Marine Metabolites and Metal Ion Chelation

16

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Contents

Abstract

This chapter covers the unique ability of marine invertebrates to bioconcentrate transition metals by up to 8 orders of magnitude above background concentrations. A brief introduction deals with the bioinorganic principles of the subject matter and the natural concentrations of metals in the environment. Next, previous metal surveys of marine invertebrates and the potential reasons for hyperaccumulation are discussed, and this is followed by a section on previously

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E. Fattorusso, W. H. Gerwick, O. Taglialatela-Scafati (eds.),
Handbook of Marine Natural Products, DOI 10.1007/978-90-481-3834-0_16,

isolated marine natural product – transition metal complexes. Studies on marine natural product complexation using physical methods are described in the context of their binding specificity and affinity. Many of these studies were conducted to try and discover potential functions for the marine natural product – transition metal complexes. Methodological aspects to discover new complexes of this type are discussed, especially hyphenated techniques utilizing elemental and molecular mass spectrometric methods. A final section proposes a rational way to uncover new marine natural product – transition metal complexes and potential methods to determine their function in nature.

16.1 Introduction

A number of metal ions are essential to life. This includes the bulk ones such as sodium, potassium, magnesium, and calcium used for osmotic balance and in the formation of biomaterials. In addition, there are trace metal ions that are critical in metalloenzymes and cofactors in enzymes and proteins [1]. Most of these are found in the first transition period such as vanadium, manganese, iron, cobalt, nickel, copper, and zinc. It is recognized that certain transition metals usually fulfill certain roles in metalloenzymes, for instance, zinc is often involved in hydrolysis enzymes and copper in oxidation enzymes [1]. An important role for metals is in oxygen transport, which in many animals is carried out by hemoglobin, containing an iron-porphyrin cofactor, and in crustaceans by the copper-containing protein, hemocyanin. An imbalance in the metal concentrations in most organisms will lead to disease or failure to thrive, and therefore, maintenance of metal homeostasis is vital to an organism's survival. For this reason, all life has developed sophisticated mechanisms for the acquisition, storage, and utilization of metal ions as well as elegant methods to keep the concentrations within an acceptable and healthy range. Metal uptake is affected by a number of factors, including speciation, chelation, pH, temperature, salt concentration, and redox potential, as well as age, sex, and physiology of the organism, yet levels are exquisitely controlled in healthy organisms [2].

The main barrier to metal uptake is the hydrophobic cell membrane, and two key mechanisms exist for internalizing biologically important metal ions (Fig. 16.1) [3]. The first is via the use of an energy pump which actively transports M^{2+} into the cell. The second is of more relevance here and involves the use of a transport ligand, often known as an ionophore, or siderophore when it is iron specific (L_t in Fig. 16.1). The ligand L_t complexes to M^{2+}, which is either in a hydrated form or in equilibrium with an external ligand L_e. Thus, the stability constant of L_t must be greater than that of L_e for the transport ligand to be effective. It must be remembered that for divalent metal ions, the stability of complexation to flexible ligands is essentially independent of the ligand and follows the Irving–Williams series shown below and that this may affect which metal is bound by such an ionophore [1].

$$Cu^{2+} > Zn^{2+} \geq Ni^{2+} > Co^{2+} > Fe^{2+} > Mn^{2+} > Mg^{2+} > Ca^{2+}$$

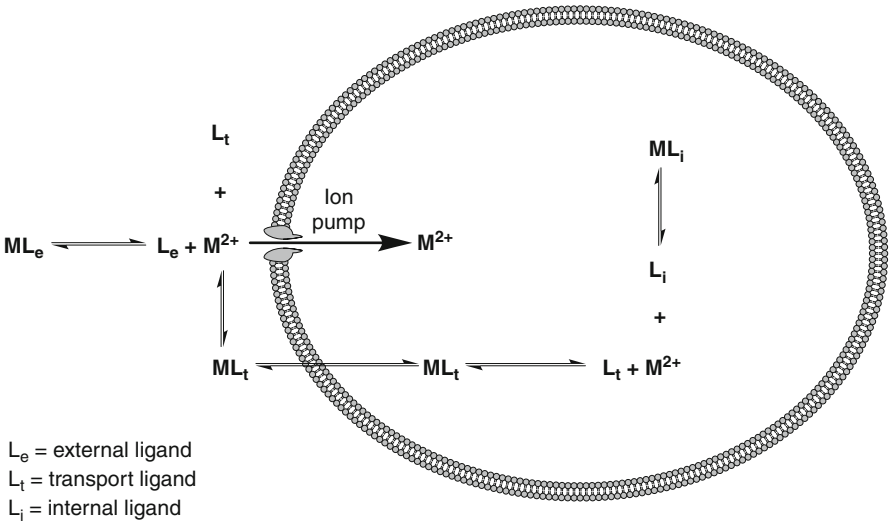


Fig. 16.1 Different routes by which a divalent metal can be taken up into a cell across the membrane [3]

The formation of an ionophore–metal complex generates a concentration gradient of the metal ions across the membrane despite the cell's already high internal metal concentration. Once inside the cell, the metal ions are bound strongly to internal ligands (L_i in Fig. 16.1) which cannot diffuse out of the cell, thus forming a kinetic trap so that there is little transport of the metal out of the cell. This implies that the stability constant of L_i must be greater than that of L_t for this to occur. There are many examples of ionophores and siderophores which allow transmembrane transport of vital metal ions.

Measurements of the concentrations of these essential metal ions in the earth's crust show that the bulk metal ions (Na, Mg, K, Ca) are present in high amounts, in the order of moles per kg (Fig. 16.2). The trace transition metal ions are present in much lower concentrations, ranging from 10^{-2}–10^{-3} mol/kg, excepting Fe, which is present at about 1 mol/kg. The biological availability of these metals in the crust is much lower than these reported concentrations as they are often not in a form that is accessible to organisms that need them. In the marine environment, the situation is different with the bulk metal ions 1–3 orders of magnitude less concentrated in open-ocean seawater and the trace metal ions, present at 10^{-9}–10^{-10} M concentrations, 7–10 orders of magnitude lower than in the earth's crust [4]. On the continental shelf, these values are slightly higher due to the input from terrestrial runoff and rivers. In the open ocean, the major input of trace metal ions is dust deposition from the atmosphere with a minor contribution from hydrothermal vents. These low concentrations are worsened by the fact that these metal ions are not present in seawater as hydrated ions, but may be complexed to an organic ligand, or present inside an organism, with some studies reporting that greater than 99% of certain

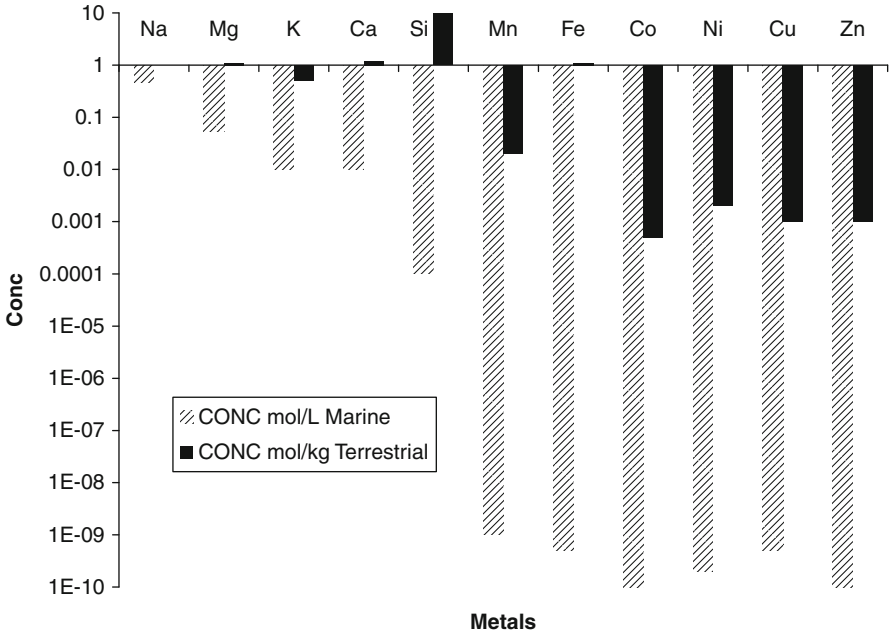


Fig. 16.2 Comparison of biologically important metal ions in marine and terrestrial environments (oceanic seawater and the earth's crust)

trace metal ions are complexed (e.g., Fe, Cu) [5]. Nutrient cycling for these metal ions is very fast compared to the bulk metal ions, exemplified by residence times of tens to hundreds of years compared to millions of years for the bulk ions. Essential nutrient ions also show a characteristic depth profile, with a very low concentration at the surface where it is heavily utilized by life in the photic zone, and then increasing to an asymptote at greater depth [4].

Given these high pressures for such vital, essential resources, marine organisms must have evolved unique mechanisms for acquisition, sequestration, and utilization of trace elements. Most research in this area has been carried out for open-ocean planktonic microorganisms which are responsible for most of the Earth's primary productivity (photosynthesis). It is known that in certain regions of the world's oceans, this productivity is limited by the scarcity of essential ions, particularly iron, and this may have an impact on the Earth's climate [6]. Iron acquisition by siderophores produced by marine microorganisms involves a number of very elegant mechanisms, and these compounds show some of the highest iron affinity constants ever recorded [7]. Very little is known about the way that marine macroorganisms, particularly invertebrates, carry out these essential functions, despite the fact that some marine invertebrates, such as sea squirts, hyperaccumulate certain metals. For this reason, this chapter will be limited to the

current understanding of the role of small molecule natural products in the complexation of metal ions in benthic, sessile marine invertebrates and will exclude the vast body of knowledge on marine microbial siderophores and ionophores.

