Tetrahedron Letters xxx (xxxx) xxx

Contents lists available at ScienceDirect

Tetrahedron Letters

journal homepage: www.elsevier.com/locate/tetlet

Promiscuous *Pseudomonas*: Uptake of non-endogenous ligands for iron acquisition

Anna R. Kaplan, William M. Wuest *

Department of Chemistry, Emory University, Atlanta, GA 30322, USA

ARTICLE INFO

Article history:
Received 17 April 2021
Revised 17 May 2021
Accepted 21 May 2021
Available online xxxx

Dedicated to Prof. Dale Boger for his contributions to the field of medicinal chemistry and continued support of the next generation of scientists.

Keywords:
Iron acquisition
Siderophores
Oxazolines
Pseudomonas aeruginosa

ABSTRACT

Iron is an essential nutrient to nearly all living beings. However, its acquisition poses a significant challenge to many organisms, including most bacteria. One of the main iron uptake strategies employed by bacteria is the uptake of siderophores, small molecules that chelate extracellular iron. The pathogenic species *Pseudomonas aeruginosa* produces two different siderophores, pyochelin and pyoverdine. *P. aeruginosa* senses the amount of bioavailable extracellular iron in order to regulate the production levels of each of these two siderophores. In previous work, we found that a series of pyochelin biosynthetic shunt products enhanced the growth of *P. aeruginosa* in iron- depleted conditions when prechelated with iron. Thus, on the basis of these results, we investigated the physiochemical and biological properties of a series of non-native oxygen counterparts to these metabolites in the current study.

Introduction

Iron is an essential nutrient playing a central role in numerous vital biological processes including photosynthesis, respiration, nitrogen fixation, oxygen transport, and DNA synthesis [1–3]. It is the fourth most abundant element on earth, however its acquisition still presents a unique challenge for many organisms including most bacteria [1–3]. For example, in densely populated terrestrial environments, such as the rhizosphere, most of the iron is present as a highly insoluble ferric oxide complex (Fe_2O_3), limiting the concentration of bioavailable iron to 10^{-9} to 10^{-18} M, which is far below the 10^{-6} M required for survival [2–4]. Another particularly sparse environment is that of mammalian host organisms, because the majority of Fe^{III} is tightly complexed and stored in various circulating proteins such as ferretin, heme, and transferrin thereby limiting the available Fe^{III} concentration to a lowly 10^{-24} M [2,3,5].

To circumvent this issue of iron limitation, bacteria have evolved several iron uptake strategies, the most common of which is the uptake of siderophores: small molecules excreted by bacteria to chelate extracellular iron and then reimported as siderophore-iron complexes [1–4,6]. Many species of bacteria produce multiple siderophores, one of which is the pathogenic bacteria *Pseudomonas aeruginosa*, which is known to colonize the lungs of immunocompromised patients [7]. The production levels of its two native siderophores, high-affinity pyoverdine and lower affinity pyochelin (structure shown in Fig. 1a) is regulated by the ferric uptake regulatory system (Fur) based on the amount of bioavailable extracellular iron (Fig 1a) [8,9]. In previous work, we investigated a series of pyochelin biosynthetic shunt products and found that when complexed with iron, these metabolites promoted growth of *P. aeruginosa* wild-type (PAO1) and a double mutant (Δ*pvdD*Δ*pchEF*) incapable of producing siderophores (Fig. 1a) [10]. Our lab has had a long standing interest in siderophores [11], particularly those with scaffolds similar to pyochelin [12]. In particular, the thiazoline/oxazoline motif akin to many siderophores [13,14], has garnered much attention [15]. Thus, we wanted to explore if exchanging the sulfur in these shunt metabolites with an oxygen (Fig. 1b) would abolish the growth enhancement effect observed previously, or if *P. aeruginosa* would in fact be able to import these complexes as xenosiderophores [9]. Herein, we synthesized a series of oxygen analogs of previously studied metabolites and investigated their physiochemical and biological properties.

