Marine Metabolites

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Marine Metabolites and Metal Ion Chelation: Intact Recovery and Identification of an Iron(II) Complex in the Extract of the Ascidian *Eudistoma gilboviride***

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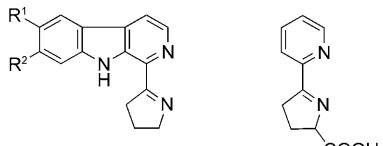
The extremely low level of biologically accessible essential trace metals in the marine environment suggests that marine organisms have developed unique mechanisms for their acquisition, sequestration, and utilization.[1] For marine invertebrates, little is known about these mechanisms, and it has been suggested that secondary metabolites may play a vital role.[2] Many marine secondary metabolites contain functional groups that can complex metals, but there is a lack of evidence whether this occurs in vivo.[3]

Ascidians are known to concentrate high levels of transition metals, but the reason behind this is not fully understood.[4,5] Vanadium has been found at highly elevated levels in ascidian blood cells,[6] and involvement of the tyrosine-derived tunichrome tripeptides has been proposed,[4] although more recent evidence indicates that the cysteine-rich peptide vanabins might be responsible for the sequestration of vanadium in *Ascidia sydneiensis*.[7] There is, however, a lack of convincing evidence to show how these metals occur in marine organisms.

Our research aims at the discovery of compounds responsible for the complexation of transition metals in ascidians and to determine the physical parameters to establish their biological function.[8–10] Herein we report the first identification of a non-covalently bound iron complex in the lipophilic extract of a marine organism. Furthermore, the discovery of such complexation agents that are selective for a single transition metal ion could have a significant application in the treatment of disease, such as in abscess formation which requires manganese or zinc for bacterial growth.[11]

As the first step to achieving these goals, we demonstrate the use of liquid chromatography with parallel inductively coupled plasma mass spectrometry/electrospray mass spectrometry (LC-ICP-MS/ES-MS) detection to uncover novel lipophilic metal complexes in the extract of the ascidian *Eudistoma gilboviride*, which is known to produce eudistomins (for example, **1**–**3**).[12] LC-ICP-MS/ES-MS simultaneously gathers both elemental and molecular information of compounds by splitting the HPLC eluent between an inductively coupled plasma mass spectrometer (ICP-MS) and an electrospray mass spectrometer (ES-MS).[13] We adapted this technique to organic extracts of ascidians to discover novel lipophilic chelating agents of biologically relevant metals.



Eudistomin G **1** R^1=H, R^2=Br
Eudistomin H **2** R^1=Br, R^2=H
Eudistomin I **3** R^1=H, R^2=H

Pyrimine **4**

Many metal chelates are unstable under reverse-phase chromatographic conditions, and therefore we employed size-exclusion chromatography (SEC) using a trimethylamine carbonate/methanol mixture as mobile phase followed by a formic acid mobile phase. We have developed this method for the HPLC separation of even moderately weak metal chelates in organic extracts (see the Supporting Information).

From a number of ascidians we selected *Eudistoma gilboviride*, as total metal analysis showed it contained very high amounts of lipophilic iron (250 $\mu g\,g^{-1}$). Following SEC-ICP-MS/ES-MS, the extracted ion chromatograms (EICs) of the different elements were compared to identify any correlations between elements, and consequently an overlap of iron peaks and two bromine peaks in the *E. gilboviride* extract was observed (Figure 1).

Analysis of the ES-MS mass spectrum of the *E. gilboviride* extract at the first iron peak (20.8 min, 240 $\mu g\,g^{-1}$ extract)

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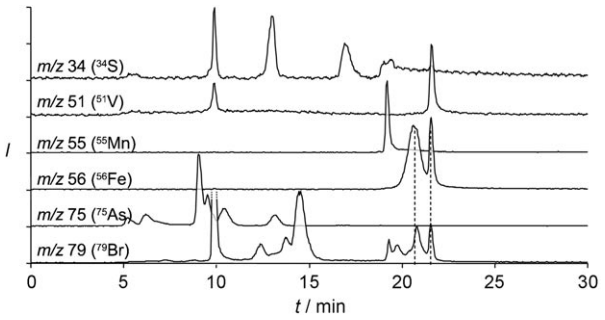


Figure 1. Selected *m*/*z* traces from ICP-MS ICP-MS/ES-MS analysis coupled online with size-exclusion chromatography of an *E. gilboviride* organic extract. Traces are offset vertically for clarity.

revealed a number of brominated compounds, and was confirmed by the bromine trace (*m*/*z* 79) using ICP-MS (Figure 2). A peak cluster at *m*/*z* 683 correlated with the iron peak (Figure 2b, (2)). Its isotopic pattern suggested that it contained two bromine atoms and one iron atom, and was possibly a $[Fe^{II}L_2]$ complex incorporating a monobrominated ligand with mass of 313, that is, **1** or **2**.[12]

