid LB media. These cultures were grown with vigorous shaking at 37 °C for 12 hours. A sample of 250 µL of each culture was transferred to 25 mL of LB media in 125 mL baffled flasks. The 25 mL cultures were buffered using 50 mM MOPS pH 7.0 and induced using 6 µM 3-*O*-C_{12}-AHL in DMSO and 30 µM C_4-AHL in water (4% v/v DMSO in culture). These cultures were grown at 37 °C for 14 hours with shaking at 200 rpm.

For small scale extractions, 5 mL of each culture was centrifuged for 20 minutes at 4,000 rcf to remove the cells. The 5 mL of cell-free supernatant was extracted with 5 mL DCM. A 1 mL sample of DCM extract was dried under reduced pressure, resuspended in 250 µL 50/50 ACN/H_2O, centrifuged at 21,000 rcf to remove the precipitants, and a 10 µL sample of the metabolites soluble in ACN/H_2O were analyzed by liquid chromatography coupled high resolution mass spectrometry (LC-HRMS) using Agilent Technologies 6520 Accurate Mass Q-TOF mass spectrometer. Data were collected under positive ion mode using electrospray ionization (ESI). The following parameters were used: gas temperature 300 °C, drying gas 10 L/min, nebulizer 45 lb/in^2, fragmentor 175 V, and skimmer 65 V. Samples were separated on a Kinetex C18 column (Phenomenex, 150 mm length, 2.6 µm particle size and 100 Å pore size) using the following method: solvent A consisted of 0.1% formic acid in water (Fisher Scientific), and solvent B consisted of 0.1% formic acid in acetonitrile (Fisher Scientific). Mobile phase was held at 2% B for 2 min, increased from 2% to 98% B over 10 min in a linear gradient, held at 95% B for 2 min before returning to 2% B over 1 min.

Isolation and structural characterization of 1

P. aeruginosa PAO1 was freshly streaked on LB plates and grown for 16 hours at 37 °C. Single colonies were picked and used to inoculate 5 mL liquid culture in LB. Cultures were grown with vigorous shaking at 37 °C for 12 hours. A 5 mL sample of this culture was transferred to 12 separate 500 mL cultures in LB in 2.8 L baffled flasks. The cultures were buffered using 50 mM MOPS pH 7.0 and induced using 6 µM 3-*O*-C_{12}-AHL and 30 µM C_4-AHL, and 4% v/v DMSO. These cultures were grown at 37 °C for 14 hours with shaking at 200 rpm. The 500 mL cultures were grown at 37 °C for 14 hours with shaking at 200 rpm. Cultures were centrifuged for 20 minutes at 4,000 rcf to remove the cells. The cell-free supernatant from each 500 mL culture was extracted twice with 250 mL of DCM each time. The DCM layer was dried under reduced pressure and resuspended in 50/50 ACN/H_2O. Compound **1** was purified from this extract using reverse-phase preparative HPLC (Agilent PrepStar) Column purification conditions are as follows: mobile phase A consisted of water, mobile phase B consisted of acetonitrile. Mobile phase was increased from 5% to 16% B over 27.5 minutes in a linear

gradient, then increased to 95% B over 22.5 minutes. A fraction eluting at 15% B that exhibited strong absorbances at 280 and 320 nm contained Compound **1** and was collected and dried under reduced pressure, and purified as a white solid (1.2 mg from 6 L of bacterial culture). The dried fraction was rerun two more times using the same HPLC gradient to yield pure Compound **1** as shown by NMR and LC-MS analysis. Characterization by 1D and 2D NMR analysis and LC-MS/MS are shown below.

^{1}H NMR (700 MHz, DMSO-d_6) δ: 10.78 (s, 1H), 6.94 (dd, J = 15.2, 7.0, 1H), 6.18 (dd, J = 15.3, 1.9, 1H), 5.97 (s, 1H), 4.22 (dd, J = 9.8, 6.2, 1H), 4.05 (q, J = 8.9, 1H), 3.41 (td, J = 9.8, 5.5, 1H), 2.61 (m, 1H), 2.13 (ddq, J = 10.0, 6.3, 1.9), 1.89 (dd, J = 6.9, 1.7, 3H).

^{13}C NMR (175 MHz, DMSO-d_6) δ: 204.22, 174.17, 163.80, 143.59, 125.24, 99.00, 65.76, 53.27, 20.18, 17.69.

HRMS (ESI) *m*/*z* $[M + H]^+$ calc'd for $C_{10}H_{13}N_2O_2$ 193.0972, observed 193.0981

Cloning of the *azeB* gene

The *azeB* gene was amplified from *Pseudomonas aeruginosa* PAO1 genomic DNA, which was isolated using Qiagen DNeasy Blood & Tissue Kit according to manufacturer instructions. PCR was performed using Q5 DNA polymerase (New England Biolabs) and the primers listed in Table S4. The PCR product was inserted into the pLIC-His vector using the ligation independent cloning method.[3] The sequence of the construct was confirmed by DNA sequencing.

Expression and purification of recombinant AzeB protein

E. coli BL21-Gold (DE3) cells were transformed with the pLIC-His-*AzeB* vector by electroporation. Starter cultures were grown overnight from a single colony in 5 mL of LB medium supplemented with 50 μg/mL ampicillin. Cultures used for expression were inoculated with 2 mL of the starter culture in 1 L of LB containing 50 μg/mL ampicillin. The expression cultures were grown at 37 °C to an OD_{600} of 0.5 when protein expression was induced by adding 0.1 mM IPTG. After induction, cells were grown for 16 hours at 16 °C and then harvested by centrifugation at 6,000 rcf for 30 minutes at 4 °C. Cells were lysed by sonication in buffer A (25 mM HEPES, 150 mM NaCl, 30 mM imidazole, pH 7.5) using a Fisher Scientific Sonic dismembrator model 500 (1.5 min total on time at 30% amplitude, in cycles of 0.5 sec pulse on and 1.5 sec pulse off). The lysed cells were pelleted by centrifugation at 21,000 rcf for 30 minutes at 4 °C. The supernatant was filtered through a 0.45 μm pore before purification by FPLC. The filtered cell lysate was purified by nickel affinity chromatography using a GE HisTrap HP Ni column. Buffer A was used as wash buffer, and Buffer B (25 mM HEPES, 150 mM NaCl, 300 mM imidazole, pH 7.5) was used as elution buffer. Proteins were eluted in a linear gradient of 0−100% Buffer B. Fractions containing AzeB were combined and concentrated to a final volume of 3.5 mL using a Millipore centrifugal filter with a 30 kDa molecular weight cutoff. The concentrated fractions were separated using size exclusion chromatography on a Superdex 200 pg column (GE HiLoad 16/600) that was pre-equilibrated with Buffer C (25 mM HEPES pH 7.5, 150 mM NaCl, 10% glycerol). Fractions containing AzeB at >90% purity were pooled and concentrated using a Millipore centrifugal filter with a 30 kDa molecular weight cutoff to a final concentration of 10−25 μM. AzeB was flash frozen in liquid nitrogen and stored at −80 °C.

***In vitro* reconstitution of AzeB activity**

A sample of 5 μM AzeB was incubated with 0.5 μM Sfp and 100 μM CoASH in 50 mM HEPES buffer pH 8 containing 8 mM $MgCl_2$ for 10 minutes at room temperature. To this reaction was added 1 mM L-serine, 1 mM L-proline or L-AZC, 1 mM crotonyl-CoA, and 4 mM ATP to a final volume of 50 μL. This reaction was incubated at room temperature for 2 hours. To quench the reaction, 50 μL acetonitrile was added, and the reaction was centrifuged at 20,000 rcf to remove precipitated protein. A 10 μL sample of supernatant was analyzed by LC-HRMS (Agilent Technologies 6520 Accurate Mass Q-TOF). Data were collected using ESI mass spectrometry under positive ion mode using the following parameters: gas temperature 300 °C, drying gas 10 L/min, nebulizer 45 lb/in^2, fragmentor 175 V, and skimmer 65 V. Samples were separated on a Kinetex C18 column (Phenomenex, 150 mm length, 2.6 μm particle size and 100 Å pore size) using the following method: solvent A consisted of 0.1% formic acid in water, and solvent B consisted of 0.1% formic acid in acetonitrile. Mobile phase was held at 2% B for 2 min, increased from 2% to 98% B over 10 min in a linear gradient, held at 95% B for 2 min before returning to 2% B over 1 min.

***In vitro* biosynthesis and characterization of 3**

An assay was conducted at a 2.2 mg-scale for production of **3** and structural characterization. A sample of 5 μM AzeB was incubated with 0.5 μM Sfp and 100 μM CoASH in
8 mM $MgCl_2$ and 50 mM HEPES pH 8 buffer for 10 minutes at room temperature. To this reaction was added 2 mM L-serine, 2 mM L-AZC, 1 mM crotonyl-CoA, and 4 mM ATP to a final volume of 10 mL. This reaction was incubated at room temperature for 4 hours. The reaction was quenched with acetonitrile and centrifuged at 20,000 rcf to remove precipitated proteins. The supernatant was filtered using a 0.2 μm filter and purified by reverse-phase preparative HPLC (Agilent Prepstar). The mixture was injected on Phenomenex Luna C18 (10 μm, 250 x 21.20 mm) and separated using a gradient of 5−95% mobile phase B over 30 minutes: mobile phase A consisted of water, mobile phase B consisted of acetonitrile). The isolated product **3** was eluted at 25% B with strong absorbances at 340 nm. Compound **3** (1.3 mg, 59% yield) was isolated as a white solid and characterized using 1D and 2D NMR experiments (Figures S13−S16). ^{13}C NMR spectrum of **3** was not obtained due to limited yield of the compound and low stability in overnight experiment.

