Pyonitrins A–D: Chimeric Natural Products Produced by Pseudomonas protegens

Emily Mevers, Josep Saurí, Eric J. N. Helfrich, Matthew Henke, Kenneth J. Barns, Tim S. Bugni, David Andes, Cameron R. Currie, and Jon Clardy

†Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 240 Longwood Avenue, Boston, Massachusetts 02115, United States
‡Structure Elucidation Group, Process and Analytical Research and Development, Merck & Co., Inc., 33 Avenue Louis Pasteur, Boston, Massachusetts 02115, United States
§Pharmaceutical Sciences Division, School of Pharmacy, University of Wisconsin—Madison, Madison, Wisconsin 53705, United States
∥Department of Medicine, University of Wisconsin School of Medicine and Public Health, Madison, Wisconsin 53705, United States
¶Department of Bacteriology, University of Wisconsin—Madison, Madison, Wisconsin 53705, United States

Supporting Information

ABSTRACT: Bacterial symbionts frequently provide chemical defenses for their hosts, and such systems can provide discovery pathways to new antifungals and structurally intriguing metabolites. This report describes a small family of naturally occurring small molecules with chimeric structures and a mixed biosynthesis that features an unexpected but key nonenzymatic step. An insect-associated Pseudomonas protegens strain’s activity in an in vivo murine candidiasis assay led to the discovery of a family of highly hydrogen-deficient metabolites. Bioactivity- and mass-guided fractionation led to the pyonitrins, highly complex aromatic metabolites in which 10 of the 20 carbons are quaternary, and 7 of them are contiguous. The P. protegens genome revealed that the production of the pyonitrins is the result of a spontaneous reaction between biosynthetic intermediates of two well-studied Pseudomonas metabolites, pyochelin and pyrrolnitrin. The combined discovery of the pyonitrins and identification of the responsible biosynthetic gene clusters revealed an unexpected biosynthetic route that would have prevented the discovery of these metabolites by bioinformatic analysis alone.

Natural product discovery increasingly relies on computational analysis of biosynthetic pathways found in genome sequences, but a few recent reports have identified metabolites that result from nonenzymatic spontaneous reactions that leave no trace in the producing organism’s genome. Examples include extremely complex metabolites, such as homodimeric A1 and the aureochaeglobosins, the former of which is formed by the dimerization of two achiral quinones to form a hexacyclic system with eight continuous stereogenic centers. A simpler recent example includes 2-amino-3-carboxy-naphthoquinone (ACNQ)—a small molecule electron shuttle produced by Shewanella oneidensis MR-1 that plays an important role in bacterial physiology and ecology. ACNQ’s important role was missed for almost two decades, as discovery efforts based on genetic approaches, such as transposon libraries, failed. This report describes still another path to this interesting class of natural products that are produced by a key “nonenzymatic” reaction.

In an extension of previous studies, we screened a library of insect-associated bacterial strains that we have accumulated over the past few years in a large-scale systematic binary screening assay for antagonism against relevant pathogens, including Candida albicans (Figure S1 and Table S1). Strains that inhibited the growth of a pathogen in the binary screening, which was determined by the presence of a zone of inhibition between the two organisms, were subsequently grown in larger scale formats, and the crude extracts were fractionated employing a two-step chromatographic approach using a highly retentive nonpolar resin (ISOLUTE ENV+) followed by chromatographic separation to generate 80 fractions per extract directly into a 96-well format for subsequent high-throughput screening (see experimental methods and Scheme S1-1 in the Supporting Information). After screening, hits were prioritized based on potency, observation of a dose response, and dereplication by LCMS and NMR analyses. For promising hits that did not appear to arise from known molecules, production scale fermentation (16 L) and reversed-phase fractionation, yielding eight fractions, were pursued to provide sufficient material for in vivo evaluation against C. albicans (Scheme S1-2). This report describes an investigation into one particularly interesting fraction, active against C. albicans, from a Pseudomonas protegens strain that led to the discovery of pyonitrins A–D (1–4; Figure 1).

