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Total Synthesis of Pyoverdin D

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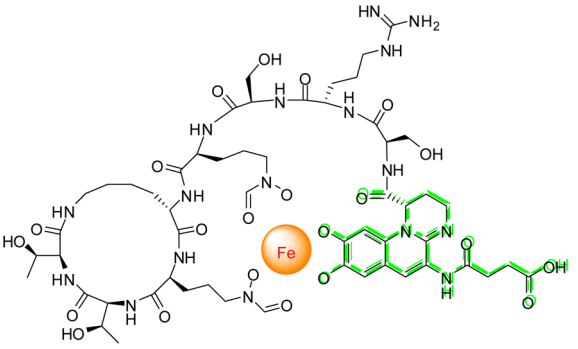
ABSTRACT



Pyoverdin D is an important siderophore that is used by the human pathogen *Pseudomonas aeruginosa* to import iron and gain a competitive advantage. This unique partially cyclic octapeptide bears four nonproteinogenic amino acids, including $^{\delta}$*N*-formyl-$^{\delta}$*N*-hydroxy-L-ornithine, and a catechol containing chiral chromophore. Here, we report the first total synthesis of pyoverdin D.

When subjected to iron starvation conditions, *Pseudomonas aeruginosa*, an opportunistic human pathogen, secretes vast amounts of its main siderophore, pyoverdin D, along with several other iron chelators.[1] Pyoverdin D is a unique, partially cyclic octapeptide that presents a number of interesting structural features, such as the side chain to backbone macrocycle that forms between the lysine residue and the C-terminal threonine carboxylate. Other important features include the presence of four nonproteinogenic amino acids found as part of the backbone of the molecule, namely two $^{\delta}$*N*-formyl-$^{\delta}$*N*-hydroxy-L-ornithine residues and two D-serine residues. In addition to these traits, the major characteristic of this class of molecules is the presence of an N-terminal dihydroxyquinoline-derived chromophore.[2,3] To date, no total synthesis for this key natural product has been reported.

The catechol-bearing chromophore, together with the two hydroxamic acids, serve as bidentate ligands for iron(III) and together form a strong octahedral complex upon chelation of this vital metal. The resulting complex has a stability constant of approximately 10^{32} M^{-1}, and once recognized by a cognate outer membrane transporter, it is immediately shuttled into the periplasm, where it dissociates and is subsequently recycled for further use. Presently, the precise regulation of these processes is not fully understood, although it is known that synchronization of population-wide siderophore synthesis is regulated through quorum sensing.[4−6] Thus far, more than 100 different pyoverdin molecules have been identified,[5] with the highly abundant pyoverdin D, in particular, having been shown by Meyer et al. to be of paramount importance not only in iron metabolism but also in terms of the producer's ability to acquire virulence.[1]

When our efforts to synthesize pyoverdin D began, the structure of the molecule had already been elucidated, not only by NMR[2,3] but also from the crystallographic data obtained on the molecule within its receptor.[7] In addition, the synthesis of the racemic protected chromophore had been described previously in a seminal study by Miller and

