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Pyonitrins A−D: Chimeric Natural Products Produced by *Pseudomonas protegens*

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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 homodimericin A[1] and the aureochaeglobosins,[2] 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.[3] ACNQ's important role was missed for almost two decades, as discovery efforts based on genetic approaches, such as transposon libraries, failed.[4,5] 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 semipurified 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

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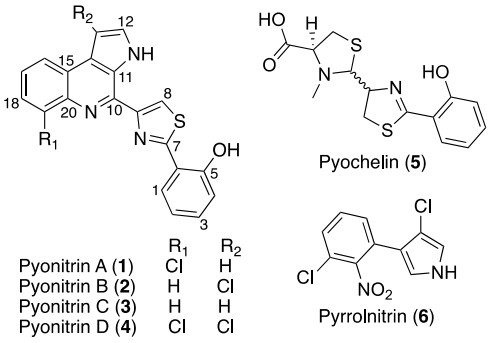

Figure 1. Structures of pyonitrin A–D (**1**–**4**), pyochelin (**5**), and pyrrolnitrin (**6**).

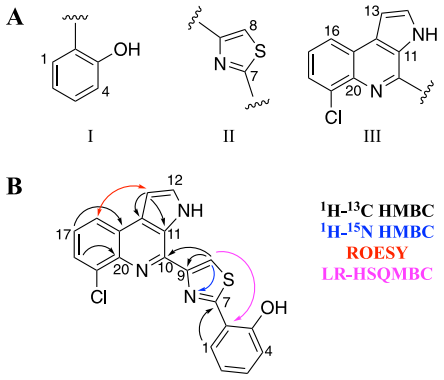

Figure 2. (A) Fragments of **1** identified by 2D NMR analysis. (B) Structure of **1** with the most relevant long-range correlations.

with pronounced hydrogen deficiencies that indicated a challenging structural analysis by NMR. The most prominent metabolite, pyonitrin A ($C_{20}H_{12}ClN_3OS$), had 16 degrees of unsaturation (observed $[M + H]^+$ 378.0460 m/z, calcd 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 ^{1}H 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 ^{1}H 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 chemical shifts ranging from 101.7 to 131.6 ppm. Therefore, based on its predicted chemical formula ($C_{20}H_{12}ClN_3OS$), 10 quaternary carbons and 2 exchangeable protons were expected. Four proton–proton spin systems were identified by examination of COSY correlations: (a) a 1,2-disubstituted phenyl ring with the typical two doublets and two triplets with large J-values (~8 Hz) integrating one proton each; (b) a 1,2,3-trisubstituted phenyl ring with two doublets and one triplet with large J-values (~8 Hz) integrating one proton each; (c) two aromatic protons coupled to each other with a small J-value (3 Hz); and (d) a singlet integrating to a single proton. Analysis of HMBC and ROESY data led to the identification of fragments I, II, and III, the latter containing spin systems b and c (Figure 2a).

Fragment **I** was identified as a 2-phenol derivative with the tetrasubstituted carbon bearing the hydroxyl group (C5) resonating at 157.3 ppm and its *ortho* carbons resonating at 117.1 (C4) and 119.9 ppm (C6), the former being a protonated carbon and the latter a nonprotonated one. Fragment **II** was identified as a 2,4-disubstituted thiazole unit linked to the 2-phenol fragment by an HMBC correlation from methine H1 and methine H8 to a quaternary carbon resonating at 164.4 ppm (C7). Based on careful examination of ^{1}H–^{13}C and ^{1}H–^{15}N HMBC correlations, carbon and nitrogen chemical shifts, and ROE data, fragment **III** was identified as a pyrrolo-quinoline unit with a chlorine located at carbon C19 and linked to the thiazole unit in fragment **II** at carbon C10 based on an observed HMBC correlation from H8 to C10 (Figure 2B). Further evidence supporting the ensemble of **1** and its long-range connectivities was gathered by acquiring an LR-HSQMBC experiment optimized for 3 Hz (see Figure S9 and Figure S10). Comparison of the HSQC data for **1** and **2** revealed that the only difference was the position of the chlorine. Upon further inspection of the HRMS data, peaks for masses of both the unchlorinated (**3**) and dichlorinated (**4**) analogs were observed in a similar polarity region but were not purified due to limited sample quantities.

