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Improved synthesis of the super antioxidant, ergothioneine and its biosynthetic pathway intermediates.

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Ergothioneine along with mycothiol, are low molecular mass redox protective thiols present in Actinomycetes, in particular mycobacteria. We report the improved chemical synthesis of ergothioneine (ESH) and biosynthetic pathway intermediates using either histidine or ESH as starting material. The detailed mechanism of ESH biosynthesis is not yet completely elucidated and substrates for enzymes in the pathway will provide valuable tools to aid this study. Particularly interesting is the PLP dependent β-lyase, EgtE, of mycobacteria, having the capability of cleaving the substrate, S-(β-amino-β-carboxyethyl)ergothioneine sulfoxide to pro-vide ESH. A synthetic route toward ESH pathway intermedi-ates also allowed the preparation of stable isotopically labelled hercynine-d_3 which was enzymatically transformed to ESH-d_3. The deuterated ergothioneine biosynthetic pathway metabolites are valuable tools for future studies.

Many gram positive bacteria, such as *Mycobacterium tuberculosis*, lack the redox protective molecule, glutathione and instead produce mycothiol and ergothioneine (**ESH**) as their principal low molecular mass thiols.[1,2] **ESH** is a thiohistidine betaine derivative with a thiol group at the C2 atom (ε-position) of the imidazole ring (**Scheme 1**). Present knowledge indicates that **ESH** may play a critical role in the *in vivo* and *in vitro* survival of mycobacteria.[3] Recently, it was found that ESH is actively secreted into culture media by *Mycobacterium smegmatis*.[4]

A structural variant of **ESH**, ovothiol A, also serve as an anti-oxidant albeit in sea urchin eggs as well as in the pathogens, *Leishmania major* and *Trypanosoma cruzi*.[5]

Humans do not synthesize **ESH**, but possess an active transport system, a cation transporter (OCTN1) with high specificity for its uptake from dietary sources.[6] The exact function of **ESH** in humans is currently under the spotlight in particular its potent antioxidant activity.[7] Recent commercial interest in **ESH** as a super anti-oxidant molecule has added even greater value to improved synthetic process development of this molecule.

In 1956, Heath *et al* elucidated ESH biosynthesis in *Claviceps purpurea*. He demonstrated that histidine or a compound closely related to histidine might be a precursor of **ESH**, subsequent publications disclosed the biosynthetic assembly of **ESH** utilizing organisms such as *Neurospora crassa* and *Mycobacterium smegmatis* with the aid of radio isotopic labelling (^{14}C and ^{35}S).[8,9,10]

Melville *et al* further established the participation of the S-(β-amino-β-carboxyethyl)ergothioneine sulfoxide as an intermediate in **ESH** synthesis by incubation of hercynine in cell-free extracts of *Neurospora crassa* in the presence of O2 and Fe^{2+}.[11]

It has now been established that **ESH** is synthesized by the sequential action of five enzymes, encoded by the genes *egtA, egtB, egtC, egtD* and *egtE* (**Scheme 1**).[12] EgtA is considered to be a γ-glutamyl cysteine ligase and catalyzes the formation of γ-glutamylcysteine. Histidine is methylated by an S-adenosylmethionine (SAM) dependant methyl transferase, EgtD, to give the trimethyl ammonium betaine, hercynine. Hercynine is then converted into S-(β-amino-β-carboxyethyl)ergothioneine sulfoxide (**II**), via an iron (II)-dependent oxidase (EgtB) which requires oxygen and γ-glutamylcysteine to produce γ-glutamylcysteinylhercynine (**I**). The exact nature of the latter transformation, in particular the sulfoxide formation, is still under investigation. Subsequently, a putative class-II glutamine amidotransamidase, EgtC, mediates the hydrolysis of the *N*-terminus glutamic acid, providing S-(β-amino-β-carboxyethyl)ergothioneine sulfoxide (**II**). Finally, EgtE, a pyridoxal 5-phosphate (PLP)-dependent β-lyase gives the final product, **ESH**.

