Published in final edited form as:

Org Lett. 2019 July 05; 21(13): 4955–4959. doi:10.1021/acs.orglett.9b01383.

Discovery and Biosynthesis of Azabicyclene, a Conserved Nonribosomal Peptide in *Pseudomonas aeruginosa*

Jon B. Patteson, Adam R. Lescallette, Bo Li*

Department of Chemistry, University of North Carolina at Chapel Hill CB 3290, Chapel Hill, North Carolina 27599-3290, United States

Abstract

Azabicyclene, an azetidine-containing natural product, was identified using quorum-sensing molecules to upregulate expression of a gene cluster highly conserved in the human pathogen *Pseudomonas aeruginosa*. Mutational studies of the gene cluster revealed essential genes for biosynthesis, including an unusual nonribosomal peptide synthetase. Reconstitution of this enzyme in vitro identified key biosynthetic intermediates. This work demonstrates a useful strategy for discovering quorum-sensing-regulated natural products. It sets the stage for understanding the biosynthesis and bioactivity of azabicyclene.

Graphical Abstract

Bacteria respond to environmental cues and coordinate their action by secreting and sensing small molecules, known as “quorum-sensing”.¹ Quorum-sensing is widespread in pathogenic bacteria and regulates many pathways involved in virulence, antibiotic resistance, and toxin production.^{2,3} Understanding quorum-sensing and the pathways it regulates is essential for controlling and preventing infectious diseases.

Pseudomonas aeruginosa is an opportunistic human pathogen and a leading cause for hospital-acquired infections.⁴ Transcriptomics studies in *P. aeruginosa* revealed several uncharacterized biosynthetic operons regulated by quorum-sensing via acylhomoserine lactones (AHLs),⁵ including a putative biosynthetic gene cluster spanning the genes

*Corresponding Author: boli@email.unc.edu.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.9b01383.

Detailed experimental procedures, supplemental figures and tables, and spectral data (PDF)

The authors declare no competing financial interest.

PA3326–PA3336 (Figure 1A). The genes in this cluster were upregulated between 4- and 87-fold by *N*-(3-oxododecanoyl)-L-homoserine lactone and *N*-butyryl-L-homoserine lactone across six disparate clinical and environmental *P. aeruginosa* isolates.⁶ Furthermore, deletion of this gene cluster in *P. aeruginosa* PAO1 cluster resulted in higher colonization in a murine lung model than the wild type bacteria.⁷ Thus, the cluster was thought to slow bacterial growth in the murine host and benefit long-term host colonization; however, its precise function is unknown.

The PA3326–PA3336 cluster encodes 11 proteins, including a nonribosomal peptide synthetase (NRPS), a flavin-dependent monooxygenase (FMO), an *S*-adenosyl-L-methionine (SAM)-dependent enzyme, and three enzymes with putative functions in fatty acid biosynthesis. We hypothesized that these proteins are responsible for synthesizing small molecules. We first analyzed the distribution of this cluster in *Pseudomonas* by bioinformatics. The genes PA3327–PA3336 in the cluster were queried against 2560 *Pseudomonas* genomes from the *Pseudomonas* genome database using MultiGeneBlast.^{8,9} This analysis revealed that the cluster is conserved in all *P. aeruginosa* genomes from this database (1644 strains total), while it was only found in 12 other *Pseudomonas* species (Table S1). Among the 12 other *Pseudomonas* species, 11 were isolated in the same clinical environment as *P. aeruginosa*.¹⁰ The conservation of this cluster in *P. aeruginosa* suggests that the small molecule(s) produced by the encoded enzymes are important for the biology of this pathogen.

