

Supporting information

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

Peguy Lutete Khonde and Anwar Jardine*

Department of Chemistry, Faculty of Science, University of Cape Town, South Africa

*Corresponding Author: anwar.jardine@uct.ac.za

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S1. Synthesis and Characterisation of Compounds

S1.1. General Procedures

All solvents were dried by appropriate techniques and freshly distilled before use. All commercially available reagents were purchased from Sigma-Aldrich and Merck and were used without further purification.

Unless otherwise stated, reactions were performed under an inert atmosphere of nitrogen in oven dried glassware and monitored by thin-layer chromatography (TLC) carried out on Merck silica gel 60-F₂₅₄ sheets (0.2 mm layer) pre-coated plates and products visualized under UV light at 254 nm or by spraying the plate with an ethanolic solution of ninhydrin (2% v/v) followed by heating.

Column chromatography was effected by using Merck Kieselgel silica gel 60 (0.040-0.063 mm) and eluted with an appropriate solvent mixtures. All compounds were dried under vacuum before yields were determined.

Nuclear magnetic resonance spectra (^1H and ^{13}C) were recorded on a Varian Mercury 300 MHz (75 MHz for ^{13}C), Varian Unity 400 MHz (101 MHz for ^{13}C), a Bruker unity 400 MHz (101 MHz for ^{13}C), or a Bruker unity 600 MHz (151 MHz for ^{13}C) and were carried out in CDCl_3 , $\text{DMSO}-d_6$ and D_2O as the solvent unless otherwise stated. Chemical shifts are given in ppm relative to tetramethylsilane (TMS, $\delta = 0.00$ ppm), which is used as internal standard. Assignments were confirmed by COSY, APT and HSQC analysis, when required. Coupling constants (J) are reported in Hertz (Hz). The spin multiplicities are indicated by the symbol s (singlet), d (doublet), dd (doublet of doublets), t (triplet), m (multiplet), q (quartet) and br (broad).

Optical rotations were obtained using a Perking Elmer 141 polarimeter at 20°C . The concentration c refers to g/100ml.

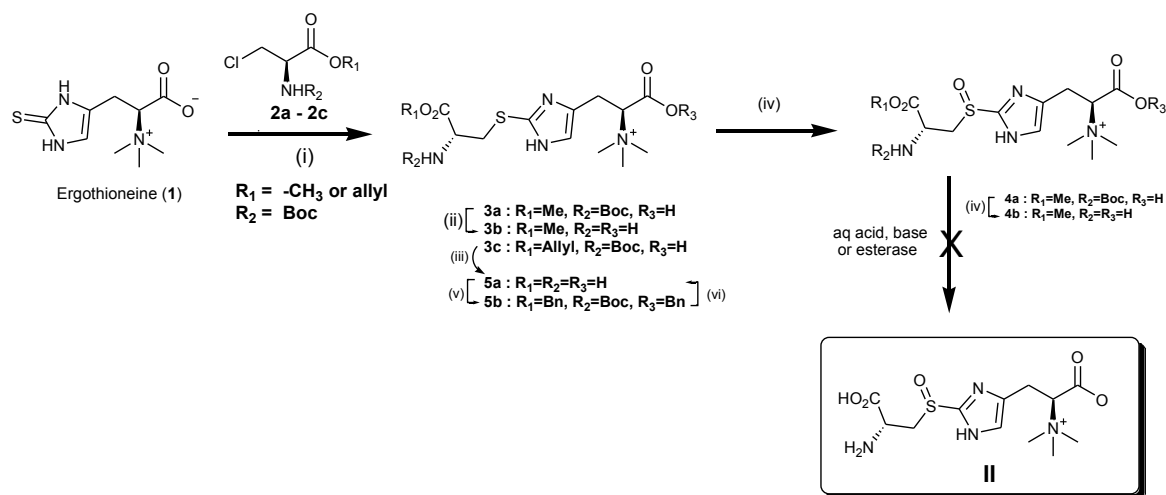
Melting points were determined using a Reichert-Jung Thermovar hot-plate microscope and are uncorrected. Infra-Red spectra were recorded on a Perkin-Elmer FT-IR spectrometer (in cm^{-1}) from 4000 cm^{-1} to 450 cm^{-1} .

Mass spectra were recorded on a JEOL GC MATE II magnetic sector mass spectrometer and the base peaks are given, University of Cape Town.