Recently, the research focus with regard to these mercapohistidines has shed light on the mechanism of C-S bond formation at the δ- or ε-positions of the imidazole ring.[13] OvoA is an iron (II) dependent sulfoxide synthase which catalyzes the first step in ovothiol A synthesis and is a homolog of EgtB. Interestingly, the substrate specificity of EgtB *vs.* OvoA in achieving C-S bond formation differs significantly. OvoA is very selective for its sulfur donor substrate and only accept L-cysteine while it prefers histidine as co-substrate. However, EgtB require γ-glutamyl-L-cysteine as sulfur donor. Furthermore, it is selective toward α-*N,N,N*-methylation on the histidine, i.e. hercynine as co-substrate. Surprisingly, OvoA switches its sulfurization pattern on the histidine ring from the δ-carbon to the ε-carbon depending on the level of α-*N*-methylation.[14] Thus, OvoA converts hercynine directly into S-(β-amino-β-carboxyethyl)ergothioneine sulfoxide (**II**) and produce a minor amount of the δ-sulfoxide (ovothiol substitution pattern) when α-*N,N*-dimethyl histidine is used as the co-substrate (**Scheme 1**).
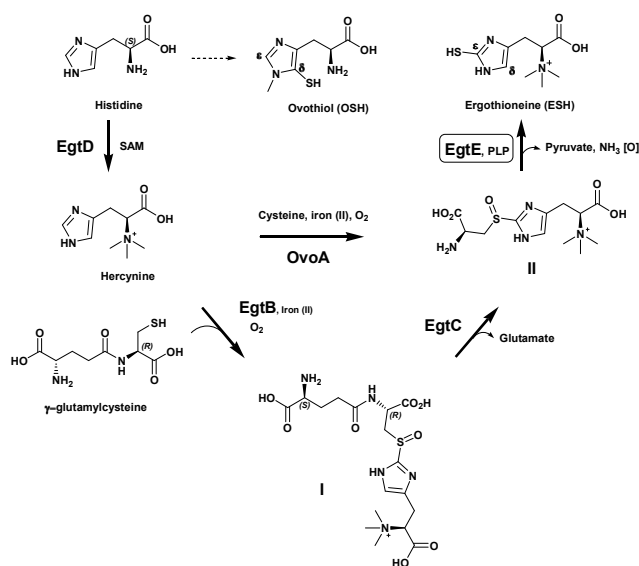
Scheme 1 ESH biosynthesis

While the enzymes EgtB and EgtC have been expressed in a functional form, EgtE is still elusive and none of the enzymes have been thoroughly studied due to the lack of readily available substrate intermediates.

Here we report the improved synthesis of the **ESH** and its biosynthetic precursor, S-(β-amino-β-carboxyethyl)ergothioneine sulfoxide (**II**). The latter sulfoxide is the substrate for the mycobacterial enzyme, EgtE. However, the absolute chirality of the sulfoxide is not known for the natural substrate or the synthetic one. Prior synthesis of intermediate (**II**) was reported in 1974 and were elaborate and irreproducible and resulted in a low overall yield of 8.5%.[15] The authors reported only the position of the aromatic proton resonance and no further structural confirmation. An optical rotation, $[\alpha]_D$ +74.4 (c = 0.5, H$_2$O), was reported and could not be reconciled with the authentic natural product $[\alpha]_D$ +9.1 (c = 0.5, H$_2$O). However, the m.p. of both natural and synthetic product was recorded as 188-190 °C. None-the-less, it was claimed that synthetic S-(β-amino-β-

carboxyethyl)ergothioneine sulfoxide (**II**) was extensively cleaved to **ESH** by crude cell free extracts of *Neurospora Crassa*.

A large number of naturally occurring sulfoxides have known absolute configurations and in some instances, the differences in biological activity of both diastereomers have been determined.[16] S-substituted cysteine sulfoxides occur almost exclusively in the R$_c$S$_s$ configuration in nature. It is therefore possible that the isolated S-(β-amino-β-carboxyethyl)ergothioneine sulfoxide (**II**) has a R$_c$S$_s$ configuration. However, the latter sulfoxide absolute chirality and its importance are yet to be established.