One of the semifurnished HPLC fractions exhibited in vivo efficacy in a mouse candidiasis assay, and all dereplication efforts failed to associate the antifungal activity with a known metabolite. A traditional bioassay-guided approach led to two related metabolites that showed weak activity against C. albicans using in vitro assays. The molecular formulas of the active metabolites indicated a small family of structural isomers.

Received: September 9, 2019
Published: October 10, 2019
with pronounced hydrogen deficiencies that indicated a challenging structural analysis by NMR. The most prominent metabolite, pyonitrin A (C_{20}H_{12}ClN_{3}O_{5}), had 16 degrees of unsaturation (observed [M + H]^{+} 378.0460 m/z, calcld 378.0462 m/z, Δ 0.5 ppm, Figure S17). The lone chlorine atom was determined based on the observed 3:1 isotopic distribution pattern in the MS analysis. A 1D ¹H NMR spectrum revealed that all protons, excluding exchangeable ones, were aromatic with chemical shifts between 6.5 and 9 ppm. The UV absorbance spectrum supported the presence of multiple conjugated aromatic systems with a strong absorbance at 320 nm. Interestingly, the HPLC elution time of pyonitrin A (1) shifted significantly if run with formic acid as an additive relative to the unacidiﬁed mobile phase, and its 1D ¹H NMR signals also shifted signiﬁcantly from sample to sample in d_{6}-DMSO suggesting that 1 contains a pH-sensitive functionality.

Structure elucidation of 1 was ultimately possible through concerted analysis of 2D NMR data. HSQC data revealed the presence of 10 aromatic methine protons, with carbon resonances ranging from 101.7 to 131.6 ppm. HSQC data revealed that all protons, excluding exchangeable ones, were aromatic with chemical shifts between 6.5 and 9 ppm. The UV absorbance spectrum supported the presence of multiple conjugated aromatic systems with a strong absorbance at 320 nm. Interestingly, the HPLC elution time of pyonitrin A (1) shifted signiﬁcantly if run with formic acid as an additive relative to the unacidiﬁed mobile phase, and its 1D ¹H NMR signals also shifted signiﬁcantly from sample to sample in d_{6}-DMSO suggesting that 1 contains a pH-sensitive functionality.

Structure elucidation of 1 was ultimately possible through concerted analysis of 2D NMR data. HSQC data revealed the presence of 10 aromatic methine protons, with carbon resonances ranging from 101.7 to 131.6 ppm. HSQC data revealed that all protons, excluding exchangeable ones, were aromatic with chemical shifts between 6.5 and 9 ppm. The UV absorbance spectrum supported the presence of multiple conjugated aromatic systems with a strong absorbance at 320 nm. Interestingly, the HPLC elution time of pyonitrin A (1) shifted signiﬁcantly if run with formic acid as an additive relative to the unacidiﬁed mobile phase, and its 1D ¹H NMR signals also shifted signiﬁcantly from sample to sample in d_{6}-DMSO suggesting that 1 contains a pH-sensitive functionality.

Structure elucidation of 1 was ultimately possible through concerted analysis of 2D NMR data. HSQC data revealed the presence of 10 aromatic methine protons, with carbon resonances ranging from 101.7 to 131.6 ppm. HSQC data revealed that all protons, excluding exchangeable ones, were aromatic with chemical shifts between 6.5 and 9 ppm. The UV absorbance spectrum supported the presence of multiple conjugated aromatic systems with a strong absorbance at 320 nm. Interestingly, the HPLC elution time of pyonitrin A (1) shifted signiﬁcantly if run with formic acid as an additive relative to the unacidiﬁed mobile phase, and its 1D ¹H NMR signals also shifted signiﬁcantly from sample to sample in d_{6}-DMSO suggesting that 1 contains a pH-sensitive functionality.

