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**ARTICLE INFO**

**ABSTRACT**

A new siderophore containing a 4,5-dihydroimidazole moiety was isolated from *Pseudoalteromonas piscicida* S2040 together with myxochelins A and B, alteramide A and its cycloadDITION product, and bromo- and dibromoalterochromides. The structure of pseudochelin A was established by spectroscopic techniques including 2D NMR and MS/MS fragmentation data. In bioassays selected fractions of the crude extract of S2040 inhibited the opportunistic pathogen *Pseudomonas aeruginosa*. Pseudochelin A displayed siderophore activity in the chrome azurol S assay at concentrations higher than 50 μM, and showed weak activity against the fungus *Aspergillus fumigatus*, but did not display antibacterial, anti-inflammatory or anticonvulsant activity.

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1. Introduction

The element iron is an essential nutrient for growth of most microorganisms as it is required for important biological processes such as nitrogen fixation, amino acid synthesis, respiration, photosynthesis, DNA biosynthesis, and the citric acid cycle.[1] However, it occurs at very low levels in most natural environments, and the concentration for instance is below 0.5 nM in oceanic oxygenated waters at pH 7.[2] In order to obtain sufficient iron for their physiological needs most microorganisms have evolved biosynthetic strategies to cope with this deficiency. One of these is the production of siderophores,[3] which are small molecular weight chelating molecules that can help transport the insoluble iron (Fe$^{3+}$) into the cell via cell membrane specific receptor proteins.[1] Once inside the cell the complexed iron$^{3+}$ is released via reduction, ligand exchange or hydrolytic mechanisms[4] to Fe$^{2+}$ soluble species.

There are three known classes of iron chelating moieties: phenols and catechols as in enterobactins,[5] hydroxamates (N-OH amides) as in the desferrioxamines,[6,7] and α-hydroxyacarboxylic acids as in achrromobactins.[8,9] A closer look at these structures shows the presence of a high number of oxygen groups that can form ligands with positively charged cations. Oxygen ligands are well known for their high affinity for iron (III) species which has possibly resulted in the evolutionary selection of these groups by the microbial biosynthetic machinery.[10] In most of the cases, each ligand is an OO′ donor and each of the siderophores is able to form tris OO′ coordination to form the stable octahedral complex of the ferric ion.[1,10]

There are not many marine derived siderophores known compared to terrestrial ones. So far, the majority of the structures belong to a family of amphiphilic compounds containing an iron(III)-binding headgroup that is appended by a series of fatty acids.[1,11] The headgroup either contains a small peptide between 4-7 amino acids as in the amphiibactins[12] or the α-hydroxyacarboxylic acid moiety in the form of an β-hydroxyaspartic acid or citric acid as in the case of the synechobactins.[13] Many microbes produce only one type of siderophore but, many, particularly infectious pathogenic ones[14,15] are known to produce multiple siderophores, including mixed-type ligands[16] as exemplified by the mycobactins that contain catechol, hydroxyamine and oxazole units.[17] Others contain thiazole as exemplified by pyochelin.[18] These features are believed to offer advantages to the microorganisms in their ability to evade the host response to continue sequestering iron from their environment in order to survive.[17,19] Furthermore, siderophores have been known to inhibit the growth of various phytopathogenic fungi such as Phytophthora parasitica,[20] Phytium ultimum,[21] and Fusarium oxysporum f.sp.dianthi.[22]

2. Results and Discussion

The crude extract of the culture of *Pseudoalteromonas piscicida* S2040 was fractionated by reversed phase solid phase extraction (SPE) before further purification by reversed phase HPLC to yield pseudochelin A (1) and six known compounds (2-7). The known compounds were identified by $^1$H NMR, HRESIMS, and MS/MS data. Pseudochelin A (1) showed a high resolution ESI MS of $m/z$ 386.172 [M+H$^+$] ($\Delta$ 0.5 ppm) from calculated for C$_{20}$H$_{24}$N$_3$O$_5$ and requiring 11 degrees of unsaturation. Interpretation of $^1$H, HMBC and edited HSQC NMR spectra (Figure S1-S4) of compound 1 indicated the presence of five methylenes, seven methines and eight quaternary carbons leading to the sub-formula C$_{20}$H$_{17}$. The presence of six methines (δ$_{\text{H}}$ 119.9, 119.8, 118.7, 118.2, 118.1 and 117.2) and six quaternary carbons (δ$_{\text{C}}$ 148.7, 147.5, 146.1, 146.1, 115.5 and 107.8) suggested the presence of two aromatic rings, which together with the presence of two additional quaternary carbons at δ$_{\text{C}}$ 170.1 and δ$_{\text{H}}$ 163.1 accounted for a total of 10 double bond equivalents. This suggested the presence of a third ring in the structure of 1 to complete the 11 degrees of unsaturation. Use of one- and two-dimensional NMR data enabled the construction of 4 substructures (Figure 1).