Two different routes to the target compound, S-(β-amino-β-carboxyethyl)ergothioneine sulfoxide (**II**) were considered. In this approach, retrosynthetic cleavage of S-(β-amino-β-carboxyethyl)ergothioneine sulfoxide (**II**) gave the β-chloro-alanine methyl ester and **ESH** (**Scheme 2, S1**) Thus, S-alkylation of a protected chloromethyl alanine ester (**2**), derived from serine, provided the core structure (**3**). The resulting sulfide (**3**) was oxidised using either *m*CPBA or H$_2$O$_2$.

Ishikawa *et al.* suggested that the reaction may possibly occur via the formation of the cyclic ethylenimine carboxylic acid intermediate produced by an intramolecular S$_N$2 reaction of the β-chloroalanine (**2**), followed by the ring opening induced by nucleophilic attack of the sulfur atom of **ESH**, giving the major product *N*-Boc methyl ester (**3a**).

Sulfoxidation reaction conditions with H$_2$O$_2$ previously investigated by Ishikawa *et al* led to an over oxidation to the sulfone in our hands and no analytical evidence were provided.[11] In order to prevent over oxidation of the sulfoxide, *m*CPBA was used. Its milder nature and potential for controlled sulfoxidation compare to hydrogen peroxide is advantageous. The sulfide methyl ester (**3a**) was subjected to S-oxidation using one equivalent of *m*CPBA to afford the sulfoxide methyl ester (**4a**). The synthetic product is most likely a mixture of R$_c$S$_s$ and R$_c$R$_s$ diastereomers. The lowest steric energy conformations (total energy 34.37 kcal/mol) of the sulfide methyl ester (**3a**) indicated potential face selectivity toward sulfoxidation, which could lead predominantly to the S$_R$ diastereoisomer sulfoxide derivative (**S1.3 & S1.4**). ^{1}H NMR spectra of the sulfoxide methyl ester (**4b**) displayed evidence of diastereoselectivity (ca. 3:1 ratio) (**Fig. S1.4.2-S1.4.3**). However, only a crystal structure, supported by CD (circular dichroism) spectra, will help establish the absolute configuration of the major chiral sulfoxide and also that of the natural sulfoxide (**II**). Deliberate oxidation of sulfide (**3b**), (**4b**) or sulfoxide (**5a**) to the sulfone (**III**) was achieved with excess oxidant.

Finally, attempted global deprotection of the Boc group and hydrolysis of the methyl ester under aqueous acidic conditions gave only the methyl ester sulfide (**3b**) or methyl ester sulfoxide (**4b**) from (**3a**) and (**4a**) respectively. Unsuccessful acid, base or esterase mediated ester hydrolysis obligated reconsideration of the synthetic route. Stable amino acid methyl esters have been reported before.[17,18] An allyl ester derivative of β-chloroserine provided the *N*-Boc allyl ester sulfide (**3c**) after S-alkylation. Sulfoxidation followed by mild RhCl(PPh$_3$)$_3$ catalysed allyl ester cleavage[19] and acid mediated Boc protecting group removal gave the target S-(β-amino-β-carboxyethyl)ergothioneine sulfoxide (**II**) in a moderate overall yield of 63 %.