^{1}H NMR (700 MHz, DMSO-d_6) δ: 9.12 (s, 1H), 8.94 (s, 1H), 8.26 (s, 1H), 6.73 (d, J = 6.9 Hz, 1H), 6.49 (dd, J = 15.2, 1.8 Hz, 1H), 4.42 (d, J = 4.8 Hz, 2H), 3.22 (t, J = 4.8 Hz, 2H), 1.83 (dd, J = 6.9, 1.7 Hz, 3H).
^{13}C NMR shifts determined from HSQC and HMBC. δ: 167.8, 154.9, 143.1, 135.9, 130.7, 130.3, 129.2, 122.8, 52.5, 28.3, 20.9.
HRMS (ESI) m/z $[M + H]^+$ calc'd for $C_{11}H_{13}N_2O_3$ 221.0921, observed 221.0918

Antimicrobial assay of purified 1 by agar diffusion

E. coli MG1655 and *B. subtilis* BSU 168 were grown overnight at 37 °C in LB media. The overnight cultures were diluted to an OD_{600} of 0.5 and 100 μL were spread on an LB plate containing 15 g/L agar. The plates were dried before paper discs were added. Purified **1** was

applied at varying concentrations to the discs, and plates were incubated for 16 hours at 37 °C before imaging.

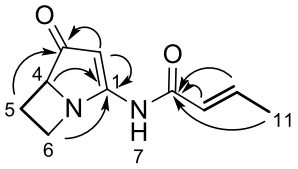
Phylogenetic analysis of AzeB-C domain

The second C domain of AzeB (AAG06715.1) was aligned with all protein sequences from the NaPDoS database,[4] and eight NRPS proteins identified in our previous work that contain C*-type domains, including LgnD (AIZ66879.1), Zmn17 (CCM44337.1), DepE (ABP57749.1), HasO (CZT62784.1), NdaA (ATP76243.1), PuwF (AIW82283.1), PuwG (AIW82284.1), and AmbE (AAG05690.1).[4-11] The alignment was performed with MUSCLE version 3.8.31.[12] A maximum likelihood tree containing bootstrap values was generated using RAxML version 8.2.4.[13] The tree was visualized using Interactive Tree Of Life (iTOL) version 3.5.4.[14]

Strain	Isolated from	Notes
Pseudomonas denitrificans strain 1332_PDEN	University of Washington, USA, intensive care unit, anatomic location not known[15]	Isolated in same clinical environment as *P. aeruginosa*
Pseudomonas denitrificans strain 148_PDEN	See above	
Pseudomonas denitrificans strain 994_PDEN	See above	
Pseudomonas denitrificans strain 466_PDEN	See above	
Pseudomonas denitrificans strain 481_PDEN	See above	
Pseudomonas denitrificans strain 518_PDEN	See above	
Pseudomonas denitrificans strain 1184_PDEN	See above	
Pseudomonas denitrificans strain 293_PDEN	See above	
Pseudomonas denitrificans strain 816_PDEN	See above	
Pseudomonas denitrificans strain 1151_PDEN	See above	
P. fluorescens strain 2-79 (NRRL B-15132)	Wheat rhizosphere isolate from Washington, USA[16]	Biocontrol agent, suppresses growth of plant pathogen *G. graminis* var. *tritici*

Table S1. Twelve non-*aeruginosa* strains of *Pseudomonas* contain the *aze* cluster.

	1				6				7			
H/C	δ_H, ppm	splitting	*J* values	δ_C, ppm	δ_H, ppm	splitting	*J* values	δ_C, ppm	δ_H, ppm	splitting	*J* values	δ_C, ppm
1				174.17				183.9				190.2
2	5.97	s, 1H		99.00	6.24	s, 1H		111.8	5.68	s, 1H		113.8
3				204.22				208				208.4
4	4.22	dd, 1H	9.8, 6.2	65.76	4.60	dd, 1H	10.2, 6.1	70.3	4.39	dd, 1H	9.8, 6.2	70.1
5a	2.61	m, 1H		20.18	2.80	m, 1H	11.8, 10.1, 8.8, 5.2	21.2	2.69	dddd, 1H	11.8, 9.9, 8.4, 5.1	Not reported
5b	2.13	m, 1H		20.18	2.43	m, 1H		21.2	2.24	m, 1H		Not reported
6a	4.05	t, 1H	8.9	53.27	4.24	ddd, 1H	9.6, 8.8, 7.3	53.5	4.08	ddd, 1H	9.8, 9.0, 7.5	51.7
6b	3.41	ddd, 1H	9.8, 9.8, 5.5	53.27	3.30	ddd, 1H	9.6, 9.6, 5.3	53.5	3.38	ddd, 1H	9.7, 9.4, 5.1	51.7
7	10.78	s, 1H										
8				163.80								
9	6.18	dd, 1H	15.3, 1.9	124.24								
10	6.94	qd, 1H	15.2, 7.0	143.59								
11	1.89	dd, 3H	6.9, 1.7	17.69								



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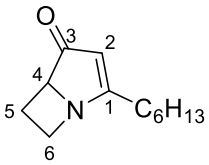

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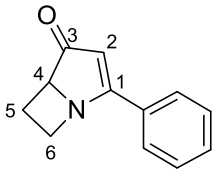


7

Key HMBC correlations

COSY correlations

Table S2. NMR shifts of **1** in DMSO-d_6 (700 MHz). The NMR shifts previously reported for synthetic compounds **6** and **7** are shown in comparison.[17]

	3				
H/C	δ_H, ppm	splitting	*J* values	δ_C, ppm	HMBC
1				130.3	
2	8.26	s, 1H		122.8	1, 3, 4, 7
3				135.9	
4				130.7	
5	3.22	t, 2H	4.8	28.3	4
6	4.42	t, 2H	4.8	52.5	
7				154.9	
8	9.12	s, 1H			2, 7, 9
9				167.8	
10	6.73	qd, 1H	17.5, 7.9	129.2	9, 12
11	6.49	ddd, 1H	16.2, 1.8	143.1	9, 12
12	1.83	dd, 3H	6.9, 1.7	20.9	10, 11
13	8.94	s, 1H			2, 3, 4

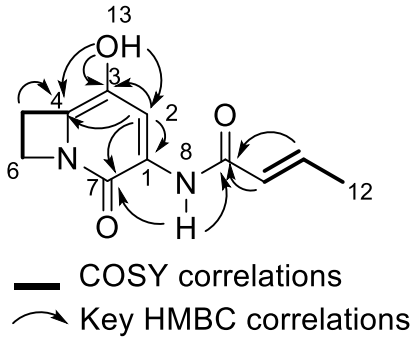


Table S3. NMR shifts of **3** in DMSO-d_6 (700 MHz).

Primer name	Sequence 5' – 3'	Purpose
3327F	TACTTCCAATCCAATGCGATGGTTCGTTTCGCTCGCTTG	*azeB* LIC cloning
3327R	TTATCCACTTCCAATGCGCTATCATGGGCGGGTCCGTTC	*azeB* LIC cloning
PW6659F	AATCGCCCTTCCTGTTCCT	Verify *azeB* insertion
PW6659R	GGTCGAACCGGAGGTATAGA	*azeB*
PW6601F	GCCCTGCTGCTGTTCTTCTA	Verify *azeC* insertion
PW6601R	ATCACGTAGTGCCAGTCGGT	*Verify azeC insertion*
PW6603F	AATGATGGCCGAGATACGAC	*Verify azeD* insertion
PW6603R	CTTCCATGAAGGCCATGAAT	*Verify azeD* insertion
PW6605F	CCTAGCCACATCGCCATC	*Verify azeE* insertion
PW6605R	GAGGAAGCGCAGGTAGGTATC	*Verify azeE* insertion
PW6607F	AACTCTACGCGAAGCGTACC	*Verify azeF* insertion
PW6607R	ATTTCCTTGGCATTCATTGG	*Verify azeF* insertion
PW6610F	CAACGCCATGGAAGAACTG	*Verify azeG* insertion
PW6610R	CGAGCATGTCGTTGCTGAG	*Verify azeG* insertion
PW6611F	AGCTGGACACTTCCGACG	*Verify azeH* insertion
PW6611R	AACAGTTCGTCCACCGTTTC	*Verify azeH* insertion
PW6613F	GATGCCGAGGAAGAAGGC	*Verify azeI* insertion
PW6613R	ATCTCGCTCTGGTGTTGGTT	*Verify azeI* insertion
PW6615F	AGTCGGCTTTCTACGAGCTG	*Verify azeJ* insertion
PW6615R	ACCATGTATTCGCTGGTGGT	*Verify azeJ* insertion

Table S4. Primers used in this study.