Figure 1. Substructure A-D of compound 1 with COSY and key HMBC correlations.

Inspection of the $^1$H and $^1$C NMR chemical shifts, $^1$H-$^1$H coupling constants and COSY data suggested the presence of two similar, but isolated aromatic spin systems (substructures A and D, Figure 1) consistent with the presence of two catechol sub-units. The adjacent units attached to these two systems however, were different. A HMBC correlation from δ$_{\text{H}}$ 7.18 to δ$_{\text{C}}$ 170.1 indicated the presence of an amide unit in substructure A. A more shielded quaternary carbon at δ$_{\text{C}}$ 163.0 that correlated to the signal at δ$_{\text{H}}$ 7.14 by HMBC suggested a different functional group was adjacent to substructure D. Long range HMBC correlations linked the proton signals at δ$_{\text{H}}$ 4.12/3.71, δ$_{\text{C}}$ 4.36 and δ$_{\text{H}}$ 7.14 to the quaternary carbon signal δ$_{\text{C}}$ 163.0 suggesting the presence of a 4,5-dihydro-imidazole unit (substructure C). The butyl chain was readily assigned by COSY and HMBC correlations (substructure B). The four substructures were assembled by long range HMBC correlations to give
pseudochelin A (1) representing a new 4,5-dihydroimidazole-catechol-type siderophore.

Table 1
NMR data for pseudochelin A (1) in CD$_3$OD

<table>
<thead>
<tr>
<th>Pos.</th>
<th>$\delta^a$, type</th>
<th>$\delta^b$ (J in Hz)</th>
<th>COSY ($^1$H-$^1$H)</th>
<th>HMBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>49.1, CH:</td>
<td>A: 4.12, dd (11.6, 11.2) 2</td>
<td>2, 3, 7$^a$</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>A: 3.71, dd (11.7, 11.5) 2</td>
<td>2, 3, 7$^a$</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>56.7, CH:</td>
<td>4.36, m</td>
<td>1A, 1B, 3A, 3B</td>
<td>1, 7$^a$</td>
</tr>
<tr>
<td>3</td>
<td>34.2, CH:</td>
<td>A: 1.86, m</td>
<td>2, 4A, 4B</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B: 1.77, m</td>
<td>2, 4A, 4B</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>21.5, CH:</td>
<td>A: 1.52, m</td>
<td>3A, 3B, 5</td>
<td>5, 6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B: 1.48, m</td>
<td>3A, 3B, 5</td>
<td>5, 6</td>
</tr>
<tr>
<td>5</td>
<td>28.8, CH$_2$:</td>
<td>1.70, m</td>
<td>4A, 4B, 6</td>
<td>3, 4, 6</td>
</tr>
<tr>
<td>6</td>
<td>38.5, CH:</td>
<td>3.45, t (6.9)</td>
<td>5</td>
<td>4, 5, 7$^a$</td>
</tr>
<tr>
<td>1'$</td>
<td>107.8, C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2'$</td>
<td>147.5, C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3'$</td>
<td>146.1, C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4'$</td>
<td>119.9, CH:</td>
<td>7.08, dd (8.1, 0.6)</td>
<td>5$^b$</td>
<td>2', 3', 6$'</td>
</tr>
<tr>
<td>5'$</td>
<td>119.8, CH:</td>
<td>6.84, dd (8.1, 8.1)</td>
<td>4', 6$'</td>
<td>1', 3$'</td>
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<tr>
<td>6'$</td>
<td>118.7, CH:</td>
<td>7.14, dd (8.1, 0.6)</td>
<td>5$^b$</td>
<td>2', 4', 5', 7$'</td>
</tr>
<tr>
<td>7'$</td>
<td>163.0, C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1&quot;</td>
<td>115.4, C</td>
<td></td>
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<td>2&quot;</td>
<td>148.7, C</td>
<td></td>
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<tr>
<td>3&quot;</td>
<td>145.9, C</td>
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<td>4&quot;</td>
<td>118.1, CH:</td>
<td>6.91, dd (8.0, 1.2)</td>
<td>5$^b$</td>
<td>2&quot;, 3&quot;, 6&quot;</td>
</tr>
<tr>
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<td>6.70, dd (8.0, 8.0)</td>
<td>4&quot;, H6&quot;</td>
<td>1&quot;, 3&quot;</td>
</tr>
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<td>6&quot;</td>
<td>117.2, CH:</td>
<td>7.18 dd (8.0, 1.2)</td>
<td>5$^b$</td>
<td>2&quot;, 4&quot;, 5&quot;, 7&quot;</td>
</tr>
<tr>
<td>7&quot;</td>
<td>170.1, C</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Coupling constants are in brackets and given in Hz.
$^a$150 MHz
$^b$400 MHz