Here, we use exogenous AHL signaling molecules to enable the discovery of a small molecule containing an azabicyclo[3.2.0]dihydropyrrolone core, azabicyclene (**1**). Its structure is identical to azetidomonamide B that was discovered by Hong et al. in parallel to our study.¹¹ We identified essential enzymes for azabicyclene biosynthesis by comprehensive mutational studies of the PA3326–PA3336 cluster (*aze*). Furthermore, we reconstituted the activity of the NRPS AzeB (PA3327) in vitro and identified a key biosynthetic intermediate produced by this enzyme.

Based on the evidence that exogenous signaling molecules upregulate *aze* genes in *P. aeruginosa*,^{5,6} we hypothesized that these molecules would also increase the production of small molecules synthesized by *aze*-encoded enzymes. A few studies on AHL-regulated gene clusters identified new bacterial metabolites by addition of exogenous AHLs or genetic engineering to increase transcription of these clusters.^{12–14} Therefore, exogenous AHLs, *N*-(3-oxododecanoyl)-L-homoserine lactone and synthesized *N*-butyryl-L-homoserine lactone (Figure S1) were added to *P. aeruginosa* cultures at the time of inoculation. Metabolite profiles of the supplemented cultures were compared to those without supplementation by liquid chromatography coupled high-resolution mass spectrometry (LC–HRMS). Two metabolites with mass-to-charge (*m/z*) ratios of 193.098 (**1**) and 207.108 (**2**) were identified at the highest levels in the AHL-supplemented culture 10–14 h after inoculation (Figure 1B, Figure S2), and both compounds' abundances were significantly increased approximately 3-fold by addition of exogenous AHLs (Figure S3). These two metabolites were detected at low levels in extracts of wild type PAO1 cultures, but they were absent in those of a mutant containing a *lacZ* insertion in the putative NRPS gene *azeB*

(PAO1 *azeB::lacZ*, obtained from a collection of *P. aeruginosa* PAO1 transposon mutants) (Figure 1B, Figures S3 and S4),¹⁵ confirming that production of **1** and **2** requires *azeB*.

To elucidate the structures of these compounds, the major species, **1**, was isolated from 6 L of PAO1 culture supplemented with exogenous AHLs. The culture supernatant was extracted with dichloromethane, and **1** was isolated using reversed-phase HPLC at a titer of 0.5 mg/L. Analyses of **1** by 1D and 2D NMR experiments support an unusual 1-azabicyclo[3.2.0]dihydropyrrolone core (Figure 1C and Figures S5–9). The NMR shifts and proton peak splitting patterns are consistent with those of the synthetic compounds containing the same core (Table S2),¹⁶ further supporting our structural assignment. The stereochemistry at C₃ was undetermined. Because of the low titer, structural characterization of **2** by NMR was not accomplished.

To determine which genes are required for the production of **1** and **2**, we also obtained individual insertional mutants of the putative biosynthetic genes *azeC–J* (PA3328–PA3335) from the same collection of mutants as PAO1 *azeB::LacZ* (Figure S10).¹⁵ Mutants of *azeB*, *azeC*, *azeF*, and *azeI* did not produce **1**, suggesting these genes are essential for production of **1** (Figure 2A and Figure S11). However, we cannot rule out the polar effects of the insertions on downstream genes to abolish compound production. *azeC* encodes an FMO protein homologous to LgnC, which is essential for the construction of the 5-membered pyrrolizidine ring in legonmycin biosynthesis.¹⁷ Thus, AzeC may catalyze a similar reaction in constructing the pyrrolizidine ring in azabicyclene. *azeI* encodes a putative acyl-carrier protein (ACP), an essential protein for fatty acid biosynthesis.¹⁸ AzeI likely serves an analogous role as the carrier protein for the biosynthesis of the crotonyl moiety. *azeF* encodes a putative cytochrome P450 and does not have an evident biosynthetic role.

