In vitro production of ergothioneine isotopologues

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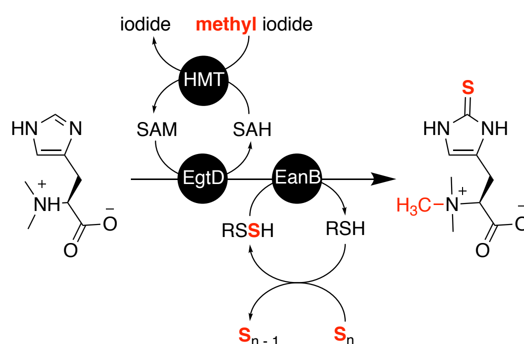
ABSTRACT: Ergothioneine is an emerging component of the redox homeostasis system in human cells and in microbial pathogens, such as *Mycobacterium tuberculosis* or *Burkholderia pseudomallei*. Synthesis of stable isotope labelled ergothioneine derivatives may provide important tools for deciphering the distribution, function and metabolism of this compound *in vivo*. We describe a general protocol for the production of ergothioneine isotopologues with programmable ^{2}H, ^{15}N ^{13}C, ^{34}S and ^{33}S isotope labelling patterns. This enzyme-based approach makes efficient use of commercial isotope reagents and is also directly applicable for the synthesis of radio-isotopologues.



INTRODUCTION

Ergothioneine (**1**, Figure 1), is increasingly recognized as an important factor in cellular redox homeostasis.[1] In the last fifteen years, a number of discoveries have shown that ergothioneine is ubiquitous in cellular live, and may participate in a broad range of biochemical processes. First indications that ergothioneine may be an active participant in human physiology came from the identification of a specific ergothioneine transporter (hETT).[2] Discovery of ergothioneine biosynthetic enzymes showed that this simple metabolite is produced by a wide variety of bacteria and almost all fungi, living under a broad range of conditions.[3] Studies with pathogenic bacteria, such as *Mycobacterium tuberculosis* showed that ergothioneine is essential for survival in their mammalian host.[4] The finding that ergothioneine participates as a cofactor in the biosynthesis of the antibiotic lincomycin A provided a first example of proteins that interact with ergothioneine to elicit biochemical function.[5] Most plants and animals do not produce ergothioneine and rely on supply from their food or environment. While ergothioneine is efficiently retained in the body, metabolized derivatives, such as Nα-trimethyl histidine (TMH) or S-methyl ergothioneine are secreted via the kidneys.[6] Oxidized forms of ergothioneine may be recycled by uncatalyzed reactions with glutathione.[7] Identification and characterization of enzymes such as ergothionase and thiourocanate hydratase revealed that ergothioneine degradation may also occur in catalyzed and oxygen-independent processes.[8] Bacterial species containing these enzymes are well represented in the gut microbiome, highlighting a possible connection between bacterial activity and the availability of nutritional ergothioneine for humans.[8b] Despite these advances, our understanding of the physiological role of ergothioneine in microorganisms, in plants and in humans is far from complete. New methodologies may be needed to address this complex biological question with chemical precision.

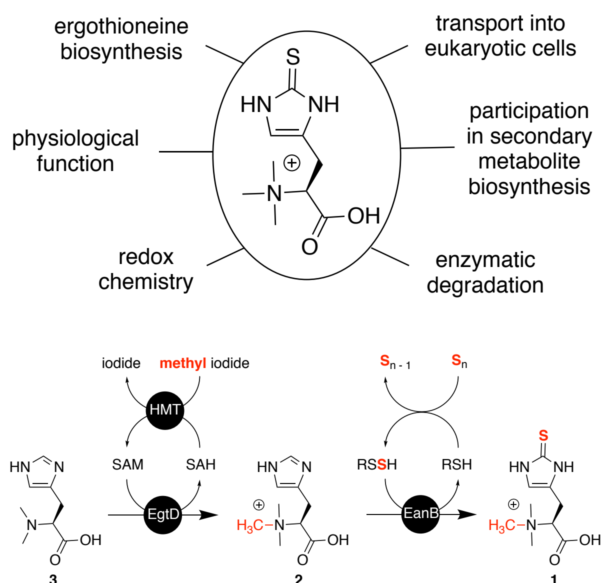


Figure 1. Top: Focus areas in ergothioneine related research. **Bottom:** One-pot synthesis of Nα-trimethylhistidine (TMH, **2**) and ergothioneine (**1**) from Nα-dimethylhistidine catalyzed by the halide methyltransferase HMT, the SAM-dependent methyltransferase EgtD and the sulfur transferase EanB.