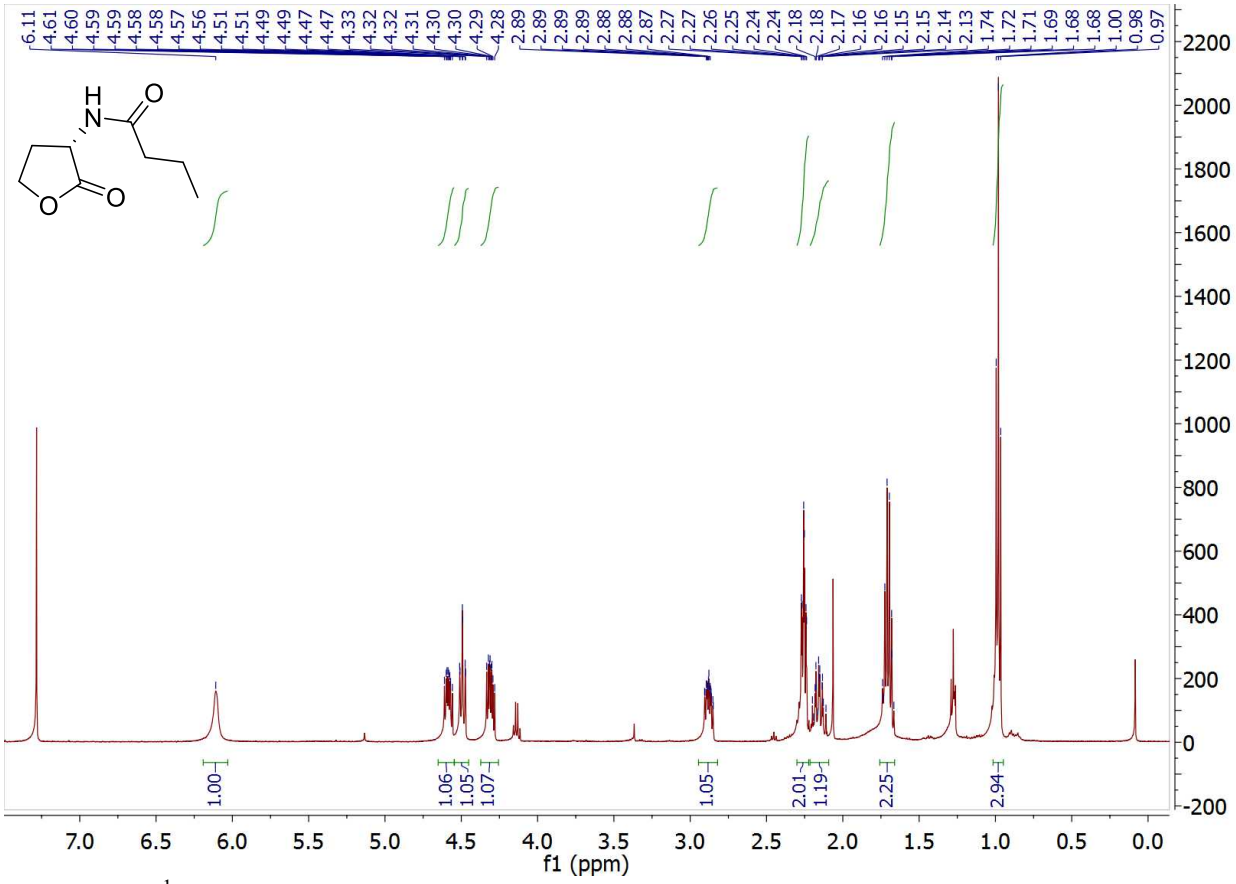


Figure S1. ^{1}H NMR spectrum of synthesized C_4-acyl-homoserine lactone in $CDCl_3$ (500 MHz).

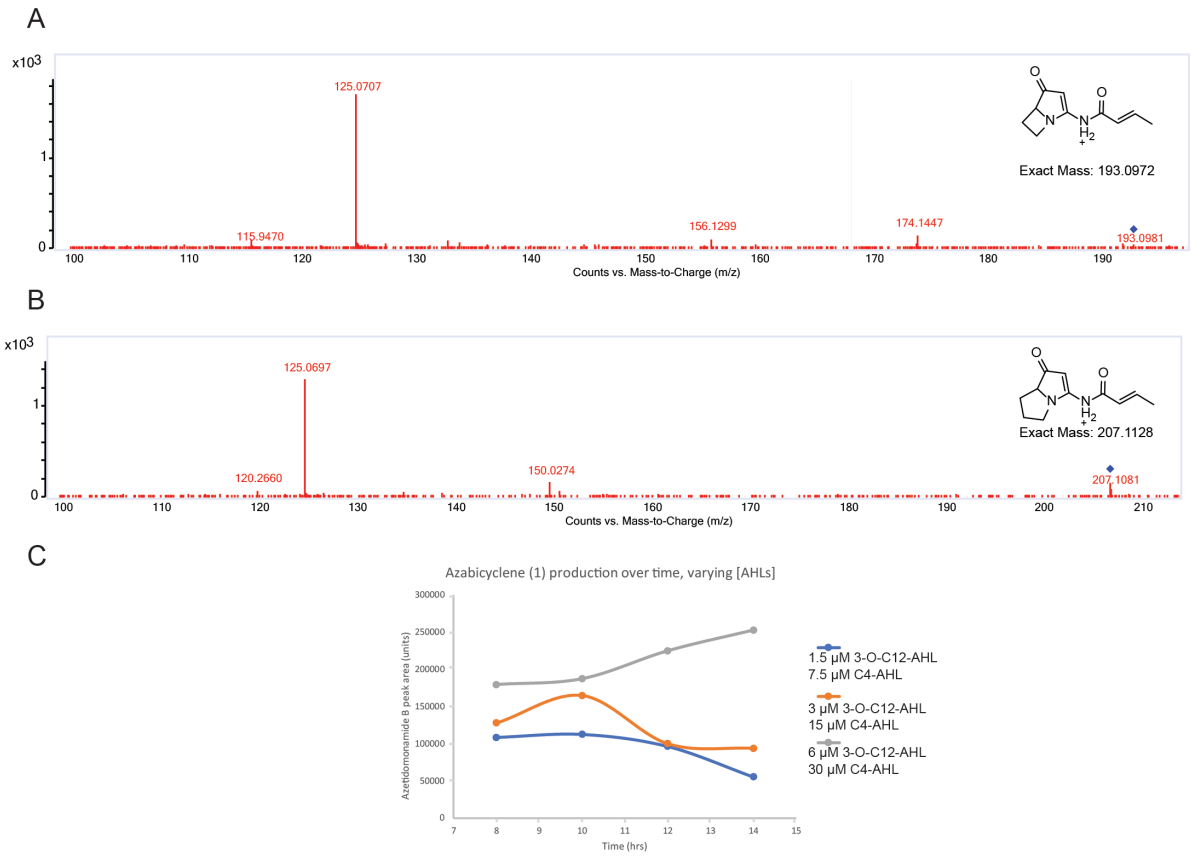


Figure S2. Production and optimization of **1** and **2**. A) Tandem mass spectrum of compound **1**. Observed 193.098, expected 193.097, 5 ppm error. B) Tandem mass spectrum of compound **2**. Observed 207.108, expected 207.113, 30 ppm error. C) The level of **1** was represented by integrated peak areas in the extracted ion chromatogram (EIC) of **1** (*m/z* 193.097 $[M+H]^+$) on the Y-axis.

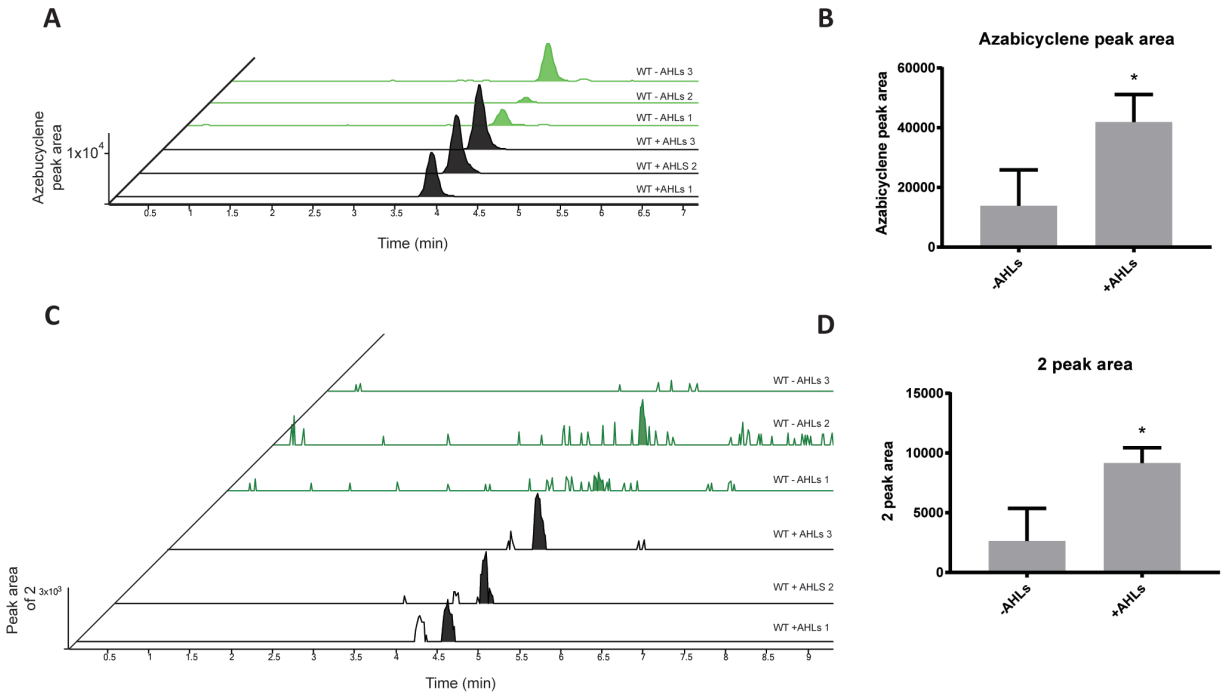


Figure S3. Production of **1** and **2** is augmented by exogenous AHLs. A) EICs of **1** (*m/z* 193.097 $[M+H]^+$) in the extracts of PAO1 wildtype strain with and without exogenous AHLs added, detected by LC-HRMS in three independent experiments. B) Peak areas of **1** from cultures with and without AHLs added. Error bars are standard deviations of three independent experiments. C) EICs of **2** (*m/z* 207.113 $[M+H]^+$) in the extracts of PAO1 wildtype strain with and without exogenous AHLs added in three independent experiments. D) Peak areas of **2** from cultures with and without AHLs added. Error bars are standard deviations of three independent experiments.

