Organic LETTERS

Letter

Cite This: Org. Lett. XXXX, XXX, XXX−XXX

pubs.acs.org/OrgLett

In Vitro Reconstitution of the Remaining Steps in Ovothiol A Biosynthesis: C−S Lyase and Methyltransferase Reactions

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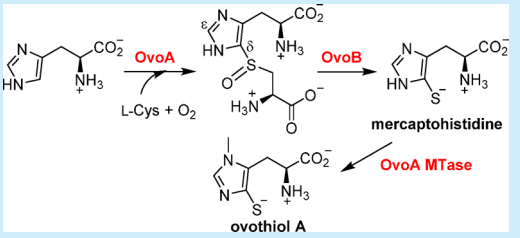
Supporting Information

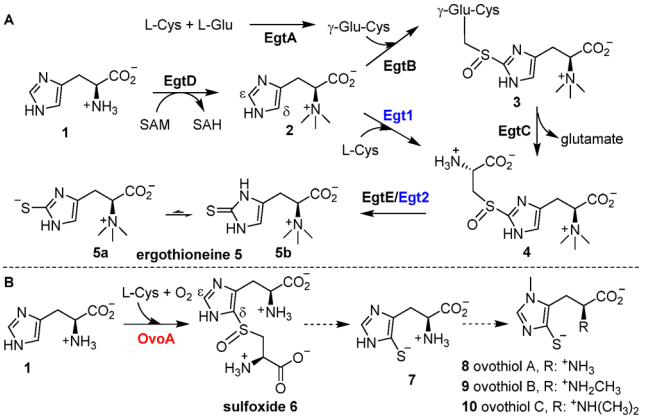
ABSTRACT: Ovothiols are thiolhistidine derivatives. The first step of ovothiol biosynthesis is OvoA-catalyzed oxidative coupling between histidine and cysteine. In this report, the remaining steps of ovothiol A biosynthesis were reconstituted *in vitro*. ETA_14770 (OvoB) was reported as a PLP-dependent sulfoxide lyase, responsible for mercaptohistidine production. OvoA was found to be a bifunctional enzyme, which mediates both oxidative C−S bond formation and methylation of mercaptohistidine to afford ovothiol A. Besides reconstituting the whole biosynthetic pathway, two unique features proposed in the literature were also examined: a potential cysteine-recycling mechanism of the C−S lyase (OvoB) and the selectivity of the *π*-*N* methyltransferase.



Sulfur-containing natural products are widely distributed in nature and include classes of compounds such as amino acids, nucleotides, enzyme cofactors, and secondary metabolites.[1−7] Ergothioneine **5** and ovothiols (**8**−**10**) are histidine derivatives with a sulfur substitution at the *ε*- and *δ*-carbons of the imidazole side chain, respectively.[8,9] Ovothiol was first isolated from the eggs and ovaries of sea urchins.[10−12] Depending on the degree of methylation at the amino group, there are three forms of ovothiol: A (**8**, unmethylated), B (**9**, monomethylated), and C (**10**, dimethylated) (Scheme 1B). The pK_a of the ovothiol thiol group (∼1.4) is significantly more acidic than other natural thiols.[13−15] As a result, ovothiol A exists predominantly in the thiolate form under physiological conditions and functions as a potent radical and peroxide scavenger.[16,17] Ovothiol-producing pathogens and marine metazoan embryos utilize this property to protect themselves from oxidative stress.[18−20] Ovothiol A has also been demonstrated to induce autophagy in human liver carcinoma cell lines, suggesting functions other than solely being a redox agent.[21]

