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On ovothiol biosynthesis and biological roles: from life in the ocean to therapeutic potential

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Abstract (100 words)

Ovothiols are sulfur-containing natural products biosynthesized by marine invertebrates, microalgae, and bacteria. These compounds are characterized by unique chemical properties suggestive of numerous cellular functions. For example, ovothiols may be cytoprotectants against oxidative stress, serve as building blocks of more complex structures and may act as molecular messengers for inter- and intracellular signaling. Detailed understanding of ovothiol physiological role in marine organisms may unearth novel concepts in cellular redox biochemistry and highlight the therapeutic potential of this antioxidant. The recent discovery of ovothiol biosynthetic genes has paved the way for a systematic investigation of ovothiol-modulated cellular processes. In this highlight we review the early research on ovothiol and we discuss key questions that may now be addressed using genome-based approaches.

This highlight article provides an overview of recent progress towards elucidating the biosynthesis, function and potential application of ovothiols.

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Ovothiol A and related 5-thiohistidines are characterized by unique chemical reactivity and complex biological functions in marine invertebrates, microalgae, and bacteria.

Background and open challenges

The extraordinary beauty of marine landscapes has always inspired scientists and artists for the huge richness in biodiversity, reflecting billions of years of evolution. Marine diversity also extends to molecular scales making the oceans one of the largest repositories for natural products with amazing structures and potentially life-saving therapeutic activities¹. One particularly interesting group of natural compounds are sulphur-containing molecules because of their ability to participate in cellular redox chemistry. Low-molecular-weight thiols, thiones, thioethers and disulfides play a key role in maintaining cellular redox homeostasis and enable organisms to survive taxing and changing environmental conditions.^{2,3} Probably one of the most abundant, yet least investigated marine sulfur compound is ovothiol A (**1**, Figure 1). This 5-thiohistidine has been isolated for the first time from sea urchin eggs, which resulted in its naming *ovothiol*.^{4,5,6} The unique antioxidant properties^{7,8,9} and the broad distribution among marine invertebrates, microalgae and proteobacteria provide reason to believe that ovothiol A and its derivatives ovothiol B and C (**2** & **3**, Figure 1) play important roles in cellular biochemistry.^{10,11}

In the past, systematic investigations on the physiological function of thiohistidines were difficult, because the origin and distribution of these compounds was obscure. The recent identification of ovothiol biosynthetic genes¹⁰ and the growing availability of sequenced genomes from bacteria, fungi and higher eukaryotes, have opened new avenues to examine several key questions, including those about i) the evolutionary origin of ovothiols ii) the distribution of ovothiol biosynthetic genes among extant marine organisms iii) the catalytic mechanisms of ovothiol biosynthetic enzymes and most importantly, iv) the physiological role of ovothiol. Answers to these questions are likely to uncover novel concepts in redox biochemistry and may also inspire the development of novel therapeutic approaches and biotechnological applications based on thio-histidines.

The objective of this article is to summarize current answers to these questions. We will also identify future challenges and discuss how bioinformatics, comparative and functional genomics, structural biology and enzymology should be combined to develop a comprehensive perspective on ovothiols in marine organisms, and to unravel the potential of ovothiol biosynthesis in the emerging research field of blue biotechnology.

Occurrence of ovothiol and related marine thiohistidines

Almost forty years ago ovothiols were recognized as common metabolites in marine invertebrates. Ovothiol A, 5(N π)-methyl thiohistidine (**1**, Figure 1) was isolated from the eggs of the sea urchins *Paracentrotus lividus*⁴, and *Arbacia lixula*, in the holothuroid, *Holothuria tubulosa*, the asteroids, *Astropecten aurantiacus* and *Marthasterias glacialis*⁵, the mottled sea star, *Evasteria stroschelii*⁷, in the eggs and biological fluids of molluscs, the common octopus *Octopus vulgaris* and the european squid *Loligo vulgaris*⁶, in the celomic fluid of the marine worm, *Platynereis dumerilii*¹². The ovothiol derivatives B and C (**2** and **3**, Figure 1) are distinguished from A by one or two additional methyl group at the α -amino function of 5-thiohistidine. These compounds were first discovered in the eggs of the spiny scallop *Chlamys hastata*⁷ and in the eggs of the purple sea urchin *Strongylocentrotus purpuratus*⁸ and *Sphaerechinus granularis*.⁵

Ovothiol A, and its N-desmethyl form 5-thiohistidine were also found as building blocks of more complex secondary metabolites from marine invertebrates. Adenochromine, an iron-binding purple pigment isolated from the common octopus (*Octopus vulgaris*) was found to consist of a dihydroxy phenylalanine core (DOPA) attached to two ovothiol A or 5-thiohistidine moieties¹³ (**4** – **6**, Figure 1). Other examples include the Discorhabdins J, K and M (**7** – **9**) isolated from marine sponges *Latrunculia brevis*¹⁴, and the cytotoxic alkaloid imbricatin (**10**) produced by the starfish *Dermasterias imbricata*¹⁵(Figure 1). In all of these compounds ovothiol or 5-thiohistidine is attached to a core structure via a thioether bond, indicating that the linkage occurred via nucleophilic attack of the thiolate onto electrophilic metabolites. For example, the biosynthesis of adenochromines has been suggested to occur via tyrosinase catalyzed oxidation of DOPA to dopaquinone (**12**, DOPA quinone), followed by nucleophilic attack of ovothiol to form intermediate **13**.¹³ The fact that isolated adenochromines are a mixture of isomers suggests that this second step is not enzyme-catalyzed. Given the nucleophilicity of thiohistidines (see below) and their high concentrations in marine organisms, thiohistidine adducts may be a common trait among marine natural products with electrophilic moieties.