LCMS analyses were carried out with a UHPLC Agilent 1290 Infinity Series (Germany), accurate mass spectrometer Agilent 6530 Quadrupole Time Of Flight (QTOF) equipped with an Agilent jet stream ionization source (positive ionization mode) (ESI^+) and column (Eclipse + C_{18} RRHD $1.8\text{ }\mu\text{m}$.2.1 X 50, Agilent, Germany).

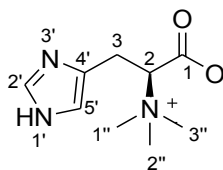
Enzymatic reactions were allowed to incubate in Nuair incubator (DH Autoflow CO_2 Air – jarcketed Incubator), and centrifuged in Eppendorf centrifuge (Model 5810R, Germany), Tygerberg Stellenbosch University, Cape Town, South Africa.

S1.2. Synthesis and characterisation of Compounds.



Scheme S1.2. 1. Synthesis of ergothioneine sulfoxide protected (4a, 4b). Reagents and conditions: (i) Et_3N / H_2O 5 h at 30°C , (**3a**); (ii) *N*-Boc deprotection: TFA, DCM / H_2O , $0-5^\circ\text{C}$ (**3b**), (**4b**) or (**II**) (iii) a) $\text{RhCl}(\text{PPh}_3)_3$, $\text{EtOH}/\text{H}_2\text{O}$ (1:1) reflux b) TFA, DCM / H_2O , $0-5^\circ\text{C}$ (**5a**); (iv) *m*CPBA, H_2O / DCM 1:1, 5 hr at 25°C (**4a**).

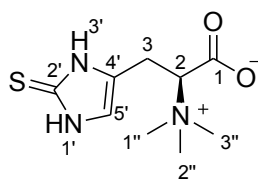
***L*-hercynine or (2*S*)-*N,N,N*-2-trimethylethanaminium-3-(1*H*-imidazol-4-yl)propanoic acid ¹**



N,N,*-*dimethyl *L*-histidine (**6**) (653 mg; 3.56 mmol) was dissolved in methanol (20 ml), and the pH was adjusted to 8-9 with a concentrated solution of NH_4OH (25 %, 80 μl), followed by the addition of methyl iodide (700 mg; 4.93 mmol). The resultant solution was allowed to stir at room temperature for 24 hours. The solvent was evaporated to dryness to afford a crude product *L*-hercynine which was recrystallized in a mixture of warm methanol and diethyl ether to yield hydroiodide salt form of *L*-hercynine as a white solid (489 mg; 42 %). Mp: $240-242^\circ\text{C}$ (with decomposition); ν_{max} KBr / cm^{-1} 3435s (N-H) 1632m (RCOOH) 1496 w (C=C Aromatic) 1335w (C-N); $[\alpha]_{\text{D}}^{20} = +48.7^\circ$ ($c = 0.9$, 5 N HCl); ^1H NMR (400 MHz, D_2O) δ 8.76 (s, 1H, H-2'), 7.51 (d, $J = 12.7$ Hz, 1H, H-5'), 4.02 – 3.83 (m, 1H, H-2), 3.49 (s, 9H, H-1''H-2''H-3''), 3.12 (dd, $J = 12.7$, 4.1 Hz, 1H, H-3a) 3.06 (dd, $J = 12.7$, 4.1 Hz, 1H, H-3b); ^{13}C NMR (101 MHz, D_2O) δ 166.8 (C-1), 135.5 (C-2'), 134.3 (C-5'), 118.3 (C-4'), 72.7 (C-2), 53.0 (C-1''C-2''C-3''), 22.3 (C-3); LRMS (EI+) m/z calculated for $\text{C}_9\text{H}_{17}\text{N}_3\text{NaO}_2^+$ 223.1 found 223.6 ($[\text{M}+\text{H}+\text{Na}]^+$; 20.4%).

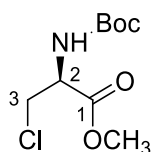
Hercynine synthesis (one pot) ²

L-histidine (293 mg; 1.6 mmol) was dissolved in NaOH (10 %, 4 ml) followed by the addition of Me₂SO₄ (0.4 ml; 532 mg; 4.2 mmol). The reaction was allowed to stir for 30 min at 0 °C and for further 30 min at room temperature. The solution was neutralized with 0.5 N HCl, and lyophilised to dryness. The residue was triturated with Et₂O, to yield the *L*-hercynine as white solid (300 mg), which was used without further purification.

Ergothioneine (1), or (2*S*)-*N,N,N*-2-trimethylethanaminium (2-mercapto-1*H*-imidazol-4-yl) propanoic acid (1).