About two decades ago, using partially fractionated cell lysate from an ovothiol-producing strain *Crithidia fasciculate*, Steenkamp and co-workers characterized ovothiol A biosynthesis and proposed that this pathway (**6** → **7** → **8**) involves a sulfoxide synthase and a PLP-dependent lyase, and a sulfur transfer process occurs prior to methylation (Scheme 1B).[22,23] In addition, Steenkamp et al. suggested the existence of an intriguing cysteine recycling mechanism in the C−S lyase step (**6** → **7** transformation, Scheme S1). Recently, two aerobic ergothioneine biosynthetic pathways were discovered (Scheme 1A): the *Mycobacterium smegmatis* pathway (EgtA−EgtE, Scheme 1A and Table S1)[24−31] and the fungal *Neurospora crassa* pathway (Egt1, Egt2, and an unidentified methyltrans-

Scheme 1. Two Aerobic Ergothioneine Biosynthetic Pathways (A) and Proposed Ovothiol Biosynthesis (B)



Received: July 24, 2018

DOI: 10.1021/acs.orglett.8b02332
Org. Lett. XXXX, XXX, XXX−XXX

ferase (MTase)) (Scheme 1A and Table S1).[32,33] In these two pathways, the two important steps are nonheme iron enzyme catalyzed oxidative C−S bond formation (EgtB, Egt1) and reductive C−S lyase reaction (EgtE, Egt2). Such a trans-sulfuration strategy differs from all other trans-sulfuration reactions reported thus far.[5,34] Based on this ergothioneine biosynthetic information, Seebeck and co-workers identified a nonheme iron enzyme OvoA in *Erwinia tasmaniensis*, which catalyzes the oxidative coupling between L-His and L-Cys in the ovothiol pathway (Scheme 1B and Table S1).[32,35−39] In this report, we reconstituted the two remaining steps of the ovothiol biosynthetic pathway: the C−S lyase and MTase.

To find the ovothiol C−S lyase, we analyzed the *E. tasmaniensis* genome and generated a protein−protein family network using Cytoscape 3.6.0[40] based on protein family information from the pfam database.[41] We identified 16 genes belonging to aminotransferase class I and II (Figure S1) as the C−S lyase candidates.[31,32] We used the String database to further analyze the relationship between OvoA and these 16 candidates based on gene co-occurrence, fusion, gene neighborhoods, and functional association information.[42] The String database analysis suggests that ETA_14770 is functionally related to OvoA in ovothiol biosynthesis (Figure S1). *E. tasmaniensis* has not been reported as an ergothioneine producer, even though ETA_14770 has been applied as an ergothioneine C−S lyase,[24] raising the question of the ETA_14770 true identity. To address this issue, we purified ETA_14770 (Figure S2) and measured the kinetic parameters by coupling ETA_14770 catalysis using ovothiol/ergothioneine sulfoxide substrates with pyruvate reduction by lactate dehydrogenase (Table 1).[43]

Table 1. Kinetic Parameters for OvoB and Egt2

enzyme	substrate	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
ETA_14770 (OvoB)	sulfoxide **4**	16.4 ± 0.6	23
ETA_14770 (OvoB)	sulfoxide **6**	56.4 ± 2.6	167
Egt2[33]	sulfoxide **4**	8.7 ± 0.1	56
Egt2	sulfoxide **6**	0.64 ± 0.02	0.4

Indeed, ETA_14770 can accept compound **4** as a substrate (Table 1, Figures S3 and S4).[31] When compound **6** was used as the ETA_14770 substrate (Figure S5)[36] in the presence of dithiothreitol (DTT) as a reductant, 5-mercaptohistidine **7** was produced (Scheme 1B and Figure S6) along with pyruvate and ammonia in a 1:1:1 ratio (Figures S7 and S8). In this reaction, DTT most likely reduces a sulfenic acid intermediate to thiol **7** as reported in our ergothioneine C−S lyases EgtE/Egt2 studies (Scheme S2).[31,33] Compound **7** was purified aerobically and isolated in its stable disulfide form (compound **7a**, Figures S9 and S10).[9,44,45] Using sulfoxide **6** as the substrate, ETA_14770 exhibited a catalytic efficiency of 167 mM^{-1} s^{-1} (Table 1 and Figure S4), which is significantly better than any other combinations listed in Table 1 (ETA_14770/sulfoxide **4**, Egt2/sulfoxide **4**, and Egt2/sulfoxide **6**, Table 1 and Figure S11). Results from this comparative kinetic characterization implied that ETA_14770 is most likely the ovothiol C−S lyase (catalyzing **6** → **7** reaction), and we named this enzyme OvoB. Due to the similarities between ergothioneine and ovothiol biosynthesis, OvoB catalysis most likely follows a mechanistic model similar to that of EgtE/Egt2 catalysis reported (Scheme S2).