With the second retrosynthetic approach, cleavage of the histidine moiety of S-(β-amino-β-carboxyethyl)ergothioneine sulfoxide (**3**) gave cysteine and 2-bromohercynine (**Scheme 2**). This route has received the most attention as it provides the required sulfurization of histidine to provide the commercially important **ESH**. 2-Bromohercynine was synthesized via the Erdelmeier method.[20] Thus, S-alkylation of 2-bromohercynine, provided the S-(β-amino-β-carboxyethyl)ergothioneine sulfide (**5a**) (**Scheme 2**). However, a large quantity of salt by-product had to be removed which hampered purification. Furthermore, the treatment of the S-(β-amino-β-carboxyethyl)ergothioneine sulfide (**5a**) with 3-mercaptopropionic acid at 90 °C for 18 hr gave **ESH**. The conversion of the sulfide (**5a**) to the bis-benzyloxy N-Boc protected ester (**5b**), allowed organic extraction and removal of salts to give a clean benzyl ester (**5b**). Global deprotection of the N-Boc benzyl ester (**5b**) was achieved by hydrogenation (Pd/C) in the presence of TFA under 50 psi hydrogen pressure to give pure S-(β-amino-β-carboxyethyl)ergothioneine sulfide (**5a**). Biphasic sulfoxidation of the sulfide (**5a**) with mCPBA in a DCM/water mixture gave the S-(β-amino-β-carboxyethyl)ergothioneine sulfoxide (**II**) in a low overall yield of 36 %.
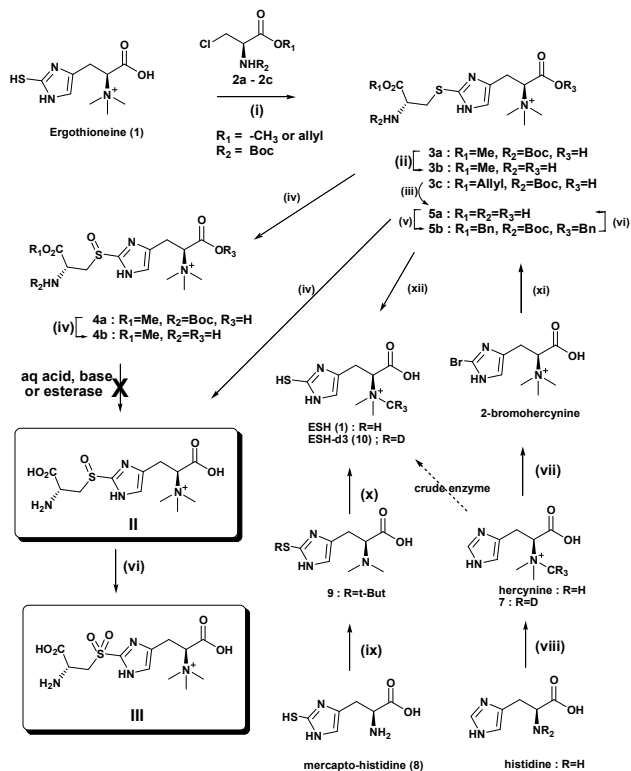

Scheme 2. Reagents and conditions: (i) Et$_3$N / H$_2$O 5 h at 30°C, (3a); (ii) N-Boc deprotection: TFA, DCM / H$_2$O, 0-5°C (3b), (4b) or (II) (iii) a) RhCl(PPh$_3$)$_3$, EtOH/ H$_2$O (1:1) reflux b) TFA, DCM / H$_2$O, 0-5°C (5a); (iv) mCPBA, H$_2$O / DCM 1:1, 5 hr at 25°C (4a) or (II) (v) a) Boc$_2$O / NaOH, H$_2$O / CH$_3$CN, rt overnight b) BnBr / DMF, rt overnight (vi) Pd/C, 3 eq TFA, H$_2$ 50 psi, rt 12h (vii) H$_2$O$_2$, 3 hr at rt (III) (viii) a) CH$_2$O, sodium triacetoxyborohydride / CH$_3$CN, 18-24 hrs at rt (6), b) NH$_4$OH, CH$_3$ I or CD$_3$I / MeOH, 24 h at rt, (7); (ix) a) t-BuOH / HCl, refluxed for 3-4 hrs, S-t-butyl mercaptohistidine; b) CH$_2$O, sodium triacetoxyborohydride / THF, 6-8 hrs at 10 °C, (9); (x) a) NH$_4$OH, CD$_3$I / MeOH 24 h, rt; b) HCl, 2-mercaptoprionic acid refluxed for 21 h (quantitative); (xi)

Cysteine HCl, H$_2$O 1 hr at 0 °C, (5a); (xii) 3-mercaptopropionic acid, HCl / H$_2$O, 19 hrs reflux.

The diastereomers of all S-substituted cysteine sulfoxides display ^{1}H NMR spectra with a characteristic ABX pattern for the S(O)CH$_2$CH(NH$_2$) methylene protons. ^{1}H NMR spectra of the S-(β-amino-β-carboxyethyl)ergothioneine sulfide (**5a**), sulfoxide (**II**) and sulfone (**III**) was consistent with the reported structures. Diagnostic ABX coupling patterns were observed for the α- and β-protons, thus giving rise to sets of doublets at about 4.0-4.5ppm and 3.3-3.6ppm respectively. Coupling constants J_{AX}, J_{BX} and J_{AB} were approx. 10, 5 and 14 Hz respectively. ^{1}H NMR cosy spectra as well as ^{13}C NMR aided complete assignment of structure.