Mutants of *azeD*, *azeG*, *azeH*, and *azeJ* showed significantly reduced production of **1** (Figure 2A and Figure S11). *azeH* encodes a FabH homologue (FabH2), a β -ketoacyl-ACP synthase in fatty acid biosynthesis.¹⁸ AzeH likely serves a similar role in the biosynthesis of the crotonyl moiety. *azeD* encodes a standalone condensation domain, *azeG* encodes a protein with no known function, and *azeJ* encodes a putative SAM-dependent enzyme. A significant decrease of **1** production due to mutation to these genes suggests that the encoded enzymes AzeD, AzeG, AzeH, and AzeJ are important but not essential for the biosynthesis of **1**. The action of these enzymes may be compensated by other enzymes of related functions in the bacterium. Surprisingly, mutation of *azeE* that encodes a FabG-like protein significantly increased production of **1** and **2** (Figure 2A). FabG catalyzes reduction of β -ketoacyl-ACP in fatty acid biosynthesis.¹⁸ PAO1 encodes a second copy of FabG in its genome, which shows affinity with AzeI based on a pull down assay,⁷ so it is possible that either FabG or AzeE could perform this reaction in **1** biosynthesis.

Mutations of most *aze* genes had similar effects on the production of **2** to their effects on **1** with the exception of the *azeJ* mutation. We observed no production of **2** in *azeB*, *azeC*, *azeF*, and *azeI* mutants, significantly reduced production in *azeD*, *azeG*, and *azeH* mutants, and higher production in *azeE* mutant (Figure 2B). A similar level of **2** was detected in the *azeJ* mutant as in the wild type, suggesting that *azeJ* is not required for the synthesis of **2** (Figure 2A,B). The *azeJ* gene encodes a putative SAM-dependent enzyme that shares 28.3%

sequence identity to the recently characterized enzyme VioH from *Cystobacter violaceus*.¹⁹ VioH synthesizes the non-proteinogenic amino acid L-azetidine-2-carboxylic acid (L-AZC) from SAM in vioprolide biosynthesis. Since **1** contains an azetidine moiety like vioprolide, and production of **1** is significantly reduced in the *azeJ* mutant, AzeJ likely catalyzes L-AZC formation in the biosynthesis of **1**. Indeed, Hong et al. recently demonstrated the activity of AzeJ as an L-AZC synthase, similarly to VioH.¹¹ Production of **2** does not depend on *azeJ*, suggesting **2** does not contain the L-AZC moiety. The mass of **2** is 14 Da larger than **1**, suggesting incorporation of a proline instead of L-AZC, consistent with the promiscuity of the adenylation domain of AzeB toward both L-AZC and L-Pro reported by Hong et al.¹¹

We identified biosynthetic intermediates produced by the *aze* insertional mutants. The *azeC* mutant accumulated two metabolites displaying *m/z* of 221.092 (**3**) and 235.108 (**4**) (Figure 3A). The difference in mass of 14 Da again suggests incorporation of L-AZC and L-Pro in the production of **3** and **4**, respectively. Thus, we hypothesized that **3** and **4** are products of the NRPS AzeB, which can be modified by AzeC and accumulate when *azeC* is inactivated. To test this hypothesis, we reconstituted the activity of AzeB in vitro. AzeB contains two modules and an N-terminal starter condensation (C) domain (Figure 3B). The structures of the final products **1** and **2** suggest that the first A domain (A₁) of AzeB activates and loads L-Ser and the second A domain (A₂) activates and can load either L-Pro or L-AZC. AzeB was purified as a 6x-His tagged fusion protein (Figure S12). The starter C domain likely catalyzes the *N*-acylation of L-Ser loaded on AzeB using crotonyl-AzeI as a substrate. Because NRPS starter C domains can also use acyl-CoAs as substrates,^{20,21} we reconstituted AzeB activity in vitro in the presence of crotonyl-CoA, L-Ser, and L-AZC and necessary cofactors and detected formation of **3** by HPLC and HRMS analysis (Figure 3C and Figure S13). Omission of AzeB or any substrate or cofactor abolished the production of **3** (Figure S7). Substitution of L-AZC with L-proline led to the formation of **4** (Figure 3C and Figure S13). Product **3** was isolated from a 2 mg scale in vitro reaction and was further characterized by 1D and 2D NMR experiments. The NMR shifts and 2D correlations support the azabicyclo[4.2.0]hydroxypyridinone structure of **3** (Figures S14–17 and Table S3). The structure of **4** likely contains the same pyridinone ring with the substitution of L-AZC with L-Pro, although **4** was not isolated for NMR analysis. Intermediates with the similar hydroxypyridinone core have been isolated in the *IgnC* deletion mutant of the legonmycin producer,¹⁷ consistent with our results. Our work provides biochemical evidence that AzeB is essential and sufficient for the formation of the pyridinone intermediates.