The synthesis of this series of compounds was achieved in a straightforward and highly efficient manner (Scheme 1). 2-

* Corresponding author.

https://doi.org/10.1016/j.tetlet.2021.153204
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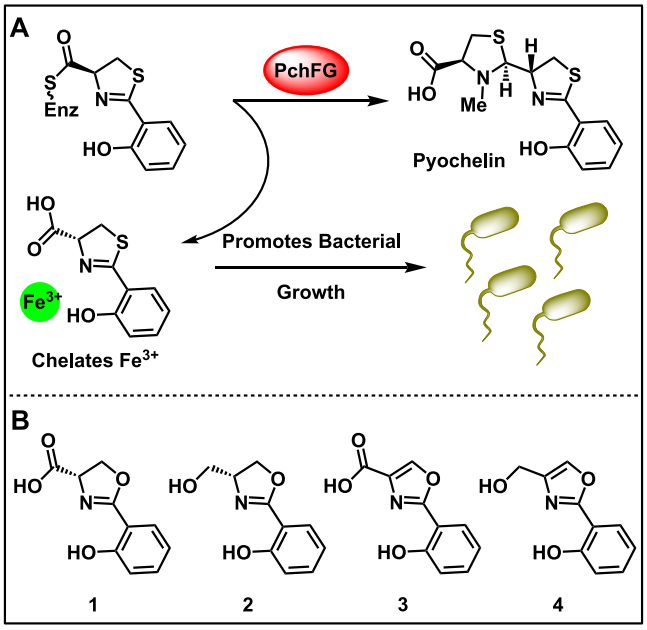
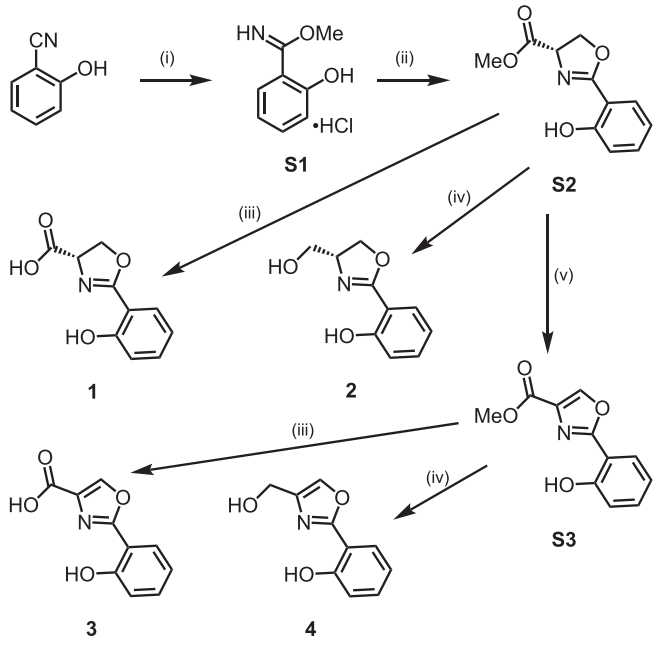


Fig. 1. (A) Summary of results from previous work; (B) Structures of compounds studied in this work.



Scheme 1. *Reagents and conditions*: (i) AcCl, MeOH, 25 °C, 16 h, 54%; (ii) L-serine methyl ester hydrochloride, Et_3N, $C_2H_4Cl_2$, 90 °C, 16 h, 45%; (iii) LiOH, THF/H_2O (1:1), 25 °C, 2 h, 50% for **1**, 84% for **3**; (iv) LAH, Et_2O, 0 °C to 25 °C, 1 h, 54% for **2**, 35% for **4**; (v) DBU, $BrCCl_3$, CH_2Cl_2, 0 °C to 25 °C, 74%.