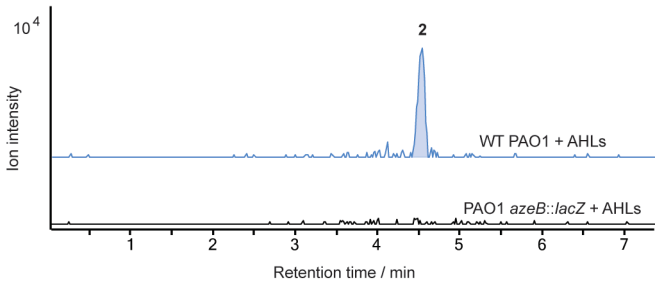


Figure S4. Production of **2** requires *azeB*. EIC of **2** (*m/z* 207.113 $[M+H]^+$) detected by LC-HRMS in the extracts of PAO1 wildtype strain and the PAO1 *AzeB*::*lacZ* mutant with AHLs added.

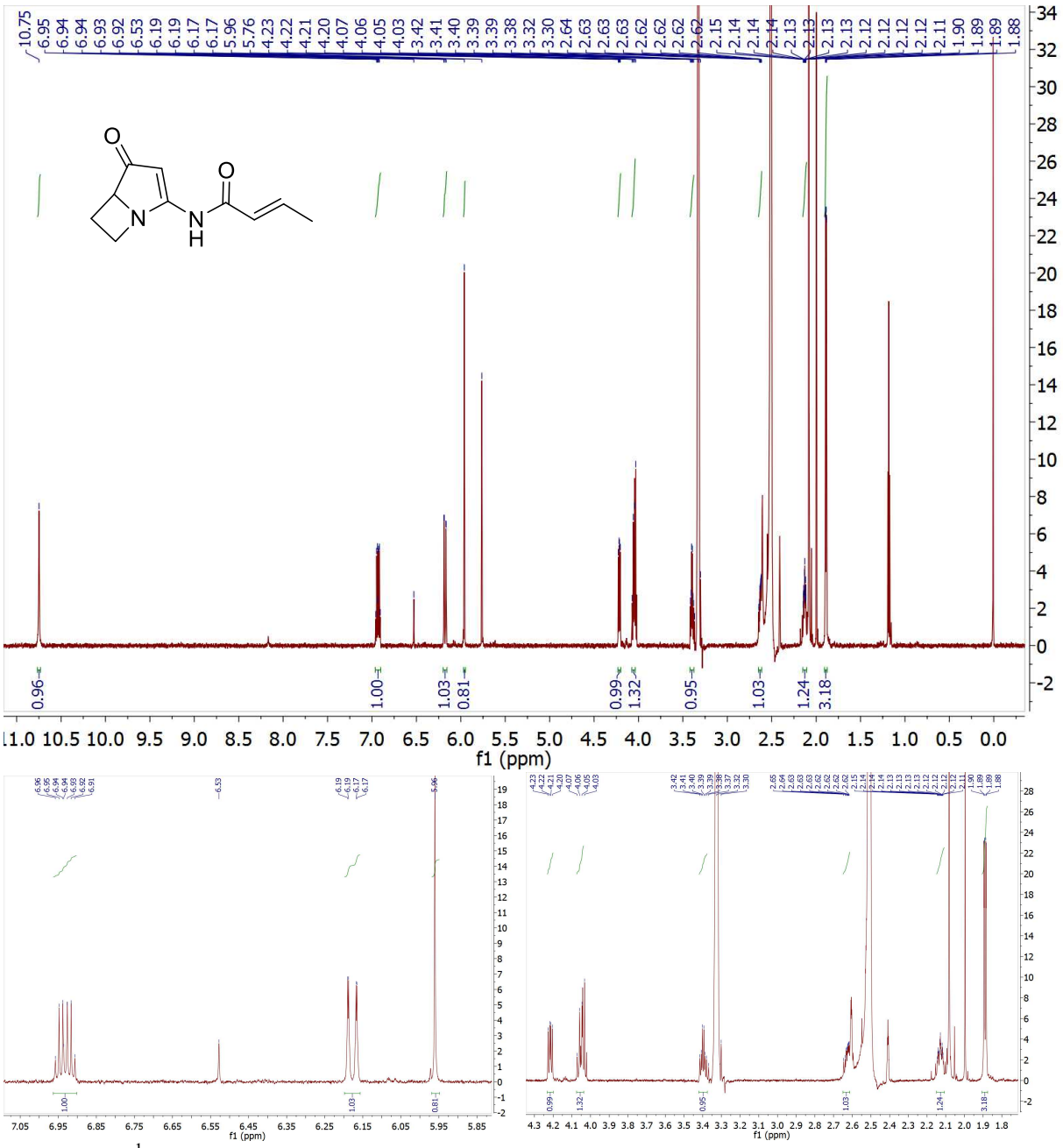


Figure S5. ^{1}H NMR spectrum of purified **1** in DMSO-d_6 (700 MHz).

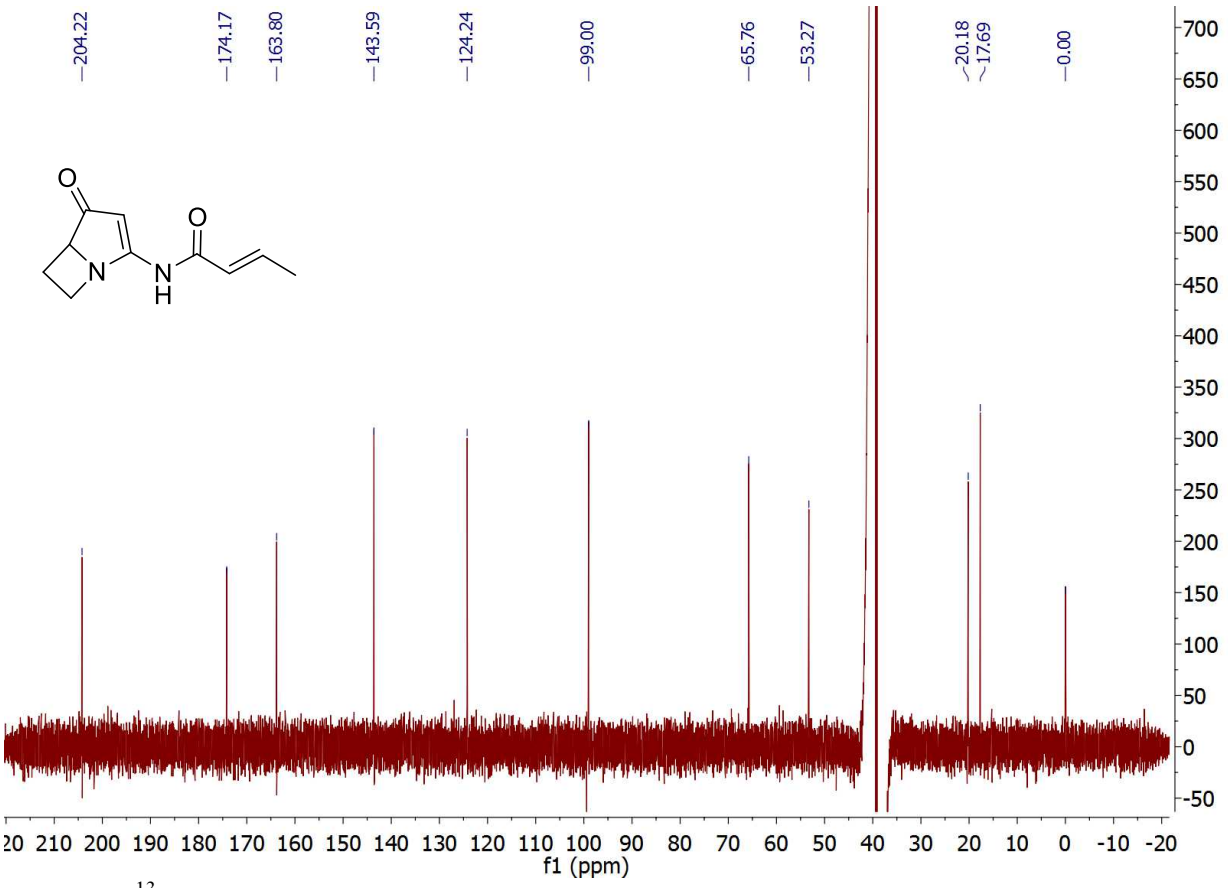


Figure 6. ^{13}C NMR spectrum of purified **1** in DMSO-d_6 (175 MHz).