16.2 Metal Surveys of Marine Invertebrates

For nearly a century, it has been known that there are extraordinary concentrations of vanadium in the blood cells of some ascidian species [8]. Only now are the processes becoming clearer by which this hyperaccumulation of vanadium is achieved by a factor of 10^7 over seawater [9]. The functional role of the vanadium to the ascidian is still undefined, and the necessity for the reported 0.35 M concentrations of vanadium in certain types of ascidian blood cells is unclear [9]. Speculation is rife with suggestions as to its true function from acting as an oxygen store [10] to playing a role in antifouling [11], but neither of these has been substantiated to date.

These findings spurred a great deal of research in the 1980s and 1990s on the possible mode of complexation of vanadium, which was proposed to be by the small molecule tunichromes (e.g., **1**), but cytochemical studies report that high concentrations of vanadium and the tunichromes do not appear in the same blood cell types [12]. Recent work shows that the vanadium is coordinated by amine nitrogen of the unusual 10.5 kDa peptide vanabins from *Ascidia sydneiensis samea*, which contain an unusual fold and nine disulfide bridges [9].

Besides vanadium, little has been reported on the complete metal complement of ascidians or other sessile marine invertebrates such as sponges, soft corals, or echinoderms. Studies reporting concentrations of other metals in sessile marine invertebrates are very limited, and many of these are collected for ascidians in Monniot's 1978 review [13]. This reports elevated concentrations of a number of transition metals in ascidian tissues, but often gives no exact concentrations or how these were measured. However, for iron, it gives a concentration of 5,100 ppm in *Pyura stolonifera*. Reports of lower iron concentrations are more common and often co-occur with elevated vanadium concentrations [8]. Some other metal concentrations that are provided in the Monniot review have been summarized in Table 16.1 [13]. For other metals that are not included in the table, either these are not significantly elevated (Sr, Zn, Sc, Eu, Th) or the concentrations are not provided explicitly (Co, Cu, Zr, Mo).

Our recent preliminary work on ascidians from the Great Barrier Reef using inductively coupled plasma mass spectrometry (ICP-MS) on HNO_3/H_2O_2 digests of the whole organism tissues shows a range of metals at concentrations significantly above the local seawater levels (Table 16.2). In particular, vanadium, iron, and arsenic were observed at high concentrations. Iron was observed at around 100 ppm or greater in 6 of the 11 organisms, while vanadium showed the highest concentrations of all the metals analyzed. The concentrations of iron ranged within 1½ orders of magnitude, while the concentrations of vanadium were highly variable,

Table 16.1 Reported concentrations of metals in ascidians from Monniot et al. [13]

Organism	Metal	Concentration
Ciona intestinalis	Sn	10^6 × seawater conc.
Microocosmus sulcatus		
Styela plicata		
Ciona sp	Mn	120 ppm
Eudistoma ritteri	Ti	1,512 ppm
	Cr	144 ppm
Pyura stolonifera	Fe	5,100 ppm
Molgula manhattensis	Nb	25–98 ppm
Styela plicata	Nb	250 ppm
	Ta	540 ppm

ranging across 5 orders of magnitude from being almost absent (0.2 mg/kg) to reaching part-per-thousand levels (2,050 mg/kg). The variability did not seem to correlate with taxonomic groups, with two species of *Didemnum* accounting for the extremes of the observed vanadium concentrations and members of two families accounting for the highest concentrations. Comparing the measured concentrations with the reported oceanic concentrations (Table 16.2) [14] of a range of metals gives the indicative bioconcentration factors shown in Fig. 16.3. The range of bioconcentration factors ranges over 7 orders of magnitude with the highest values observed for Fe (1.6×10^7), Mn (2.2×10^6), and V (1.0×10^6). Biologically important metals are highly concentrated which suggests active acquisition is occurring, but also nonessential metals such as Cd and U are being concentrated by 3–4 order of magnitude. Clearly, much more work is required to fully appreciate the extent of metal concentration by ascidians, including the range of metals and the bioconcentration factors.

These surveys can mark the beginning of investigations into possible complexation of the accumulated metals by secondary metabolites. A total metals analysis of the organic extract of the organism can guide the search for chelating agents. For example, the high iron present in the organic (CH_2Cl_2/MeOH) extract of *Eudistoma gilboviride* (Fig. 16.4) led us to discover Fe^{II} chelates that comprised 0.4% w/w of the extract [15]. This study is described in more detail later in the chapter.

Apart from the Monniot review and our own work reported above, we have been unable to locate more recent broad studies encompassing other sessile marine invertebrate phyla reporting on the concentrations of a range of metals in environmentally noncontaminated environments. Filter-feeding invertebrates, mainly sponges, have been used as bioindicators for metal pollution; however, this represents the organisms' responses to high metal loads and not their native state. The Mediterranean sponge *Crambe crambe* was shown to accumulate Cu and Pb, and could be regarded as a useful bioindicator for these metals [16]. A 12-year longitudinal study on twelve metals in the Mediterranean sponge *Spongia officinalis* showed spatial and temporal variation in their concentrations indicating a significant reduction in heavy metals after a sewage plant came

Table 16.2 Preliminary investigation of metal concentrations in 11 ascidians from the Great Barrier Reef and oceanic concentrations [14] of those metals presented in ppm (mg of metal per kg of organism, dry weight or mg per kg of seawater). Values are measurements of single HNO_3/H_2O_2 digests of ground whole dry organism

Species	*Leptoclinides* sp	*Aplidium* sp	*Didemnum* sp *1*	*Didemnum* sp *2*	*Didemnum* sp *3*	*Polysyncraton* sp	*Didemnum* sp *4*	*Polycarpa* sp	*Didemnum* sp *5*	*Didemnum* sp *6*	*Lissoclinum patella*	Oceanic metal concentrations
Li	0.9	1.8	1.4	2.1	2.7	1.2	0.5	1.5	0.3	0.3	1.6	1.8×10^{-1}
V	31	970	2,050	82	0.7	0.9	0.7	6.2	0.2	0.7	0.4	2.0×10^{-3}
Cr	0.7	9.0	4.2	1.7	1.4	2.8	7.4	5.4	1.0	1.2	0.8	2.1×10^{-4}
Mn	1.3	43	19	3.4	2.5	8.7	11	19	1.3	0.9	3.1	2.0×10^{-5}
Fe	52	480	270	220	29	160	97	100	14	14	43	3.0×10^{-5}
Co	0.3	0.8	0.3	0.3	0.2	0.3	0.2	0.3	0.1	0.2	0.1	1.2×10^{-6}
Ni	1.5	7.3	3.0	3.3	0.9	1.9	3.6	5.6	2.0	1.8	1.7	4.8×10^{-4}
Cu	5.9	4.7	5.2	7.1	0.8	3.2	1.5	15	1.5	1.6	4.1	1.5×10^{-4}
Zn	23	24	8.2	20	7.1	7.0	0.2	5.7	0.1	0.3	4.3	3.5×10^{-4}
As	8.9	9.5	11	120	1.6	8.7	4.6	15	6.0	4.5	6.7	1.2×10^{-3}
Se	1.0	1.8	1.6	4.9	0.2	1.1	1.0	2.1	0.3	0.2	0.7	1.6×10^{-4}
Rb	1.1	1.6	2.5	3.5	0.5	1.5	1.9	2.2	0.3	0.5	2.4	1.2×10^{-1}
Mo	0.6	1.6	1.6	1.7	0.2	1.0	0.7	4.6	0.5	0.4	0.3	1.0×10^{-2}
Cd	0.2	0.2	0.7	1.6	0.2	1.1	0.5	0.2	0.0	0.2	0.5	7.0×10^{-5}
Ba	5.2	5.6	6.3	1.1	9.2	6.3	8.6	7.2	4.9	10	3.4	1.5×10^{-2}
U	3.4	1.6	2.5	1.5	0.2	3.1	1.8	0.9	2.9	3.9	1.8	3.2×10^{-3}

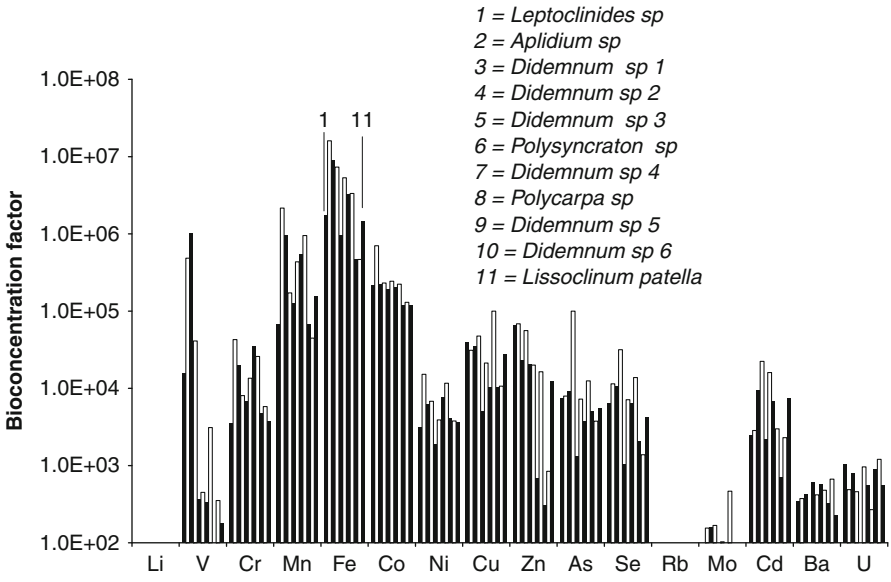


Fig. 16.3 Indicative bioconcentration factors for 11 ascidians from the Great Barrier Reef using information given in Table 16.2