Structure elucidation of 1 was ultimately possible through concerted analysis of 2D NMR data. HSQC data revealed the presence of 10 aromatic methine protons, with carbon resonances ranging from 101.7 to 131.6 ppm. HSQC data revealed that all protons, excluding exchangeable ones, were aromatic with chemical shifts between 6.5 and 9 ppm. The UV absorbance spectrum supported the presence of multiple conjugated aromatic systems with a strong absorbance at 320 nm. Interestingly, the HPLC elution time of pyonitrin A (1) shifted signiﬁcantly if run with formic acid as an additive relative to the unacidiﬁed mobile phase, and its 1D ¹H NMR signals also shifted signiﬁcantly from sample to sample in d_{6}-DMSO suggesting that 1 contains a pH-sensitive functionality.

Structure elucidation of 1 was ultimately possible through concerted analysis of 2D NMR data. HSQC data revealed the presence of 10 aromatic methine protons, with carbon resonances ranging from 101.7 to 131.6 ppm. HSQC data revealed that all protons, excluding exchangeable ones, were aromatic with chemical shifts between 6.5 and 9 ppm. The UV absorbance spectrum supported the presence of multiple conjugated aromatic systems with a strong absorbance at 320 nm. Interestingly, the HPLC elution time of pyonitrin A (1) shifted signiﬁcantly if run with formic acid as an additive relative to the unacidiﬁed mobile phase, and its 1D ¹H NMR signals also shifted signiﬁcantly from sample to sample in d_{6}-DMSO suggesting that 1 contains a pH-sensitive functionality.
examined whether prnD encoding the enzyme responsible for the ultimate transformation from 12 to 6 had acquired a mutation that prevented the oxidation. While a Gly159Ser mutation in PrnD was detected (Figure S26), structural modeling suggested that the mutated residue is located at the surface of the protein. However, this mutation may still interfere with the catalytic function of PrnD (Figure S28), as the natural abundance of 6 is quite low (0.028% in crude extract, Table S4). We therefore propose that the pyonitrins arise from the premature release of the known pyochelin shunt product dihydroaerugenoic acid (7) from the NRPS protein PchE followed by reduction to aeruginaldehyde, a known cell-to-cell signaling molecule and disease mediator (8)(Figure 3).10,12 The remaining fragment needed to construct the pyonitrins, building blocks 11 or 12, likely originated in intermediates from pyrrolnitrin biosynthesis. It seems likely that during pyonitrin A (1) biosynthesis aeruginaldehyde (8) and the amine moiety of 11 or 12 undergo a spontaneous Pictet–Spengler condensation. Hence, the last step during pyonitrin biosynthesis likely involves the generation of the imine intermediate 12 (13 or 14), similar to the proposed malleobactin biosynthesis, followed by an intramolecular electrophilic aromatic addition of the imine carbon onto the pyrrole ring, followed by rearomatization to yield the isolated pyonitrins (1-4).12 This hypothesis was confirmed by incubating dechloroaminopyrrolnitrin, obtained from the chemical reduction of 6, or recombinantly produced aminopyrrolnitrin with aeruginaldehyde (8), isolated from the native producer, in an abiotic mixture using culture conditions that yielded the pyonitrins. Monitoring the reaction by HR-LCMS revealed conversion of dechloroaminopyrrolnitrin and 8 to pyonitrin D (4) within 24 h while the reaction with natural substrate, aminopyrrolnitrin, was significantly faster likely due to mesomeric effects from the chlorine atom (Figure S29). Bacterial natural product biosynthesis is a highly regulated process. Even though the number of reports about spontaneous reactions during natural product biosynthesis is steadily increasing, spontaneous reactions remain a relatively rare phenomenon. This seems to be particularly true for the type of condensation reported here. In fact, all Pictet–Spengler condensations involved in bacterial natural product biosynthesis reported to date are enzyme catalyzed and involve either the action of an unusual module-encoded condensation domain13 or a dedicated Pictet–Spenglerase (PS).14 To the best of our knowledge, this is the first report of a spontaneous Pictet–Spengler condensation during natural product biosynthesis, which is likely due to the highly activated character of the nucleophile involved in the reaction.