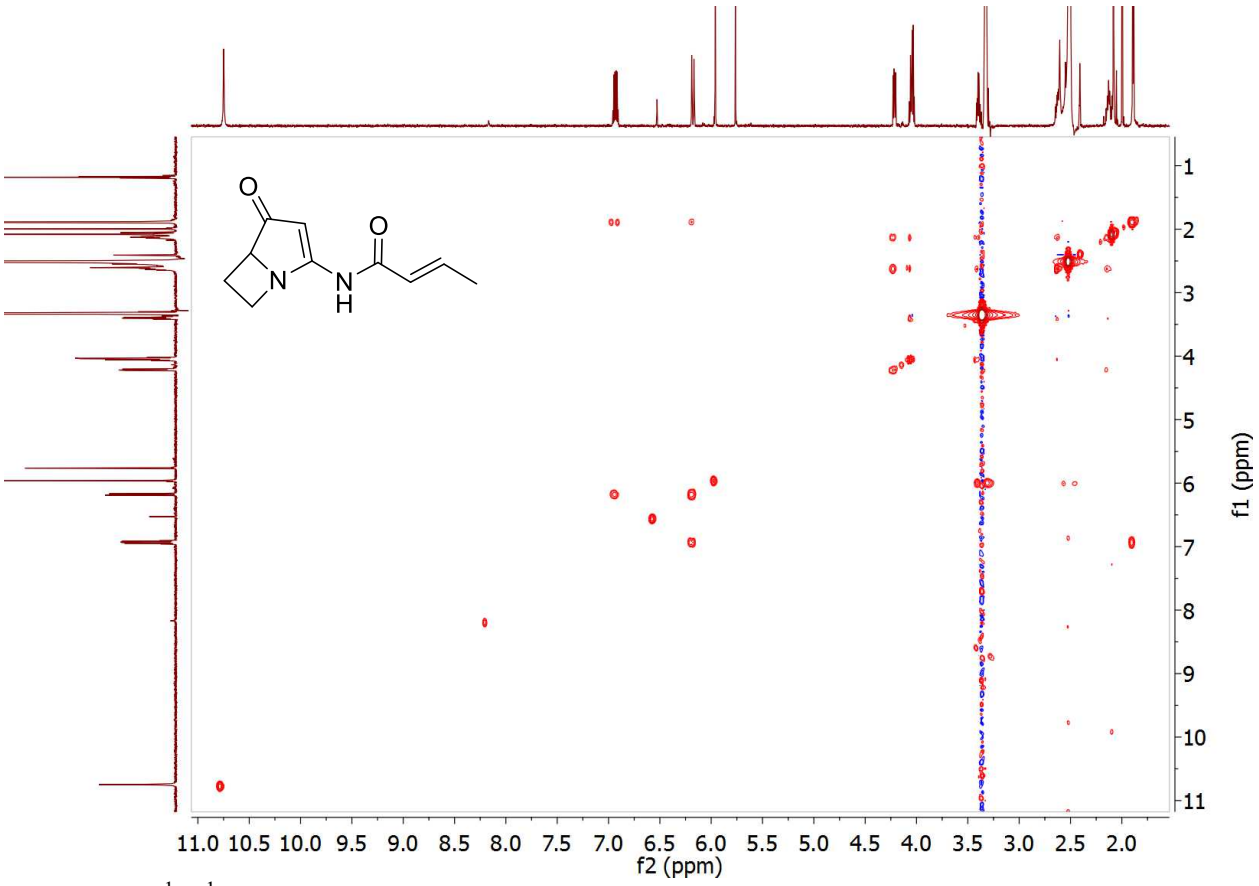


Figure S7. (^{1}H,^{1}H) COSY NMR of purified **1** in DMSO-d_6 (700 MHz).

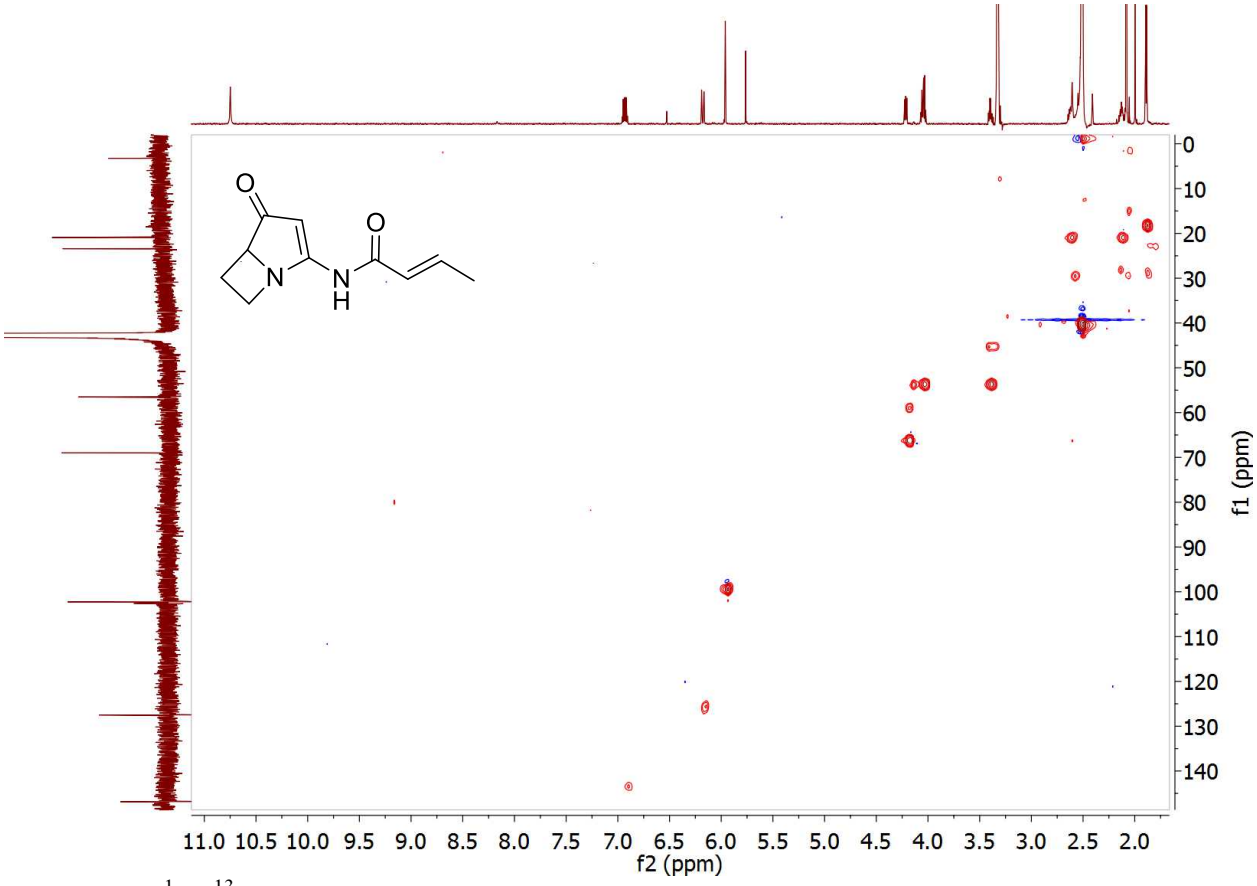


Figure S8. (^{1}H, ^{13}C) HSQC NMR spectrum of **1** in DMSO-d_6 (700 MHz).

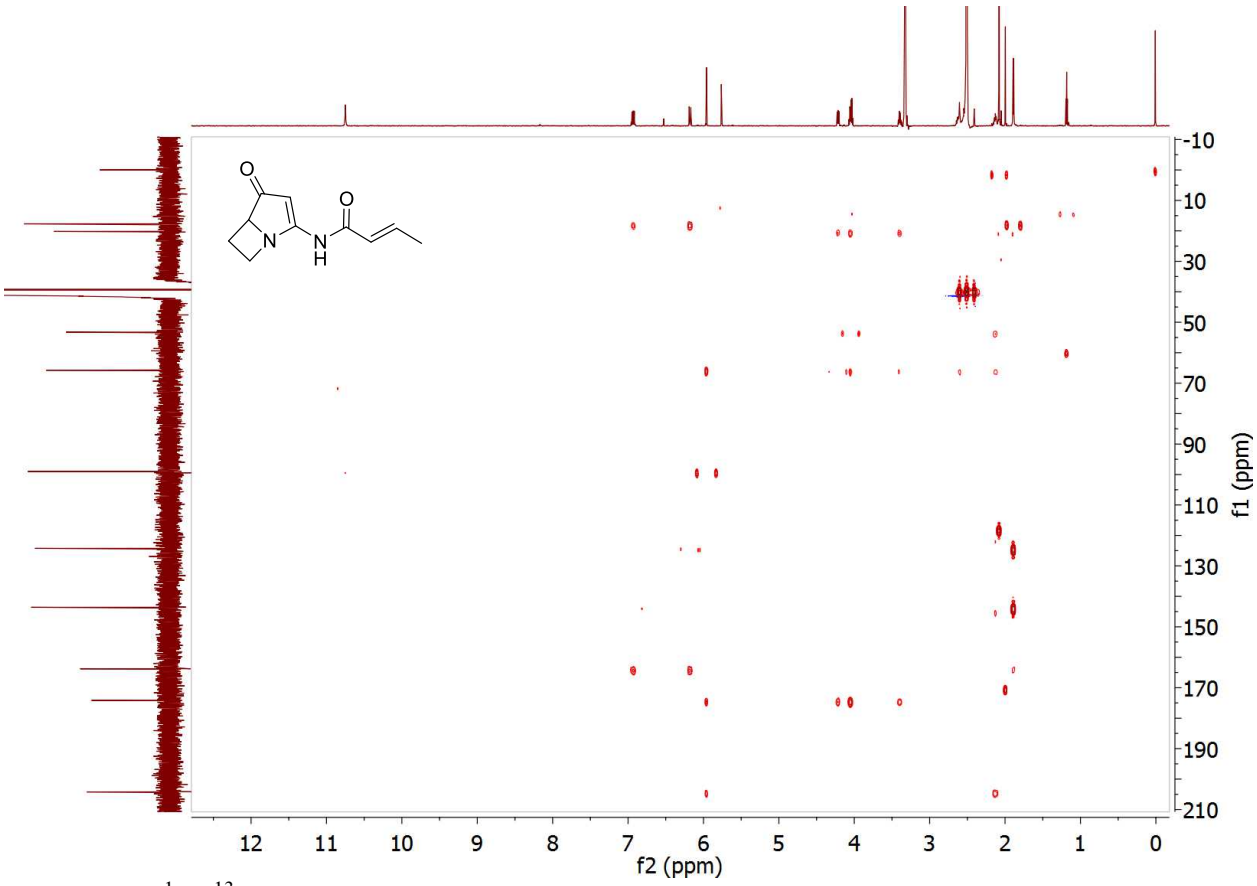


Figure S9. (^{1}H, ^{13}C) HMBC NMR spectrum of **1** in DMSO-d_6 (700 MHz).

