

# Synthesis and Investigation of the Abiotic Formation of Pyonitrins A–D

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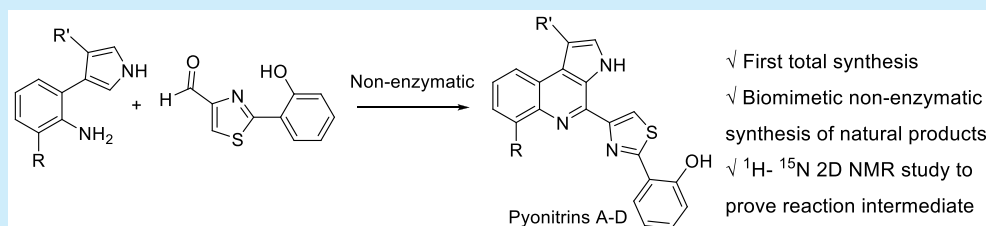
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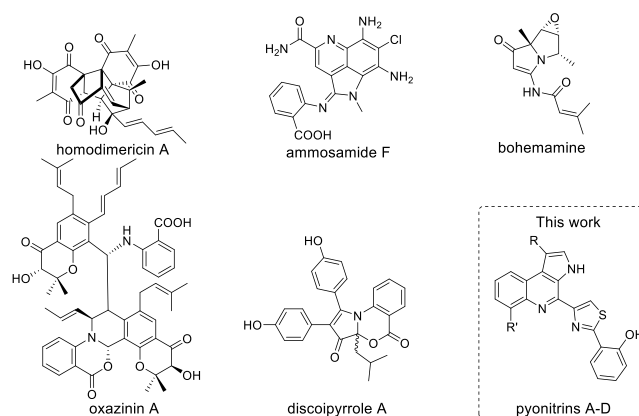


**ABSTRACT:** Pyonitrins A–D are recently isolated natural products from the insect-associated *Pseudomonas protegens* strain, which were isolated from complex fractions that exhibited antifungal activity via an *in vivo* murine candidiasis assay. Genomic studies of *Pseudomonas protegens* suggested that pyonitrins A–D are formed via a spontaneous nonenzymatic reaction between biosynthetic intermediates of two well-known natural products pyochelin and pyrrolnitrin. Herein we have accomplished the first biomimetic total synthesis of pyonitrins A–D in three steps and studied the nonenzymatic formation of the pyonitrins using  $^{15}\text{N}$  NMR spectroscopy.

The pyonitrins are a family of alkaloids recently discovered from an insect associated strain of *Pseudomonas protegens*. The molecules were identified from a powerful assay platform to identify microbial-derived natural products with efficacy in an *in vivo* model of *Candida albicans* infection.<sup>1</sup> *Candida* species commonly cause bloodstream infections that resulted in 34,800 hospitalizations and 1,700 deaths of patients in the year 2017 in the United States.<sup>2</sup> In the assay, partially purified fractions were able to inhibit *C. albicans* growth. Bioassay guided purification using *in vitro* antifungal activity led to isolation of less than 0.5 mg of purified pyonitrin A (1) and B (2) and only trace quantities of pyonitrin C (3) and D (4). The *in vivo* activity could not be fully confirmed due to the limited quantities.

In addition to the potential *in vivo* biological activity, our interest in these molecules was inspired by their proposed biosynthetic origin. Portions of the pyonitrins are structurally related to the known microbial metabolites pyrrolnitrin (5) and pyochelin (6). In the initial publication, Mevers et al. identified independent and functional biosynthetic gene clusters for 5 and 6. Based on the organizational structure of the biosynthetic gene clusters and the qualitative analysis of intermediates, the proposal was made that aerugaldehyde (7), a shunt metabolite of the biosynthetic pathway of (6), and an aminopyrrolnitrin derivative (8) undergo nonenzymatic imine formation, followed by a spontaneous cascade cyclization/oxidation (Figure S1).<sup>1</sup> Preliminary evidence for the biosynthetic proposal was provided via a microscale reaction of 7 and 24 in fermentation media to provide 3.<sup>1</sup>

Our laboratory has been interested in the study of natural products that involves nonenzymatic or spontaneous reactions in their formation, such as the discoipyrrole, bohemamines, and ammosamides (Figure 1).<sup>3</sup> As part of these previous studies, we have used isotope labeling approaches to validate the nonenzymatic reactions and further understand the cascade



**Figure 1.** Examples of bioactive natural products that utilize one or more nonenzymatic transformations in their formation.