Isotope labeling is a powerful approach to identify, localize and quantify specific metabolites within complex media.[9] For example, labeling with radio-isotopes was an essential tool in the early phase of ergothioneine research. Administering ^{14}C-labeled histidine to rats was used to demonstrate that animals by themselves do not produce ergothioneine.[10] Labeling studies with ^{35}S- or ^{14}C containing precursors was used to decipher the biosynthetic logic of ergothioneine production by mycobacteria and fungi.[11] More recently, uniform stable isotope labelling in the context of untargeted metabolite profiling provided first evidence that cyanobacteria produce ergothioneine.[12] This finding was confirmed later based on genetic and more analytical evidence.[3a, 13]

Similar strategies may open new ways to examine the metabolic fate of ergothioneine in oxidatively stressed cells, to quantify the rate of ergothioneine degradation by pathogenic and commensal members of the human microbiome, or to identify new ergothioneine derivatives within complex media. Ergothioneine isotopologues are accessible via chemical synthesis.[14] However, total synthesis with rare isotopes is often expensive and the production of radioactive compounds requires specific safety measures. As a more efficient alternative we developed an enzyme-catalyzed one-pot cascade reaction that produces ergothioneine from histidine, methyl iodide and elemental sulfur (Figure 1). This approach is particularly versatile because all starting materials are commercially available in various isotopic compositions. To develop this methodology we combined a system that enables preparative N-methylation of histidine,[15] with a sulfur transferase (EanB) that can attach sulfur onto the imidazole ring of Nα-trmethylhistidine.[16] As a demonstration of scope, we used this methodology to produce eight different ergothioneine isotopologues.

Results and Discussion

EanB-catalyzed sulfurization of TMH. The biosynthesis of ergothioneine can occur through different pathways.[3b] Mycobacteria require five enzymes to methylate histidine and to mediate O_2-dependent transfer of a sulfur atom from cysteine to Nα-trimethylhistidine (TMH, **2**, Figure 1).[3a] Fungi and many proteobacteria require only three enzymes, but sulfur insertion occurs via the same oxidative chemistry.[3b] A number of strictly anaerobic bacteria an archaea produce ergothioneine just in two steps: trimethylation of histidine by a S-adenosylmethionine (SAM)-dependent methyltransferase, followed by sulfurization by an O_2-independent sulfur transferase.[16a] The crystal structure of this sulfur transferase (EanB) from *Chlorobium limicola* revealed an active site cysteine (Cys412, Figure 2) that accepts an additional (sulfane)[17] sulfur atom to form a hydropersulfide. The sulfane sulfur is then transferred onto carbon 2 of the imidazole ring of the substrate TMH.[16b] By analogy to related sulfur transferases,[18] we initially thought that a pyridoxal 5'-phosphate (PLP) dependent cysteine desulfurase is required to extract and transfer sulfur atoms from free cysteine to EanB.[16a] Upon further analysis we realized that soluble polysulfides generated from partially oxidized hydrogen disulfide can also serve as the primary sulfur donor.[16b, 19] This finding has been confirmed recently by an independent study.[20]

Measuring the rates of ergothioneine production at concentrations of Na_2S ranging from 1 to 50 mM showed that the enzyme reaches saturation at 5 mM (entries 2 – 6, Table 1). At this concentration EanB catalyzes TMH sulfurization at a rate of 1 min^{-1}, which is two-fold faster that previously determined as maximal velocity under single turnover conditions (entry 1).[16] It is possible that attachment of additional sulfur atoms to cysteine residues outside of the catalytic center increase the activity of this enzyme. Despite the comparatively low turnover rate, EanB may be useful for preparative applications because it is remarkably robust. In a test reaction containing 50 mM Na_2S, 4.4 uM, EanB completely converted 2 mM of TMH to ergothioneine, corresponding to nearly 500 turnovers per enzyme (Figure S4).