Compounds **4** and **6** are structurally distinct in several aspects. First, the sulfoxide functionality is at the ε-carbon and δ-carbon of the imidazole ring of compounds **4** and **6**, respectively. Second, in compound **4**, the amine group is trimethylated, while in compound **6**, the primary amine group is unmethylated. To understand the selectivity of the ETA_14770 (OvoB)-catalyzed reaction, we obtained the crystal structure of PLP-bound OvoB at 2.7 Å resolution through a molecular replacement method (Figure 1 and Table S2). The final model of OvoB is comprised of residues 11 to 389, with the N-terminal 10 residues disordered. Similar to our recently reported ergothioneine C−S lyase Egt2,[33] the OvoB central catalytic domain (residues 94−256) has a classic seven-stranded β-sheet wrapped within six α-helices, which is a typical folding feature in type I PLP-dependent enzymes (Figure 1A and Figure S12).[46] The N-terminal (residues 11−93) and C-terminal domains (residues 257−389) form a lid that shelters the PLP-binding pocket and the active site (Figure S12). The active site is located at the dimer interface, formed by the catalytic and C-terminal domains of one monomer along with the N-terminal and C-terminal domains of the other monomer (Figure S12). In the active site, an internal aldimine between the PLP cofactor and K240 was observed. The PLP pyridine ring and the aromatic ring of Y125 form a $\pi-\pi$

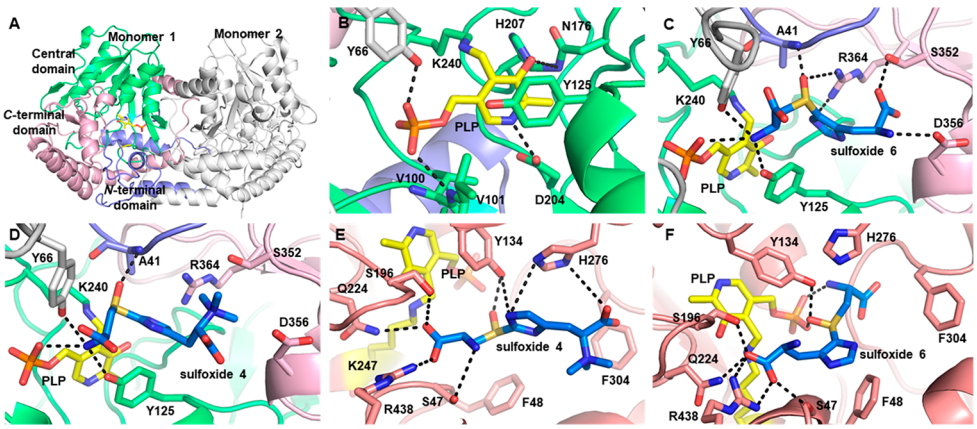


Figure 1. OvoB structural analysis. (A) Overall structure of OvoB. (B) The active site of OvoB shows the interaction network of residues around the PLP cofactor (yellow stick). (C) Model of the OvoB•substrate **6** (blue stick) binary complex. (D) Model of the OvoB•sulfoxide **4** (blue stick) binary complex. (E) Our reported structure of the Egt2•substrate **4** binary complex (salmon). (F) Model of the Egt2•substrate **6**.

DOI: 10.1021/acs.orglett.8b02332
Org. Lett. XXXX, XXX, XXX−XXX

stacking interaction. The orientation of the PLP pyridine ring is anchored by a side chain of N176, D204, and H207. The phosphate group of PLP forms hydrogen bonds with side chain of Y66 and the main chain amide of V100 and V101 (Figure 1B).