The pyonitrins were isolated based on weak in vitro antifungal activity following significant in vivo activity in the initial screen of the refined fractions. Quantification of the in vitro activity against C. albicans revealed that pyonitrin A or B exhibited only minor inhibitory activity at 50 mg/mL, and unfortunately, we did not have enough isolated material to evaluate the purified compounds in dose response studies or the in vivo mouse Candidiasis system. The lack of potent in vitro efficacy suggests that there is a disconnect between our observed in vivo and in vitro results, as no other HPLC fraction exhibited in vitro activity. Possible explanations for the lack of in vitro activity are that the pyonitrins work synergistically with other metabolites in the extract, a metabolite in the semipurified fraction acts as a “pro-drug” and is thus inactive.

Figure 3. Biosynthetic gene clusters of pyochelin and pyrrolnitrin and model for pyonitrin biosynthesis. (A) Biosynthetic gene clusters for pyochelin (pch) and pyrrolnitrin (prn). Red: core NRPS gene; green: transporter; brown: regulator; light blue: dehydrogenase; dark blue: salicylic acid biosynthetic genes; gray: thioesterase; purple: halogenase, orange: pyrrolnitrin synthase, light green: oxygenase. (B) biosynthetic proposal for pyonitrin production. A: adenylation domain; Cyc: cyclase; MT: methyltransferase; TE: thioesterase, white circles: peptidyl carrier protein; red: reduction; ox: oxidation; proteins are color-coded according to genes in panel A.
in the *in vitro* assays, or a minor contaminant was responsible for the observed *in vivo* activity. Teasing this apart will be part of future efforts.

Biosynthetic enzymes can be slow compared to most other enzymes, and as a result, reactive species disassociate from pathways and subsequently react with each other. Whether the current coupling is a chance occurrence or a purposeful biosynthetic assembly is not clear, but the pyonitrins derive their chimeric structures from two pathways joining at the metabolomic level. This is in contrast to many other natural products that are the result of what appears to be the joining of two pathways at the genetic level. It is therefore tempting to speculate that successful chimeric metabolites fused on the metabolic level might facilitate joining the biosynthetic machineries at the genetic level to ensure coregulation and joined gene transfer.

**ASSOCIATED CONTENT**

**Supporting Information**
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.9b09739.

Experimental methods, MS chromatograms, NMR spectra, and biological data (PDF)

**AUTHOR INFORMATION**

*Corresponding Author*
*jon_clardy@hms.harvard.edu*

**ORCID**
Emily Mevers: 0000-0001-7986-5610
Jon Clardy: 0000-0003-0213-8356

**Author Contributions**
V.E.M., J.S., and E.J.N.H. contributed equally.

**Notes**
The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

This work was funded by U19 AI109673 and R01AT009874 (J.C.). We thank both the Analytical Chemistry Core (ACC) facility in the Biological Chemistry and Molecular Pharmacology department within Harvard Medical School for analytical support and the East Quad NMR facility at Harvard Medical School for NMR support. We also thank the Small Molecule Screening Facility within the University of Wisconsin Carbone Cancer Center (P30 CA014520) for initial biological evaluation. E.J.N.H. gratefully acknowledges funding from a Postdoc Mobility fellowship granted by the Swiss National Science Foundation.

**REFERENCES**

(1) Mevers, E.; Sauri, J.; Liu, Y.; Moser, A.; Ramadhar, T. R.; Varlan, M.; Williamson, R. T.; Martin, G. E.; Clardy, J. Homodimericin A: A Complex Hexacyclic Fungal Metabolite. *J. Am. Chem. Soc.* 2016, 138 (38), 12324–12327.

(2) Yang, M.-H.; Gu, M.-L.; Han, C.; Guo, X.-J.; Yin, G.-P.; Yu, P.; Kong, L.-Y. Aureochaeloglobosins A-C, Three [4 + 2] Adducts of Chaetoglobosin and Aureonitol Derivatives from *Chaetomium globosum*. *Org. Lett.* 2018, 20 (11), 3345–3348.