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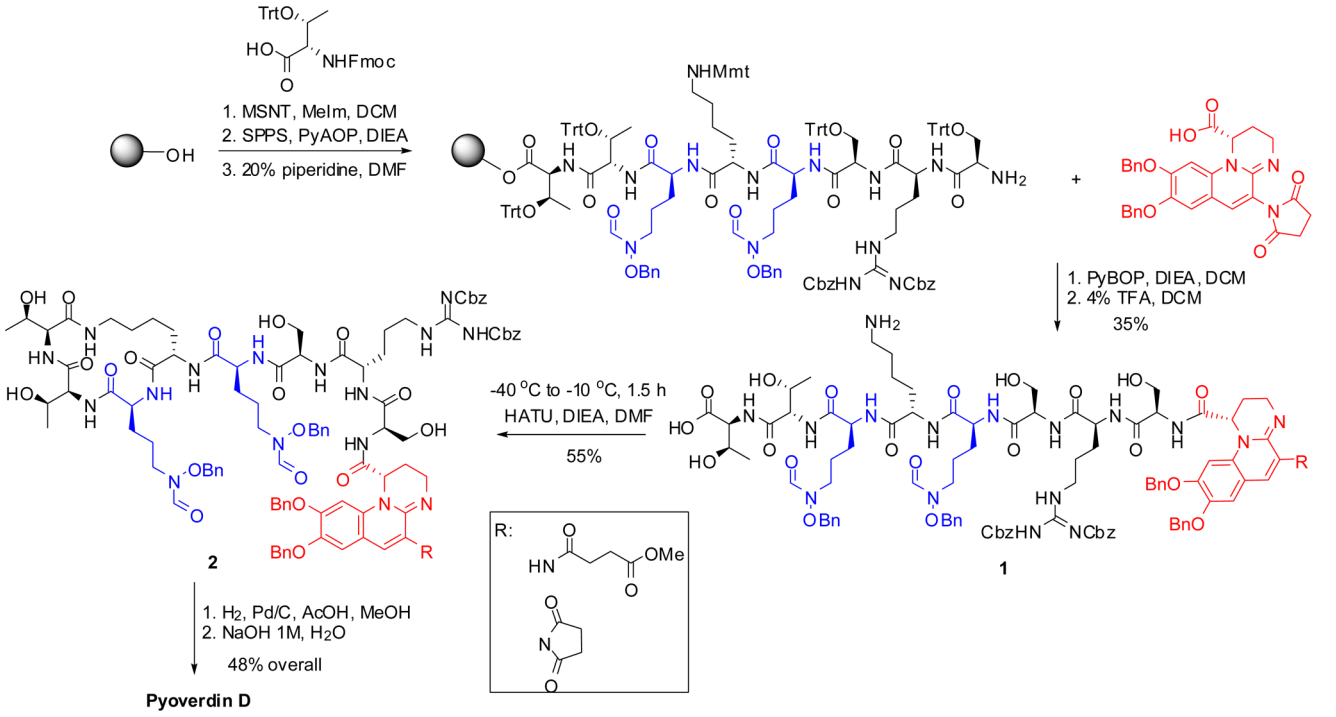
Scheme 1. Total Synthesis of Pyoverdin D



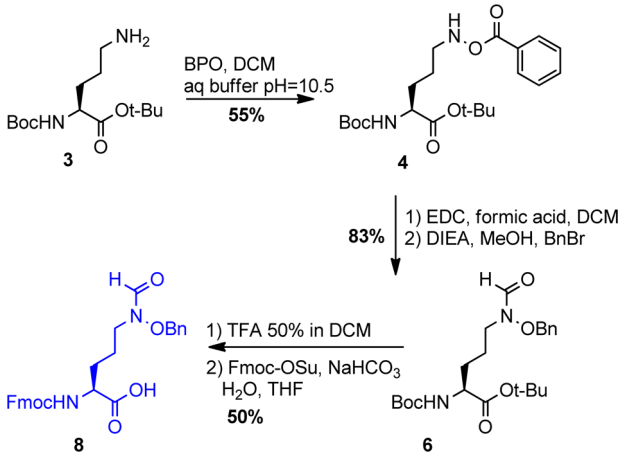
co-workers.[8] Nevertheless, most of the chemical research regarding pyoverdin D had focused on derivatization of pyoverdins obtained through fermentation.[9−12] Indeed, the lack of a synthetic pathway for pyoverdin D has thus far narrowed the scope of derivatization sites available on the molecule, thereby limiting the questions that can be answered by using such molecular tools. With this in mind, we started the total synthesis of pyoverdin D. We chose to employ a solid-phase peptide synthesis (SPPS) approach,[13,14] where the introduction of different or even nonproteinogenic amino acids is possible during backbone build-up.[15]

Here, we present a concise synthetic route for pyoverdin D that can serve as the basis for further efforts aimed at the synthesis of a variety of related pyoverdins and analogs. Such syntheses will further extend our ability to study the important and still enigmatic relationship between iron signaling and *P. aeruginosa* behavior at the single and multicellular levels.