Based on the results from mutational analysis of *aze* genes and in vitro reconstitution of AzeB, we propose a biosynthetic pathway for azabicyclene **1** (Figure 4). Malonyl-CoA is loaded onto the ACP AzeI by FabD of fatty acid biosynthesis, where it is extended by the FabH homologue AzeH to generate β -ketoacyl-AzeI. The 3-ketoacyl-AzeI is reduced to the β -hydroxyacyl-AzeI by AzeE or FabG in fatty acid biosynthesis. Dehydration of the β -hydroxyacyl-AzeI intermediate forms crotonyl-AzeI, which may be catalyzed by a hydroxyacyl dehydratase located outside of the *aze* cluster. Crotonyl-AzeI is then incorporated and condensed with L-Ser and then L-AZC on the AzeB assembly line, the product of which is further modified and rearranged to form **3**. AzeC likely catalyzes Baeyer–Villiger ring expansion followed by decarboxylation of **3** to form azabicyclene.

Activation of AzeB L-Pro instead of L-AZC directs the pathway through intermediate **4** to produce **2**. Mutational studies suggest that AzeD, AzeF, and AzeG are important for azabicyclene biosynthesis, but their functions remain to be characterized.

We assessed the antibacterial activity of azabicyclene against *E. coli* MG1655 and *B. subtilis* BSU168. No growth inhibition was observed when up to 20 μ L of 8.3 mM azabicyclene (32 μ g total) was applied in disk diffusion assays (Figure S18). The role that azabicyclene plays in the biology of *P. aeruginosa* remains to be determined.

We showed that *aze* is conserved in all sequenced genomes of *P. aeruginosa* and demonstrated that *aze*-encoded enzymes produce a novel small molecule, azabicyclene. Thus, azabicyclene likely plays a conserved role in the biology of *P. aeruginosa*. Azabicyclene is a new and unique member of pyrrolizidine alkaloid natural products. It contains a rare 4-membered azetidine moiety that is only found in a small subset of natural products, including antifungal vioprolides and the plant siderophore nicotianamine.^{19,22} Our discovery of azabicyclene will facilitate studies of the biological role of *aze* and help identify the biological targets of azabicyclene.

Mutational studies revealed that the NRPS AzeB and FMO AzeC are essential for the biosynthesis of azabicyclene. Homologues of AzeB and AzeC were identified in the biosynthetic gene clusters for legonmycins and brabantamides.^{17,23–25} Our work marks the first in vitro reconstitution of the class of NRPSs in pyrrolizidine alkaloid biosynthesis and provides biochemical evidence that the NRPS AzeB is essential and sufficient for synthesizing pyridinones. Conversion of L-Ser-L-AZC or L-Ser-L-Pro dipeptide to pyridinones likely entails a 2,3-dehydration of the L-Ser side chain followed by a cyclization (Figure S19). The 2,3-dehydration of β -hydroxyamino acids has been identified in nonribosomal peptide biosynthetic pathways for the nonproteinogenic amino acid methoxyvinylglycine and the β -lactam antibiotic norcardicin, and both dehydrations are likely catalyzed by noncanonical C domains.^{26,27} Indeed, phylogenetic analysis of the second C domain of AzeB shows that it groups with the C domain in methoxyvinylglycine biosynthesis (Figure S20). The unusual chemistry catalyzed by AzeB will be the focus of future studies.