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reactions that take place in the complex chemical environment of microbial fermentations. Due to the synthetic tractability of **7** and **8**, the exciting preliminary biological activity, and the spontaneous reactivity, we carried out a total synthesis of **1–4** and utilized a  $^{15}\text{N}$ -labeled version of **8** to further study the nonenzymatic imine formation and subsequent spontaneous cascade.

Retrosynthetically, we planned to synthesize pyonitrins A–D via a Pictet–Spengler reaction between **8** and **7**, the biogenesis products of pyrrolnitrin and pyochelin, respectively (Figure 2).

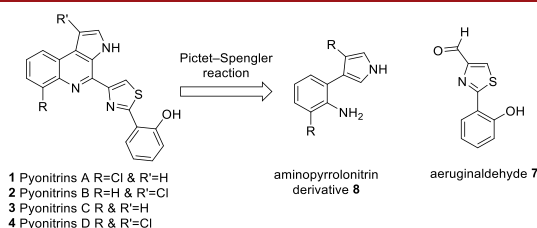
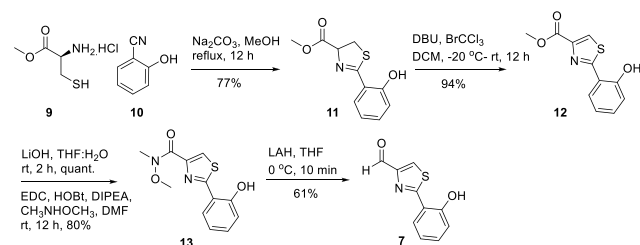


Figure 2. Retrosynthetic analysis based on biogenesis of pyonitrins A–D.

Synthesis of **7** was started from condensation of L-cysteine methyl ester hydrochloride (**9**) and 2-hydroxy cyanobenzene (**10**) to afford thiazoline derivative (**11**) in 77% yield.<sup>4</sup> Dehydrogenation of **11** with bromotrichloromethane and DBU at  $-20\text{ }^\circ\text{C}$  gave thiazole (**12**) as a sole product in quantitative yield.<sup>5</sup> The methyl ester was hydrolyzed to the acid using lithium hydroxide and transformed into a Weinreb amide (**13**) using standard conditions. Reduction of **13** with LAH afforded **7** in excellent yield (Scheme 1).

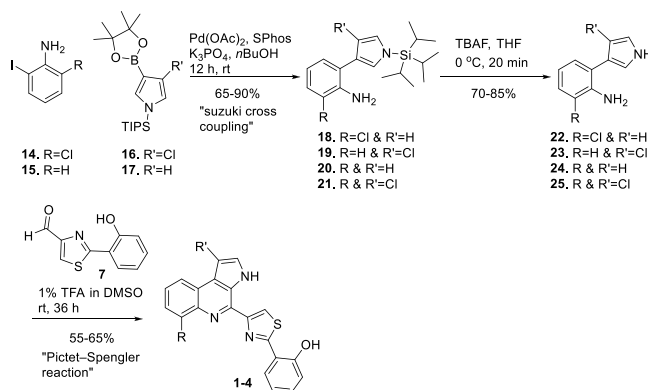
### Scheme 1. Synthesis of Aeruginaldehyde **7**