Further supporting evidence of the structure came from MS/MS fragmentation data (Figure S6).

The absolute stereochemistry of compound 1 was determined to be S by comparing the experimental circular dichroism spectrum with the theoretical spectrum calculated using self-interaction corrected time-dependent density functional theory within the real-space time-propagation framework (S1). The best fit was obtained between the experimental spectrum and the CD spectrum simulated for the 2S configuration (Figure 2).

Pseudochelin A can potentially exist in two tautomeric forms in solution as shown in Figure 3. Correlation of $^{13}$C experimental and calculated chemical shift values did not show any significant difference between the two structures based on the regression coefficient values[26] (Figure 4).

Pseudochelin A (1) complexes iron (III) as colour changes were observed after the addition of 1 to Fe$^{3+}$ solutions (Figure S7), and the appearance of a broad absorption maximum at 572 nm in the UV spectrum in MeOH (Figures S8, S9). It also showed a HRESIMS m/z of 439.0827 (M+H)$^+$ ($\Delta$ 0.4 ppm) from calculated for C$_{20}$H$_{21}$N$_3$O$_5$Fe suggesting three donor groups were involved in the formation of the ferric-pseudochelin A complex. The identity of these donor groups have not been determined yet even though any combination of three of the four catechol units is possible based on known binding mechanism of
catechol-type siderophores.[3,27] Analogous compounds such as agroactin and parabactin are known to utilize the nitrogen in the central oxazoline ring to form \( \Lambda \), cis coordination compounds.[28] The metal binding properties of pseudochelin A is currently being investigated and will be discussed elsewhere.

Pseudochelin A is likely to originate from non-ribosomal peptide synthetase (NRPS) assembly line, a similar biosynthetic pathway to the one reported for myxochelins A and B.[23] where the aldehyde intermediate \( 2 \) is generated through the reduction of the peptidyl carrier protein (PCP)-bound thioester in the NRPS assembly line. The transamination on \( 2 \) yields myxochelin B (3) which can undergo cyclization to produce 1 (Scheme 1).

![Scheme 1. Plausible biogenetic pathway of pseudochelin A](image)

The four crude SPE fractions of *Pseudoalteromonas piscicida* S2040 were initially screened for antibacterial and antifungal activities. All fractions showed minor inhibitions (14-16%) of *Staphylococcus aureus* MRSA-MB539. The fraction (SPE 100) showed a 62% growth inhibition of *Pseudomonas aeruginosa* while the fraction (SPE 25) showed a minor inhibition (12%) of *Candida albicans*. Myxochelin A showed a 62% growth inhibition of *Pseudomonas aeruginosa*. Further evaluation of pseudochelin A-Fe\(^{3+}\) complex was measured by HPLC using a Phenomenex HPLC C4 column (50 x 4.6 mm, 5 µm, 300 A) and using a linear gradient from 0 to 100% (MeOH in water) in 15 minutes. All solvents used in this study were either of HPLC or LCMS grade.

### 3.2 Bacterial strain

*Pseudoalteromonas piscicida* S2040 was isolated during the global research cruise Galathea 3 due to its bioactivity against the human pathogen *Staphylococcus aureus* NCTC 8325 and the fish pathogen *Vibrio anguillarum* 90-11-287 (serotype O1).[35] S2040 was obtained from a copepod sampled from surface water off the Northwestern coast of Australia (-16.06 N, 119.354 E).