We used native quorum-sensing molecules to enable the identification of new natural products regulated by quorum-sensing. This work highlights the utility of small molecule elicitors for identifying natural products.²⁸ This workflow can be applied to study other uncharacterized biosynthetic gene clusters under quorum-sensing regulation in *P. aeruginosa* as well as other known human pathogens. Identifying the natural products synthesized by enzymes encoded in these gene clusters will reveal new chemistry and lead to greater understanding of the biology of infectious bacteria.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

We thank the Manoil lab and Conlon lab for providing the insertional mutants of *P. aeruginosa* PAO1. This work is supported by the National Science Foundation (CHE1654678) and National Institutes of Health (DP2HD094657) and a Molecular and Cellular Biophysics Training Grant (T32GM008570 to A.R.L.). J.B.P. acknowledges support from the Dean's Graduate Fellowship and Ralph W. Bost Fellowship from the University of North Carolina at Chapel Hill.

REFERENCES

- (1). Papenfort K; Bassler BL Quorum Sensing Signal-Response Systems in Gram-Negative Bacteria. *Nat. Rev. Microbiol* 2016, 14, 576–88. [PubMed: 27510864]
- (2). Rutherford ST; Bassler BL Bacterial Quorum Sensing: Its Role in Virulence and Possibilities for Its Control. *Cold Spring Harbor Perspect. Med* 2012, 2, 1–25.
- (3). Carlson-Banning KM; Sperandio V Enterohemorrhagic *Escherichia coli* Outwits Hosts through Sensing Small Molecules. *Curr. Opin. Microbiol* 2018, 41, 83–88. [PubMed: 29258058]
- (4). Raman G; Avendano EE; Chan J; Merchant S; Puzniak L Risk Factors for Hospitalized Patients with Resistant or Multidrug-Resistant *Pseudomonas aeruginosa* Infections: A Systematic Review and Meta-Analysis. *Antimicrob. Resist. Infect. Control* 2018, 7, 79. [PubMed: 29997889]
- (5). Wagner VE; Bushnell D; Passador L; Brooks AI; Iglewski BH Microarray Analysis of *Pseudomonas aeruginosa* Quorum-Sensing Regulons: Effects of Growth Phase and Environment. *J. Bacteriol* 2003, 185, 2080–2095. [PubMed: 12644477]
- (6). Chugani S; Kim BS; Phattarasukol S; Brittnacher MJ; Choi SH; Harwood CS; Greenberg EP Strain-Dependent Diversity in the *Pseudomonas aeruginosa* Quorum-Sensing Regulon. *Proc. Natl. Acad. Sci. U. S. A* 2012, 109, E2823–E2831. [PubMed: 22988113]
- (7). Chen W; Wang B; Gruber JD; Zhang Y-M; Davies C Acyl Carrier Protein 3 Is Involved in Oxidative Stress Response in *Pseudomonas aeruginosa*. *Front. Microbiol* 2018, 9, 1–13. [PubMed: 29403456]
- (8). Medema MH; Takano E; Breitling R Detecting Sequence Homology at the Gene Cluster Level with Multigeneblast. *Mol. Biol. Evol* 2013, 30, 1218–1223. [PubMed: 23412913]
- (9). Winsor GL; Griffiths EJ; Lo R; Dhillon BK; Shay JA; Brinkman FSL Enhanced Annotations and Features for Comparing Thousands of *Pseudomonas* Genomes in the *Pseudomonas* Genome Database. *Nucleic Acids Res.* 2016, 44, D646–53. [PubMed: 26578582]
- (10). Roach DJ; Burton JN; Lee C; Stackhouse B; Butler-Wu SM; Cookson BT; Shendure J; Salipante SJ A Year of Infection in the Intensive Care Unit: Prospective Whole Genome Sequencing of Bacterial Clinical Isolates Reveals Cryptic Transmissions and Novel Microbiota. *PLoS Genet.* 2015, 11, e1005413. [PubMed: 26230489]
- (11). Hong Z; Bolard A; Giraud C; Prevost S; Genta-jouve G; Deregnacourt C; Susanne H; Jeannot K; Li Y Azetidine-Containing Alkaloids Produced by a Quorum-Sensing Regulated Nonribosomal Peptide Synthetase Pathway in *Pseudomonas aeruginosa*. *Angew. Chem., Int. Ed* 2019, 58, 3178–3182.
- (12). Seyedsayamdost MR; Chandler JR; Blodgett JAV; Lima PS; Duerkop BA; Oinuma K-I; Greenberg EP; Clardy J Quorum-Sensing-Regulated Bactobolin Production by *Burkholderia thailandensis* E264. *Org. Lett* 2010, 12, 716–719. [PubMed: 20095633]
- (13). Ishida K; Lincke T; Behnken S; Hertweck C Induced Biosynthesis of Cryptic Polyketide Metabolites in a *Burkholderia thailandensis* Quorum Sensing Mutant. *J. Am. Chem. Soc* 2010, 132, 13966–13968. [PubMed: 20853892]
- (14). Duerkop BA; Varga J; Chandler JR; Peterson SB; Herman JP; Churchill MEA; Parsek MR; Nierman WC; Greenberg EP Quorum-Sensing Control of Antibiotic Synthesis in *Burkholderia thailandensis*. *J. Bacteriol* 2009, 191, 3909. [PubMed: 19376863]
- (15). Held K; Ramage E; Jacobs M; Gallagher L; Manoil C Sequence-Verified Two-Allele Transposon Mutant Library for *Pseudomonas aeruginosa* PAO1. *J. Bacteriol* 2012, 194, 6387–6389. [PubMed: 22984262]