Finally, ovothiol A was also identified as a major low-molecular weight thiol in the protozoan *Crithidia fasciculata*.¹⁶ This discovery came as a surprise because it was the first indication that ovothiol A may not be limited to marine species. Indeed, the observation that pathogenic trypanosomatids such as *Trypanosoma cruzi* and *Leishmania donovani* produce ovothiol raised interesting questions concerning possible roles of this metabolite in human disease.^{17,18} The

identification of *C. fasciculata* as an ovothiols producing organism was an important prerequisite to study the biosynthetic origin of ovothiols (see below).

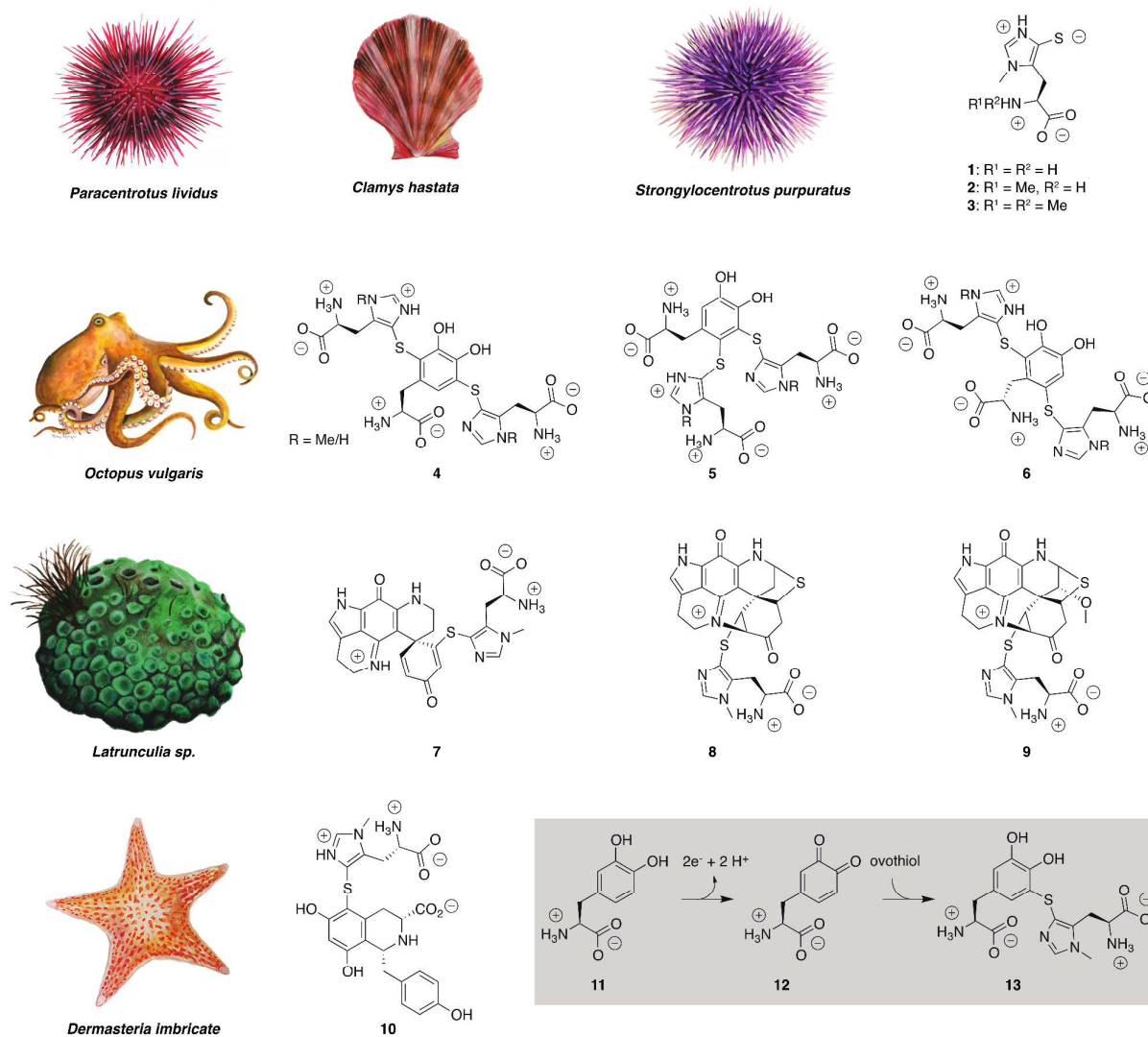


Figure 1. Ovothiols A, B and C (**1 – 3**) and thiohistidine containing marine secondary metabolites adenochromines (**4 – 6**), discorhabdins (**7- 9**) and imbricatine (**10**). Box: proposed mechanism for tyrosinase catalyzed adenochromine biosynthesis.¹³