The pyonitrins are structurally related to both pyochelin (**5**) and pyrrolnitrin (**6**), two well-studied *Pseudomonas* spp. metabolites.[6,7] In **1**–**4**, the salicylic acid and thiazole rings are identical to those of pyochelin (**5**) and the chlorinated aromatic ring is quite similar to that of pyrrolnitrin (**6**). These observations indicate that the pyonitrins are some sort of metabolic chimera produced by the biosynthetic machineries of **5** and **6**, and we therefore sought to define the terms of the merger. Significant amounts of pyochelin were observed in HPLC fractions neighboring those for the pyonitrins, but only small quantities of pyrrolnitrin were observed. However, we were also able to detect significant amounts of the pyrrolnitrin biosynthetic intermediates, dechloroaminopyrrolnitrin (**11**) and aminopyrrolnitrin (**12**). These results suggest that the pyochelin and pyrrolnitrin pathways were largely or completely intact. We sequenced the genomic DNA of the pyonitrin producer, assembled the producer's genome, and used antiSMASH 4.0 analysis[8] to identify the biosynthetic gene cluster (BGC) for pyonitrin production. AntiSMASH analysis revealed an intact pathway for pyochelin and pyrrolnitrin biosynthesis separated by 89 kb (Figure 3; Tables S5–S6); there was no indication of a merged biosynthetic pathway that might be responsible for pyonitrin production. We then compared the genomes of sequenced *P. protegens* strains to the pyonitrin producer (99.9% 16S rRNA identity) to study if a rearrangement in the genome sequence affects either of the two pathways, as **1**–**4** were not reported from any other *Pseudomonas* strain although both BGCs are widely distributed. Comparative genomic analyses with other *P. protegens* strains did not show any genomic rearrangement events effecting the two BGCs, either (Figure S27).[9] During pyrrolnitrin biosynthesis, tryptophan is first chlorinated by PrnA to yield 7-chlorotrypthophan (**10**), followed by an oxidative rearrangement and a putative second chlorination step to yield **11** and **12**, respectively.[11] Compound **12** is then proposed to be oxidized to yield pyrrolnitrin.[6] Since only traces of pyrrolnitrin were observed, while the intermediates **11** and **12** were detected in significant amounts in LC-MS experiments, we

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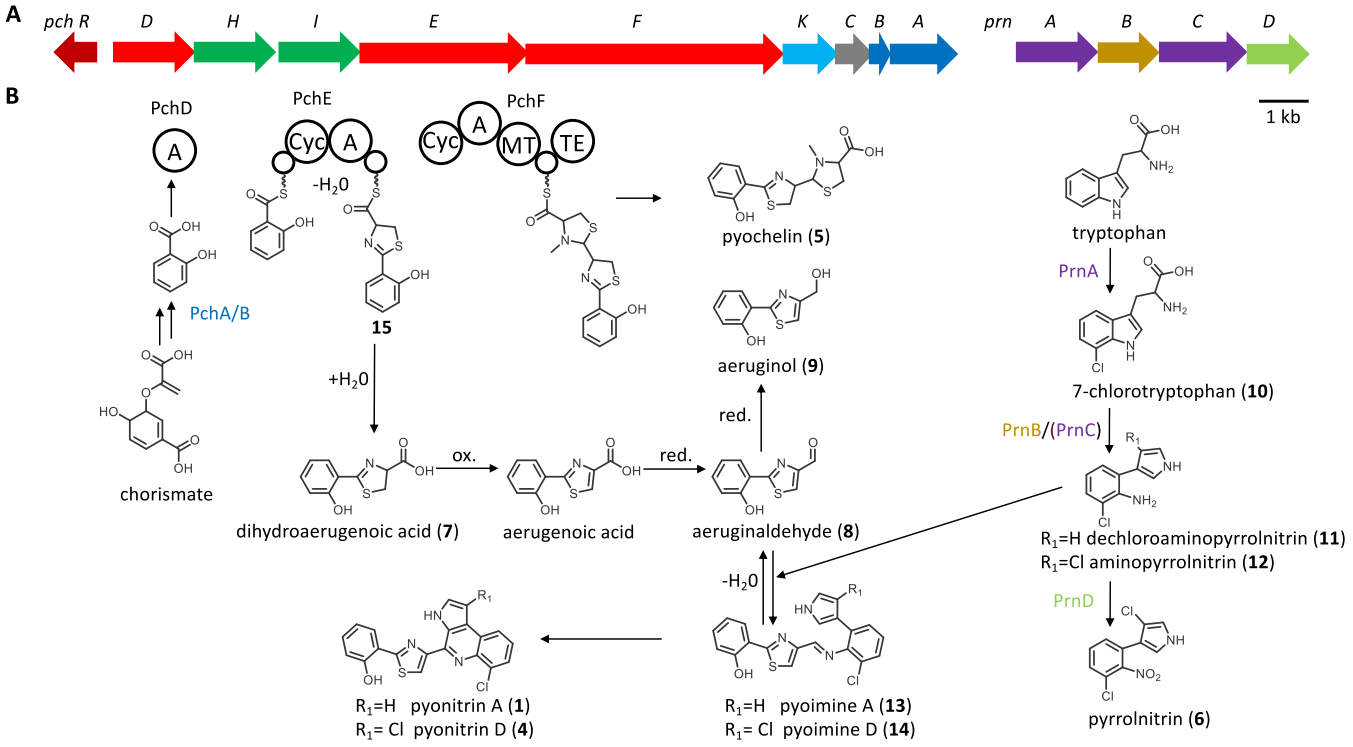

Figure 3. Biosynthetic gene clusters of pyochelin and pyrrolnitrin and model for pyonitrin biosynthesis. (A) Biosynthetic gene clusters for pyochelin (*pch*) and pyrronitrin (*prn*). Red: core NRPS gene; green: transporter; brown: regulator; light blue: dehydrogenase; dark blue: salicylic acid biosynthetic 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.

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 rearomatizatio 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 amino-pyrrolnitrin 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 domain[13] 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

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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)

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Notes

The authors declare no competing financial interest.

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