- (16). Miaskiewicz S; Gaillard B; Kern N; Weibel JM; Pale P; Blanc A Gold(I)-Catalyzed N-Desulfonylative Amination Versus N-to-O 1,5-Sulfonyl Migration: A Versatile Approach to 1-Azabicycloalkanes. *Angew. Chem., Int. Ed* 2016, 55, 9088–9092.
- (17). Huang S; Tabudravu J; Elsayed SS; Travert J; Peace D; Tong MH; Kyeremeh K; Kelly SM; Trembleau L; Ebel R; Jaspars M; Yu Y; Deng H Discovery of a Single Monooxygenase That Catalyzes Carbamate Formation and Ring Contraction in the Biosynthesis of the Legonmycins. *Angew. Chem., Int. Ed* 2015, 54, 12697–12701.
- (18). Cronan JE; Thomas J, Chapter 17 Bacterial Fatty Acid Synthesis and Its Relationships with Polyketide Synthetic Pathways In *Methods in Enzymology*; Academic Press, 2009; Vol. 459, pp 395–433. [PubMed: 19362649]
- (19). Yan F; Muller R Class I Methyltransferase VioH Catalyzes Unusual S-Adenosyl-L-Methionine Cyclization Leading to 4-Methyl-azetidinecarboxylic Acid Formation During Vioprolide Biosynthesis. *ACS Chem. Biol* 2019, 14, 99–105. [PubMed: 30540188]
- (20). Kraas FI; Helmetag V; Wittmann M; Strieker M; Marahiel MA Functional Dissection of Surfactin Synthetase Initiation Module Reveals Insights into the Mechanism of Lipoinitiation. *Chem. Biol* 2010, 17, 872–880. [PubMed: 20797616]
- (21). Imker HJ; Krahn D; Clerc J; Kaiser M; Walsh CT *N*-Acylation During Glidobactin Biosynthesis by the Tridomain Nonribosomal Peptide Synthetase Module GlbF. *Chem. Biol* 2010, 17, 1077–1083. [PubMed: 21035730]
- (22). Higuchi K; Suzuki K; Nakanishi H; Yamaguchi H; Nishizawa N-K; Mori S Cloning of Nicotianamine Synthase Genes, Novel Genes Involved in the Biosynthesis of Phytosiderophores. *Plant Physiol.* 1999, 119, 471–480. [PubMed: 9952442]
- (23). Schimming O; Challinor VL; Tobias NJ; Adihou H; Grun P; Poschel L; Richter C; Schwalbe H; Bode HB Structure, Biosynthesis, and Occurrence of Bacterial Pyrrolizidine Alkaloids. *Angew. Chem., Int. Ed* 2015, 54, 12702–12705.
- (24). Schmidt Y; Van Der Voort M; Crüsemann M; Piel J; Josten M; Sahl HG; Miess H; Raaijmakers JM; Gross H Biosynthetic Origin of the Antibiotic Cyclocarbamate Brabantamide A (Sb-253514) in Plant-Associated *Pseudomonas*. *ChemBioChem* 2014, 15, 259–266. [PubMed: 24436210]
- (25). Johnston CW; Zvanych R; Khyzha N; Magarvey NA Nonribosomal Assembly of Natural Lipocyclocarbamate Lipoprotein-Associated Phospholipase Inhibitors. *ChemBioChem* 2013, 14, 431–435. [PubMed: 23401283]
- (26). Long DH; Townsend CA Mechanism of Integrated B-Lactam Formation by a Non-Ribosomal Peptide Synthetase During Antibiotic Synthesis. *Biochemistry* 2018, 57, 3353–3358. [PubMed: 29701951]
- (27). Patteson JB; Dunn ZD; Li B In Vitro Biosynthesis of the Nonproteinogenic Amino Acid Methoxyvinylglycine. *Angew. Chem., Int. Ed* 2018, 57, 6780–6785.
- (28). Okada BK; Seyedsayamdost MR Antibiotic Dialogues: Induction of Silent Biosynthetic Gene Clusters by Exogenous Small Molecules. *FEMS Microbiol. Rev* 2017, 41, 19–33. [PubMed: 27576366]