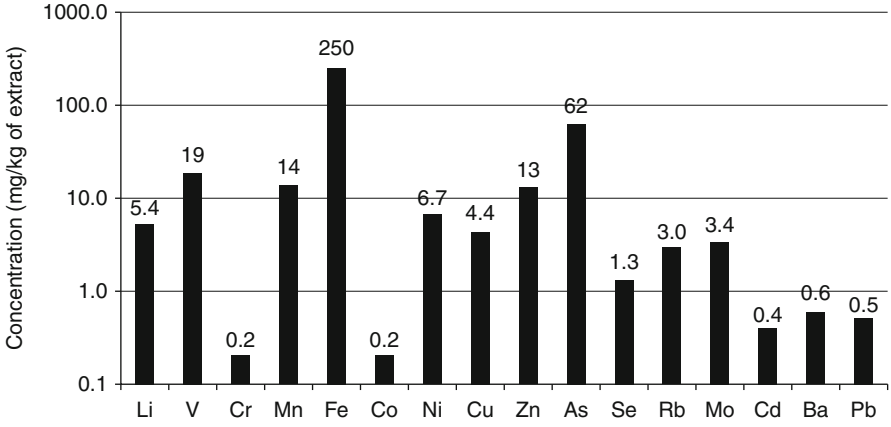


Fig. 16.4 Concentration (mg/kg) of a selected range of metals in the organic extract of the Great Barrier Reef ascidian *Eudistoma gilboviride*. Values are results of an HNO_3/H_2O_2 digest of a CH_2Cl_2/MeOH extract of the dried organism. Metals that were analyzed but present at less than 0.05 mg/kg are not shown (Be, Ga, Ag, Te, Tl, Bi, and U) [15]

into operation [17]. One final report is worthy of note. The deepwater sponge *Tedania charcoti* collected in Prydz Bay, Antarctica, was shown by ICP-atomic emission spectroscopy to have extraordinary levels of Cd (15,000 ppm) and Zn (5,100 ppm) [18].

16.3 Why Do Marine Invertebrates Concentrate Metals?

Concentration of metals by up to 7 orders of magnitude from seawater by these invertebrates is a significant feat, and it is assumed that a number of specialized metal ligands and metal transport proteins are involved in this process, such as the vanabins mentioned previously. Internal transport proteins have been well defined in other organisms such as transferrin and ferritin for iron and the amino-terminal Cu and Ni binding motif in a number of Cu and Ni transport proteins [1]. The involvement of natural small molecule organic ligands has often been speculated (i.e., L_t in Fig. 16.1), but there is little concrete evidence that these are involved in the uptake and possible storage of nutrient metals for marine invertebrates, as has been shown for bacterial siderophores and ionophores [1]. A small number of marine natural products have been isolated complexed to transition metals, and a variety of roles have been speculated for the metal in these complexes [19].

The most obvious role of marine invertebrate compounds with the potential to complex metals is that they sequester metals for the organism's metabolism (i.e., L_t or L_i in Fig. 16.1). Some of the known compounds will be catalogued in the next section, but proof of their function as siderophores or ionophores in the producing organism is still lacking. An indication that marine siderophores differ from their terrestrial counterparts is exemplified by alterobactin (**2**), isolated from the open-ocean marine bacterium *Alteromonas luteoviolacea*, which has one of the highest stability constants ever reported [7]. This compound is secreted in response to low iron environments and complexes iron via the catecholate and β-hydroxyaspartate moieties.

It is possible that metal complexation is a way to imbue the natural product with biological activity and thus allow it to play a role in preventing predation and fouling, but this has not been shown conclusively so far. Some invertebrate marine natural products may complex a redox-active metal to confer reactivity to the ligand–metal complex. A terrestrial example is the anticancer agent bleomycin, derived from the microbe *Streptomyces verticillus,* which requires the presence of Fe(II) or Cu(I) and molecular oxygen to enable sequence-selective DNA binding and cleavage [20]. This hypothesis has been tested for very few marine natural products, the most significant of which is the copper-mediated nuclease activity of tambjamine E (**3**) isolated from the ascidian *Atapozoa* sp. [21]. It appears that a 2:2 complex of **3** with Cu(II) in the presence of oxygen, but in the absence of an external reducing agent, gives rise to stoichiometric DNA cleavage via a mechanism involving a reactive oxygen species. The pyridoacridine alkaloid ascididemnin (**4**) from the ascidian *Didemnum* sp. looks like a suitable candidate for binding to transition metals, especially Cu(II), and it was initially suggested that the mechanism by which it induced topoisomerase-II-mediated DNA cleavage involved a ascididemnin–Cu complex which was capable of generating reactive oxygen species [22]. However, the presence of Cu was shown not to be required and reactive oxygen species are produced by direct reduction of the iminoquinone moiety.

It is possible that the presence of a nonredox active metal in a complex with a marine natural product confers a preferred bioactive conformation to the complex. A protein example of this is the ubiquitous calmodulins, which change conformation upon binding to Ca, thus allowing the complex to bind to specific proteins and elicit a specific response [23]. A final suggestion is that the metal ions are necessary for templating the biosynthesis of macrocyclic ligands, but there has been no direct evidence for this hypothesis although there is some synthetic evidence that the presence of metal ions may assist such reactions [24].

The examples already discussed show that certain functional groups (e.g., hard/soft ligands) and molecular features (e.g., macrocycles, structural constraints) are often encountered in organic metal ligands. Learning from the metalloproteins, the presence of residues such as histidine, methionine, cysteine, tyrosine, aspartic and glutamic acid, and their derivatives in a natural product may suggest that the compound is involved in metal binding. Care needs to be taken that the coordination environment these represent is taken into consideration. Typical functional groupings found in siderophores and ionophores such as catecholates, hydroxamates, and β-hydroxyaspartates also imply a role of the compound in metal complexation. Complexation sites such as those present in heteroaromatics such as **3** and **4** are also very suggestive of a role in metal complexation. A number of moieties expected in metal-complexing natural products have been summarized in Fig. 16.5. More difficult to predict are conformations of macrocycles which present carbonyl, amide, or other functional groups in a coordination environment ideal for metal binding.

Based on such principles, a large number of marine metabolites are proposed to function as metal ligands; however, only very few cases have been substantiated. Most of the structural types that are suspected of being able to complex to metal ions have been catalogued in the influential review by Michael and Pattenden [19]. Rather than duplicating this information, we will give a brief survey of the structural classes which have proposed complexing abilities, followed in the next section by those in which either the metabolite was isolated as a metal complex or for which complexation studies have been carried out.

We have already seen examples in which obvious chelating functionalities are present such as the tunichrome **1** which contains three trihydroxyphenylalanine-derived substructures [8]. There is now evidence that these compounds are not involved in the complexation of vanadium, but it is possible that they are involved in the complexation of iron [12]. Similar moieties, in this case a dihydroxyphenol in addition to two β-hydroxyaspartates, are present in **2**, which has been shown to be a strong iron-complexing agent [7]. Tambjamine **3** has characteristic pyrrole functionality which predisposes it to bind to copper [21]. Ascididemin **4** has a bipyridyl "bay" region, which again makes it a likely candidate for complexing to transition metals, but the initial isolation study showed it did not complex iron(II) [25]. The eudistomins from *Eudistoma* sp. (e.g., **5**) have long been suspected of

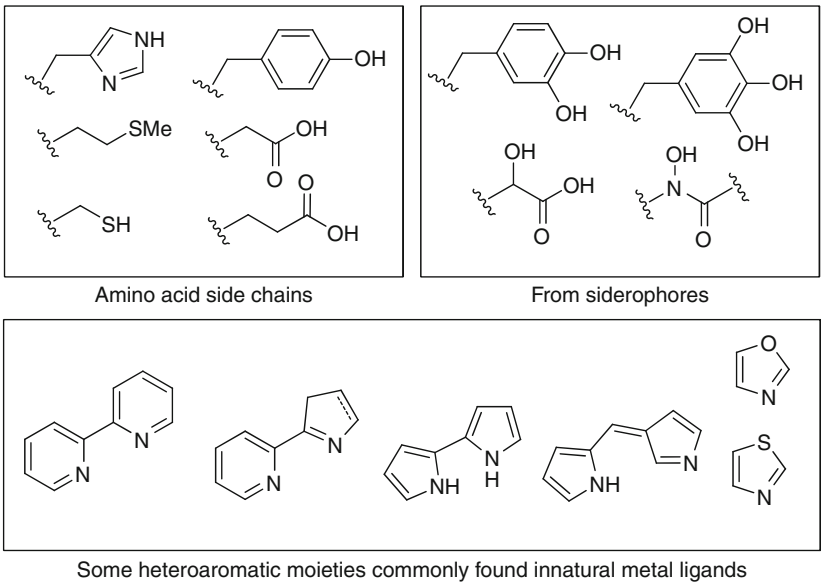
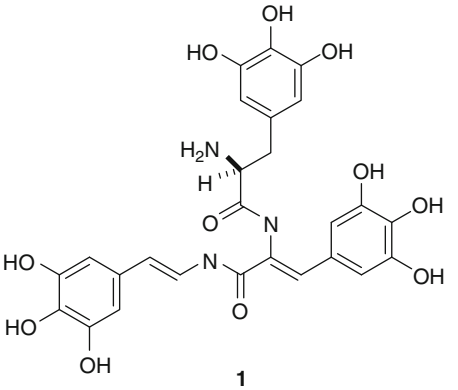