Biochemical properties and biological functions of ovoidiol

Because the thiol group of ovoidiol A is attached to the 5-position of the imidazole ring, its pK_a is much lower ($pK_{a,SH} = 1.4$) than that of cysteine ($pK_{a,SH} = 8.4$) and that of cysteine derivative such as glutathione ($pK_{a,SH} = 8.7$), trypanothione ($pK_{a,SH} = 7.4$), mycothiol ($pK_{a,SH} = 7.9$), coenzyme A ($pK_{a,SH} = 9.8$) or bacillithiol ($pK_{a,SH} = 8.0$).²⁰ This unique property has led to the belief that ovoidiol A may be a particularly efficient scavenger of peroxides.^{9,20} On the other hand, the disulfide of ovoidiol A is less stable than the disulfide of glutathione. The two-electron redox potential of ovoidiol A is considerably more positive ($E^\circ = -0.09$ V vs. the standard hydrogen electrode) than that of glutathione ($E^\circ = -0.26$ V).²⁰ Hence, the more reactive ovoidiol A and the more reductive glutathione could cooperate to protect sea urchin eggs from hydrogen peroxide induced damage.^{7,21} According to this model, ovoidiol A would first reduce peroxides by nucleophilic attack. The resulting ovoidiol A disulfide would be reduced by glutathione and the resulting glutathione disulfide would then be recycled by NAD(P)H dependent glutathione reductases.²⁰ Most ovoidiol producing organisms contain at least one additional cysteine derivative as the major redox buffer. Therefore, peroxidase activity may be a general function of ovoidiol. However, a quantitative analysis of the peroxidase activities of ovoidiol A and trypanothione ($E^\circ = -0.24$ V vs. the standard hydrogen electrode) in trypanosomatids challenged this idea.¹⁸ This study demonstrated that trypanothione is slightly more reactive toward hydrogen peroxide than ovoidiol A, and that the contribution of non-enzymatic peroxide reduction under physiological conditions is negligible. An alternative function of ovoidiol A in trypanosomatids may be the reduction of nitrosothiols.² This suggestion is particularly appealing because pathogenic trypanosomatids have to survive high concentrations of nitric oxide produced by macrophages in order to establish and sustain infection. Depending on how important this mechanism is, trypanosomal ovoidiol A biosynthesis may emerge as a target for novel anti-infective therapeutics.

The significant nucleophilicity also predestinates ovoidiols as potent scavengers of carbon electrophiles. As indicated above, uncatalyzed ovoidioylation is a likely fate of electrophilic metabolites in cells with high ovoidiol content. The side chain of ovoidiol is also characterized by a remarkably low one-electron redox potential ($E^\circ = 0.45$ V)²² similar to that of selenocysteine ($E^\circ = 0.43$ V).²³ Consequently, ovoidiols may also act as free radical scavengers. A particularly interesting option is that ovoidiol could quench oxygen superoxide ($E^\circ = 0.89$ V) or radicals on proteins and nucleic acids ($E^\circ > 0.9$ V). Unlike the thiyl radicals of cysteine-derivatives which can damage proteins and nucleic acids, ovoidiol radicals are benign and possibly auto-quench by disulfide bond

formation. Moreover, thiohistidines are potent ligands for soft transition metals such as copper, zinc, cadmium, or mercury.²⁴ Therefore, it is also pertinent to investigate their role in metal trafficking and detoxification.

Finally, we would like to mention possible roles of ovothiol that do not directly derive from its chemical properties. Several observations indicate that ovothiol may also be involved in chemical signaling. For example, ovothiol A was reported to act as a pheromone that is secreted by the male marine worm *P. dumerilii*. Exposure to the reduced form of ovothiol A, induces the mature females to release their eggs.¹² Another example is imbricatine (**10**) from *D. imbricate*. This secondary metabolite induces a “swimming response” in sea anemone, *Stomphia coccinea*, when they get in contact with the sea star.¹⁵ Another intriguing involvement of ovothiol in chemical signaling has been observed in the green microalgae *Dunaliella salina*. According to the reported observations the oxidized form of ovothiol A inhibits the light activated ATPase activity of the chloroplast coupling factor CF1.²⁵

Description of the mechanistic and structural basis for ovothiol biosynthesis.

Pioneering experiments on cell-free extracts from *C. fasciculata* revealed that ovothiol biosynthesis starts with oxidative coupling of the side chains of cysteine and histidine to form a sulfoxide conjugate (**14**, Figure 2). This intermediate is cleaved in a pyridoxal 5-phosphate reaction to produce 5-thiohistidine (**15**) which is methylated at the proximal imidazole nitrogen to form ovothiol.²⁶ A subsequent study identified the gene coding for the first enzyme in ovothiol biosynthesis by genome comparisons.¹⁰ *In vitro* reconstitution of OvoA from the plant bacterium *Erwinia tasmaniensis*, and from the pathogenic eukaryote *Trypanosoma cruzi* confirmed that this enzyme indeed catalyzes oxidative coupling of cysteine and histidine. The most surprising result of this finding was that ovothiol production is a much more common trait than previously thought. marine bacteria, including *Shewanella*, *Photobacteria*, and *Pseudoalteromonas* encode ovoA homologs consistent with the idea that ovothiol is a marine metabolite. However, ovothiol biosynthesis also occurs in terrestrial species living under very different conditions, such as the iron (III) reducing bacterium *Geobacter bemidjensis*, the human pathogen *Trichomonas vaginalis*, the pathogen *Erwinia amylovora*, or the iron oxidizing *Acidithiobacillus ferrooxidans*. The broad distribution of ovothiol in many ecological niches raises the possibility that this metabolite can support cellular life in many different ways. The occurrence of ovoA genes in organisms that live under predominantly anaerobic conditions, such as *G. bemidjensis* or *T. vaginalis* is of particular interest. Since OvoA-catalyzed ovothiol biosynthesis requires oxygen (see below), it seems possible

that this biosynthetic pathway is active even at very low oxygen pressures, and that ovothiols may support the oxygen tolerance of anaerobic organisms. Oxygen-independent biosynthesis of the thiohistidines has also been observed,²⁷ but this pathway is catalyzed by an entirely different enzyme-type.