(3) Mevers, E.; Su, L.; Pischany, G.; Baruch, M.; Cornejo, J.; Hobert, E.; Dimise, E.; Ajo-Franklin, C. M.; Clardy, J. An Elusive Electron Shuttle from a Facultative Anaerobe. *eLife* 2019, 8, No. e48054.

(4) Myers, C. R.; Myers, J. M. *Shewanella oneidensis* MR-1 Restores Menaquinone Synthesis to a Menaquinone-Negative Mutant. *Appl. Environ. Microbiol.* 2004, 70 (9), 5415–5425.

(5) Newman, D. K.; Kolter, R. A role for Excreted Quinones in Extracellular Electron Transfer. *Nature* 2000, 405 (6782), 94–97.

(6) Tripathi, R. K.; Gottlieb, D. Mechanism of Action of the Antifungal Antibiotic Pyrrolnitrin. *J. Bacteriol.* 1969, 100 (1), 310–318.

(7) Ankenbauer, R. G.; Cox, C. D. Isolation and Characterization of *Pseudomonas aeruginosa* Mutants Requiring Salicylic Acid for Pyochelin Biosynthesis. *J. Bacteriol.* 1988, 170 (11), 5364–5367.

(8) Weber, T.; Blin, K.; Duddela, S.; Krug, D.; Kim, H. U.; Bruccoleri, R.; Lee, S. Y.; Fischbach, M. A.; Müller, R.; Wohlleben, W.; Breitling, R.; Takano, E.; Medema, M. H. antiSMASH 3.0 – A Comprehensive Resource for the Genome Mining of Biosynthetic Gene Clusters. *Nucleic Acids Res.* 2015, 43 (W1), W237–W243.

(9) Serino, L.; Reimmann, C.; Visca, P.; Beyeler, M.; Chiesa, V. D.; Haas, D. Biosynthesis of Pyochelin and Dihydroaeruginic Acid Requires the Iron-Regulated pchDCBA Operon in *Pseudomonas aeruginosa*. *J. Bacteriol.* 1997, 179 (1), 248–257.

(10) Ye, L.; Cornelis, P.; Guillenyn, K.; Ballet, S.; Hammerich, O. Structure Revision of N-Mercapto-4-formylcarbostyril Produced by *Pseudomonas fluorescens* G308 to 2-(2-Hydroxyphenyl)thiazole-4-carbaldehyde [aeruginaldehyde]. *Nat. Prod. Commun.* 2014, 9, 789–794.

(11) Hammer, P. E.; Hill, D. S.; Lam, S. T.; Van Pée, K. H.; Ligon, J. M. Four Genes from *Pseudomonas fluorescens* That Encode the Biosynthesis of Pyrrolnitrin. *Appl. Environ. Microbiol.* 1997, 63 (6), 2147–2154.

(12) Trottmann, F.; Franke, J.; Ishida, K.; Garcia-Altares, M.; Hertweck, C. A Pair of Bacterial Siderophores Releases Siderophores Reases and Traps an Intercellular Signal Molecule: An Unusual Case of Natural Nitrone Bioconjugation. *Angew. Chem., Int. Ed. 2019*, 58 (1), 200–204.

(13) Koketsu, K.; Watanabe, K.; Suda, H.; Oguri, H.; Oikawa, H. Reconstruction of the Saframycin Core Scaffold Defines Ducle Pictet-Spengler Mechanisms. *Nat. Chem. Biol.* 2010, 6, 408–410.

(14) Chen, Q.; Ji, C.; Song, Y.; Huang, H.; Ma, J.; Tian, X.; Ju, J. Discovery of McbB, an Enzyme Catalyzing the β-carboline Skeleton Construction in the Marinacaroline Biosynthetic Pathway. *Angew. Chem., Int. Ed.* 2013, 52 (38), 9980–9984.