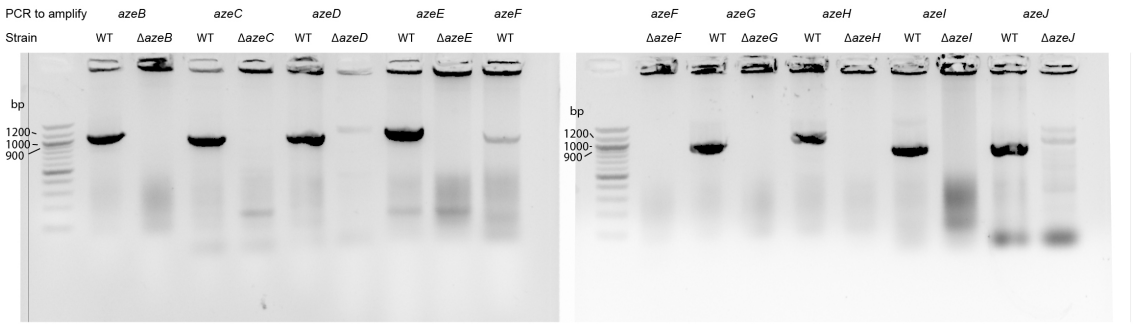


Figure S10. Verification of PAO1 insertional mutants from the Manoil Collection.[2] Products of PCRs using specific primers are shown for each insertional mutant in comparison with PAO1 wildtype. Expected band lengths from left to right are: 1084 (*azeB*), 1023 (*azeC*), 1045 (*azeD*), 1236 (*azeE*), 1070 (*azeF*), 945 (*azeG*), 1143 (*azeH*), 843 (*azeI*), and 868 (*azeJ*).

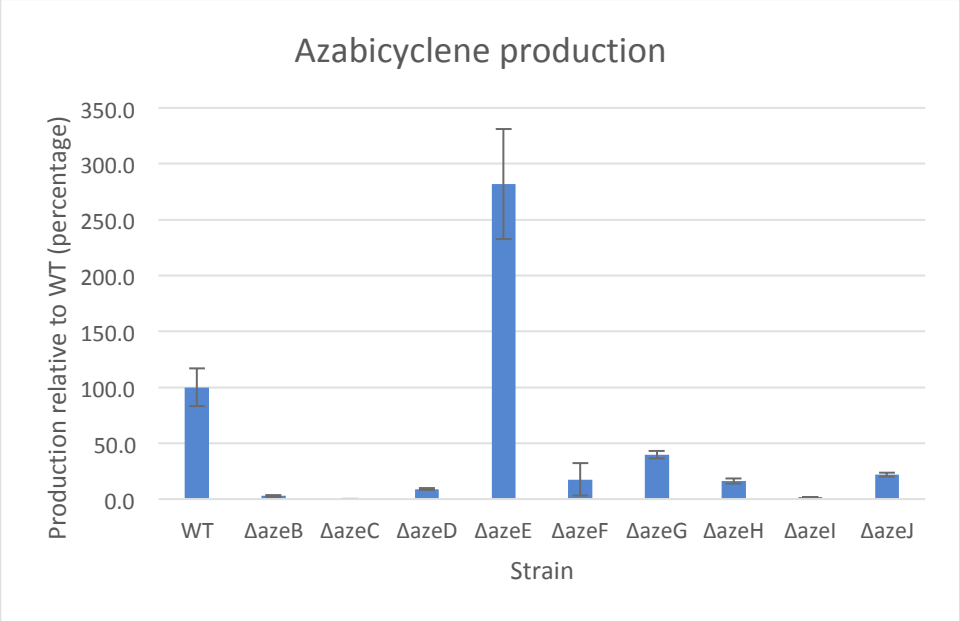


Figure S11. Production of azabicyclene (**1**) in PAO1 wildtype and insertional mutants in the *aze* pathway. Production in PAO1 wildtype is set to 100%. Each mutant is normalized relative to the amount produced by wildtype. Error bars are standard deviations of two duplicate cultures for each mutant. Cultures were grown in duplicate on three separate occasions, resulting in similar results. One representative set of cultures are shown.

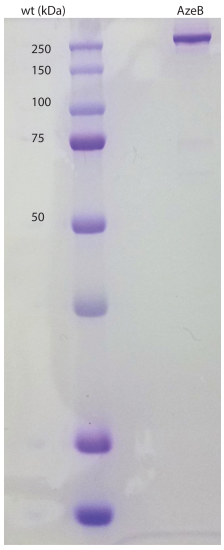


Figure S12. SDS-PAGE analysis of 6xHis-AzeB (expected molecular weight, 262 kDa).

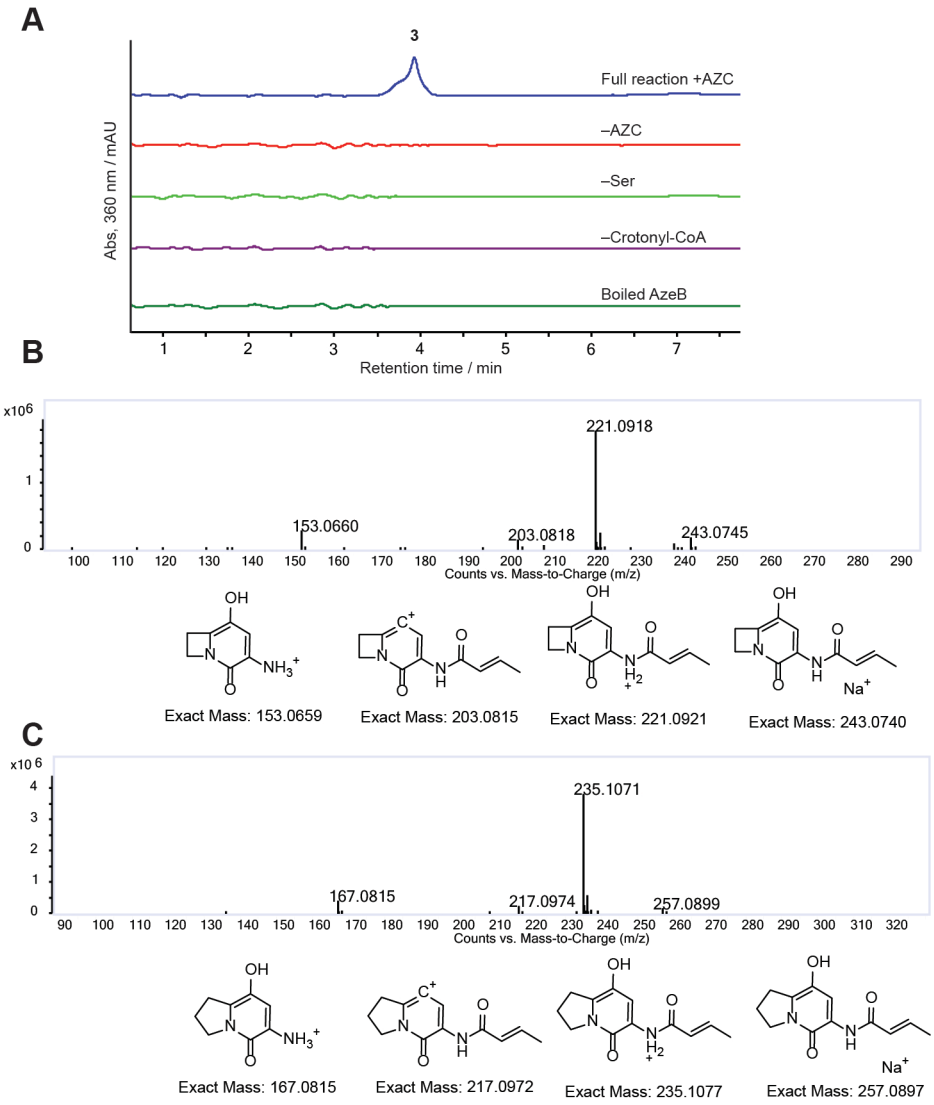


Figure S13. HPLC and MS analysis of production of **3** and **4** from *in vitro* reaction containing AzeB. A) HPLC traces of production of 3 from AzeB with negative controls. B) Mass spectrum of compound **3** from *in vitro* reaction of AzeB. Observed 221.092, expected 221.092, 0 ppm error. C) Mass spectrum of compound **4** from *in vitro* reaction of AzeB. Observed 235.107, expected 235.108, 4 ppm error.