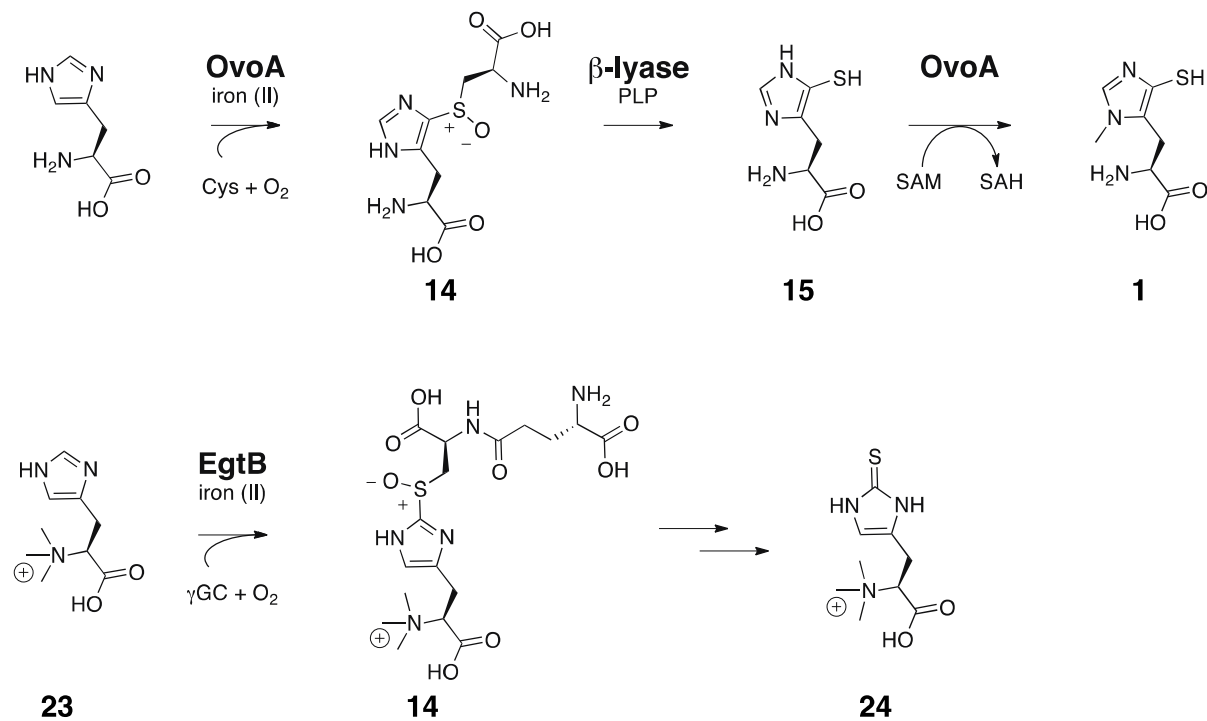


Figure 2. The sulfoxide synthases OvoA (top) and EgtB (bottom) catalyze analogous oxidative C-S bond forming reactions to produce key intermediates in ovethiol and ergothioneine biosynthesis.

OvoA is a bifunctional protein consisting of an unusual iron-dependent sulfoxide synthase at the N-terminus and a S-adenosyl methionine-dependent (SAM) methyltransferase at the C-terminus. Crystal structures of OvoA are not yet available. However, homology models of the N-terminal part of OvoA have been constructed based on the crystal structure of EgtB from *Mycobacterium thermoresistibile*.^{28,11,29} EgtB is a sulfoxide synthase that contributes to the biosynthesis of ergothioneine (**24**, Figure 2).³⁰ Instead of cysteine and histidine, this enzyme accepts N α -trimethyl histidine (**23**) and γ -glutamyl cysteine as substrates. Instead of attaching sulfur to the imidazole

5-position, EgtB attaches the sulfur to the 2-position (**16**, Figure 3). These differences, compounded by limited sequence identity between OvoA and EgtB (25 %) make detailed interpretation of the OvoA homology model difficult.

The overall structure of the sulfoxide synthase module of OvoA is characterized by a four-helix bundle (DinB_2 domain) and a domain that is structurally most related to that of copper-dependent formylglycine-generating enzymes.³¹ The iron-binding active site is housed in a deep cleft between these two domains. So far, EgtB and OvoA are the only known enzymes with this two-domain architecture. Finding homologous enzymes that catalyze different reactions, or understanding why this particular protein architecture is exclusive to thiohistidine biosynthetic enzymes, is an intriguing challenge for future research.

OvoA coordinates iron (II) by a three-histidine facial triad. After recruiting the substrates histidine, cysteine and oxygen to the three remaining coordination sites, the enzyme initiates carbon-sulfur bond formation, followed by oxygen atom transfer onto the thioether function (Figure 3). Because the attached oxygen atom is not retained in the final product, and is irrelevant for the two following reactions, sulfoxidation seems to occur only to return OvoA to the initial ferrous state. The mechanism by which sulfoxide synthases mediate oxygen activation and guide the activated intermediates along this complicated reaction trajectory are not yet clear. Structural and kinetic observations based on the ergothioneine biosynthetic sulfoxide synthase EgtB from *M. thermoresistibile* are suggestive of the catalytic mechanism depicted in Figure 3. Important tenets of this model are the following: binding of all three substrates produces an iron (III)-superoxo species (**17**, Figure 3); proton transfer from an active site tyrosine (Tyr377) to this species facilitates oxidation of the substrate cysteine to a thiyl radical (**18**) which then attacks the imidazole ring of the second substrate (A, Figure 3).^{32,33} Oxygen transfer and deprotonation of this intermediate (**19**) complete the reaction cycle. This model has been challenged by two independent computational studies.