Fig. 16.5 Functional groups often encountered in natural metal ligands

being capable of chelating iron [26], due to their resemblance to the Fe(II) chelator pyrimine (**6**) isolated from a *Pseudomonas* sp. [27]. Etzionin (**7**) isolated from an unidentified Red Sea ascidian [28] contains a hydroxamate functionality as well as a terminal amine, and the combinations of these moieties are reminiscent of the potent siderophore deferoxamine [29]. Compounds containing small heterocycles (e.g., oxazoles, oxazolines, thiazoles, thiazolines) are often suspected of being capable of chelating metals. One such example is ulapualide (**8**), isolated from the nudibranch *Hexabranchus sanguineus*, containing an array of three oxazoles. A molecular mechanics study was carried out with **8** complexed to a dummy metal ion and was used to predict its relative stereochemistry [30]. This structure also typifies the large macrocycles with a wealth of chelating functionality which make them appear ideal candidates for metal complexation. Other macrocycles such as the oxime-containing iodotyrosine-derived bastadins from the sponge *Ianthella basta* (e.g., **9**) [31] are also suspected of complexing metals, but there has been no definitive proof. Similarly, polyether macrolides such as the bryostatins derived from the bryozoan *Bugula neritina* (e.g., **10**) apparently display a perfect coordination environment for the binding of metals, but this has not been substantiated despite being suggested when its structure was first reported [32].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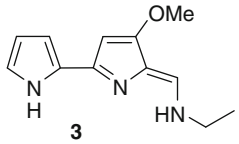
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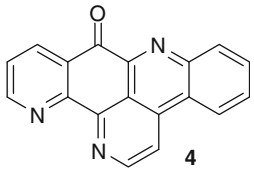
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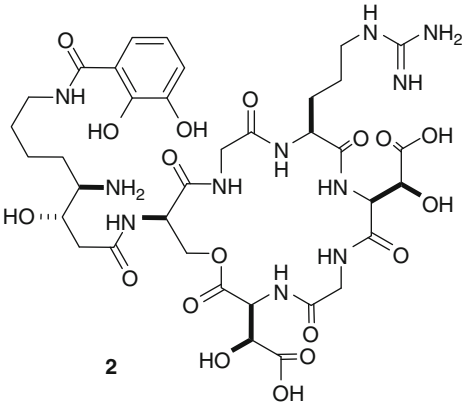
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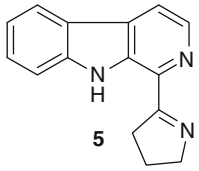
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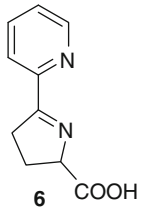
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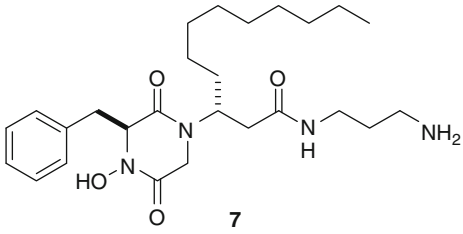
6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N
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NH2
HO
N
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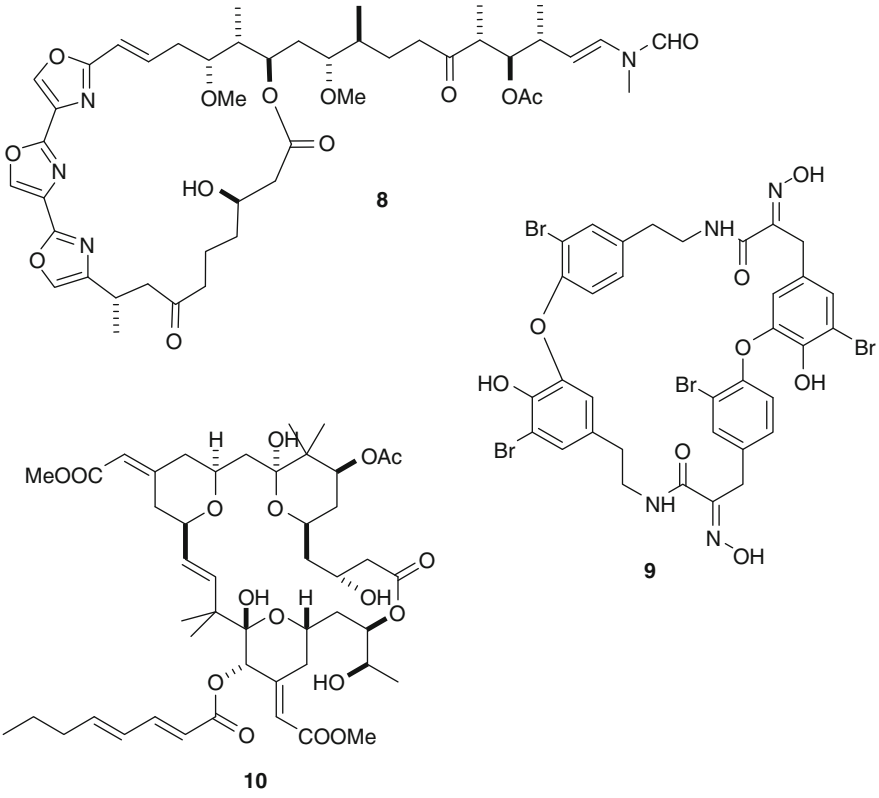


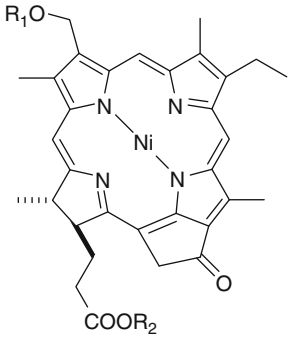
16.4 Marine Natural Products Isolated Containing Metals

A survey of the literature and databases reveals very few marine invertebrate natural products which have been isolated as metal complexes. Compared to the last comprehensive survey by Michael and Pattenden which highlighted compounds **11–13**, only a few more complexes have been reported (**14, 16**) [19]. In addition to this, a few marine microorganism-derived metal complexes have been isolated, but these are beyond the scope of this review. Of the previously reviewed compounds, the nickel-containing porphyrin tunichlorin (**11a**) was originally isolated from the Caribbean ascidian *Trididemnum solidum* [33] and again later from the South Pacific Ocean sea hare *Dolabella auricularia* [34]. Subsequently, a large

number of acyl tunichlorin analogues (**11b**) were isolated from *Trididemnum solidum* where the acyl chains ranged from $C_{14:0}$ to $C_{22:6}$ [35]. Zinc complexes have been isolated from calcareous sponges; the first one was the clathridine–zinc complex (**12**) isolated from the Mediterranean sponge *Clathrina clathrus* [36]. A second zinc-complexing compound was isolated from a Fijian *Leucetta* sponge, the zinc complex of isonaamidine C (**13**) [37].

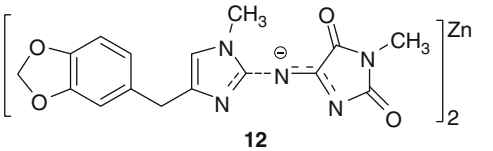
The unique geodin A, a macrocyclic polyketide lactam tetramic acid, was isolated as its magnesium complex (**14**) from the Australian sponge *Geodia* sp. [38]. The 2:1 complex was originally observed by mass spectrometric methods, and the stoichiometry was confirmed by atomic absorption spectroscopy. Geodin A is related to the tetramic acid magnesidin A (**15**) produced by the marine *Vibrio gazogenes*. Magnesidin A (**15**) was isolated as its magnesium complex but was found to be unstable to chromatographic methods using TFA [39]. This raises the point that although a marine natural product may exist as a metal complex in vivo, the extraction and isolation procedures may destroy the complex. The complexes reported above have all been stable to the work-up and chromatographic conditions, but it is likely that many others do not survive. Additional evidence for this comes from the existence of several zinc-free clathridines and naamidines from calcareous sponges, which may have been present as their zinc complexes in vivo but are destroyed during extraction and isolation procedures. To take this into account, we developed an isolation procedure using size exclusion chromatography which was mild enough to enable weak complexes to survive the purification process [15]. Application of this methodology, which is described in detail in Sect. 16.6, enabled the recovery of the 3:1 eudistomin–iron complex (**16**) from the Australian ascidian *Eudistoma gilboviride*, whose metal complement we had previously determined (Fig. 16.4). The methodology also enabled us to calculate that 0.4% of the organic extract of the organism was composed of the complex **16** and its analogues and that this represented 75% of the total lipophilic iron found in *E. gilboviride* and therefore a significant proportion of the organism's total iron content [15]. In addition, a reexamination of the crude extract by UV spectroscopy indicated that complex **16** was indeed present at this stage of the isolation procedure. It is possible that the formation of this complex is an artifact of sample preparation, but its concentration and presence in the original extract seem to suggest that it plays an important role in the organism's physiology as iron chelator. As mentioned previously, the eudistomin core structure (**5**) resembles the known iron chelator pyrimine (**6**) which also forms a 3:1 complex with iron(II) [27]. Further studies with pyrimine (**6**) showed that its complexes with Cu(II), Fe(II), and Mn(II) acted as efficient superoxide dismutase mimetics, with Cu(II) giving the best results [40]. It is possible, therefore, that the role of complex **16** in *E. gilboviride* is for the

removal of reactive oxygen species which may be generated in the high light environment it inhabits.