The synthesis of Ergothioneine (**1**) was carried out as reported by *Trampota et al.*³

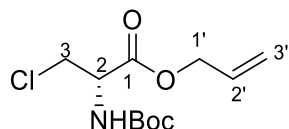
107 mg of *L*-(+)-ergothioneine (**1**) was synthesized. Mp: 276-279 °C literature 275-277 °C³; $[\alpha]_D^{20} = +138.21^\circ$ (*c* = 1; H₂O)³; R_f silica gel 0.3 (methanol/water 9:1); ¹H NMR (400 MHz, D₂O) δ 7.05 (m, 1H, H-5'), 3.76 (dd, *J* = 11.7, 4.0 Hz, 1H, H-2), 2.88 (m, 2H, H-3), 2.75 (s, 9H, NMe₃); ¹³C NMR (101 MHz, D₂O) δ 177.0 (C-1), 161.2 (C-2'), 128.9 (C-4'), 103.4 (C-5'), 60.6 (C-2), 55.6 (NMe₃), 24.9 (C-3); ν_{max} (KBr)/cm⁻¹ 3138s (NH) 1746s (RCOOH), 1995m (N=C-S), 1403s (C=C aromatic ring); HRMS (ESI⁺): *m/z* 230.0963 [M]⁺. Calculated for C₉H₁₆N₃O₂S⁺, found 230.0958 [M]⁺.

(*S*)-Methyl 2-(*tert*-butoxycarbonylamino)-3-chloropropanoate (2a) ⁴

2, 4, 6-Trichloro-[1, 3, 5] triazine (1.87 g; 10.1 mmol) was added portion wise in DMF (2ml) at an internal temperature was maintained at 25 °C. After the formation of a white solid, the reaction was monitored (TLC) until complete disappearance of TCT, thereafter CH₂Cl₂ (25 ml) was added, followed by the addition of N-Boc *L*-serine methyl ester (2.22 g; 10.1 mmol). The resultant mixture was allowed to stir at room temperature, monitored (TLC pet-ether: EtOAc, 1:1) until completion (ca 4 h). The reaction mixture was diluted with water (20 ml) and washed with saturated aqueous sodium carbonate solution (2 × 15 ml) followed by 1 N HCl and brine.

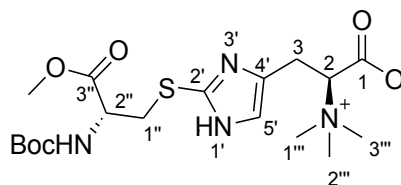
The combined organic phases were dried (Na_2SO_4), filtered and concentrated under vacuum. The residue was purified by flash column chromatography (petroleum ether: EtOAc 1:1) and was crystallized and recrystallized in a mixture of petroleum ether (40-60°C) and ethyl acetate to afford (*S*)-methyl 2-(*tert*-butoxycarbonylamino)-3-chloropropanoate (**2a**) as a white solid (736 mg; 31 %). Mp: 59-61 °C; ν_{max} (KBr) / cm^{-1} 3362s (NH) 2991s + 2943s (C-H, aliphatic) 1739s 1294s (RCOOMe) 809s + 852m (C-Cl); $[\alpha]^{20}_{\text{D}} = +32.5^\circ$ ($c = 0.15$; CHCl_3) Lit $[\alpha]^{20}_{\text{D}} = +37.8^\circ$ ($c = 0.15$; CHCl_3)⁵; ^1H NMR (300 MHz, CDCl_3) δ 5.43 (s, 1H, NH), 4.69 (br.s, 1H, H-2), 3.96 (dd, $J = 4.6$ Hz, 3.4 Hz, 1H, H-3a), 3.84 (dd, $J = 4.6$ Hz, 3.4 Hz, 1H, H-3b), 3.80 (s; 3H, OCH_3), 1.46 (s, 9H, Boc); LRMS (EI)⁺ m/z calculated for $\text{C}_9\text{H}_{15}\text{Cl}^{35}\text{NO}_4$ 236.1 found 235.8 ($[\text{C}^{35}\text{M}-\text{H}]^+$; 5.3 %), 138.0 ($[\text{C}^{35}\text{M}^+-\text{Boc}]$; 11 %), 140.0 ($[\text{C}^{37}\text{M}^+-\text{Boc}]$; 2.6 %).