One study based on density functional theory (DFT)³⁴, and one based on combined quantum and molecular mechanics (QM/MM)³⁵ explored different sequences of the elementary steps and an alternative role of Tyr377. The DFT study concluded that species **17** directly transfers an oxygen atom to the sulfur atom of γ -glutamyl cysteine (**25**, Figure 3), and consequently, that the imidazole ring of N α -trimethyl histidine is attacked by the sulfinyl rather than the thiyl radical (**26**). One problem of this mechanism is that the stereochemistry of the resulting sulfoxide product (**27**) is inconsistent with the empirically determined structure of the EgtB product **16**.³² The QM/MM study raised the possibility that Tyr377 could transfer a hydrogen atom to species **17** to produce an iron

hydroperoxo species and a tyrosyl radical (**28**). It is not yet clear, whether this species can directly lead to C-S bond formation (**29**), or whether it may be an off-pathway species that equilibrates with species **17**.

Arbitration between these competing proposals may come from parallel characterization of distant EgtB homologs such as OvoA. Despite low sequence similarity and different physiological functions, OvoA shares key functional features with EgtB. For example, OvoA can also accept TMH as a substrate and attaches the cysteine sulfur atom to the 2-position instead of the 5-position on the imidazole ring.³⁶ The resulting product is an ergothioneine precursor. Inherent EgtB-like side activity of OvoA may have enabled certain cyanobacteria to recruit an ancient OvoA-homolog for ergothioneine biosynthesis.³⁷ Characterization of an OvoA homolog from *Microcystis aeruginosa* revealed that this enzyme lost its ability to make ovothiols and instead became a proficient EgtB ortholog. Functional adaptation required so few mutations that ergothioneine making and ovothiol making OvoA homologs cannot be distinguished based on global sequence similarity. Facile transition between EgtB- and OvoA-like activity suggests that the catalytic mechanisms of the two types of sulfoxide synthase are essentially the same.

A recent study confirmed that OvoA contains a structurally equivalent active site tyrosine (Tyr417) that plays the same pivotal role as Tyr377 in EgtB.³⁸ This study also showed that this Tyr residue could be substituted with the non-natural tyrosine derivative 2-amino-3-(4-hydroxy-3-(methylthio)phenyl)propanoic acid (MtTyr) using stop-codon suppression technology. This non-natural mutation resulted in decreased sulfoxide synthase activity, suggesting that even minimal perturbation at this site interferes with catalysis. The ability to modulate the active sites of sulfoxide synthases with atomic precision may prove an invaluable tool to decipher the catalytic mechanism of these enzymes.

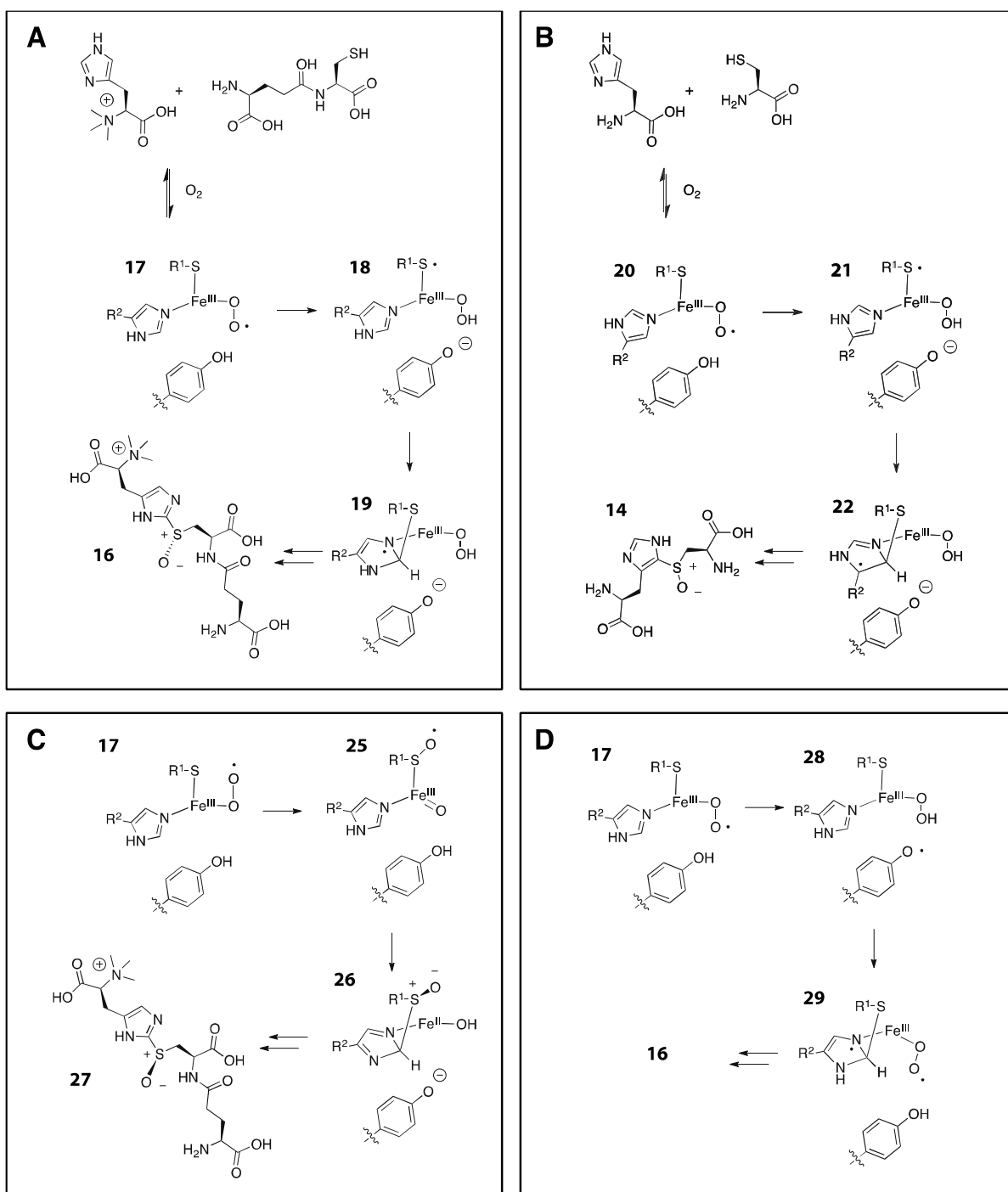


Figure 3. Proposed reaction mechanism of EgtB (A). A similar mechanism could be formulated for OvoA (B). Alternative models of EgtB catalysis proposed based on DFT (C) or QM/MM calculations (D).