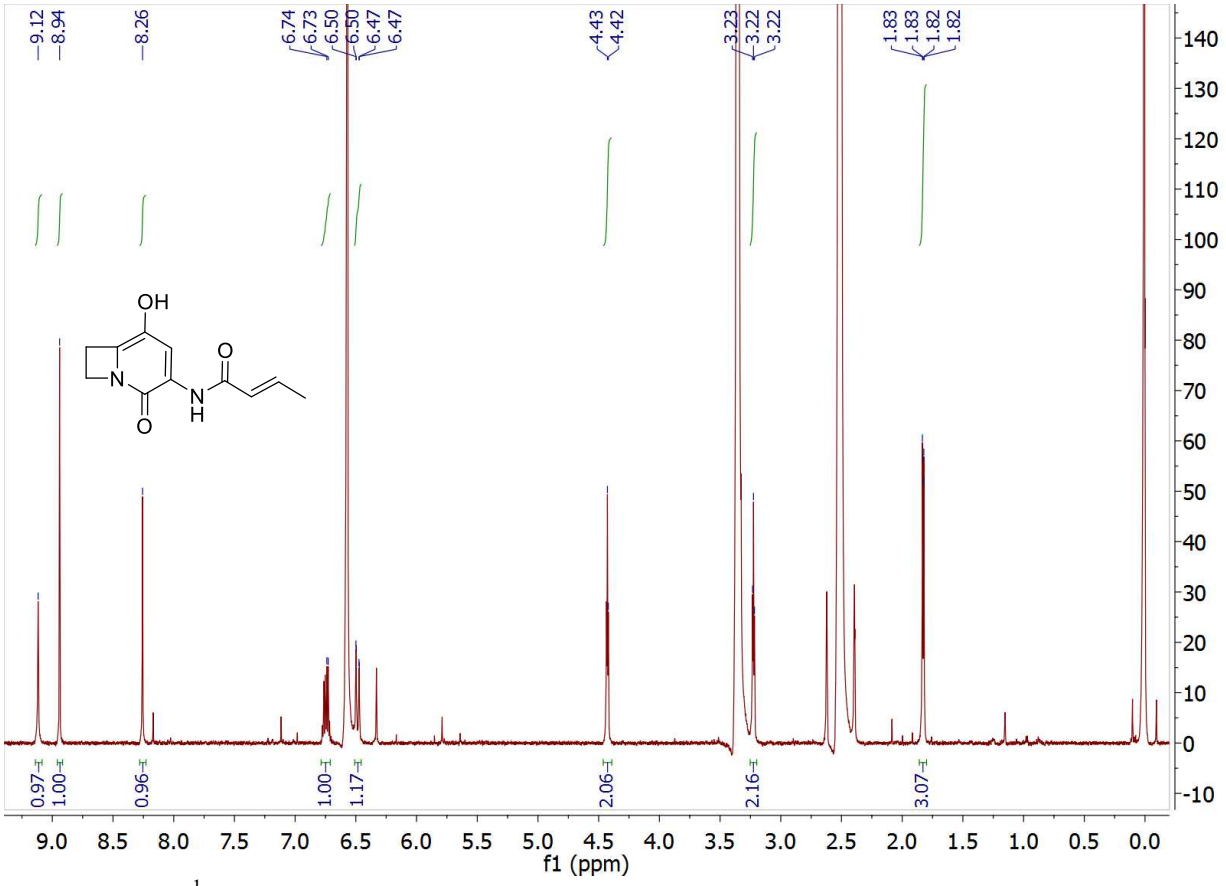


Figure S14. ^{1}H NMR spectrum of **3** in DMSO-d_6 (700 MHz) generated from *in vitro* reconstitution of AzeB.

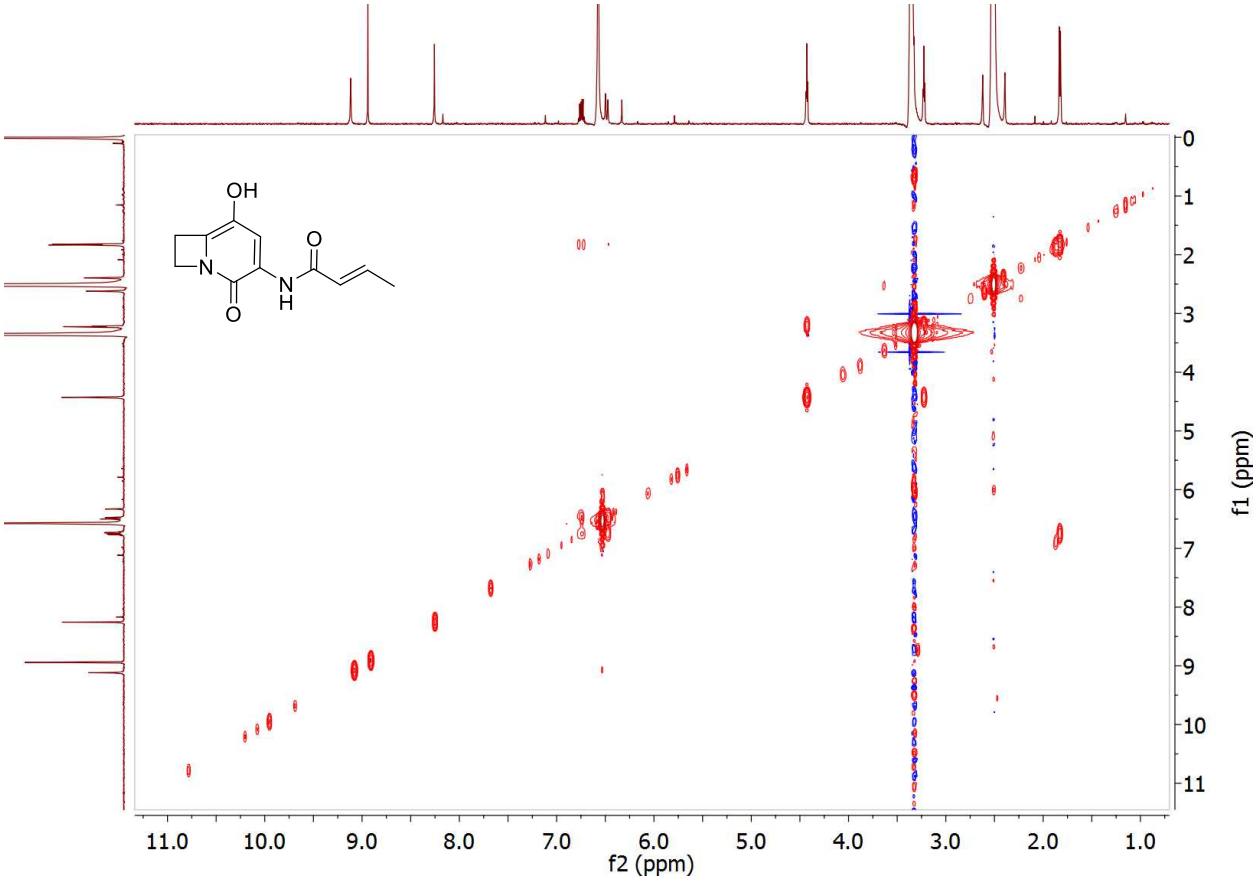


Figure S15. (^{1}H,^{1}H) COSY NMR spectrum of **3** in DMSO-d_6 (700 MHz) generated from *in vitro* reconstitution of AzeB.

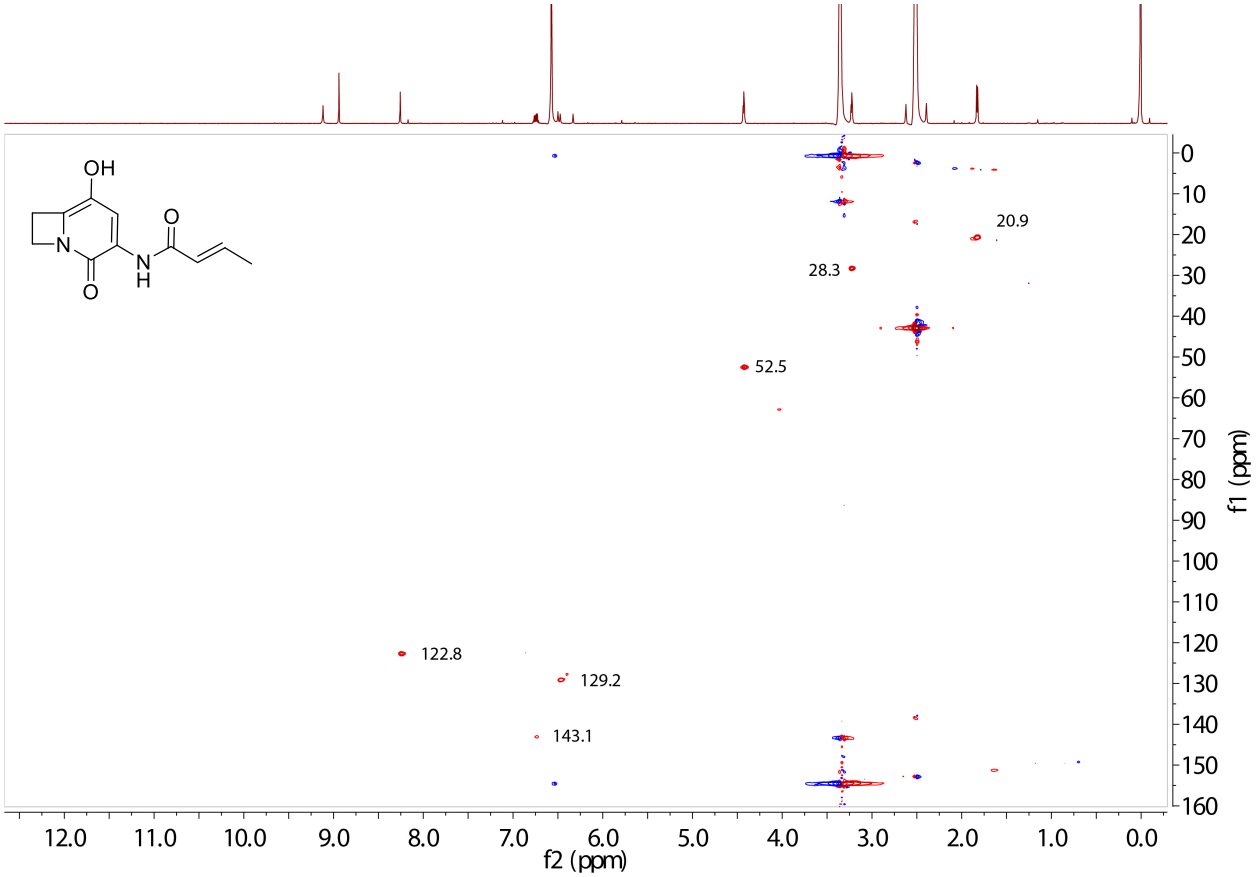


Figure S16. (^{1}H, ^{13}C) HSQC NMR spectrum of **3** in DMSO-d_6 (700 MHz) generated from *in vitro* reconstitution of AzeB.