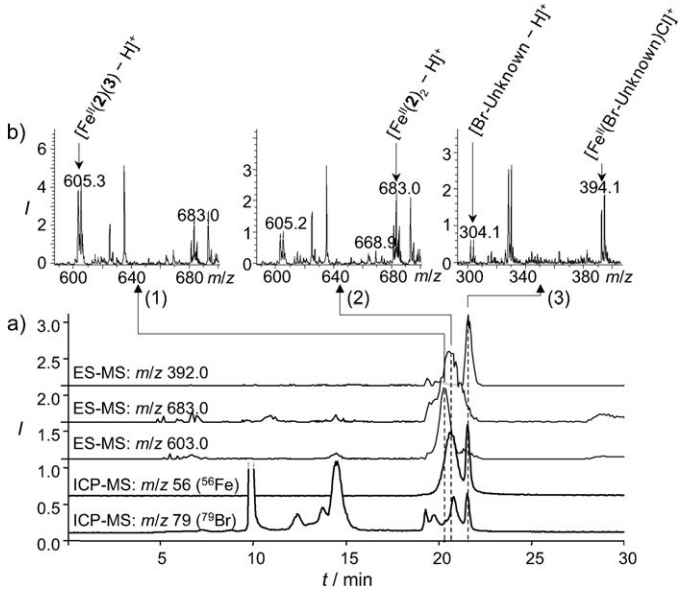


Figure 2. Iron(II) chelates identified in an *E. gilboviride* organic extract by SEC-ICP-MS/ES-MS. a) Selected ICP-MS and ES-MS EICs; b) ES averaged mass spectra and identifications for 1) 20.4–20.6 min, 2) 20.7–20.9 min, and 3) 21.6–21.7 min (complexes not identified).

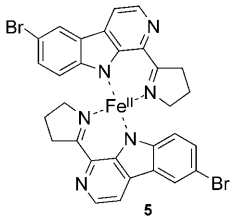
Compound **2**, containing 22% **1** (this inseparable mixture is hereafter referred to as **2** for simplicity) was isolated from the *E. gilboviride* extract and used to confirm the presence of $[Fe^{II}(\mathbf{2})_2]$ in the extract. Iron(II) and iron(III) complexes of **2** were prepared and then analyzed by direct-injection ES-MS. The **2**/$FeCl_2$ (2:1 molar ratio) solution gave rise to the same peak cluster at *m*/*z* 683 as the extract by SEC-ICP-MS/ES-MS, in addition to the chloride-containing species $[Fe^{II}(\mathbf{2})_2]Cl$. A small peak cluster near *m*/*z* 499, which corresponded to $[Fe^{II}(\mathbf{2})_3]^{2+}$, was also present. $[Fe^{II}(\mathbf{2})_2]$ was the major MS^2 fragment of both the $[Fe^{II}(\mathbf{2})_2]Cl$ and $[Fe^{II}(\mathbf{2})_3]$ peaks. Consecutive MS^n fragmentation of $[Fe^{II}(\mathbf{2})_2]$ showed consecutive loss of two bromine ions before disintegration of the iron–ligand complex. Direct-injection ES-MS of **2**/$FeCl_3$ (2:1 molar ratio) showed only one peak cluster, which centered around *m*/*z* 477 and was identified as $[Fe^{III}(\mathbf{2})]Cl_2(H_2O)_2$. These data support the identification of the iron complex in *E. gilboviride* as $[Fe^{II}(\mathbf{2})_2]$ and discounts the possibility that iron(II) was reduced from iron(III) during the electrospray process.

The prepared complexes were also analyzed by SEC-ICP-MS/ES-MS. The ICP-MS (Fe,Br), ES-MS (*m*/*z* 683), and UV/Vis ($\lambda_{600\,nm}$) chromatograms all clearly indicated that the $[Fe^{II}(\mathbf{2})_2]$ eluted close to the expected retention time. Conversely, $[Fe^{III}(\mathbf{2})]Cl_2(H_2O)_2$ dissociated into free ligand and unbound iron(III). If the *E. gilboviride* extract was spiked with the prepared solution of $[Fe^{II}(\mathbf{2})_2]$, the peaks at 20.8 min corresponding to iron and bromine increased, which confirmed that this extract did contain $[Fe^{II}(\mathbf{2})_2]$.

As **1** and **2** are inseparable chromatographically and both have the same *m*/*z*, we could not distinguish between $[Fe^{II}(\mathbf{1})_2]$, $[Fe^{II}(\mathbf{2})_2]$, and $[Fe^{II}(\mathbf{1})(\mathbf{2})]$, but we propose that these are all present as a statistical mixture in the organism. Support for the existence of mixed complexes is provided by the presence of the iron(II) complex containing one equivalent of **3** and one equivalent of **2** or **1** at *m*/*z* 603 (Figure 2b, (1)). The identification of these complexes also led to the discovery of $[Fe^{II}(\mathbf{3})_2]$ at *m*/*z* 525.

Minor peaks corresponding to $[Fe^{II}(\mathbf{2})_3]^{2+}$ (*m*/*z* 499, fragmenting to the expected *m*/*z* 683 for $[Fe^{II}(\mathbf{2})_2]$) were also observed in the SEC-ICP-MS/ES-MS of both the prepared complex and the extract, indicating that the 1:3 iron(II)/eudistomin complex is at least partially stable under chromatographic conditions.