The second reaction in ovothiol biosynthesis is catalyzed by a PLP- dependent β -lyase.³⁹ Comparison of OvoA encoding genomes from many organisms did not reveal any gene coding for a β -lyase exclusively dedicated to ovothiol biosynthesis. Given that β -elimination of cysteine S-conjugates is a fairly simple and a very common reaction, it is possible that most organisms use unspecific β -lyases to support ovothiol production. The last step is methylation of 5-thiohistidine (**15**) at N π of the imidazole ring. This SAM-dependent reaction is catalyzed by the C-terminal domain of OvoA (Misson, Liao Seebeck, unpublished). The methyltransferases that can methylate the α -amino group of ovothiol A to form ovothiol B and C (Figure 1) are not yet known.

Based on what we currently know about ovothiol A-biosynthetic enzymes, we can conclude that ovothiol emerges from a remarkably simple biosynthetic pathway that starts with a very complicated carbon-sulfur bond forming reaction. Cellular ovothiol production depends on a single polypeptide that encodes both essential enzymatic activities. This simplicity may have facilitated dissemination of this biological trait by horizontal gene transfer. Once fully understood this enzymatic machinery should provide a robust platform for the biotechnological production of this potential therapeutic natural product. An alternative way to produce ovothiol would be total chemical synthesis. The different synthetic routes explored so far remain cumbersome.^{40,41,42,43} Most importantly, two recent studies showed that 5-thiohistidine (**15**) and ovothiol A can be made from the natural precursor L-histidine following relatively simple 2- and 5-step syntheses.^{43,44}

Insight into the evolutionary history of the ovothiol biosynthetic pathway.

Definitive identification of ovothiol biosynthetic enzymes cleared the view over the distribution of ovothiol production activity across the tree of life. Close analysis of this distribution provides an alternative approach to understanding the cellular function of ovothiol.

The distribution of ovoA genes among microorganisms follows a very complex pattern. Bacteria and lower eukaryotes inhabiting very different ecological niches appear to have acquired this gene by horizontal gene transfer driven by evolutionary pressures that may be different for each individual genus or species. The most interesting question here is as to when and in what species OvoA-like sulfoxide synthases diverged from EgtB-like enzymes to make ovothiol instead of ergothioneine. Because this event may date back almost two to three billion years, this will be a challenging question to answer solely based on sequence comparisons.

The evolutionary history of ovothiol biosynthesis in metazoans is interesting for another reason. Analysis of metazoans' genomes revealed that *ovoA* is highly conserved in marine metazoans, from Porifera to Placozoa, Cnidaria, protostomes and deuterostomes.¹¹ Among deuterostomes, *ovoA* is conserved in Echinodermata and Hemichordata, Cephalochordata, Urochordata, and in Chondrichthyes (Figure 4). Interestingly, the *ovoA* gene has been lost at least at two key events in metazoan evolution (red cross, Figure 4). One such event occurred in ancestral ecdysozoa (nematodes and arthropods) rendering most of terrestrial species – insects, for example – unable to make ovothiol. This pattern indicates that loss of ovothiol production may be related to the transition from an aquatic to a terrestrial life-style.

Another gene-loss event traced to the last common ancestor of Osteichthyes fish.¹¹ This loss does not necessarily mean that fish lost their appetite for ovothiol. In fact, ovothiols have been reported to be found also in the eggs of the rainbow trout, *Salmo gairdneri*, as well as from the eggs of the Coho salmon, *Oncorhynchus kisutch*.⁴⁵ Most likely these higher animals acquire ovothiol through their nutrition, which in the case of salmon includes ovothiol producing mollusks and crustaceans. In a way, it seems that bony fish simply out-sourced ovothiol production down the food chain but still rely on this antioxidant as a micro-nutrient or a vitamin. This interpretation highlights an interesting analogy to the distribution of ergothioneine and ergothioneine producing genes in terrestrial species. A growing number of observations document the physiological importance of ergothioneine in human physiology.⁴⁶ However, higher eukaryotes including most plants and animals do not synthesize ergothioneine on their own, but they acquire this metabolite from their nutrition by way of an ergothioneine-specific transporter protein.⁴⁷ Meanwhile, production of ergothioneine is almost exclusively a bacterial and a fungal activity. If ovothiol is indeed a micro-nutrient for bony fish, there should be a specific ovothiol transporter protein. Identification of the corresponding gene would pave the way to examine the localization, regulation and developmental significance of ovothiol in higher animals. It is clear that such an investigation would produce important insight as to how ovothiol may be exploited as a therapeutic compound. A comprehensive overview of the evolutionary history of metazoan *ovoA* genes may also help to identify other events of *ovoA* gene loss, duplication and diversification. A third objective for future investigations may be addressing the question as to when and from where the *ovoA* gene entered the metazoan lineage.