### 3.3 Culture

S2040 was routinely grown on Marine Agar 2216 (MA) at 25°C. For the initial screening, S2040 was pre-cultured overnight in 5 mL SCM (2% sea salts (Sigma-Aldrich\([36]\), S4), 0.3% casamino acids, 0.4% mannose) at 200 rpm and 25°C. A 500 mL baffled Erlenmeyer flask containing 150 mL SCM was inoculated with 750 µl pre-culture and incubated at 200 rpm and 25°C. For the initial screening, S2040 was pre-cultured overnight in 5 mL SCM (2% sea salts (Sigma-Aldrich\([36]\), S4), 0.3% casamino acids, 0.4% mannose) at 200 rpm and 25°C. A 500 mL baffled Erlenmeyer flask containing 150 mL SCM was inoculated with 750 µl pre-culture and incubated at 200 rpm and 25°C. After 48 h, 1.8 g sterilized Diaion® HP-20 resin (Sigma-Aldrich) was added to the culture and incubation was continued for 24 h.

Resin and cells were separated by centrifugation for 20 min at 3059 x g. The resin was washed with MilliQ water and extracted with MeOH. The cell pellet was extracted with EtOAc/MeOH (8:2, v/v), and combined with the resin extract. The crude extract was dried under nitrogen. Upscaling was performed accordingly in 2 L SCM in 10 L Nalgene flasks and upon addition of 24 g Diaion® HP-20 resin (Sigm-Aldrich)[36]

### 3.4 Fractionation and isolation

The crude sample was fractionated on a C18 solid phase extraction (SPE) column using 25% MeOH in water (SPE 25), followed by 50% MeOH (SPE 50), then by 100% MeOH (SPE 100). Finally, the column was flushed with 100% methanol containing 0.05% trifluoroacetate (SPE 100+TFA). The compounds of interest were detected in the SPE 50 fraction by LC-MS and \(^1\)H NMR analysis. Purification of this fraction was
carried out by reversed phase C18 HPLC using a mixture of MeOH, water and TFA (0.05%) as an eluent with a linear gradient from 0-100% MeOH over 25 min and a solvent flow of 2 mL/min to yield 1 (6.3 mg), 2 (2.1 mg) and 3 (2.5 mg). Purification of the SPE 100 fraction was carried out by reversed phase C18 HPLC using a mixture of MeOH and water as eluent with a three step gradient from 60-80% MeOH over 10 min, followed by 80-100% MeOH over 15 min, and finally by 100% MeOH over 20 min. In addition, the solvent flow rate was reduced from 2.0 to 1.4 mL/min in the first step and then increased in the third step from 1.4 to 2.0 mL/min to yield 4 (2.3 mg), 5 (2.1 mg) and a mixture of 6 and 7 (3.5 mg).

Pseudochelin A (1), Brownish oil, 6.3 mg; [α]25D -51.3 (c 1.01 MeOH); IR (cm⁻¹) 3240, 2945, 2867, 1675, 1640, 1625, 1590, 1565, 1460, 1355, 1248, 1200, 1140, 840, 800, 725 (Figure S11); ¹H NMR and ¹³C NMR (MeOD-δ6) see Table 2; HRESIMS m/z 386.172 [M+H]+ (Δ 0.5 ppm) from calculated for C20H24N3O5.

3.4 Biological activities

All the SPE fractions of S2040 were tested for their ability to inhibit the growth of Gram-negative and Gram-positive bacteria (Escherichia coli MB2884, Acinetobacter baumannii MB5973, Pseudomonas aeruginosa PAO-1), and methicillin-resistant Staphylococcus aureus MRSA, MB5393), fungi (Aspergillus fumigatus ATCC46645) and yeast (Candida albicans MY1055) following previously described methodologies.[37,38] Briefly, each compound was serially diluted in DMSO with a dilution factor of 2 to provide 10 concentrations starting at 64 μg/mL for all the assays. The MIC was defined as the lowest concentration of an assay.[40] In all experiments performed in this work the RZ' factor obtained was between 0.87 and 0.98.

Furthermore, the SPE fractions were tested for their anti-inflammatory activity by inhibition of TNFα production in LPS-stimulated monocytes (THP-1 cells) using sELISA.[41]

The pure compound pseudochelin A (1) was re-evaluated in all the above assays. The siderophore activity was performed using the chrome azurol S assay.[42] The zebrafish larvae assay for anticonvulsant activity was carried out using previously described methods (S2).

Acknowledgements

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Supplementary data

Supplementary data (HRESIMS, MS/MS, UV, IR, and NMR including ¹H, ¹³C, COSY, HSQC and HMBC data of 1 associated with this article can be found in the online version).

References and notes

[36] Sigma-Aldrich (n.d.).
[39] (n.d.).