11a $R_1 = R_2 = H$

11b R_1 = acyl, R_2 = H or Me



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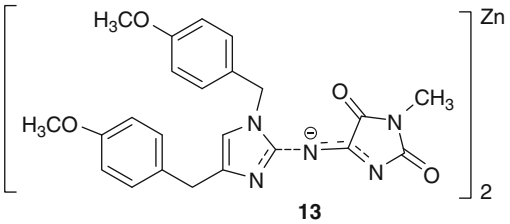

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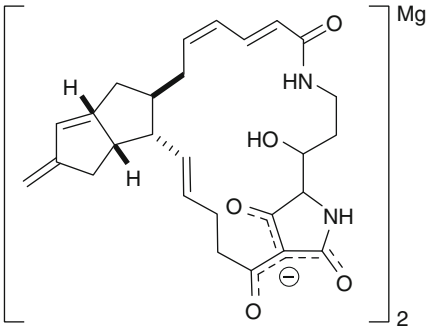

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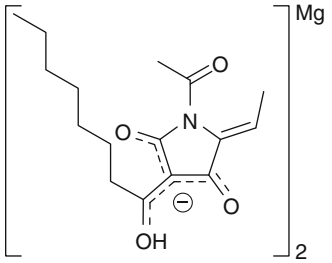

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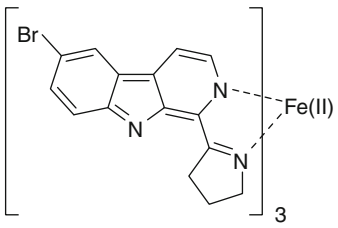

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16.5 Marine Natural Product Complexation Studies

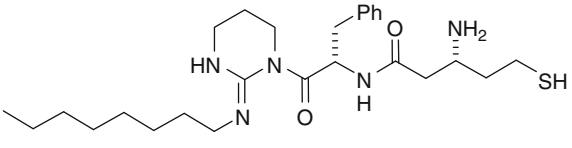
In the preceding sections, we have seen a limited number of authentic complexes isolated from marine invertebrates and some speculation based on the combined functionality and conformation of a molecule presenting a coordination environment suitable for complexing metals. The eventual aim of any studies based around determining metal chelation by marine natural products is to predict their function and hence their benefit to the producing organism. The nuclease activity determined for the copper complex of tambjamine (**3**), mentioned above, may indeed be its natural function, and it may thus act as a means of chemical defense. Similarly, the potential role of the eudistomin–iron complex **16** as a superoxide dismutase appears to make sense in its normal environment, yet neither of the assertions regarding these complexes' native function is easy to confirm.

As a first step toward understanding the function of marine metabolite–metal complexes, the basic structural and physicochemical parameters need to be determined. Several research groups have tried to confirm suspected complexation ability of marine natural products using spectroscopic and other methods. A useful parameter in such work is the binding selectivity for a particular metal ion, and then its binding affinity must also be measured. The ligand atoms involved in the complexation must also be determined so that the complete coordination environment and eventually the full three-dimensional structure of the complex in the solid or solution state should be solved. In this section, we will discuss illustrative examples of how combined physical, spectroscopic, and theoretical methods can be used to confirm the metal-complexing ability of some of the suspected ligands derived from marine invertebrates.

The simplest studies just involve one or two techniques to determine the metal complexation ability of a potential ligand, and this was the case for the highly unusual peptide caledonin (**17**), isolated from the New Caledonian ascidian, *Didemnum rodriguesi* [41]. The structural features of **17**, a penicillamine-like β-amino acid at one end and a hydrophobic chain at the other, made the investigators suspect that it was involved in metal ion transport across membranes. ^{1}H NMR titration studies of **17** with Zn(II) resulted in a 2:1 ligand to metal complex which was confirmed by mass spectrometric studies. Changes in the ^{1}H NMR spectrum on complexation to Zn(II) suggested that the terminal thiol and amino groups were involved in the binding.

Kuanoniamine D (**18**) contains a bipyridyl-type "bay" chelating functionality, and this was investigated in more detail using a variety of techniques and a range of metals which provided evidence for the formation of 2:1 ligand–metal complexes [42]. ^{1}H NMR titrations increasing the amount of $ZnCl_2$ showed line broadening of the signals at H2, H3, and H11 in **18** which sharpened and remained unchanged once a 2:1 ratio was attained. Kuanoniamine D fluoresces with λ_{ex} 350 nm and λ_{em} 524 nm, and the emission peak was greatly attenuated upon addition of excess Zn(II), Fe(II), and Cu(II) and was completely quenched on addition of Co(II). Fluorescence titrations could therefore be used to confirm the ligand to metal

ratio for Cu(II) and Co(II) in the complex and determine stability constants of 2.5×10^{10} M^{-2} for Co(II) and 1.3×10^{10} M^{-2} for Cu(II).



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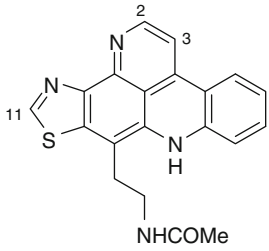

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The original isolation paper of ascididemin (**4**) indicates that it does not bind to iron(II) [25]. The compound was subsequently reisolated from a *Lissoclinum* sp., measurements of the metal content in the ascidian extract showed elevated levels of several transition metals (Cu, Zn, Fe), and the mass spectrum showed an isotope pattern consistent with a **4**-CuOAc complex, which suggested that **4** might complex copper [43]. As ascididemin is fluorescent (λ_{ex} 219 nm, λ_{em} 295, 334 nm), a fluorescence quenching study was carried out (Fig. 16.6), and the fluorescence quenching mechanism and efficiency were evaluated. This showed that the fluorescence quenching efficiency is significantly greater for the Cu(II) complex than the Co(II) or Ni(II) ones.

An early complexation study was initiated by the realization that the cyclic depsipeptide jasplakinolide (Fig. 16.7a, **19**), isolated from the Fijian sponge, *Jaspis* sp., resembled several other depsipeptide ionophores such as the enniatins [44]. Consequently, Inman and coworkers conducted ^{1}H NMR titrations of a 200-mM solution of **19** in CD_3CN with Li^+, Na^+, and K^+. Only Li^+ generated significant changes in the spectrum, which were used to calculate a binding constant of 60 M^{-1} for the 1:1 complex of **19** with Li^+. Significant conformational changes were observed via the change in a number of coupling constants, implying changes in dihedral angles of **19**. A molecular mechanics study using explicit Li^+ revealed a conformation consistent with these dihedral angles that allowed the carbonyls at C1, C10, and C14 to bind to the Li^+ with reasonable Li–O bond lengths, and that Li–O distance was too long for the carbonyl at C17. A more recent study took a different approach and first determined the solution conformation of **19** in CD_3CN using NOE-restrained molecular dynamics calculations (Fig. 16.7b) [45]. This was followed by the determination of the solution structure of 16 mM **19** in CD_3CN in the presence of 1 equivalent of Li^+ which was modeled using NOE restraints without the use of an explicit Li^+. Again, large changes in the conformation on formation of a 1:1 ligand to metal complex were evident, as is clear from the resulting minimum energy structure represented in Fig. 16.7c. This conformation appeared very different from that determined in the earlier study [44], perhaps as an effect of the much lower concentration employed in the more recent study [45].

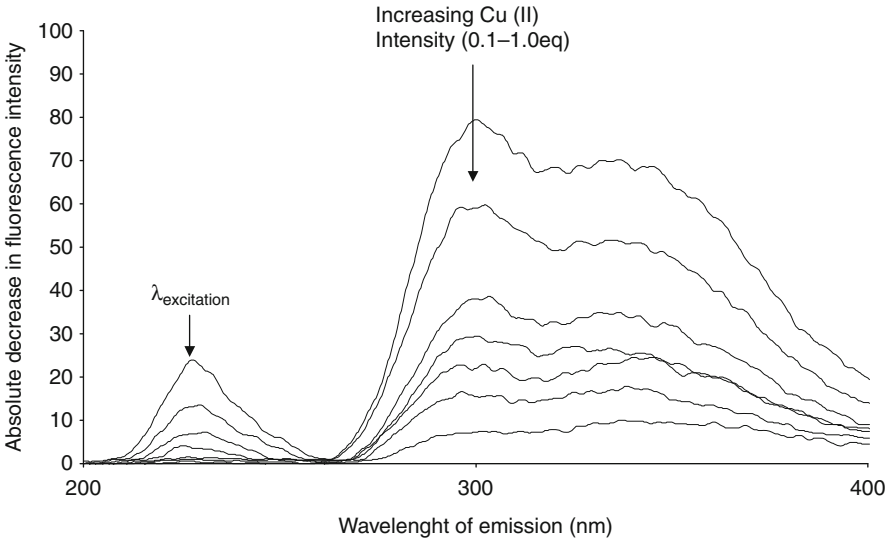


Fig. 16.6 Titration of ascididemin (**4**) with increasing amounts of Cu(II) monitored by fluorescence spectroscopy showing fluorescence quenching with increasing amounts of Cu(II). Excitation wavelength set at 219 nm and emission monitored at 295 and 334 nm

A full conformational search without NOE restraints in the presence of explicit Li^+ found a low-energy conformation that was similar to the solution conformation in Fig. 16.7c. An overlay of the calculated and experimentally determined chelating structures is shown in Fig. 16.7d. This then suggests that at this concentration, the Li^+ binds outside the macrocycle, involving the carbonyls at C14 and C17, and the π-electrons of the β-tyrosine. A comparison of these two studies thus casts light on another difficulty, that weak complexes such as this may display different modes of coordination depending on the concentration.