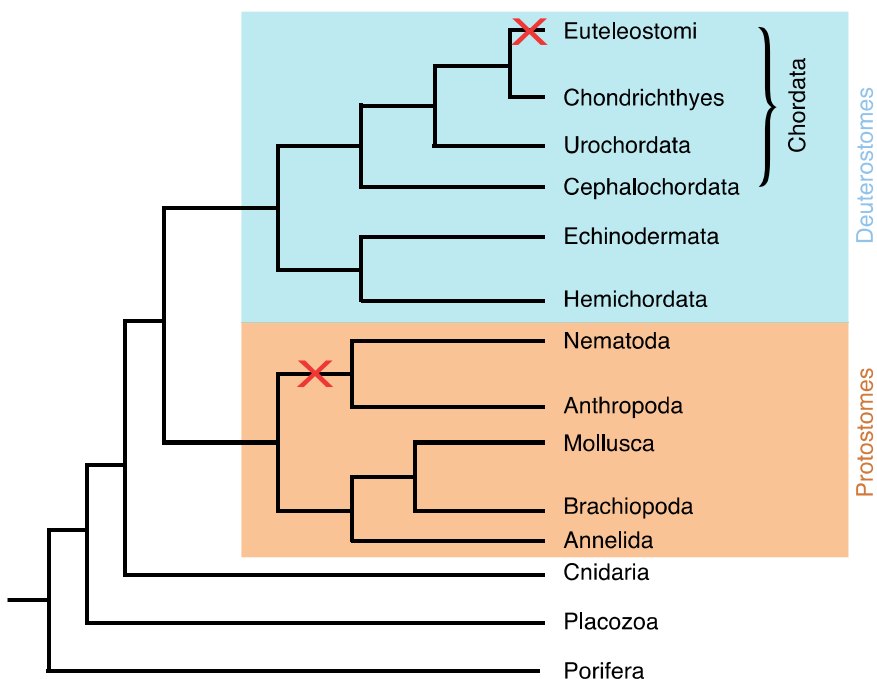


Figure 4. Scheme of phylogenetic distribution of OvoA in metazoans.

Unravelling the biological roles of ovothiols

The genomic structure of - such as the exon–intron boundaries - and transcriptional regulation of the ovoA gene *P. lividus* has been characterized recently.¹¹ This analysis highlighted that the biosynthesis of ovothiol is important during fertilization as well as during larval development. After the consumption of maternal ovoA transcript, embryos start ovoA transcription at the pluteus stage, the time when larva starts to reorganize its body plan to go in metamorphosis. Throughout sea urchin development, ovoA transcription is regulated by metals and toxic algal blooms.¹¹ This regulatory link between chemical stress and ovothiol production makes much sense. Sea urchins are broadcast spawners, meaning they release gametes in the seawater column where fertilization and embryo development occur. In this environment vulnerable embryos and larvae are exposed to many kinds of organic or inorganic toxins which can induce oxidative stress. Hence, active up-regulation of ovoA in the pluteus stage suggests that ovothiol protects cells not only during the post-fertilization oxidative burst, but also in later stages, regulated by redox-sensitive pathways.

Recently ovoA transcription was also found to be up-regulated in *Nematostella vectensis* (starlet sea anemone), in response to dispersant and/or sweet crude oil exposure alone or combined with ultraviolet radiation (UV).⁴⁸ These data confirm the involvement of this metabolic pathway in the defence mechanisms from environmental factors and highlight the importance of these sulphur-containing amino acids for UVA protection in area more exposed to radiation such as the poles.

In *Euglena gracilis*, a highly complex alga belonging to the green plants, different ovoA transcripts have been identified through the analysis of its transcriptome.⁴⁹ In particular, a complete transcript covering all the three characteristic domains of OvoA, has been found during algal cell growth in dark conditions, whereas two different sequences, one covering the OvoA N-terminal domain and one covering the OvoA C-terminal domain were detected in both dark and light transcript sets. It will be very intriguing to investigate in the future whether this pathway can be finely regulated at the transcriptional level depending on the light dark cycle, or if these different transcripts encode for different enzymatic activities. Overall these studies suggest that ovothiol may be involved in plasticity response of organisms to the changing conditions of the environment.

The increasing availability of new genomes and of relevant amounts of transcriptomic data for different marine species, for what concerns both adult tissues, tissues subject to biotic and abiotic stress, and larval stages, will allow in the next future to comparatively investigate the tissue distribution of OvoA transcript, its regulation under different conditions and to give insight into ovothiol function. Moreover, thanks to the fundamental developments of advanced gene technology applied to marine organisms⁵⁰, all the old theories on the biological function of ovothiol and related marine thiohistidines may be revised, opening new concepts and ideas.

Therapeutic potential of ovothiols

Discovery of compounds with therapeutic potential is one of the main objective of natural product research. In this quest the ocean has emerged as a nearly unlimited resource. Since its discovery, ovothiol has already attracted some interested as a possible therapeutic.⁵¹ However, because the proper roles of ovothiol are still somewhat obscure, we believe that the therapeutic potential of ovothiol is still largely underappreciated. In addition, because humans and other terrestrial vertebrates do not biosynthesize or require ovothiol, it is possible that OvoA is a valid target to treat infections by pathogenic microorganisms, including *Trypanosoma*, *Leishmania* or *Trichomona*. Advances in these directions clearly hinge on progress in understanding of ovothiol and ovothiol

biosynthetic enzymes in their cellular context. In the following we will summarize the current approaches to valorizing ovothiol.