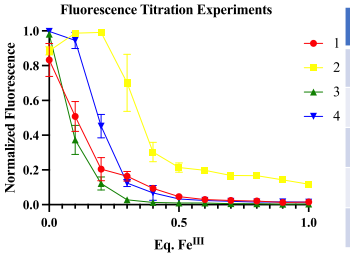
Hydroxybenzonitrile was converted to imidate **S1**, followed by condensation with L-serine methyl ester hydrochloride to give oxazoline **S2** [16]. This material was then hydrolyzed or reduced to give carboxylic acid **1** and primary alcohol **2**, respectively. Desaturation of the oxazoline was achieved utilizing methodology developed by Wipf and Williams, furnishing oxazole **S3** [17,18]. In the same fashion as with the oxazolines, hydrolysis and reduction afforded **3** and **4**, respectively.

The iron-binding properties of this series was then investigated qualitatively and quantitatively. Upon addition of a solution of each corresponding compound to a solution of $FeCl_3$, (both in methanol) a distinct color change was observed, changing from yellow to purple. Consistent with previous findings [10], the degree of color change was concentration dependent, as the addition of increasing amounts of compound to $FeCl_3$ yielded solutions with a darker shade of purple (Fig. S1). It should be noted that this effect was more obvious for some compounds and less obvious for others.

Quantification of the iron-binding properties of these compounds was achieved using fluorescence titration experiments. Fe^{III} was added in 0.1 M equivalent increments to a solution of each compound in methanol until a full molar equivalent had been added. The emission spectra were then recorded after each addition, allowing us to generate fluorescence titration curves (Fig. 2a). Similar to previous work [10], these spectra showed that the emission signals of all compounds were quenched with different amounts of Fe^{III}, indicating that the stoichiometry with which they each bind iron is different. These spectra were then extrapolated to determine ligand-to-Fe^{III} binding ratios (L:Fe^{III}), as well as putative dissociation constant (K_d) values (Fig. 2b, S2). It should be mentioned that the K_d values were calculated in prism and serve as a ballpark estimate of the true K_d value of each compound. Notwithstanding, these K_d values are within 1–2 orders of magnitude and are thus relatively comparable to those of pyochelin [19–21].

Finally, a preliminary biological investigation of this series was performed. In our previous work, we tested our series of metabolites against a panel of *Pseudomonads* (*P. aeruginosa*, *P. fluorescens*, *P. putida*, and *P. syringae*) as well as other clinically relevant species including (methicillin-resistant) *Staphylococcus aureus*, *Enterococcus faecalis*, and *Escherichia coli*, with the hypothesis that they may be acting through a mechanism similar to that of yersiniabactin, wherein chelation of iron would inhibit the growth of a given bacteria [22]. However, no such inhibition was observed for any of the compounds against any bacterial species. Because these oxygen-containing counterparts, unlike those studied in our previous work, are not endogenous to *P. aeruginosa*, we hypothesized that they could garner this inhibitory effect. Albeit, no growth effect was observed, thus we moved on to elucidate potential growth-enhancing effects of these molecules on *P. aeruginosa*.

All four compounds were incubated (as free ligands) with both wild type PAO1 and the previously mentioned double knockout mutant **Δ*pvdD*Δ*pchEF*** in iron depleted media and growth to stationary phase was monitored up to 20 h (Fig. 3). Unsurprisingly, there was no noticeable influence on growth, consistent with previous findings. However, again similar to our previous work, when these compounds were prechelated with Fe^{III}, a marked increase in growth was observed [10]. This enhancement of growth was similar to that resulting from incubation with the same concentration of free Fe^{III} (125 μM) in the case of PAO1 and greater for **Δ*pvdD*Δ*pchEF***, thereby indicating that this observed enhancement



Compound	L:Fe^{III}	K_d (mM)
1	2:1	40.81
2	1:1	553.7
3	3:1	23.14
4	2:1	113.5
Pyochelin[19-21]	1:1/2:1	2.0-5.0

Fig. 2. (A) Fluorescence titration curves of **1**–**4** titrated with 1.0 equivalents of $FeCl_3$ (each titration was performed in triplicate); (B) Table of binding stoichiometries and calculated K_d values for **1**–**4** and pyochelin [19–21].

