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Formation of the Chromophore of the Pyoverdine Siderophores by an Oxidative Cascade

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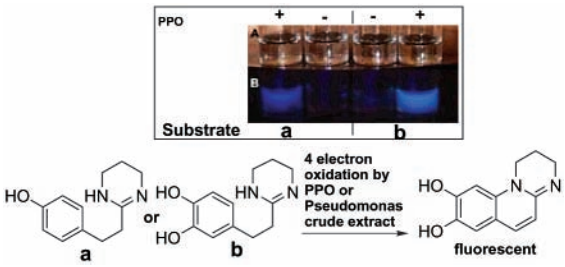
ABSTRACT



The pyoverdine chromophore is formed in a reaction involving a two-electron oxidation, a conjugate addition, and a second two-electron oxidation. This oxidative cascade can be carried out with polyphenol oxidase (PPO), MnO_2, and cell-free extracts from *Pseudomonas aeruginosa*.

Pathogenic *pseudomonas aeruginosa* and related *Pseudomonads* secrete a pyoverdine siderophore[1] (**1**) under iron starvation conditions (Figure 1).[2] This siderophore is a virulence factor that functions by complexing Fe^{3+} prior to transport into the cytoplasm via the transmembrane protein, FpvA.[3] Pyoverdines are species-specific peptides consisting of 6−19 amino acid residues, containing D-amino acids, one or more hydroxamates, and the characteristic pyoverdine chromophore.[4]

Incorporation studies have demonstrated that the pyoverdine chromophore is derived from tyrosine (**2**)[5] and L-2,4-diaminobutyric acid (**3**).[6] It has also been demonstrated that the *pvc* gene cluster is essential for the biosynthesis of the chromophore (Scheme 1).[7]

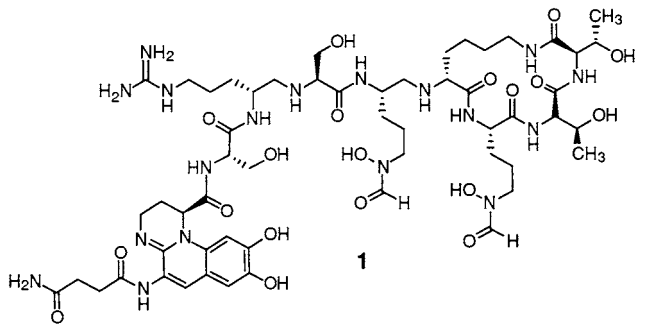


Figure 1. Structure of a pyoverdine. In subsequent structures, the peptide and the bisamide substituents on the chromophore are represented as R groups.

Several compounds (**4**−**8**, Figure 2), which are related to the pyoverdines, have been isolated from various *Pseudomonads*. Of these, it has been demonstrated that **4** is a precursor

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(1) Pseudobactin and fluorescein are two altenative names used for pyoverdine, particularly in the older literature.

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(3) Schalk, I. J.; Kyslik, P.; Prome, D.; Dorsselaer A. V.; Poole, K.; Abdallah, M. A.; Pattus, F. *Biochemistry* **1999**, *38*, 9357−9365.
(4) Fuchs, R.; Budzikiewicz, H. *Curr. Org. Chem.* **2001**, *5*, 265−288.
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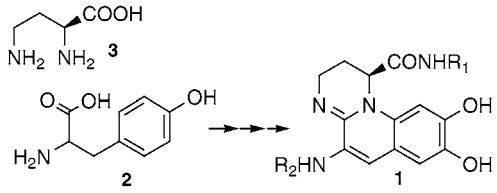
Scheme 1. Precursors to the Pyoverdine Chromophore



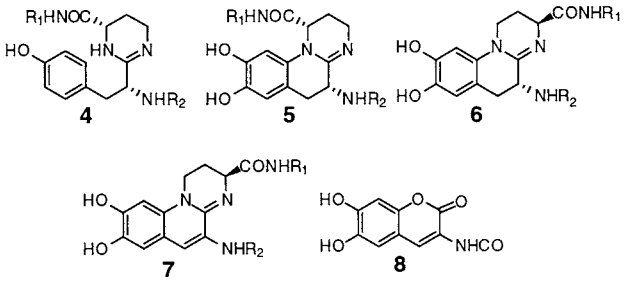


Figure 2. Isolates that are biogenically related to pyoverdine.