The first study to investigate the biological activity of ovothiol derivatives demonstrated that the ovothiol derivative 1-methyl-2-[3-trifluoromethyl-phenyl]-4-mercapto-imidazole (MFP-4MI) is a neuroprotective agent for the mammalian brain. Mice exposed to oxidative stress caused by magnesium deficient diet and treated with the reduced form of MFP-4MI resulted in anticonvulsant protection when exposed to an auditory signal inducing a sound-produced seizure. Moreover, the compound inhibited cell death in grey and white matter and reduced brain injury.⁵² The finding that the oxidized form of the compound, exhibited *in vivo* cerebroprotective properties similar to those of the reduced form, led to the identification of several recycling mechanisms consistent with the conversion of the oxidized to the reduced compound, and providing strong evidence that recycling (thiol-disulphide exchange) properties of the oxidized form are involved in modulating neuroprotective processes in the same way of the antioxidant reduced form.

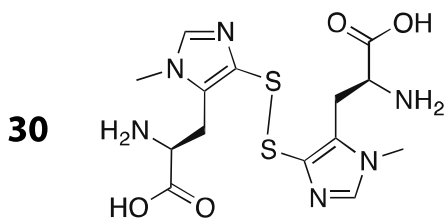


Figure 5. Disulfide of ovothiol A (30)

Recently, ovothiol A (1), purified from *P. lividus* eggs in its disulfide form (**30**, Figure 5), have been also shown to induce a decrease in cell proliferation with the concomitant activation of an autophagic process in human hepatocarcinoma cell lines, Hep-G2⁵³, thus suggesting a potential role of the molecule in the regulation of tumor cell growth. Autophagy is a finely regulated catabolic process, often associated to nutrient deprivation, which cells can adopt to survive under stress conditions or self-suicide. Changes in the autophagic process are the cause of many pathological conditions, including liver diseases and some types of cancer.⁵⁴ Whereas it has been previously demonstrated ovothiol A, administered in the form of disulfide in human cells, is reduced by the GSH/GSSG system within the cell⁵² this may not be the case of Hep-G2, in which ovothiol could not enter cancer cells. This hypothesis was supported by the finding that ovothiol was not detected inside in the cells, and was not able to reduce reactive oxygen species.⁵³ In this case, it is very likely that ovothiol A interact with a membrane surface protein, and subsequently induce a molecular pathway leading to autophagy. Considering that ovothiol A is a modified amino acid, it will be very interesting to

its potential involvement in recycling of amino acids inside the cells and in inducing protein synthesis, two processes both involved in cell proliferation and autophagic mechanisms.

However, ovoidiol A plays pleiotropic activities depending on cellular systems and different molecular targets affected. When evaluating the effects of the marine-derived ovoidiol A in an *in vitro* cellular model of pathology associated to chronic low-grade inflammation and oxidative stress, the molecule adopts a different behavior, resembling that of an antioxidant.⁵⁵ Diabetes is one of the most widespread pathology associated with oxidative stress and vascular chronic inflammation, alterations underlying the development of cardiovascular disease. Endothelial dysfunction is the main cause of cardiovascular disease development and is characterized by an increased expression of endothelial adhesion molecules and the recruitment of monocytes in the intima, a pivotal and critical event in promoting atherosclerosis. In an *in vitro* model of hyperglycemia-induced endothelial dysfunction, that is human umbilical vein endothelial cells from women suffering from gestational diabetes and from healthy mothers, ovoidiol A in its disulfide form was rapidly taken up by both cell types, and partially reduced inside, most likely by redox exchange with GSH/GSSG cellular system.⁵⁵ In this case, in fact, ovoidiol A seems to act as an antioxidant partially scavenging ROS and peroxynitrite and making NO freely to modulate endothelium functionality. The maintenance of endothelial NO bioavailability is indeed considered beneficial to endothelial functions and more in general to vascular health because it counteracts the pro-inflammatory response in the early stages of atherosclerosis, especially during chronic hyperglycemia. This effect was more evident when cells were stimulated with Tumor Necrosis Factor- α (TNF- α), and ovoidiol A induced a significant down-regulation of adhesion molecules, responsible for the reduction of monocyte–endothelium interaction.⁵³ These results pointed to the potential anti-atherogenic properties of the natural antioxidant ovoidiol A, and highlight its therapeutic potential in pathologies related to cardiovascular diseases associated to

Conclusions

Ovothiol A (1) and its derivatives B (2) and C (3) are fascinating sulfur natural products with unique chemical properties. 5-thiohistidines behave as excellent nucleophiles and as reducing agents that in principle may protect cells from reactive oxygen and nitrogen species, electrophilic organic compounds and thiophilic metals. However, elucidating the biological role of ovothiol in marine organisms will also require systematic analysis of the distribution and regulation of proteins that make, transport and interact with ovothiol. The discovery of ovothiol biosynthetic genes showed that ovothiol production is a frequent trait in marine bacteria and metazoans. Description of the transcriptional activity of the *ovoA* gene in larval stages of sea urchins indicated that protection by ovothiol is not limited to the post-fertilization events. Identification of ovothiol as a regulatory factor in green microalgae suggests that ovothiol can interact with proteins - likely by forming mixed disulfide bonds with surface cysteines. Finally, finding of ovothiol from salmon – a bony fish that encodes no *ovoA* gene – suggests that higher animals may acquire ovothiol as a micro-nutrient, presumably through an ovothiol-specific transporter protein. These are intriguing leads, suggesting that ovothiol is a ubiquitous and highly integrated component in marine biochemistry. Advances in these directions will illuminate the complex role of ovothiol. This research will also address the question as to, why ovothiol is much more abundant in marine ecosystems than in terrestrial ecosystems. Also, mechanistic appreciation of cellular ovothiol function would provide a platform to evaluate and validate the therapeutic potential of this marine natural product.

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Conflicts of interest

There are no conflicts of interest to declare.

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