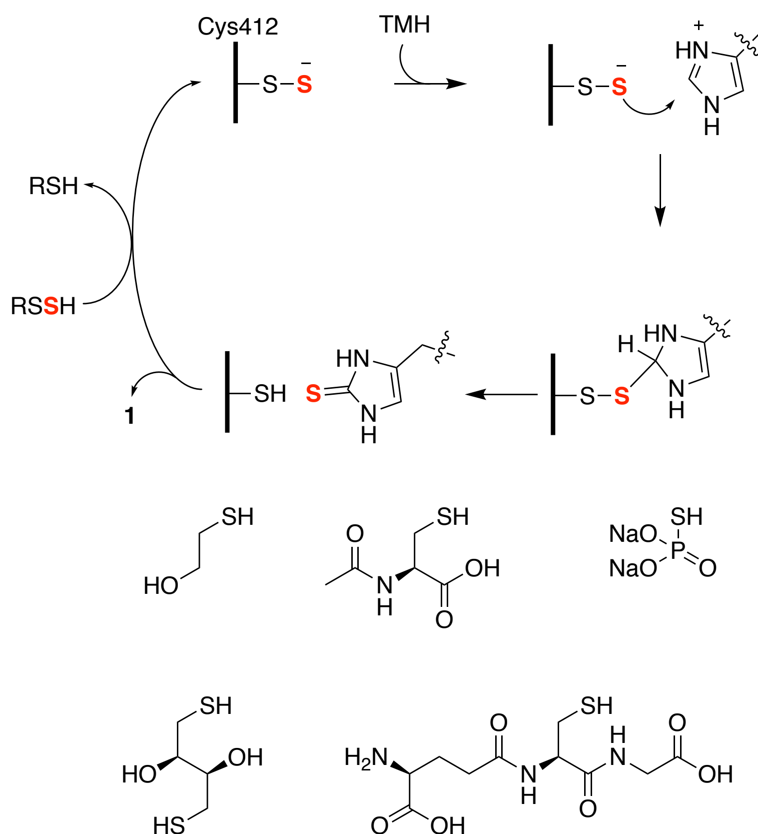


Figure 2. Top: Mechanism of EanB-catalyzed sulfurization of TMH. An active site cysteine (Cys412) accepts a sulfur atom from a hydropersulfide (R-S-SH) or a low weight polysulfide (R-S_n-SH). The sulfane sulfur in the active site of EanB is added to the imidazole ring of TMH in the second half-reaction.[16] **Bottom:** Tested sulfur carriers (R-SH): β-mercapto ethanol (βME), N-acetyl cysteine (NAC), thiophosphate, 1,4-dithiothreitol (DTT) and glutathione (GSH).

Table 1[a] Rates of EanB-catalyzed sulfurization of TMH using Na_2S and S_8 as sulfur donor

entry	Conditions	Rate, min^{-1}
1	EanB (single turnover)	0.6
2	EanB + 1 mM Na_2S	0.04
3	EanB + 2.5 mM Na_2S	0.13
4	EanB + 5 mM Na_2S	0.95
5	EanB + 10 mM Na_2S	1.0
6	EanB + 50 mM Na_2S	1.2
7	EanB + 10 mg S_8	0.01
8	EanB + 10 mg S_8+ 1 mM Na_2S	0.98

[a] Observed rates for EanB-catalyzed conversion of TMH to ergothioneine. Reaction conditions: 100 mM phosphate buffer pH 8.0, 100 mM NaCl, 0.5 mM TMH, 2.2 µM EanB, pH 8.0, 25 °C. The corresponding [**1**] vs. time plots are shown in Figure S3. The production rates are associated with an estimated error of less than 20 % of the given values.