Synthesis of pyonitrins A–D began with Suzuki–Miyaura cross-couplings of the pyrrolepinacolboronate esters (**16–17**) and the corresponding aniline (**14–15**) derivatives in the presence of catalytic palladium diacetate (SPhos,  $\text{K}_3\text{PO}_4$ , aq.  $n\text{BuOH}$  at rt) to obtain compounds **18–21** in 65–90% yield.<sup>6</sup> Final preparation of the aminopyrrolnitrin analogs **22–25** with differing chlorination patterns was achieved by removal of the silyl protecting group using TBAF. With the coupling partners in hand we turned our attention to the final Pictet–Spengler reaction. To mimic the aqueous fermentation conditions, we attempted the Pictet–Spengler reaction of **22** and **7** in water at room temperature for 36 h under acidic conditions (10% TFA).<sup>7</sup> Under these conditions, **1** precipitated out of the aqueous solution with an overall yield of 56%. While conditions for the Pictet–Spengler reaction could clearly be optimized, we were looking for conditions that would be compatible with NMR studies, such as  $^1\text{H}$ – $^{15}\text{N}$  HMBC NMR spectroscopy, to resolve the reaction pathway. As the aqueous

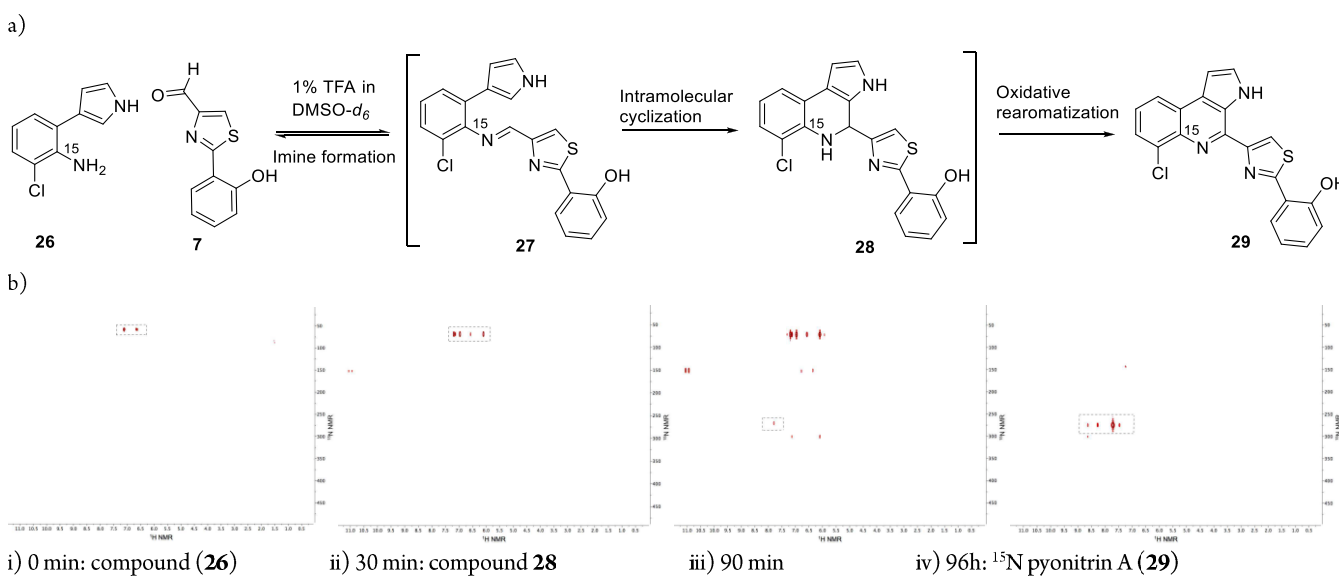
conditions led to precipitation of **1**, we looked to the use of organic solvent to keep the product in solution. The Pictet–Spengler reaction of **22** and **7** in DMSO with TFA (1%) at room temperature for 36 h led to the production of pyonitrin A in 61% yield. The structure of synthetic pyonitrin A was confirmed by comparison of our NMR and high resolution mass spectral data with the literature report (Table S1).<sup>8</sup> The synthesis of the remaining pyonitrins B–D from the corresponding aminopyrrolnitrins **23**, **24**, and **25** and **7** was achieved using the developed Pictet–Spengler reaction protocol (1% TFA in DMSO solvent for 36 h) (Scheme 2).