Improved synthesis of ESH

Here we report the improved synthesis of the ESH via the biosynthetic precursor, S-(β-amino-β-carboxyethyl)ergothioneine sulfide. The latter sulfide and its sulfoxide (II) are substrates for the mycobacterial enzyme, EgtE that produces ESH. Our process starts with a commercially available N-benzyl protected histidine rather than the unprotected form (key difference). We discovered that the bromination of the N-benzyl protected hercynine intermediate was achieved with N-bromosuccinimide (NBS) to give a more stable N-benzyl protected 2-bromohercynine derivative using DMF as solvent in 90% yield. The advantage of the latter intermediate is that subsequent process steps is near quantitative, relatively simple, all at room temperature, shortened and allow an overall synthesis yield of 80%. Thus, our process is at least 2 times better in overall yield than any prior patented or published process.[20] The high yields of the intermediate products also allow viable isotopic labelling steps to be performed. Isotopes are usually very expensive and are advantaged by high yield conversions. The final step involves a biomimetic pyridoxal phosphate (PLP) mediated cleavage of the sulfide or sulfoxide substrates as well as with crude enzymatic extracts of M. smegmatis to give ESH. (Scheme 3)
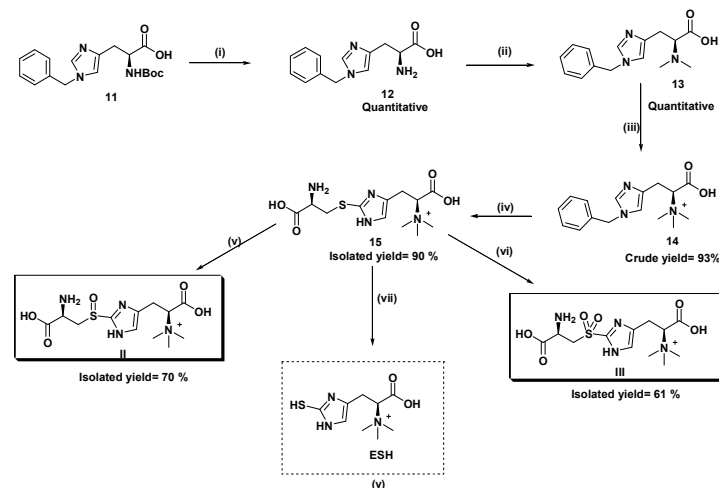
Scheme 3. Improved synthesis of sulfoxide (II), sulfone (III) and ESH. : (i) TFA, DCM, rt overnight; (ii)CH$_2$O, NaBH(OAc)$_3$, CH$_3$CN, 24 h at rt; (iii) MeI, THF, 24 h at rt; (iv) (a) NBS (2.5 eq), DMF, dark at rt, b) L-Cysteine (2.5), DMF, 24 h at rt; (v) p-toluene sulfonic acid (cat),

H_2O_2 (2.4 eq); (vi) boric acid (cat), H_2O_2 (4.8 eq); (vii) C-S enzyme lyase or PLP non enzymatic cleavage.

Previously, the biosynthesis pathway of **ESH** has been elucidated utilising radiolabeled intermediates. Advances in synthetic procedures now made it possible to synthesize the same intermediates incorporating stable isotopes. These intermediates are valuable internal standards in the quantitation of pathway metabolites during external stimuli or drug treatment. Here we also report the synthesis of **ESH**-d_3 (**10**) and demonstrate its biosynthesis from deuterated hercynine.

Hercynine-d_3 (**7**) was synthesized in a two-step reaction starting with the commercially available L-histidine (**Scheme 2**). The first step involved reductive amination using aqueous formaldehyde and sodium triacetoxyborohydride to give N,N-dimethyl histidine (**6**). The second step involved the quaternarization of the crude N,N-dimethyl histidine (**6**) using methyl-d_3 iodide under basic conditions to give the hercynine-d_3 (**7**). Characteristic HRMS peaks indicating M+3 for hercynine-d_3 (**7**) were obtained (**S3.2.1**).