We also sought to obtain the structure of the OvoB•substrate **6** binary complex. This effort was unsuccessful, however, due to the high turnover number of OvoB. To probe the substrate selectivity of OvoB, we modeled substrates **4** and **6** separately into the OvoB structure (Figure 1C and D) and compared them with the reported structure of ergothioneine C−S lyase Egt2•compound **4** binary complex and Egt2•compound **6** docking model (Figure 1E and F).[33] In the OvoB•compound **6** docking model, the histidine carboxyl group of substrate **6** forms a salt bridge with R364 and a hydrogen bond with S352, while the amino moiety forms a hydrogen bond with the side chain of D356. The orientation of the histidine imidazole ring is loosely defined through a hydrogen bond with R364. The sulfoxide of substrate **6** forms a hydrogen bond with the side chain of R364, and this position is further strengthened through a hydrogen bond with the main-chain amide of A41. The amino group of the cysteine moiety forms hydrogen bonds with the side chains of Y66 and Y125 and the PLP phosphate group. To explain substrate preference of OvoB for sulfoxide **6** over sulfoxide **4**, we modeled sulfoxide **4** into the OvoB structure (Figure 1D). In this model, the cysteine amino group maintains hydrogen bonds with the side chain of Y66 and Y125 and the PLP phosphate group. However, due to the difference in C−S bond locations (ε-position in **4** vs δ-position in **6**), most of the interactions predicted for the histidine portion in the OvoB•**6** complex are absent in the OvoB•**4** complex. The sulfoxide forms only one hydrogen bond with the main-chain amide of A41, while the interactions between the R364 side chain to both sulfoxide and histidine imidazole ring predicted in the OvoB•**6** complex are not observed in the OvoB•**4** complex. In addition, in our reported Egt2•**4** binary complex,[33] the *N*-trimethylamine of sulfoxide **4** forms a cation−π interaction with F304 (Figure 1E), and this interaction is not observed in OvoB either (Figure 1C and D). We also generated the model of the Egt2•substrate **6** complex (Figure 1F). Similar to the OvoB case, the difference in C−S bond location in the substrate dictates its interaction with Egt2. The histidine imidazole of compound **4** forms hydrogen bonds with Y134 and H276, while these interactions are absent in the model Egt2•substrate **6** complex. These docking results are consistent with the selectivity of OvoB for compound **6** and Egt2 toward compound **4**, respectively. Notably, OvoB exhibits a larger active site (~700 Å calculated by Dogsite)[47] relative to that of Egt2 (~600 Å).[33]

In 2001, using cell lysate of *C. fasciculate*, an ovothiol-producing strain, as the catalytic system and in the presence of DTT as the reductant, Vogt et al. reported that L-Cys was observed as one of the products in ovothiol A C−S lyase reaction, and a mechanistic model was offered to explain this unexpected result (Scheme S1).[22] Our OvoB structural characterization indicated that the active site of OvoB is slightly larger than that of Egt2, suggesting a possibility of accommodating additional molecules in the OvoB active site for the proposed cysteine regeneration activity. With the establishment of the *in vitro* catalytic system, we repeated the OvoB reaction using [3′-^{13}C]-labeled sulfoxide **6a** (structural information in Figure S13). ^{13}C NMR analysis revealed the production of only [3-^{13}C]-pyruvate (Figure S13), without any detectable [3-^{13}C]-Cys. This result suggested that OvoB does not support cysteine regeneration, and the observed L-Cys production in the Vogt report is most likely a result from other enzymes in the cell lysate mixture. In our studies, we also observed that PLP alone is capable of mediating the C−S lyase reaction in the presence of DTT to afford thiol **7**, pyruvate, and ammonia in a 1:1:1 ratio (k_{cat} = 0.31 ± 0.03 s^{-1}, Figures S8, S14, and S15). However, this activity is nearly two orders of magnitude less active than that of OvoB.