(*S*)-allyl 2-(*tert*-butoxycarbonylamino)-3-chloropropanoate (2c**)⁶**



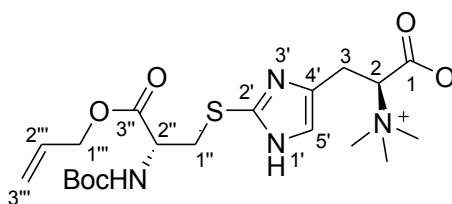
2, 4, 6-Trichloro-[1, 3, 5] triazine (577 mg; 3.13 mmol) was added portion wise in DMF (1ml) at an internal temperature was maintained at 25 °C. After the formation of a white solid, the reaction was monitored (TLC) until complete disappearance of TCT, thereafter CH_2Cl_2 (20 ml) was added, followed by the addition of *N*-Boc *L*-serine allyl ester (768 mg; 3.13 mmol). The resultant mixture was allowed to stir at room temperature, monitored (TLC pet-ether: EtOAc, 2:1) until completion (ca 10 h) The reaction mixture was diluted with water (20 ml) and washed with saturated aqueous sodium carbonate solution (2×15 ml) followed by 1 N HCl and brine. The combined organic phases were dried (Na_2SO_4), filtered and concentrated under vacuum. The residue was purified by flash column chromatography (petroleum ether: EtOAc 2:1) to afford (*S*)-allyl 2-(*tert*-butoxycarbonylamino)-3-chloropropanoate (**2c**) as a yellow oil (729 mg; 2.76 mmol; 89 %); $[\alpha]^{20}_{\text{D}} = +23.94^\circ$ ($c = 0.35$, CH_2Cl_2); R_f 0.75 (hexane/EtOAc 2: 1); ^1H NMR (400 MHz, CDCl_3) δ 5.91 – 5.75 (m, 1H, H-2'), 5.37 (brs, 1H, NH), 5.27 – 5.12 (m, 2H, H-3'), 4.52 - 4.45 (m, 2H, H-1') 4.39 - 4.30 (m, 1H, H-2), 3.96 (dd, $J = 8.1$, 3.8 Hz, 1H, H-3a), 3.91 (dd, $J = 8.1$, 3.8 Hz, 1H, H-3b), 1.45 (s, 9H, Boc); ^{13}C NMR (101 MHz, CDCl_3) δ 171.3 (C-1), 154.3 (C=O Boc), 114.1 (C-2'), 72.4 (C-3'), 70.1 (C(Boc)), 63.7 (C-2), 52.7 (C-1'), 30.5 (C-3), 28.4 (3C, (Boc)); LRMS (EI)⁺ m/z calculated for $\text{C}_6\text{H}_8\text{ClINO}_2$ 161.0 for Cl^{35} and 162.0 for Cl^{37} found 160.1 ($[\text{M Cl}^{35}\text{-Boc} - \text{H}]^+$; 30 %), 162.1 ($[\text{M Cl}^{37}\text{-Boc} - \text{H}]^+$; 10 %); 133.0 ($[\text{M Cl}^{35} - \text{Boc} - \text{CH}_2=\text{CH}_2 - \text{H}]^+$; 100 %).

(2S)-N,N,N-2-trimethylammonium-3-[2-((2R)-2-(*tert*-butoxycarbonylamino)-3-methoxycarbonyl)-ethylthio)-1*H*-imidazol-4-yl] propanoic acid (3a**)**⁷



N-Boc- β -chloro-*L*-alanine methyl ester (**2a**) (93 mg; 0.39 mmol) was dissolved in a mixture of DCM: H₂O (50:50; 4 ml) followed by the addition of Et₃N (0.4 mL) which adjusted the pH of the solution to 9-10. The resulting mixture was allowed to stir at room temperature for 30 min and subsequently followed by the addition of ergothioneine (**1**) (89 mg; 0.34 mmol). The solution was allowed to stir at 30 °C for 5 hours. The solvent was removed under high vacuum to dryness and the crude product was purified by reverse chromatography (C18) to afford (2*S*)-*N,N,N*-2-trimethylammonium-3-[2-((2*R*)-2-(*tert*-butoxycarbonylamino)-3-methoxycarbonyl)-ethylthio)-1*H*-imidazol-4-yl] propanoic acid (**3a**) as a pale yellow solid (92 mg; 0.20 mmol; 51 %); ¹H NMR (400 MHz, D₂O) δ 5.95 (s, 1H, H-5'), 4.51 - 4.43 (m, 1H, H-2), 4.27 - 4.19 (m, 2H, H-1''), 4.10 (dd, *J* = 10.0, 4.0 Hz, 1H, H-3a), 4.05 (dd, *J* = 10.0, 4.0 Hz, 1H, H-3b), 3.36 - 3.25 (m, 1H, H-2''), 2.49 (s, 3H, OCH₃), 1.59 (s, 9H, H-1''' H-2''' H-3'''), 1.56 (s, 9H, Boc); LRMS *m/z* calculated for C₁₈H₃₁N₄O₆S⁺ 431.2 found 431.8 ([M⁺]; 2.5 %).