The convergent synthetic route leading to pyoverdin D generation employed in this study is depicted in Scheme 1. The family of known pyoverdin molecules is derived from either a linear or a partially cyclic peptide bearing two metal-chelating amino acids and possessing a chelating chromophore at the N terminus.[8,9,16]

With these conserved features in mind, and based on the elegant work of Miller and co-workers, we developed a relatively simple strategy that can serve as a general methodology for the syntheses of those pyoverdin molecules that carry a similar chromophore. In order to enable an SPPS approach to synthesize the peptide moiety, representing a facile combinatorial method for analog

Scheme 2. Synthesis of $^{\delta}N$-Formyl-$^{\delta}N$-benzyloxy-L-ornithine



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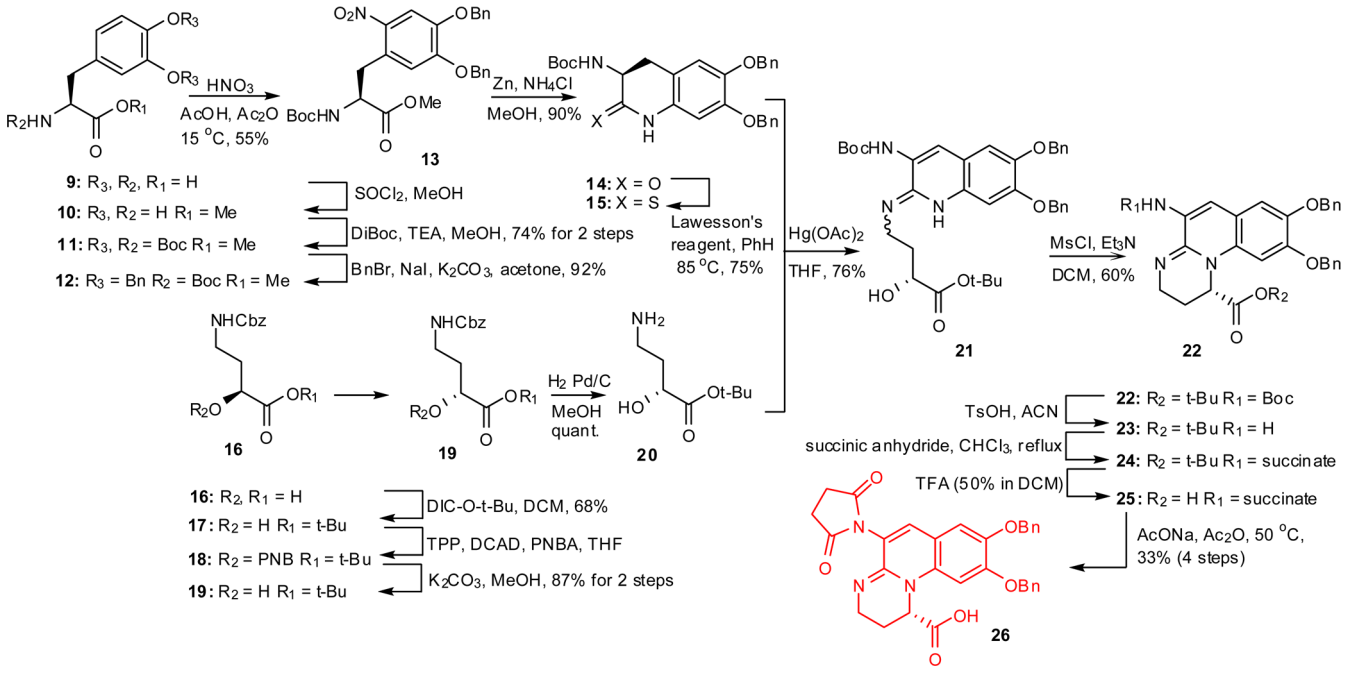
Scheme 3. Synthesis of the Protected Chromophore



preparation, we first developed a succinct method for the synthesis of 9-fluorenylmethyloxy carbonyl (Fmoc)-protected[17] $^{\delta}$*N*-formyl-$^{\delta}$*N*-hydroxy-L-ornithine.