### Scheme 2. Synthesis of Pyonitrins A–D (**1–4**)



In natural product biosynthesis, the Pictet–Spengler condensation is typically a highly regulated enzyme catalyzed process, and **1–4** are the first examples of the nonenzymatic Pictet–Spengler condensation in natural products.<sup>1</sup> Similar to our previous work on nonenzymatic condensation/cyclization transformations, such as the discoipyrroles, our goal was to monitor the formation and disappearance of key intermediates in the nonenzymatic formation of the pyonitrins in real time. We wanted to exploit the large chemical shift range of  $^{15}\text{N}$  NMR due to the electronic interactions involving the lone pair of electrons on the nitrogen atoms.<sup>9</sup> To this end, we have utilized heteronuclear multiple-bond correlation (HMBC) NMR experiments to identify  $^1\text{H}$ – $^{15}\text{N}$  heteronuclear correlation of key intermediates formed in real time. In order to create a probe for both structural changes and intermolecular interactions in compounds that contain nitrogen atoms with changing chemical environments, an  $^{15}\text{N}$ -labeled starting material was required. As a result, we began our NMR study of the abiotic formation of the pyonitrins with the synthesis and utilization of  $^{15}\text{N}$ -labeled aminopyrrolnitrin (**26**), whereby the aniline contained the  $^{15}\text{N}$  isotope label.

All the NMR experiments were performed using **26** (1 equiv) and **7** (1 equiv) in 1% TFA in 700  $\mu\text{L}$  of  $\text{DMSO-}d_6$ . Experimentally we began by carrying out the Pictet–Spengler condensation with **26** and conducted continuous reaction monitoring in 30 min intervals for 48 h using  $^1\text{H}$ – $^{15}\text{N}$  HMBC. **26** has a  $^{15}\text{N}$  shift of 57.8 ppm and a strong correlation to aromatic protons of the aniline ring at 6.60 and 7.09 ppm (Figure 3). Immediately (30 min) after addition of **26** and **7** in 1% trifluoroacetic acid in  $\text{DMSO-}d_6$ , a new, strong signal appeared at  $\delta^{15}\text{N}$  70.7 ppm with HMBC correlation to protons at 6.09, 6.57, 6.95, 6.96, 7.13, and 7.18 ppm suggesting the formation of a 1,2-dihydroquinoline moiety (**28**). The cross peak with a  $^1\text{H}$ – $^{15}\text{N}$  shift of 11.00 and 151.73 ppm corresponds to nitrogen of the pyrrole on **28**. This was

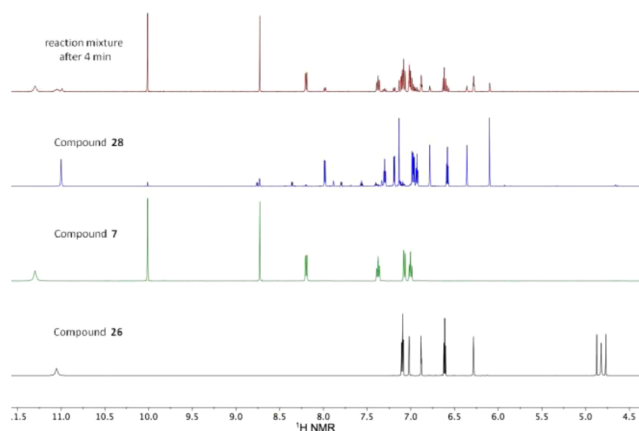


**Figure 3.** (a) Formation of pyonitrin A along with the intermediates. (b) Representative time points from <sup>1</sup>H-<sup>15</sup>N HMBC experiments.

surprising since we expected to see the formation of the imine intermediate (27) prior to cyclization. After 90 min, the <sup>1</sup>H-<sup>15</sup>N HMBC correlations showed a new <sup>15</sup>N signal at 270 ppm appears with correlations to protons at 8.70, 8.34, 7.78, 7.54 ppm, representing formation of <sup>15</sup>N-labeled pyonitrin A (29). These were the only intermediates observed in the reaction. We stopped the NMR experiment after 48 h, and the ratio of the product to 28 was determined to be 1:2 via a <sup>1</sup>H NMR (Figure 3 and video S1). The reaction was allowed to continue for an additional 48 h to obtain complete conversion to 29.