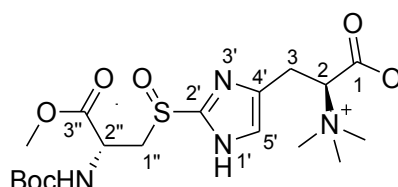
(2S)-N,N,N-2-trimethylammonium-3-[2-((2R)-2-(*tert*-butoxycarbonylamino)-3-allyloxycarbonyl)-ethylthio)-1*H*-imidazol-4-yl] propanoic acid (3c**)**⁷



N-Boc- β -chloro-*L*-alanine allyl ester (**2c**) (728 mg; 2.76 mmol) was dissolved in a mixture of THF:H₂O (66:34; 15 ml) followed by the addition of Et₃N (0.5 ml) which adjusted the pH of the solution to 9-10. The solution was allowed to stir at room temperature for 30 min and subsequently followed by the addition of ergothioneine (**1**) (729 mg, 2.76 mmol). The solution was allowed to stir at 30 °C for 5 hours. The solvent was removed under high vacuum to dryness and the crude product was purified by reverse chromatography (C18) to afford (2*S*)-

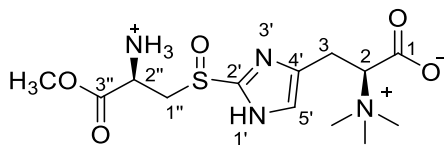
N,N,N-2-trimethylammonium-3-[2-((2*R*)-2-(*tert*-butoxycarbonylamino)-3-allyloxycarbonyl)-ethylthio)-1*H*-imidazol-4-yl] propanoic acid (**3c**) as yellow hygroscopic solid (1.12 g; 2.27 mmol; 82 %). Mp: 195-197 °C; ν_{\max} (CH₂Cl₂)/cm⁻¹ 3350m (NH) 1732s + 1163s + 1046 (RCOOR) 895s (N=C-S); ¹H NMR (300 MHz, DMSO) δ 6.88 (brs, 1H, H-5'), 5.98 – 5.80 (m, 1H, H-2'''), 5.32 (dd, *J* = 10.3, 1.5 Hz, 1H, H-3'''a), 5.20 (dd, *J* = 10.3, 1.5 Hz, 1H, H-3'''b), 4.95 (m, 1H, H-2''), 4.58 (t, *J* = 7.8 Hz, 1H, H-2), 4.07 (m, 2H, H-1''), 3.66 (t, *J* = 4.8 Hz, 2H, H-1'''), 3.05 (s, 9H, NMe), 2.98 (m, 2H, H-3), 1.38 (s, 9H, Boc); ¹³C NMR (101 MHz, DMSO) δ 171.1 (C-3''), 155.7 (C-1, C=O(Boc)), 132.8 (C-2', C-3'''), 117.7 (C-2''', C-4'), 111.4 (C-5'), 78.7 (C-2), 65.0 (C(Boc)), 61.6 (C-1'''), 56.8 (C-2''), 45.8 (C-1''), 44.2 (NMe₃), 28.5 (3C(Boc)), 18.9 (C-3).

(2*S*)-*N,N,N*-2-trimethylammonium-3-[2-((2*R*)-2-(*tert*-butoxycarbonylamino)-3-methoxycarbonyl)-ethylsulfinyl)-1*H*-imidazol-4-yl] propanoic acid (4a**)**