ESH-d_3 (**10**) was synthesized in two sequential reaction steps starting with the S-$tert$-butyl protected 2-mercapto-histidine (**9**), derived from mercapto histidine (**8**).[21] Selective N-methylation with methyl-d_3 iodide, followed by S-$tert$-butyl deprotection using 2-mercaptopropionic acid ($tert$-butyl scavenger) in HCl gave **ESH**-d_3 (**10**). Characteristic HRMS peaks indicating M+3 for the **ESH**-d_3 (**10**) were obtained (**S3.2.2**).

We compared the enzymatic and non-enzymatic PLP mediated synthesis of **ESH** from the synthetic sulfoxide (**II**). To this end, crude *M. smegmatis* cell free extracts were isolated from cultures grown and harvested at the late exponential phase, characterised by high enzymatic activity.[10]

The crude enzymatic transformation of the **ESH** biosynthetic pathway precursors, including sulfide and sulfoxide variants were evaluated by the concomitant production of **ESH** in excess of basal levels as determined by LCMS. **ESH** precursor metabolites hercynine-d_3 (**7**), hercynyl cysteine methyl ester sulfoxide (**4b**), (β-amino-β-carboxyethyl)ergothioneine sulfide (**15**) and (β-amino-β-carboxyethyl)ergothioneine sulfoxide (**II**) were incubated with the crude cell free extract at 37° C, pH = 7.4 for 1 day, and analyzed by LCMS.

The control reaction containing only the crude *M. smegmatis* cell free extract was also treated under the same conditions as the four metabolites. The concentration of **ESH** thus obtained was **5.70 (±0.30)** ng /ml, which was equated to that of endogenous **ESH**. This concentration was above the limit of detection (0.78 ng/ml), thus any increase in the concentration of **ESH** in the experiment above 1 ng/ml is considered significant enough to be ascribed to basal levels or biotransformation of the respective substrates by the crude endogenous enzymes of the **ESH** pathway.

When hercynine-d_3 (**7**) was used as substrate, as expected, no change in baseline **ESH** was observed. However, as expected, the LCMS analysis of the mixture revealed the production of **ESH**-d_3 (**10**). The HRMS (ESI+) displayed a peak at m/z 233.1161 corresponding to $[M+d_3]^+$ (**S4.2.1**). Peak values at m/z 230.0958 $[M]^+$ or at m/z 231.0980 $[M+H]^+$ belonging to the natural **ESH** were not present in the chromatogram. This clearly shows that

the crude *M. smegmatis* enzymes (dialyzed extract) transformed the hercynine-d_3 (**7**) into **ESH**-d_3 (**10**). This proved that the isolated crude enzyme extract were fully functional in the reconstitution of **ESH** synthesis.

(β-amino-β-carboxyethyl)ergothioneine sulfoxide (**II**) biosynthetically produced the highest concentration of **ESH** (22.6 ng/ml) (**Fig. 2a**). The (β-amino-β-carboxyethyl)ergothioneine sulfide (**15**) appeared to be almost as good a substrate as the (β-amino-β-carboxyethyl)ergothioneine sulfoxide (**II**) (19.2 ng/ml **ESH**). It is well known that PLP-dependent transformations can also undergo enzyme free conversion albeit with a much slower rate and specificity.[22] Thus, the non-enzymatic treatment of the sulfide (**15**) with 50 mM PLP at 37 °C resulted in an efficient formation of **ESH** (96.3 ng/ml) (**Fig. S4.5.1**). However, under the same conditions, the sulfoxide (**II**) produced no **ESH** at all (**Fig. S4.5.2**).
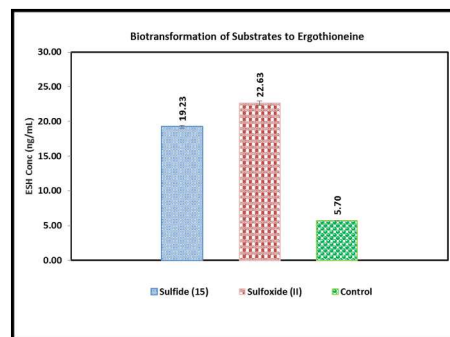

Figure 2a In vitro reconstitution of ESH; 100 μl reactions containing 20 mM Tris HCl pH= 7.4, 20 mM NaCl, 0.2 Mm $FeSO_4$.7 H_2O, 0.5 mM mercaptoethanol, 83 μl of crude M. smeg enzymes and 50 mM of either (a) S-(β-amino-β-carboxyethyl)ergothioneine sulfide (**15**); (b) S-(β-amino-β-carboxyethyl)ergothioneine sulfoxide (**II**) and (c) control. The crude enzyme reactions were incubated for 1 day at 37° C followed by analysis by LC/MS.