The cyanobactins, exemplified by the patellamides, **20**, and westiellamide, **21**, are posttranslationally modified ribosomal cyclic peptides containing azole moieties isolated from cyanobacteria. The patellamides, **20**, were originally isolated from the ascidian *Lissoclinum patella* but were subsequently shown to be produced by the symbiotic cyanobacterium *Prochloron didemni* [46, 47]. One study showed elevated levels of copper and zinc in the tissues of *Lissoclinum patella* [48], adding ecological significance to the previously observed crystal structure of ascidiacyclamide **23** with two equivalents of copper and a bridging carbonate (Fig. 16.8a, b) [49]. These authors studied these complexes by a number of techniques and found that in the solid state the Cu···Cu distance was 4.43 Å, whereas a separate study showed this to be 3.7 Å in solution using EPR measurements coupled with molecular simulations [50]. The key findings of many studies, by a number of authors using a range of techniques, including mass spectrometry,

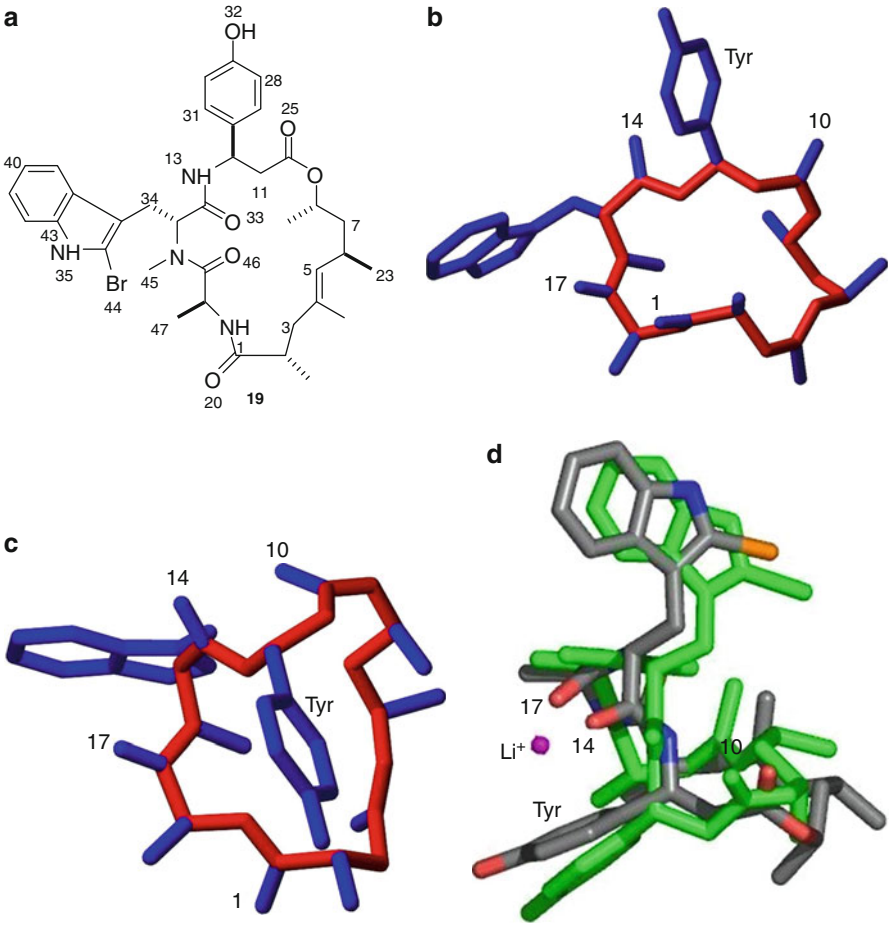
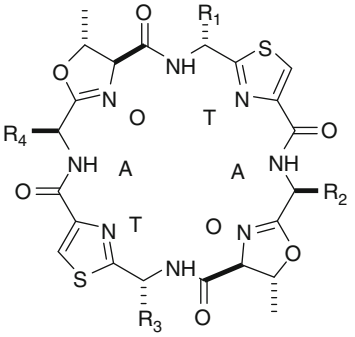


Fig. 16.7 (**a**) Structure of jasplakinolide; (**b**) NOE-restrained molecular dynamics structure of jasplakinolide in CD_3CN; (**c**) NOE-restrained molecular dynamics structure of jasplakinolide with 1 equivalent of Li^+ in CD_3CN; (**d**) Overlay of the structure in (**c**) with a Li^+ docked minimized jasplakinolide obtained by conformationl searching

ultraviolet, circular dichroism, NOE-restrained molecular dynamics calculations, and magnetic susceptibility have been recently summarized [24]. This intensive research was prompted by Hawkins' original suggestion that the patellamides (**20**) could complex two equivalents of copper via the thiazole (T), amine (A), and oxazole (O) nitrogens, as later confirmed by many of these studies.[26] A cooperative binding mechanism was demonstrated in which the binding of one Cu(II) ion unfolded the patellamide molecule to present a second TAO-binding site ready to accept the second Cu(II) ion (Fig. 16.8c) [51]. The presence of elevated

levels of copper in *Lissoclinum patella* and the ideal coordination environment presented for the complexation of two Cu(II) ions suggested that this complex played an important role in the ecology of the organism, whereas the role of a Zn–patellamide complex was deemed less important due to the lower stability of the complexes formed [48, 51]. The distance between the two copper centers in the patellamide–dicopper complexes tantalizingly suggested that they might have a role in activating dioxygen, similar to the dicopper proteins, but this has not been verified to date [52].



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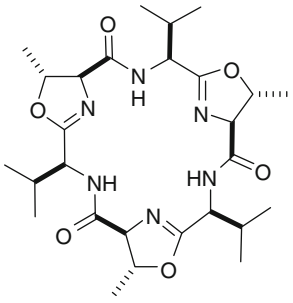

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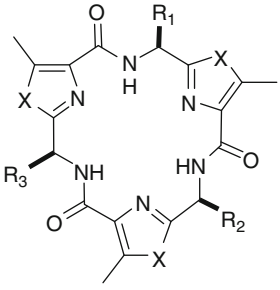


22, X = NMe, O or S

The study of westiellamide (**21**) was initiated by the unusual sandwich complex generated with four Ag(I) ions (Fig. 16.8d) [53]. The near-planar structure of **21**, upon complexation with Ag, is modified by the rotation of the oxazoline rings perpendicular to the macrocycle, with the coordination environment for the fourth Ag(I) ion generated by the carbonyl groups pointing toward the center of the sandwich. A detailed study of these complexes of **21** and analogues **22** has been published recently, showing that the compounds are highly preorganized for Cu(II)

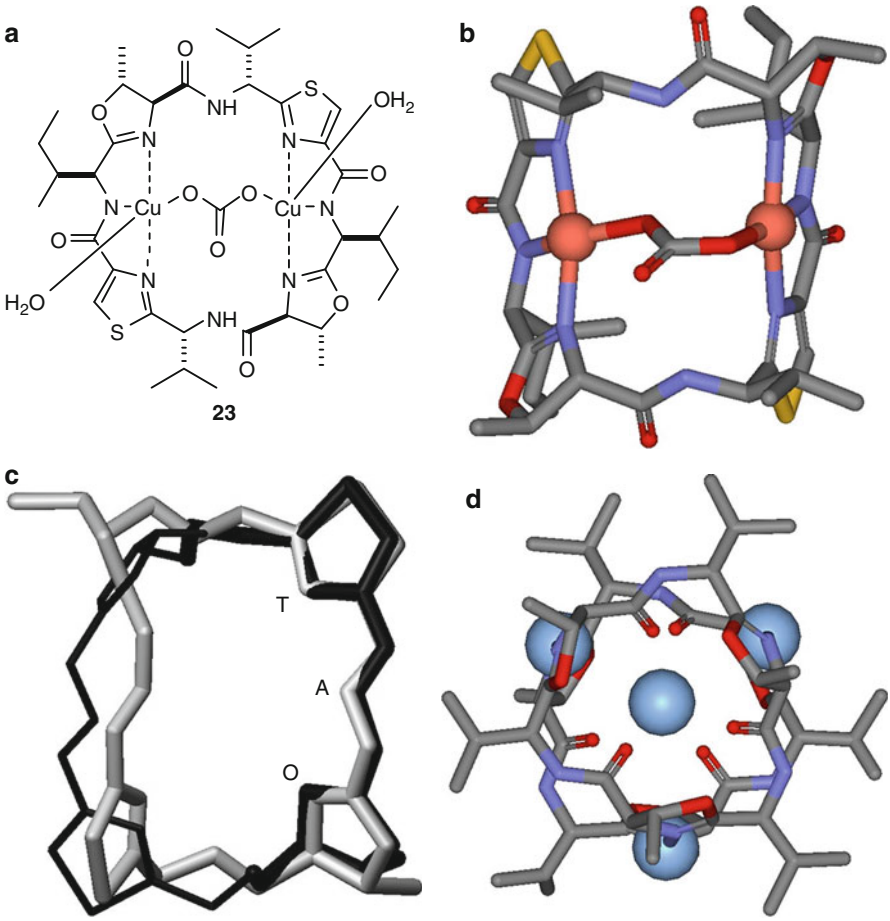
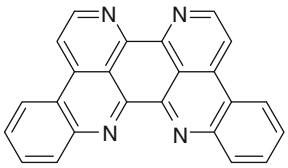


Fig. 16.8 (**a**) Ascidiacyclamide–Cu_2CO_3 structure. (**b**) Crystal structure of the ascidiacyclamide–Cu_2CO_3 complex, Cu depicted as spheres. (**c**) Overlay of the patellamide C–Cu NOE-restrained molecular dynamics structure (*black*) with the ascidiacyclamide crystal structure (*gray*) showing the TAO nitrogens in the correct configuration to accept the second Cu ion. (**d**) Sandwich complex of two westiellamide molecules with four Ag ions, Ag depicted as spheres

coordination [54]. These examples show that although some of these complexes may form with biologically relevant metals, in other cases, novel complexes can be formed using other metal ions. Such is the case for eilatin (**24**), isolated from the Red Sea ascidian *Eudistoma* sp. which forms complexes with ruthenium and two

bipyridyl units (**25**) [55]. These complexes have anti-HIV activity in vitro by binding to DNA and RNA.