The Fmoc SPPS approach developed also required the enantiopure preparation of a chromophore with suitable protecting groups. Following synthesis of the peptide, our design proceeded with the incorporation of the chromophore, cleavage of the linear-protected pyoverdin from the resin, formation of the macrolactam, and global deprotection. We commenced with the synthesis of properly protected formylhydroxyornithine (Scheme 2).

Our strategy was based on a similar synthetic route designed by Fennell et al.[18] where a protected formylhydroxylysine was synthesized. We started from commercially available Boc-L-Orn-OtBu (**3**) which was oxidized by treatment with dibenzoyl peroxide to furnish **4**. Formylation was achieved via coupling with formic acid, using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) as the coupling reagent, to obtain the $^{\delta}$*N*-formylated product that was further reacted to give **6** through one-pot solvolysis and benzylation reactions. Deprotection of the *tert*-butyloxycarbonyl (Boc) and *tert*-butyl ester moieties by exposure to trifluoroacetic acid (TFA) provided the free amine and carboxyl moieties (**7**). Subsequent exposure to Fmoc-OSu gave **8** with an overall yield of 23%. With the hydroxamate-containing amino acid in hand, we next set out to synthesize the chromophore. Our synthesis of the protected dihydroxyquinoline was inspired by the synthetic route published by Miller and co-workers. The Boc protecting group was incorporated early in the synthesis, since its bulkiness was proven to be crucial for the synthesis of the chromophore.[8] The synthetic pathway is depicted in Scheme 3, starting from commercially available L-DOPA (**9**), which was treated with thionylchloride in methanol to furnish **10**. The α-amine was then treated with Boc anhydride and Et_3N in methanol to generate **11**.

The dibenzyl-protected catechol was synthesized by treatment of **11** with benzyl bromide in the presence of K_2CO_3 and NaI in acetone. The nitration of **12** was carried out under relatively mild acidic conditions (1.5 equiv of nitric acid in an acetic acid/acetic anhydride mixture at 15 °C) to prevent cleavage of the acid-labile Boc group, affording **13**. The next step, one-pot reduction and cyclization to form the quinoline moiety, was achieved by treating **13** with zinc dust and NH_4Cl in a THF/methanol mixture. The resulting amine and **14** were then extracted from the reaction mixture and heated in methanol at reflux temperature to give the Boc-protected quinoline **14**.[19]

Thionation was performed by reacting **14** with Lawesson's reagent[20] to give the thioquinoline **15**. The thioquinoline was reacted with freshly prepared 4-amino-2-hydroxybutanoate (**20**) in the presence of mercuric acetate, which not only led to ligation of the two fragments but also to the expected oxidation of the C7−C8 bond, generating the fluorescent scaffold as a mixture of E and Z isomers (**21**). After mesylation of the alcohol, we observed that both of the isomers had collapsed to the same product upon the concomitant one-pot cyclization, yielding