The pKa value of the amino acid α-hydrogen usually is in the range 20-30 and many enzymes effectively increase the α-hydrogen acidity with the aid of coenzyme, PLP. PLP-dependent enzymes exist in their native state as an internal aldimine (Schiff base) (**S-5.1-5.3**) with the ε-amino group of a lysine residue present in the catalytic site. The amino acid substrate displaces the lysine from the internal aldimine to form a new aldimine, termed external aldimine. The formation of such an external aldimine can reduce the pKa value of the α-proton of the substrate from 30 to as low as 6.[22]

PLP-dependent enzymes have been differentiated on the basis of their tertiary structures, more specifically according to fold type I–V. EgtE is classified as a fold type V which is found mainly in enzymes that catalyze β-replacement and β-elimination reactions. In the absence of a crystal structure of EgtE, structure homology analysis amongst mycobacteria is not possible. However, a putative β-lyase from *E. tasmaniensis* appeared to catalyze this step but has a sequence similarity with EgtE of *M.Smeg* and *M.tb* of only 14%.[12] Thus, the proposed mechanism of this C-S β-lyase utilizing (β-amino-β-carboxyethyl)ergothioneine sulfide (**15**) as substrate was based on that of the well characterized, cystalysin from *Treponema denticola* which shares 31% sequence identity with the β-lyase

from *E. tasmaniensis*.[23] It may well be that actinomycetes has similar β-lyases at work.[24]

We also observed a rather facile non-enzymatic (PLP only) β-elimination with (β-amino-β-carboxyethyl)ergothioneine sulfide (**15**). However, the conversion of the natural substrate, (β-amino-β-carboxyethyl)ergothioneine sulfoxide (**II**) to **ESH** is not that straight forward. Elimination of an unstable (β-amino-β-carboxyethyl)ergothioneine sulfinate (**IV**) is envisaged, whereby self-condensation leads to the thiosulfinate, which in turn, decompose to **ESH** and an equivalent amount of a relatively stable (β-amino-β-carboxyethyl)ergothioneine sulfinic acid (**V**). The latter sulfinic acid can subsequently be reduced to the thiol, **ESH**, with the aid of excess mercaptoethanol, but may be difficult under the current experimental conditions.[25] Note that the (β-amino-β-carboxyethyl)ergothioneine sulfoxide (**II**) did not produce **ESH** (in the absence of mercaptoethanol).

Efforts are underway to purify and crystallize EgtE, which would allow a better understanding of enzyme-substrate binding, specificity and the potential for inhibitor design. An EgtD deletion mutant of *M. smegmatis* and a mycothiol-deficient mutant did not affect their susceptibility to antibiotics.[4] However, the **ESH**/mycothiol-deficient double mutant was significantly more sensitive to peroxide than either of the single mutants lacking either ESH or mycothiol, suggesting that both thiols play a role in protecting *M. smegmatis* against oxidative stress. Thus, an inhibitor of **ESH** synthesis will be valuable in drug susceptibility studies of mycobacteria.

Supporting Information

Experimental materials, methods, and figures. This material is available free of charge via the Internet at http://pubs.acs.org."

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Notes

The authors declare no competing financial interest.

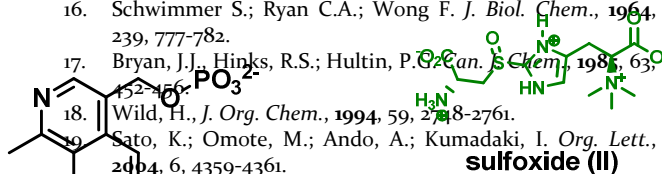## ACKNOWLEDGMENT

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TOC Graphic

histidine   hercynine