to pyoverdine,[8] that **5** is readily oxidized to pyoverdine in a nonenzymatic reaction,[9] and that **8** is secreted by a pyoverdine mutant overexpressing the *pvc* gene cluster.[10] Several mechanistic proposals for the formation of the chromophore have been described, based on isolation studies,[11,12] labeling studies,[5] and mutant complementation.[13]

We suggest a new mechanistic proposal for the formation of the pyoverdine chromophore that is consistent with the formation of **4**−**8** by related chemistry (Figure 3).[14−16] In

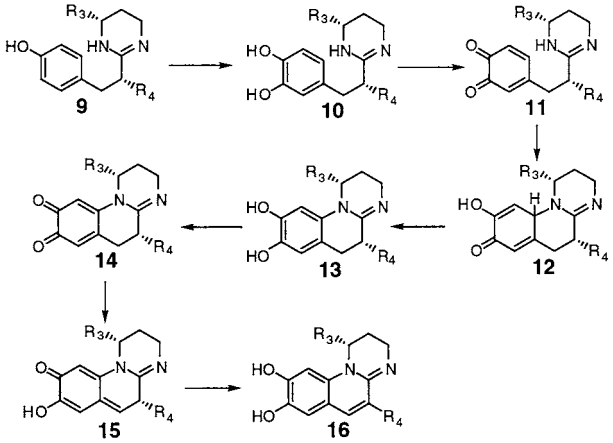
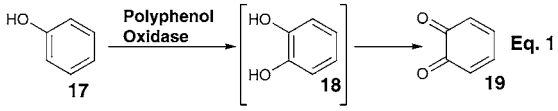


Figure 3. Mechanistic proposal for the formation of the pyoverdine chromophore.

this proposal, hydroxylation of **9** would give catechol **10**. Oxidation of **10** to the *o*-quinone **11** followed by a conjugate addition would give **12**. A facile tautomerization reaction would then reestablish the catechol functionality in **13** and set up the system for a second two-electron oxidation to give *o*-quinone **14**. A sequence of two tautomerizations yields **16**. Analogous oxidative cascades have been observed for the phenoxazinone synthase catalyzed formation of the phenoxazinone chromophore in actinomycin biosynthesis,[17] the polyphenol oxidase catalyzed formation of 5,6-dihydroxyindole in melanin biosynthesis[18] and in the biomimetic synthesis of styelsamine.[19]

To test this mechanistic proposal, phenol **20**, the simplest analogue of **9**, was synthesized[20] and incubated with polyphenol oxidase. Polyphenol oxidase is an enzyme known to hydroxylate a variety of phenols to the corresponding catechols and also to oxidize catechols to the corresponding *o*-quinones (eq 1).[21]



Two major products were formed in this reaction, which were characterized by ^{1}H NMR, MS, UV−vis, and fluorescence spectroscopy. These were shown to be structures **21** and **22**.[22] Polyphenol oxidase catalyzed oxidation of catechol **23** also gave a mixture of **21** and **22** supporting the

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(20) See the Supporting Information.

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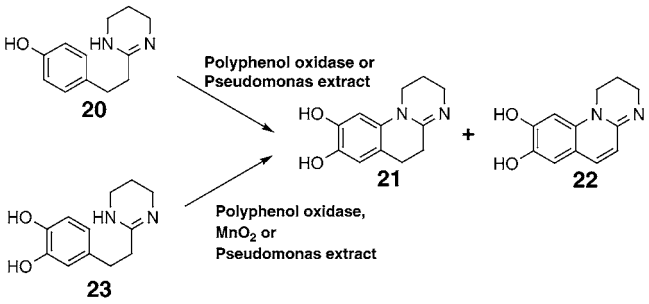
(22) A solution of 3 mg of **20** in 15 mL of 40 mM KPi at pH 6.8 was incubated with polyphenol oxidase (40 μg) until maximum fluorescence was observed. At this point, the reaction was quenched with methanol and filtered through a membrane by centrifugation (5000 Da cutoff) to remove the precipitated protein. The methanol was removed under reduced pressure followed by removal of the water by lyophilization. The fluorescent product was purified by cation exchange chromatography (Amberlite CG-50, 6 g) using a gradient of 0.001 M HCl to 0.2 M HCl and all the fluorescent fractions were lyophilized to give 22. (200 μg, 6%) as a green solid: ^{1}H NMR (400 MHz, D_2O) 7.62 (d, J = 8.4, 1H), 6.99 (s, 1H), 6.97 (s, 1H), 6.50 (d, J = 9.6, 1H), 4.05 (br t, 2H), 3.32 (br t, 2H), 2.05 (br m, 2H); fluorescence em (1:1 MeOH/H_2O, pH 7.5, ex 390, λ_{max}) 445; UV−vis (1:1 MeOH/H_2O, λ) pH 9.5; 406 ($\epsilon = 1 \times 10^5$), 235, 213, pH 7.5; 391, 265, 233, pH 3.5; 359, 308, 248, 220. MS ESI (m/z, M + H$^+$) 217; daughter ion 217 (MS MS, m/z, M$^+$) 217, 189 (retro Diels−Alder), 160 (loss of nitrile). A second, light green fluorescent fraction was isolated which corresponded to **21** (see the Supporting Information). Incubation of **21** with polyphenol oxidase gives **22**.