After successfully reconstituting the OvoB activity *in vitro*, we sought to identify the remaining π-*N*-MTase in ovothiol A biosynthesis. Our bioinformatic analysis of the *E. tasmaniensis* genome garnered 52 MTases; however, none of these is functionally related to OvoA based on String database analysis. Interestingly, pfam analysis revealed that OvoA has three domains: the iron-binding DinB_2 and formylglycine (FGE)-sulfatase domain for sulfoxidation activity and the uncharacterized MTase domain.[32] Recently, Seebeck and co-workers suggested the possibility of having the OvoA MTase domain being responsible for π-*N*-methylation (**7** → **8**, Scheme 1B).[48] In ergothioneine biosynthesis, the fungal *N. crassa* Egt1 gene also encodes three domains, a DinB_2 and FGE-sulfatase domains for C−S bond formation and a MTase domain. Similarly, the Egt1 gene was proposed as a bifunctional enzyme, responsible for both sulfoxidation and methylation in ergothioneine biosynthesis.[49] However, the proposed MTase activity in Egt1 was not observed in our *in vitro* Egt1 characterization.[32] To test whether OvoA functions as a bifunctional enzyme, we examined OvoA MTase activity using thiol **7** and *S*-adenosylmethionine (SAM) as the substrates. OvoA indeed methylates compound **7** to afford ovothiol A. The kinetic parameters of OvoA MTase activity were measured using coupled assays commercially available from Promega (MTaseGlo, Promega). In this coupled assay, the side-product *S*-adenosylhomocysteine (SAH) was converted to ATP through a series of enzymatic transformations (Scheme S3).[50] The amount of ATP was then quantified by luminescence intensity generated from ATP-involved luciferase catalysis. Through this assay, OvoA kinetic parameters were measured: k_{cat} of 3.05 ± 0.17 min^{-1} and K_m of 7.81 ± 1.18 μM for SAM at saturating concentration of compound **7** and k_{cat} of 3.37 ± 0.19 min^{-1} with K_m of 7.03 ± 1.15 μM for compound **7** at saturating SAM concentration (Figures S16 and S17). To examine the regioselectivity of the methylation reaction, compound **8** was isolated and characterized (Figure S18). Similar to thiol **7**, ovothiol A was isolated as a dimer. Upon the addition of DTT, the reduced form of ovothiol A was obtained (Figure S19). The methylation of ovothiol A **8** on π-*N* position instead of the τ-*N* position was confirmed by 2D COSY NMR spectroscopy (Figure S20). This OvoA-catalyzed *N*-methylation reaction is among the very few reported cases of histidine side-chain *N*-methylation. Thus, far, carnosine *N*-methyltransferase 1 is the only reported structure on such a type of enzymes.[51] The identification of OvoA as an *N*-MTase could pave the way for future structure−functional studies on this subclass of MTases.

In summary, we have biochemically reconstituted the remaining two steps in ovothiol A biosynthesis (C−S lyase and MTase) and established the ovothiol A pathway *in vitro*, which could facilitate future mechanistic studies of these enzymes and ovothiol A production through metabolic engineering.

DOI: 10.1021/acs.orglett.8b02332
Org. Lett. XXXX, XXX, XXX−XXX

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.8b02332.

Experimental details, NMR spectra of products, and kinetic study results (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The invention related to this work was made with Government Support under Contract No. CHE-1309148 awarded by the National Science Foundation to P.L., and the government has certain rights in the invention. The work here is also partially supported by the Strategic Priority Research Program (B) of the CAS (XDB20000000 to J. Z.), National Science Foundation of China (31430002 and 31320103911 to L.Z.), and Shanghai Science and Technology Committee (17DZ1205402). N.N. is partially supported by the Warren−McLoed Fellowship from the Boston University Marine Program. P.H. is supported by a fellowship from the China Scholarship Council. We thank the staff of the beamline BL19U1 of the National Center for Protein Science (Shanghai, China) for X-ray data collection.

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DOI: 10.1021/acs.orglett.8b02332
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