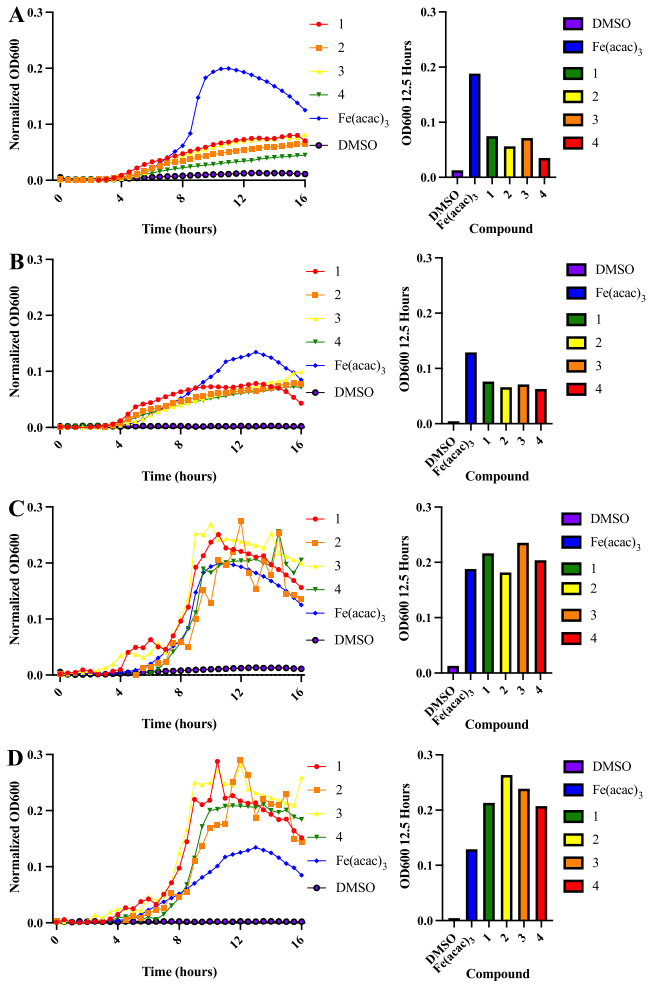


Fig. 3. Growth curves of PAO1 (A,C) and Δ*pvdD*Δ*pchEF* (B,D) incubated with 125 μM of either free ligand (A,B) or prechelated complexes (C,D) and bar graphs showing the OD_{600} value for each compound after 12.5 h of growth.

is likely due to uptake of the ligand-Fe^{III} complexes themselves and not just free Fe^{III}. Further, the enhanced growth observed in both PAO1 and Δ*pvdD*Δ*pchEF* were similar, allowing us to reasonably conclude that these complexes are likely imported directly into the cells with no recomplexation of iron with the native siderophores produced by PAO1.

In summary, a series of four oxazoline/oxazole compounds were synthesized and investigated physiochemically and biologically. All four compounds demonstrated affinity for iron both qualitatively and quantitatively. In addition, while none of these compounds exerted an antibiotic effect against any of the bacteria tested, the ligand-Fe^{III} complexes of each compound did enhance the growth of both PAO1 and Δ*pvdD*Δ*pchEF* in iron-depleted media. As we discussed in our prior work, further experimentation will need to be conducted to fully elucidate the mechanism by which these complexes are imported into the cell, the transport machinery involved, and ultimately the biological mechanism of these compounds. However, one claim that can be made is that *P. aeruginosa* is fairly promiscuous in its uptake of iron-chelated small molecules in iron-depleted laboratory conditions. Future work is focused on determining if this phenomenon is specific to *Pseudomonas* or if it applies to other pathogenic bacteria such as *Acinetobacter baumanni.*

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We are grateful to the NIH (GM119426) and NSF (CHE2003692) for funding this work. The NMR instrumentation used in this work was funded by the NSF (CHE1531620). A.R.K. acknowledges the National Science Foundation Pre-Doctoral Fellowship (DGE1937971). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. We also thank Prof. Steve Diggle (Georgia Tech) for the *P. aeruginosa* Δ*pvdD*Δ*pchEF* strain used in the study and Prof. Steve Diggle and Prof. Marvin Whiteley (Georgia Tech) for fruitful discussions.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tetlet.2021.153204.

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