When compared to the media fermentation conditions, a 1% solution of TFA in DMSO-*d*<sub>6</sub> is far too acidic and might promote the cyclization of 27 to 28. We decided to lower the percentage of TFA and redo the reaction in order to try and detect the formation of 27. With samples of <sup>15</sup>N-labeled aminopyrrolnitrin 26, aeruginaldehyde 7, pyonitrin A, and 1,2-dihydroquinoline 28 we reran the experiment using only <sup>1</sup>H NMR. The experimental design was as follows: Using 26 (1 equiv) and 7 (1 equiv) in 0.05% TFA in DMSO-*d*<sub>6</sub>, an array of 1 scan <sup>1</sup>H NMR experiments were run and the spectra were compared against the starting materials and products, including the dihydroquinoline intermediate 28 (Figure 4). Interestingly, as soon as the starting materials were added together, we could immediately see the formation of 28 and not 27 like we expected. This might be due to the fact that as soon as the imine intermediate is formed, the inherent reactivity and proximity of the imine allow for immediate cyclization to 28.

We believe this is significant in context to the biological system, since the imine intermediate that is formed from the reaction of aminopyrrolnitrin and aeruginaldehyde is not observed in the study. The low probability that two biosynthetic intermediates come together in the bacterial milieu to form the imine intermediate makes the inherent reactivity of the two starting materials imperative for pyonitrin formation. Interaction between the two starting materials in the biological system and subsequent imine formation is the limiting factor in the formation of pyonitrin A. As a result, potential reversibility of imine formation would hinder conversion to the final product. The fast and spontaneous



**Figure 4.** Real time investigation of imine 27 formation by comparison of <sup>1</sup>H NMR of starting materials 26, 7 and intermediate 28.

cyclization of the imine drives formation of the pyonitrin A from any aminopyrrolnitrin and aeruginaldehyde molecules.

A number of microbial fermentations lead to the co-occurrence of aldehydes and amines. Many of these, however, do not lead to the production of a new chemical. A vast number of aldehyde and amine reactions are in equilibrium with the imine product. Depending on the conditions, the equilibrium can lie with the starting materials. In order for these reactions to proceed efficiently, especially in the case of starting materials in low concentrations, a strong driving force is needed to shift the equilibrium toward the imine. In the case of the pyonitriins, irreversible cyclization causes the equilibrium to shift toward a novel natural product.

In conclusion, we have accomplished the first biomimetic total synthesis of antifungal chimeric natural product pyonitriins A–D in short synthetic sequence. We have utilized the Suzuki–Miyaura cross-coupling and Pictet–Spengler reactions as the key steps in our synthesis. The developed route is scalable and highly amenable for the synthesis of diverse library of compounds around the pyonitrin scaffold to study the structure–activity relationship (SAR). The biological evaluation of the pyonitriins A–D against the *Candida albicans*

is under progress. The nonenzymatic nature of pyonitrin formation was explored using NMR as a sensitive tool. Key intermediates, such as the 1,2-dihydroquinoline, were shown, and others, such as the proposed imine intermediate, were not seen. By utilizing  $^1\text{H}$ - $^{15}\text{N}$  NMR, potential mechanisms of pyonitrin formation were elaborated and studied and provide a framework with which other interesting nonenzymatic reactions can be explored.

## ■ ASSOCIATED CONTENT

### SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.orglett.0c00098>.

General procedures, data tables, NMR spectra, and additional data (PDF)

Time-lapse video showing the development of the  $^1\text{H}$ - $^{15}\text{N}$  HMBC spectrum during the Pictet–Spengler reaction of  $^{15}\text{N}$ -labeled aminopyrrolnitrin **26** and **7** (MP4)

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### Notes

The authors declare no competing financial interest.

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