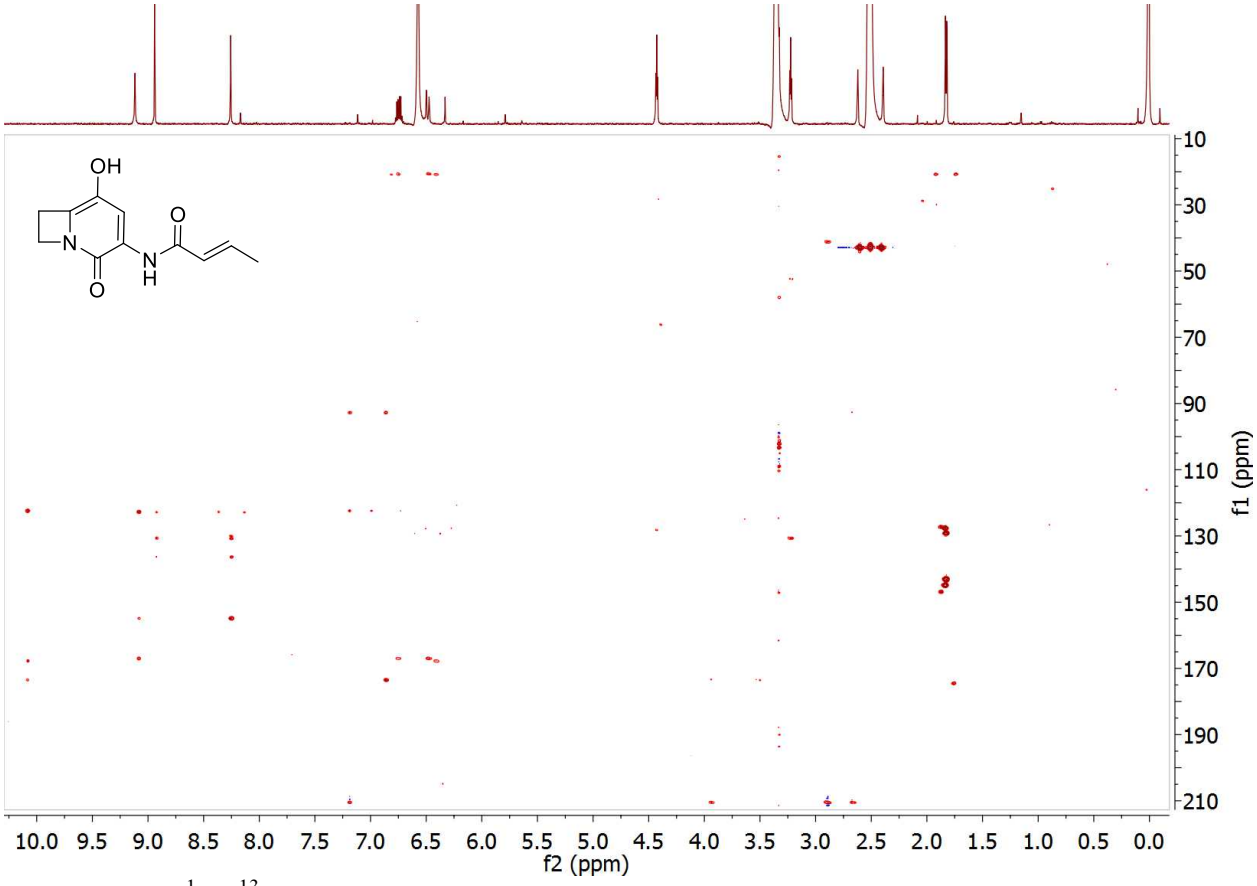
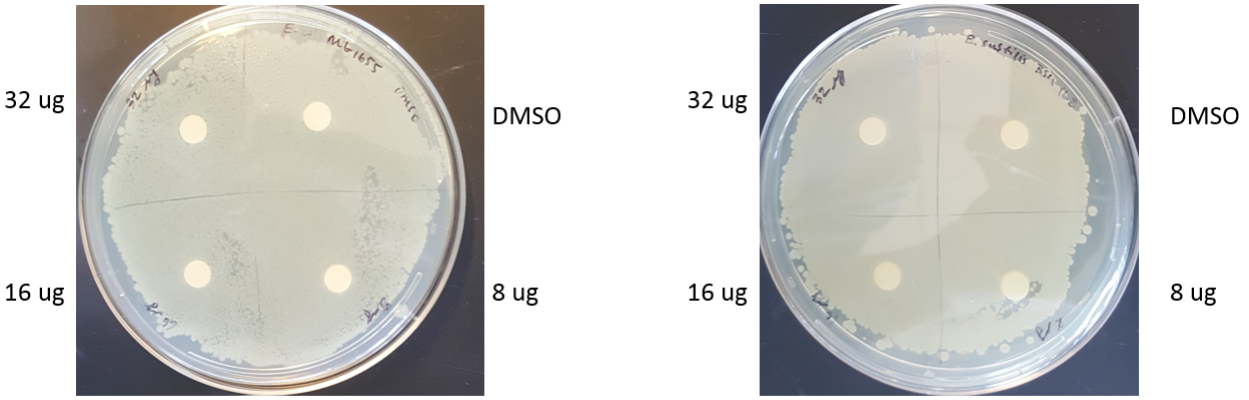


Figure S17. (^{1}H, ^{13}C) HMBC NMR spectrum of **3** in DMSO-d_6 (700 MHz) generated from *in vitro* reconstitution of AzeB. The additional cross-peaks are due to the instability of **3** during the overnight NMR experiment.



E. coli MG1655 *B. Subtilis* BSU 168

Figure S18. Growth inhibition assay of **1** against *E. coli* MG1655 and *B. subtilis* BSU 168 by agar disc diffusion. Experimental details are described in supplemental methods.

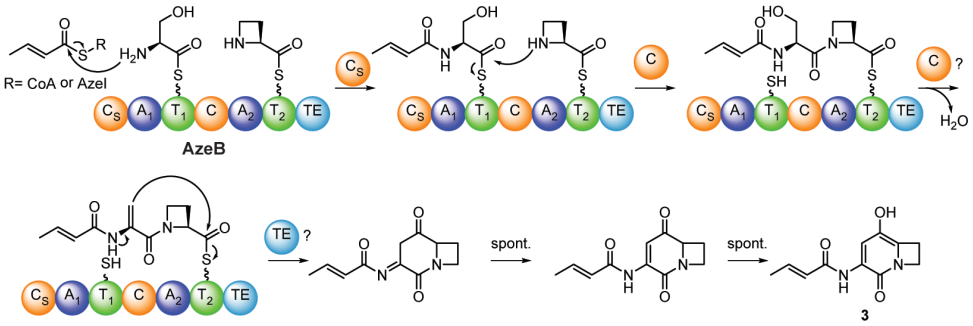


Figure S19. Proposed reactions catalyzed by AzeB.

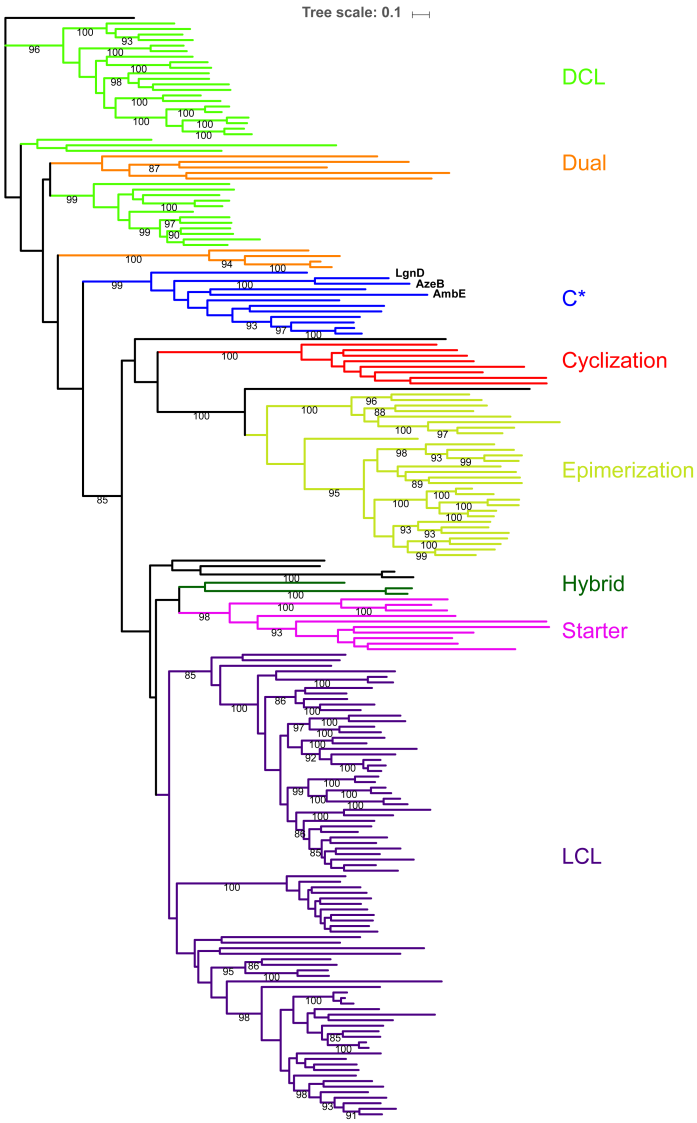


Figure S20. Bioinformatic analysis of the second C domain of AzeB reveals that it belongs to a unique group of C domains (C*). Proteins in the phylogenetic tree include AzeB (AAG06715.1), AmbE-C* (AAG05690.1), the C domains from the NaPDoS database, and seven C* domains from the MIBiG database.[18] From top to bottom in the C* group: DepE_C (ABP57749.1, FK228 biosynthesis), LgnD_C (AIZ66879.1, legonmycin biosynthesis), AzeB_C, Bleom7_C2 (AAG02359.1, bleomycin biosynthesis), AmbE_C (AAG05690.1), Bleom4_C2 (AAG02355.1, bleomycin biosynthesis), Zmn17 (CCM44337.1, zeamine biosynthesis), HasO_C (CZT62784.1, hassallidin biosynthesis), NdaA (ATP76243.1, nodularin biosynthesis), McyA_C (BAA83992.1, microcystin biosynthesis), PuwF_C (AIW82283.1, puwainaphycin biosynthesis), and PuwG_C (AIW82284.1, puwainaphycin biosynthesis). Abbreviations: LCL: condensation between two L amino acids, DCL: condensation between an L and D amino acid, Dual: condensation and epimerization, Starter: acylation to variety of molecules, Hybrid: condensation of amino acid to polyketide. Tree scale represents average expected percentage (0.1=10%) of amino acid substitutions per site.

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