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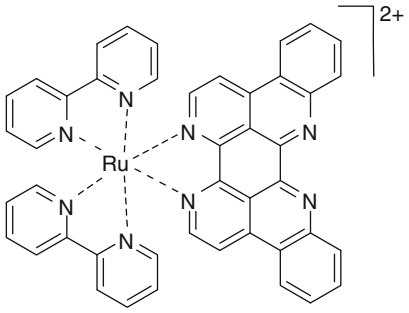

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16.6 Methods to Identify Metal Complexes from Marine Invertebrates

From the preceding discussion, it is clear that metal-complexing agents from marine invertebrates are underinvestigated. The complexes that have been studied, either isolated as complexes, or ligands studied in the presence of metals show excellent selectivity, high affinity, and some fascinating biological activities. For this reason, efficient methods for the recovery of intact complexes or potential ligands need to be developed. Much work has been done on developing rapid colorimetric methods for the discovery of iron ligands such as the chrome azurol S (CAS) assay [56], but there is a dearth of similar assays for other metals of interest in addition to which a more general method, able to cope with a range of metals, would be preferable.

The method of choice for the detection of trace elements in solution is ICP-MS (inductively coupled plasma mass spectrometry), an elemental mass spectrometric technique, which is able to detect most elements in solution below concentrations of 1 ng/mL (ppb). The method is not only sensitive but also has the ability to detect multiple elements simultaneously which enables the screening of extracts or acid-digested tissues for a series of elements of interest. These can be the classical transition elements: metalloids such as arsenic and selenium, and nonmetals such as bromine, iodine, phosphorus, and sulfur. Hence, the ICP-MS analysis of extracts from marine organisms can quickly establish which samples contain significant quantities of elements of interest.

ICP-MS uses a hard ionization source that completely destroys, atomizes, and ionizes every compound, so with this technique, it is intrinsically impossible to determine intact molecules. However, when chromatographic separation in the form of HPLC (high-performance liquid chromatography) is interfaced to ICP-MS, molecular information can be gained indirectly; different molecules

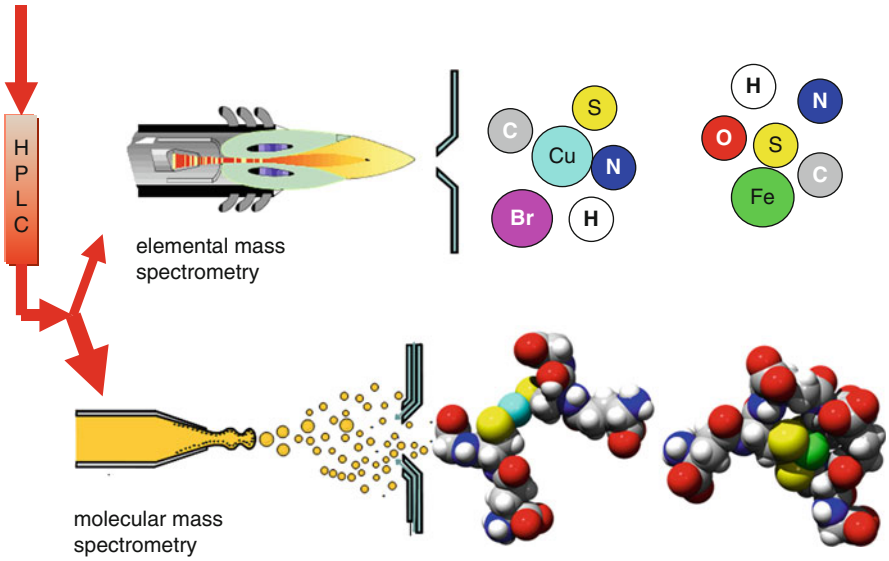

Fig. 16.9 HPLC hyphenated simultaneously to ICP-MS and ES-MS to identify and quantify metal complexes in extracts of biological marine specimens

containing trace elements are separated by HPLC, and element-specific detection is achieved with ICP-MS. The molecular identification relies on retention time comparison with standard compounds. This technique can also be used to screen for unknown compounds and can give information whether or not different compounds containing a certain element occur in a sample extract. However, HPLC-ICPMS alone cannot identify novel compounds where no standard compound is available. For molecular identification of unknown compounds in a sample extract, molecular mass spectrometric methods such as electrospray ionization mass spectrometry (ES-MS) are needed. Electrospray is a soft ionization technique that is able to ionize molecules, complexes, and even unstable compounds without fragmentation. When HPLC is interfaced to ES-MS, compounds can be separated and detected, but it is very difficult to identify the metal-containing species from an ES mass spectrum. Here, the combination of ICP-MS and ES-MS has proven ideal for the detection of labile metal compounds in complex extract matrices. The combination of both MS detectors capitalizes on their complementary analytical features, and recently HPLC has been coupled simultaneously to both detectors. Figure 16.9 illustrates this innovative arrangement of interfacing HPLC in parallel to ICP and ES mass spectrometers.

All metal compounds in an extract can be detected simultaneously, and elemental as well as molecular information can be collected. While the ICP-MS identifies the "needle in the haystack" by giving direct information about the retention time of the molecules containing the element of interest (which could be characteristic for the ligand such as bromine in **9** and **16** or for the complexed metal-like zinc in **12**

and **13** or iron in **16**), ES-MS provides molecular information in the same time window. This enables the identification of traces of metal complexes in a crude biological extract, without having a standard at hand. This system is especially useful for the identification of target species with unknown elemental composition and structure and which are potentially labile during chromatography. Since only one chromatographic system is used, the system can be tailored for the identification of certain complexes. This has been demonstrated for the identification of labile arsenic and mercury phytochelatin complexes, which were separated on a reverse-phase column in a methanol/formic acid mobile phase [57, 58].

However, the major disadvantage of all MS-based techniques is that they have to be linked to chromatography or capillary electrophoresis. Ion-exchange chromatography and capillary electrophoresis typically use buffers with high aqueous content, which are not generally suitable for natural products due to solubility issues. Reverse-phase HPLC is the most commonly used form of LC-MS for analysis of natural products, but it tends to result in dissociation of all but strong chelates. To enable LC-MS of even weak natural product chelates, we investigated size-exclusion chromatography (SEC) with a natural product friendly solvent system. Contrary to other forms of chromatography, SEC involves minimal interaction between the analytes and the stationary phase, which helps to prevent dissociation of metal complexes. We combined the use of a poly(methyl methacrylate) SEC-HPLC column with a methanolic buffer. However, analyte interactions with residual hydrophilic sites on the stationary phase resulted in chelate dissociation and excessive retention. Through much trial and error, we developed a buffer (50 mM trimethylamine carbonate, pH 8.8) that was able to prevent the dissociation of weak chelates [15]. Although some chelates were still strongly retained on the column, these could be eluted with 100 mM formic acid in methanol with minimal complex dissociation.

This SEC-HPLC method was coupled to both ICP-MS and ES-MS to investigate organic extracts of ascidians for chelates. As previously mentioned, cyclic peptides produced by the ascidian *Lissoclinum patella,* such as the patellamides (**20**), are able to chelate copper with stability constants in the region of 1×10^4–3×10^5 [48, 59, 60]. These copper complexes were successfully observed using SEC-HPLC-ICP/ES-MS in an extract of *L. patella* that had been spiked with copper. The unspiked extract of *L. patella* was also analyzed by this method, but no complexes were observed, which suggested that these cyclic peptides were not complexed to copper in the organism.