Figure 1.

aze-encoded enzymes produce the small molecule azabicyclene, **1**. (A) *aze* gene cluster and putative functions of *aze* genes. Blue: nonribosomal peptide synthetase; red: oxidase; purple: fatty acid biosynthesis; green: hypothetical proteins; gray: Clp protease; white: transporter. (B) Extracted ion chromatograms (EICs) of **1** (m/z 193.097 [$\text{M} + \text{H}$] $^+$) from the extracts of PAO1 and PAO1 *azeB::lacZ* with or without AHLs supplementation. Experiments were conducted independently three times yielding similar results (Figure S3). (C) Structure of **1** and key (^1H , ^{13}C) HMBC NMR correlations.

Figure 2. Mutational studies of the *aze* cluster reveal essential enzymes for the production of **1** and **2**. (A) EICs of **1** (m/z 193.097 $[M + H]^+$) from the extracts of cultures of PAO1 wild type and mutants supplemented with AHLs. (B) EICs of **2** (m/z 207.113 $[M + H]^+$) from the extracts of cultures of PAO1 wild type and mutants supplemented with AHLs.

Figure 3.

AzeB is essential and sufficient for the formation of the pyridinone intermediates **3** and **4**. (A) EICs of **3** (m/z 221.092 [$M + H$]⁺) and **4** (m/z 235.108 [$M + H$]⁺) from the extracts of PAO1 wild type and mutant cultures supplemented with AHLs. (B) AzeB domain organization and substrates. Condensation (C), adenylation (A), thiolation (T), and thioesterase (TE). (C) In vitro reconstitution of AzeB activity and analysis of the reaction mixture by HPLC.

Figure 4.
Proposed biosynthesis of **1** and **2**.