For preparative applications Na_2S is not an optimal reagent because it is toxic, hygroscopic and prone to oxidation upon storage. Therefore, we explored whether elemental sulfur (S_8) – a more stable and cheaper form of sulfur – could also serve as a reagent for the EanB reaction. Indeed, reactions containing suspended S_8 in addition to 1 mM Na_2S produced ergothioneine at the same rate as a reaction containing 50 mM Na_2S (entry 8, Table 1). By contrast, reactions containing either S_8 or 1 mM Na_2S were 100- and 25-fold less productive (entries 7 and 2). These observations suggest that Na_2S acts as a sulfur mobilizing catalyst. As a drawback, mixing Na_2S with S_8 makes the origin of the sulfur in ergothioneine ambiguous. In search for sulfur carriers that avoid this problem we compared the rates of TMH consumption and ergothioneine production in reactions containing S_8, 1 mM TMH, 10 uM EanB and 10 mM of either β-mercaptoethanol (βME), N-acetyl cysteine (NAC), dithiothreitol (DTT), glutathione (GSH) or one of the non-thiol reducing agents thiophosphate (Na_3PO_3S) and sodium borohydride ($NaBH_4$, Figure S5). From this comparison GSH and βME emerged as the most efficient sulfur transfer reagents. βME was selected for further protocol development because of its relatively low cost. In the presence of S_8 and 0.3 – 10 mM βME the rate of EanB catalyzed sulfurization of TMH is dependent on [βME]. In the absence of elemental sulfur no ergothioneine formed, showing that βME is not consumed as a sulfur donor (Figure S6).

Ergothioneine biosynthesis from histidine, methyl iodide and S_8. In a next step we combined this simplified system for TMH sulfurization with an enzyme cascade that produces TMH from histidine or Nα-dimethlyhistidine (DMH). We have previously shown that the histidine-specific methyltransferase EgtD from *Mycolicibacterium smegmatis* catalyzes three cooperative

methylation steps converting histidine to TMH.[15, 21] Each methylation converts one equivalent of SAM to S-adenosylhomocysteine (SAH). In the cascade reaction SAH is methylated back to SAM by a halide methyltransferase (HMT) which uses methyl iodide as methyl donor (Figure 1). This system is remarkably efficient. A reaction containing 20 uM HMT, 20 uM EgtD, 40 uM SAH, 3 mM DMH, 3.3 mM MeI in phosphate buffer at pH 8 converted 3 mM DMH to TMH within one hour (Figure 3, left). A similar reaction containing 3 mM histidine and 10 mM MeI was complete within 8 h. Addition of 20 uM EanB, 10 mM βME, and 1 mg S_8 to these solutions converted all TMH to ergothioneine within seven days (Figure 3, right). Removal of the enzymes by heat denaturation and filtration produced a solution containing ergothioneine iodide and βME dissolved in phosphate buffer. The final product was purified by ion exchange-HPLC (IE-HPLC, Figure S7) and analyzed by high resolution electrospray ionization mass spectrometry (HR-ESI-MS) and ^{1}H NMR (Figure S8).

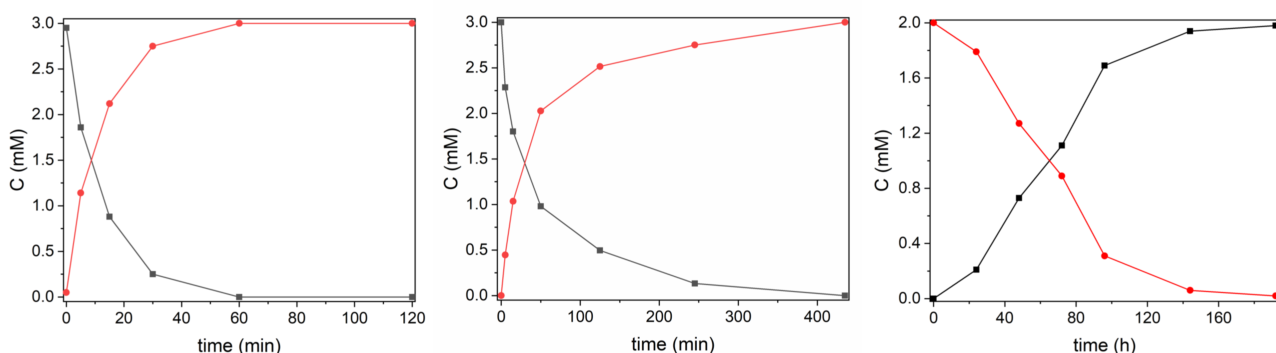


Figure 3. Methylation of DMH (**left**, black) or L-histidine (**middle**, black) to TMH (red). Reaction conditions: 20 uM HMT, 20 uM EgtD, 40 uM SAH, 3 mM DMH (or 3 mM histidine), 1.1 equivalents (or 3.3 equivalents) of MeI in 20 mM phosphate buffer, pH 8, 25°C. **Right:** Sulfurization of TMH (red) to ergothioneine (black): Reaction conditions: 20 uM EanB, 10 mM βME, ~0.5-1 mg S_8, 2 mM TMH in phosphate buffer, pH 8, 25°C. The reactions were followed by IE-HPLC.