intermediacy of this compound. In addition, polyphenol oxidase catalyzed the oxidation of **21** to **22**. Products **21** and **22** were also obtained by oxidizing **23** with manganese dioxide.[23] The formation of the chromophore **22**, by the polyphenol oxidase catalyzed oxidation of **20** and **23**, as well as the isolation of **21** supports the proposed oxidative cascade for the formation of the pyoverdine chromophore (Scheme 2).

Scheme 2



To test **20** and **23** as possible substrates for the pyoverdine biosynthetic enzymes, these compounds were treated with cell free extract from *P. aeruginosa*, grown under iron limiting conditions to induce the pyoverdin biosynthetic genes. Under these conditions, **20** and **23** were oxidized to **22** (Figure 4) by cell-free extract. The oxidation of **23** to **22** was 5 times faster than the oxidation of **20** to **22**. The activity was not detected when the cell-free extract was omitted and reduced to 4% when the cell-free extract was boiled. To further eliminate the possibility that the oxidation was mediated by free transition metal ions in the cell-free extract, the assay was repeated using cell-free extract from cells grown in the presence of 40 μM Fe(III), conditions known to repress expression of the pyoverdine biosynthetic genes.[7] Under these conditions, the production of **22** was decreased by at least a factor of 330.[24] Finally, cell-free extract prepared from a strain mutated in an essential transcriptional regulator

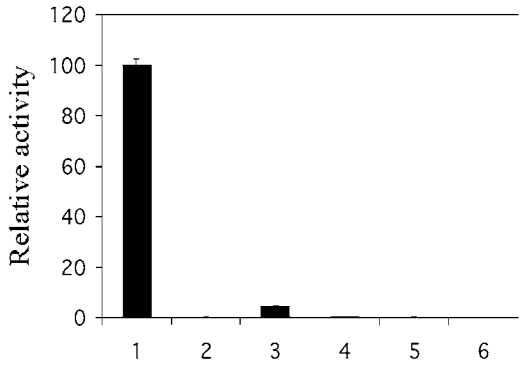


Figure 4. Oxidation of **20** to **22** by cell free extract from *Pseudomonas aeruginosa*: (1) **20** and cell-free extract from cells grown in the absence of Fe(III); (2) same as 1, without cell-free extract; (3) same as 1 except that the cell free extract was boiled; (4) **20** and cell-free extract grown in the presence of 40 μM Fe(III); (5) **20** and cell-free extract from pvdS$^-$ cells grown in the absence of Fe(III); (6) **20** and cell-free extract from pvdS$^-$ cells grown in the presence of 40 μM Fe(III). The assay procedure is described in the Supporting Information. Each activity is an average of four independent experiments.

(the sigma factor *pvdS*) for pyoverdine biosynthesis[25] did not catalyze the oxidation of **20** to **22**, even when the medium did not contain Fe(III). This is the first direct confirmation that the chromophore producing enzymes are regulated by PvdS. These observations also confirm that the highly truncated version of **9** is a substrate for the enzyme(s) involved in the biosynthesis of the pyoverdine chromophore.

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Supporting Information Available: Procedures for the synthesis and chemical and enzymatic oxidation of **20**, **21**, and **23**. This material is available free of charge via the Internet at http://pubs.acs.org.

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