Many novel natural product chelates that exist undetected may be discovered by SEC-HPLC-ICP/ES-MS. A crude extract of *Eudistoma gilboviride*, in which we had discovered high levels of iron (Fig. 16.4), was analyzed using this method. Peaks in the ES mass spectrum were detected that coeluted with Fe peaks in the ICP-MS chromatogram (Fig. 16.10). Despite only using low resolution ES-MS, we were able to identify complexes of Fe(II) with eudistomin H (**16**) and its analogues. The incorporation of high-resolution ES-MS would enhance this method's potential for discovery of novel chelates manyfold. While the presence of these novel Fe(II)–eudistomin chelates were identified in the organic extract, the question remains as

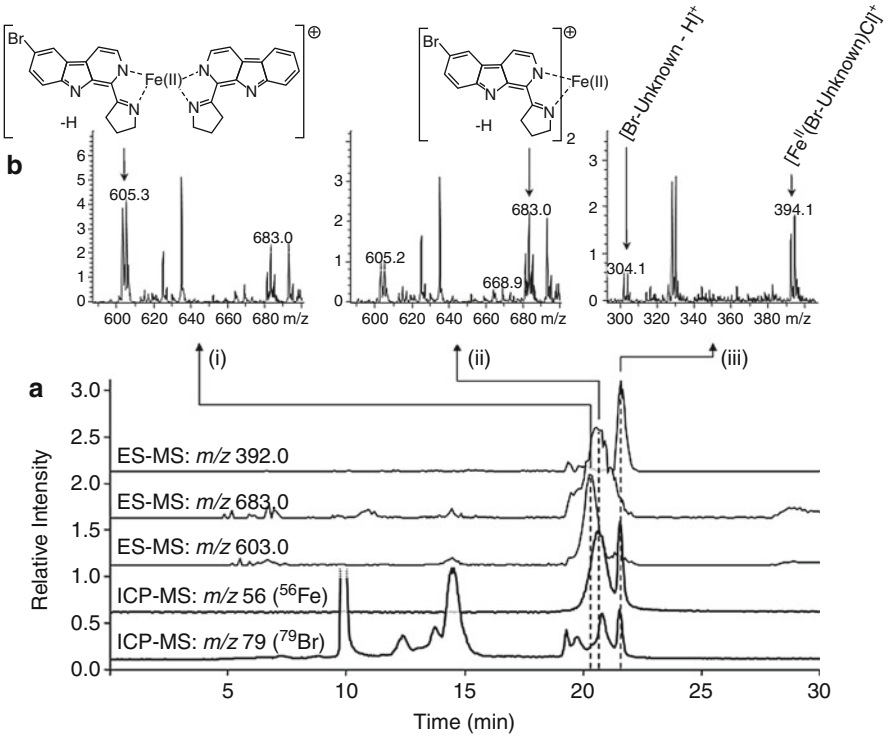

Fig. 16.10 Iron(II) complexes identified in a organic extract of the ascidian *Eudistoma gilboviride* by size exclusion chromatography coupled in parallel to ICP-MS and ES-MS. (**a**) Selected ICP-MS (Fe, Br) and ES-MS (603, 683, 392 *m*/*z*) extracted ion chromatograms. (**b**) Mass spectra and identifications for peaks at 20.5, 20.8, and 21.6 min [15]

to whether they were present in the organism itself or were formed during the extraction process.

If the metal complexes dissociate during chromatographic separation, other methodologies which do not rely on separation techniques have to be employed, such as electrochemical (EC) detection. These techniques are usually applied to aqueous solutions but have a potential to be used for nonaqueous extracts. EC techniques can thus detect complexes with different stability constants by tuning experimental parameters and can distinguish between free metal ions, labile metal complexes, and inert stable complexes. Although it cannot directly identify the molecular structure of the complex, it is possible to determine the conditional stability constant of the complex. So far, no study has been published which applied EC techniques for the identification of metal complexes in biological extracts. Although EC methods are well-established methods in oceanography, the reason why these are not widely used for the analysis of metal-containing natural products might be the severe matrix problems when they are used in nonaqueous solution.

In order to overcome this problem, and to guarantee the integrity of the metal complex during analysis, direct analytical methods need to be employed which do not rely on the extraction step in the first place, methods that can be used to determine whether a complex is actually present in the organism itself. These are elemental and molecular imaging methods. One type of analysis is based on the absorption of synchrotron radiation. In particular, X-rays generated by tuneable synchrotron radiation can be applied to the specimen, and the absorption is then recorded as a function of the energy. X-ray absorption near edge spectroscopy (XANES) and extended X-ray absorption fine structure (EXAFS) give information about the element and the redox state as well as the bond length to the ligands. For example, it is possible to identify whether arsenic is trivalent or pentavalent and whether it binds to ligands via oxygen or sulfur using the fresh specimen without any extraction steps. EXAFS analysis is often only used for the identification of the redox state of the metal. For example, the identification of the different redox ions in different ascidians (*Ascidia ceratodes* and *Ascidia nigra*) gave evidence of subgeneric differences and was the basis for further investigations to identify vanadium(IV)-binding proteins in ascidians [9, 61].

The drawback of these synchrotron radiation (SR)-based methods is that they cannot be used as routine analysis for screening analysis since beamtime at a synchrotron is limited and very expensive. SR methods can reveal whether any novel metal complexes are disintegrated or formed de novo during the sample preparation and the analysis. The complementary use of XANES/EXAFS methods compared to MS-based methods has never been applied to the identification of novel marine products, but was successfully employed for the identification of arsenic complexes in plants [62].

Another approach is to analyze marine organisms directly using elemental and molecular imaging techniques. Micro-XANES analysis makes it possible to record an element spectrum on a spot the size of 100 μm. Rastering the target in the laser beam enables the construction of an image which contains semiquantitative data of the element distribution in the organism with additional information of its redox state or binding ligands. This can be used to identify the organ in which the most interesting metal complexes are located. Where synchrotrons are not available, other laboratory-based techniques such as LA-ICP-MS (laser ablation ICPMS) can be used for generating elemental maps of the organism. For this analysis, it is necessary to produce thin sections, by microtoming, of the organism, and by interrogating it with a laser beam of variable energy, the material is ablated and transported into the ICP-MS. Multielement maps can be constructed on specimen size up to 10 cm^2 with a spatial resolution as low as 10 μm.

Although these techniques give vital information about the spatial distribution of metals and metalloids, no metal complexes can be identified. Hence, again molecular information is a necessary addition to the elemental mapping. In recent years, new surface molecular imaging methods have been developed which can assist to gain information on small molecules and metal chelates. After a surface application of a matrix of easily ionizable substances, soft ionization of the molecules can

easily transfer the charge to molecules to form molecular ions which can be transported into a mass spectrometer for analysis. This technique is called MALDI-TOF-MS (matrix-assisted laser desorption ionization time of flight mass spectrometry). This method has recently been used to identify secondary metabolites produced by marine cyanobacteria and sponges [63]. The newest development is the use of DESI (desorption electrospray ionization) MS for the localization of natural products directly on the marine organism without applying any matrix on the surface) [64].

If elemental and molecular imaging techniques are used with adjacent thin sections of one organism, metal and ligand maps can be matched and superimposed using image manipulation techniques. This has never been demonstrated with marine organisms, but its potential was demonstrated when it was successfully used to identify metal–protein complexes in a liver abscess [65].

In summary, a variety of analytical methodologies can be used for screening analysis of metal-containing natural products in extracts. To choose the best methodology, readers may refer to a recent review which critically appraises different methodologies for the determination of trace element compounds in biological samples [66].

16.7 Conclusion – A Proposal for a Structured Search for Marine Natural Products Containing Metals

Besides developing the tools to more efficiently discover complexes and ligands from marine invertebrates, it is also worth thinking carefully about how a constructive search for these might be structured. There are some structural features and general considerations which are important when looking for complexes and ligands from marine invertebrates, and these could form the search parameters for a directed search.

The first parameter to consider is the metal content of the invertebrate tissues and organic extracts. A high concentration of metals in the organic extract suggests that they are complexed by an organic ligand. Interest in the eudistomins (e.g., **5**) was aroused by the high content of Fe in the extract of *Eudistoma gilboviride* (Fig. 16.4), which led to the discovery of the eudistomin–iron complex **16** [15]. Therefore, the screening of the organisms to look for transition metals (e.g., Mn, Fe, Co, Ni, Cu, Zn, Mo) in organic extracts will be very productive, but as has been discussed before, few studies have addressed this.

In assessing compounds with potential metal-complexing properties, structural features of known compounds can be very suggestive of good metal ligands. Initially, this may include looking for hard/soft ligands, groups which are involved in metal binding in metalloproteins such as histidine, methionine, cysteine, tyrosine, aspartic and glutamic acid, as well as the coordination environment these present (Fig. 16.5). Additionally, we can look for typical functional groupings found in siderophores and ionophores such as catecholates, hydroxamates, and

β-hydroxyaspartates as well as those present in heteroaromatics (Fig. 16.5). If we are considering the functions of these complexes, such as the generation of reactive oxygen species by the tambjamine (**3**)–copper complex, then the metal center should be coordinatively unsaturated to allow a substrate to bind.

Cyclic compounds have a greatly reduced conformational space compared to acyclic compounds, often allowing them to present ideal coordination environments for metal complexation. This, together with constraining features such as azole rings and prolines, should be given priority as search parameters. Other features such as disulfide bridges also reduce the conformational space accessible.

If we are considering the potential biological functions of these metal complexes, we should look for groups with potential for involvement in redox processes. Some of the compounds have signatures implying the possibility of redox involvement such as disulfide bridges. Similarity to known metalloprotein active sites may also be considered an important search parameter to ascribe a function to a complex, such as the similarity of the patellamide dicopper complexes (Fig. 16.8a, b) to dicopper proteins. A similarity search according to substructures is likely to be less successful than a similarity search based on key coordinating atoms in the active site. One final feature to look out for is protection of the coordination environment by lipophilic groups which might be important for controlling substrate access.

These search parameters, when combined, may lead to the discovery of a number of exciting new complexes from marine invertebrates with associated biological functions and potential for medical or biotechnological applications.

16.8 Study Questions

1. Suggest reasons why marine invertebrates might hyperaccumulate metals. What evidence is there to support each theory?
2. Many marine natural products contain functionality that suggests an ability to complex transition metals. What functional groups and structural features might be important in complexing transition metals? From the marine natural product literature or databases, identify four such structures and propose which metals they might bind to and why.
3. Which physical methods might you use to determine the binding specificity and affinity of marine natural products for transition metals?
4. If you were presented with an organic invertebrate extract that was identified as containing high levels of zinc, what steps would you take to isolate and determine the structure of the zinc complexed to the marine natural product?
5. Draw a flowchart explaining the steps that need to be taken to carry out a structured search for marine natural product metal complexes. You should explain the choice of your steps and the methods to be used at each stage.

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