Production and characterization of ergothioneine isotopologues. The same procedure was applied to generate a number of exemplary ^{2}H, ^{13}C and ^{15}N labelled ergothioneine isotopologues with ionic masses (m/z) ranging from 230 to 240 (Figure 4). The synthesis of derivatives **4** and **5** required deuterated or ^{13}C-labelled methyl iodide as reagents. Derivative **6** was produced from deuterated DMH that was preparted by reductive amination with deuterated formaldehyde and sodium cyanoborodeuteride ($NaBD_3CN$). Each of the three derivatives were isolated by IE-HPLC with yields of around 70 % (Figure S9, Table S2). Derivatives **7** and **8** were obtained with similar yields using elemental ^{33}S and ^{34}S. HR-ESI-MS analysis confirmed the faithful incorporation of the heavy sulfur isotopes, without competition by ^{32}S derived from the sulfur carrier βME. Because this one-pot reaction requires minimal manipulation, the same protocol should also be applicable to the preparation of radio-isotopologues without production of excessive radioactive waste. It is also worth noting that that the reaction conditions can be optimized to completely consume MeI or

DMH, ensuring that most of the ^3H or ^{14}C labels are incorporated into the probe. Excess elemental sulfur can be removed simply be centrifugation. Efficient depletion of the hot reagents may render post-synthesis purification unnecessary.

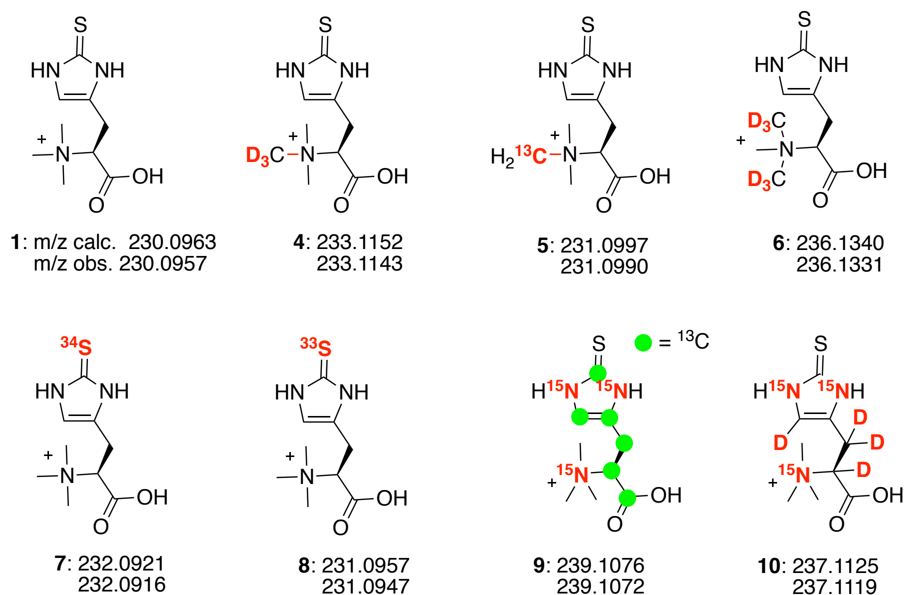


Figure 4. Ergothioneine isotopologues synthesized by *in vitro* biocatalysis. Expected masses were calculated with *enviPat*.[22]

CONCLUSSION

In vitro biocatalysis offers new options in the generation of isotopically labelled natural products. In this report we describe a one-pot cascade reaction that produces ergothioneine from commercially available isotope reagents. This synthesis entails two enzyme catalyzed group-transfer steps that are driven by two cyclic regeneration reactions. In the first reaction the methyl donor SAM is regenerated by HMT from SAH and MeI. In the second reaction the hydropersulfide in EanB is regenerated by βME that delivers sulfane sulfur from S_8. The enzymes required for this process can be accessed by standard protocols for recombinant protein production and purification. The resulting ergothioneine derivatives can be purified efficiently by IE-HPLC. Given the ease of implementation we anticipate that the protocol described here will prove useful as a tool in the many approaches to study the intriguing and multifaceted biology of ergothioneine.

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