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the protected chromophore **22**. Treatment of **22** with *p*-toluenesulfonic acid (TsOH) in acetonitrile,[21] provided the free amine **23**,[22] which was treated with succinic anhydride in chloroform at 50 °C to provide **24**. The *tert*-butyl ester was then cleaved by exposure to 50% TFA, forming **25**, which was further treated with sodium acetate in acetic anhydride at 60 °C to facilitate the formation of the succinimide (**26**) from the open succinate at a 33% overall yield. Finally, the synthesis of pyoverdin D (Scheme 1) was carried out on super acid-labile Rink acid resin[23] using Fmoc chemistry. Double loading of the resin by treatment with 1-(mesitylene-2-sulfonyl)-3-nitro-1,2,4-triazole (MSNT) and 1-methylimidazole (MeIM) in CH_2Cl_2 had been reported to minimize racemization.[24] The peptide was elongated using (benzotriazol-1-yloxy)-tripyrrolidinophosphoniumhexafluorophosphate (PyAOP)[25] as the coupling reagent.[26] The chromophore was then coupled to the peptide, followed by treatment with 4% TFA, yielding the linear pyoverdin D skeleton (**1**) as a mixture of a succinimide and a methyl succinate (probably due to washings with MeOH after cleavage).

We performed the cyclization reaction in solution phase, as possible formation of cross-links on the solid support was probable due to the steric hindrance promoted by the bulky protecting groups. Cyclization was achieved by treating the semiprotected linear peptide with 10 equiv of HATU[27] and *N*,*N*-diisopropylethylamine (DIEA) in DMF,[28] affording the partially cyclic octapeptide **2**.

Further deprotection by catalytic hydrogenation followed by basic hydrolysis furnished pyoverdin D. HPLC analysis (Supporting Information) of synthetic and natural pyoverdin D (extracted and purified from wild-type *P. aeruginosa* strain PAO1) showed the synthetic compound and the extracted pyoverdin to be identical while detailed NMR (^{1}H NMR, TOCSY and ROESY) and CD analyses showed that the extracted and synthetic samples perfectly matched each other (Figures 1−3, Supporting Information).

We also tested the biological activities of the synthetic and natural samples using a *P. aeruginosa* strain lacking the pyoverdin synthetase (PAO6606) and thus handicapped in its ability to take up iron through the pyoverdin pathway. Figure 1 shows that this strain does not grow under iron-limited conditions, effected through addition of human transferrin, a high-affinity iron chelator that is present in human plasma, unless pyoverdin is added to the medium. The concentration-dependent responses in growth stimulation were nearly identical for synthetic and natural pyoverdin D.

In summary, we report the first total synthesis of pyoverdin D, with its identity and configuration confirmed by comparison with extracted and purified samples from wild-type *P. aeruginosa*. Our versatile and modular synthetic strategy can be applied to generate a large number of pyoverdin analogs, related siderophores and drug conjugates, enabling the pursuit of questions that carry major significance for understanding bacterial survival and competition for resources, as well as potential crosstalk between population-wide signaling of cell density and iron availability.

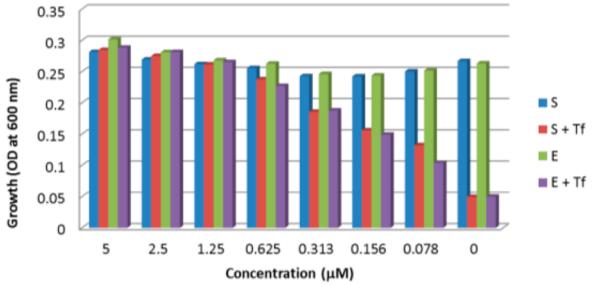

Figure 1. Effects of synthetic (S) and extracted (E) pyoverdin D on growth of the pyoverdin- and pyochelin-deficient *P. aeruginosa* strain PAO1ΔpvdDΔpchEF (in CAA medium incubated at 37 °C for 8 h), in the presence and absence of 4 μM human apotransferrin (Tf). The transferrin effectively depletes the medium of available iron and prevents growth of the double mutant in the absence of pyoverdin D. Other siderophores are able to chelate iron under iron-rich conditions, enabling growth of pyoverdin-deficient *P. aeruginosa* strains.

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Supporting Information Available. Detailed experimental procedures, characterizations, and copies of ^{1}H and ^{13}C NMR spectra of new compounds.This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.

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