To complement the experimental analysis of the metal-containing species, theoretical calculations of the UV spectra for the two possible symmetrical 2:1 ligand/metal complexes (**5** and **6**) were performed.[14] Despite the inherent approximations involved in these calculations and the fact that they



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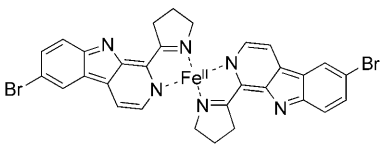

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were performed in the gas phase, a considerable difference was observed between the spectra of the two binding modes. A comparison of the experimentally obtained $[Fe^{II}(\mathbf{2})_2]$ spectrum with the calculated spectra for both complexes (Figure 3) indicated that complex **6** has the correct coordination, which is similar to that of the *Pseudomonas*-derived iron(II) binding agent pyrimine (**4**).[15] A titration of **4** with iron(II) indicates the stable complex to be $[Fe^{II}(\mathbf{4})_3]$, which has a similar UV spectrum to a 1:2 mixture of iron(II)/**2**

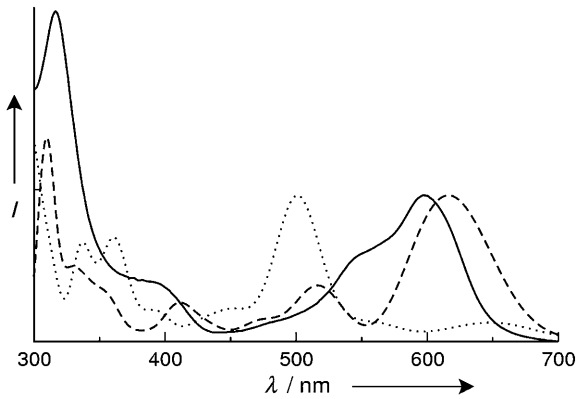

Figure 3. Overlay of experimental absorption spectrum of $[Fe^{II}(\mathbf{2})_2]$ (——) with calculated spectra for model complexes **5** (•••••) and **6** (– – –).

(Supporting Information, Figure S2).[15] This result suggests that $[Fe^{II}(\mathbf{2})_3]$, and not $[Fe^{II}(\mathbf{2})_2]$, is in fact the species present in both the prepared complex and the organism extract. The UV spectrum of the crude organism extract is highly similar to the prepared complex between 500–700 nm (Supporting Information, Figure S3,4) which provides evidence that it contains eudistomin/iron(II) complexes. Quantifying the SEC-ICP-MS/ES-MS data, we estimate that 0.4% of the extract is composed of eudistomin/iron(II) complexes and that 75% of the total lipophilic iron in *E. gilboviride* is bound by eudistomins G–I.

Using these tools it is now possible to examine the question of whether such complexes play an integral part in an organism's physiology or whether they are artifacts of the sample preparation. A comparison with the pyrimine/iron(II) complex suggests eudistomin/iron(II) complexes might indeed have a physiological role in iron(II) binding, and we can speculate that they are produced by microorganisms associated with *E. gilboviride*.[16] We contend that ascidians provide an excellent resource for the discovery of novel biochelating agents with high specific affinities for single transition metals which may serve a distinct ecological purpose as antimicrobials and might have utility as novel antibiotics.[11]

Experimental Section

Eudistoma gilboviride (Family Polycitoridae, Order Enterogona) was collected in August 2006 at Roxburgh Reef on the Great Barrier Reef at 18.25.727°S 147.03.521°E, and frozen at −20°C. The specimen was identified by Chris Battershill and a voucher is retained at the Australian Institute of Marine Science (no. 26485). A solvent extract was made using CH_2Cl_2/MeOH (1:1 v/v). Eudistomins were isolated from the extract using solvent partitioning and C18 HPLC and identified using 1D and 2D NMR spectroscopy. For SEC-ICP-MS/ES-MS, the mobile phases were: A) trimethylamine carbonate (50 mM) in methanol, pH 8.8, B) formic acid (100 mM) in methanol, and C) acetylacetone (100 mM) and formic acid (100 mM) in methanol. The column was a Tosoh Bioscience TSK-gel SuperAW2500 HPLC column (6.0 mm internal diameter, 150 mm length, 4 μm particle size) used at 10°C. Samples were injected (25 mg mL^{-1} in methanol, 50 μL) onto the column and were eluted with mobile phase A, B, C, B, and A for 5, 17, 17, 15, and 15 minutes, respectively, at a flow rate of 0.45 mL min^{-1}. ICP-MS/ES-MS instrumentation and conditions were as reported previously.[17] Solutions of **2** and $FeCl_2$ or $FeCl_3$ were prepared in degassed methanol and combined without delay to make a 200 μM solution of $[Fe^{II}(\mathbf{2})_2]$ or Fe^{III} complex of **2**.

All theoretical calculations were carried out using the ORCA 2.6.04 software package.[14]

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