(2*S*)-*N,N,N*-2-trimethylammonium-3-[2-((2*R*)-2-(*tert*-butoxycarbonylamino)-3-methoxycarbonyl)-ethylthio)-1*H*-imidazol-4-yl] propanoic acid (**3a**) was dissolved in a mixture of H₂O: DCM (50:50; 4 ml) followed by the addition of *m*CPBA (13 mg; 0.06 mmol; 77 %). The solution was allowed to stir at 0-5°C for 3-5 hours until completion (TLC). The solution was partitioned in a separator funnel, water layer was returned and organic layer discarded. Water layer was treated with amberlyst (H⁺ form) to neutral and then extracted with DCM to remove any organic impurities. Water layer was returned and lyophilised to dryness. The residue was purified by reverse phase chromatography (C18) to afford (2*S*)-*N,N,N*-2-trimethylammonium-3-[2-((2*R*)-2-(*tert*-butoxycarbonylamino)-3-methoxycarbonyl)-ethylsulfinyl)-1*H*-imidazol-4-yl] propanoic acid (**4a**) as a pale yellow crystal (25 mg; 0.06 mmol; 96 %). ¹H NMR (400 MHz, D₂O) δ 6.10 (s, 1H, H-5') 4.07 (brs., 1H, H-2) 3.89 (m, 2H, H-3) 3.77 (s, 3H, OCH₃) 3.72 -3.67 (m, 1H, H-2'') 3.60 (dd, *J* = 11.72, 6.41 Hz, 1H, H-1''a) 3.42 (dd, *J* = 11.72, 6.41 Hz, 1H, H-1''b) 2.98 (s, 9H, NMe₃) 1.47 (s, 9H, Boc); MS (EI⁺) *m/z* calculated C₁₈H₃₁N₄O₇S⁺ 447.2 found 450.4 ([M + 3H]⁺, 1.5 %)

***S*-(β-amino-β-carboxyethyl)ergothioneine methyl ester sulfoxide or (2*S*)-*N,N,N*-2-trimethylammonium-3-[2-((2*R*)-2-amino-2-methoxycarbonyl) ethylsulfinyl)-1*H*-imidazol-4-yl] propanoic acid (4b).**



(2*S*)-*N,N,N*-2-trimethylammonium-3-[2-((2*R*)-2-(*tert*-butoxycarbonylamino)-3-methoxycarbonyl)-ethylsulfinyl)-1*H*-imidazol-4-yl] propanoic acid (**4a**) (45 mg, 0.10 mmol) was dissolved in distilled H₂O (1 ml) followed by the addition of trifluoroacetic acid (1 ml) with cooling in a ice bath. The solution was allowed to stir at 0-5°C until completion (monitored by TLC). The solvent was concentrated by lyophilisation and the residue was extracted with DCM to remove any by product and lyophilised to dryness. The crude product was crystallized in ethanol to afford (2*S*)-*N,N,N*-2-trimethylammonium-3-[2-((2*R*)-2-amino-2-methoxycarbonyl)ethylsulfinyl)-1*H*-imidazol-4-yl] propanoic acid (**4b**) as a pale yellow and hygroscopic crystal (25 mg; 0.07 mmol; 70 %). Mp: 108-110°C; ν_{\max} (KBr)/cm⁻¹ 3409s (RNH₂) 2086m (N=C-S) 1688vs (COOH) 1434s (C=C aromatic ring) 1206s (C-N) 1142s (S=O); $[\alpha]^{20}_{\text{D}} = -30.26^\circ$ ($c = 0.39$, H₂O); ¹H NMR (400 MHz, D₂O) δ 7.38 (brs, 1H, H-5'), 4.08 (dd, $J = 9.9$, 4.7 Hz, 2H, H-1'' major diastereoisomer), 4.02 (dd, $J = 9.6$, 4.6 Hz, 2H, H-1'', minor diastereoisomer), 3.89 (d, $J = 4.2$ Hz, 1H, H-2), 3.86 (s, 3H, OCH₃), 3.77 (dd, $J = 11.5$, 4.2 Hz, 1H, H-3a), 3.67 (dd, $J = 11.05$, 4.2 Hz, 1H, H-3b), 3.45 – 3.40 (m, 1H, H-2''), 3.05 (s, 9H, NMe₃); ¹³C NMR (101 MHz, D₂O) δ 162.6 (C=O), 162.9 (C=O), 163.3 (C=O), 163.6 (C=O), 120.9 (C-2'), 118.0 (C-4'), 115.1 (C-5'), 72.2 (C-2), 66.0 (C-1'' major diastereoisomer), 62.9 (C-1'' minor diastereoisomer), 63.8 (OCH₃), 43.8 (C-2''), 43.4 (NMe₃), 36.5 (C-3); HRMS (ESI⁺): m/z 348.1462 [M+H]⁺. Calculated for C₁₃H₂₄N₄O₅S⁺ found 348.1465 [M+H]⁺.

S1.3. 3 D ChemBioDraw Ultra 11.0 modelling of *m*CPBA oxidation of sulfide (3a) to sulfoxide (4b) (milder oxidation)

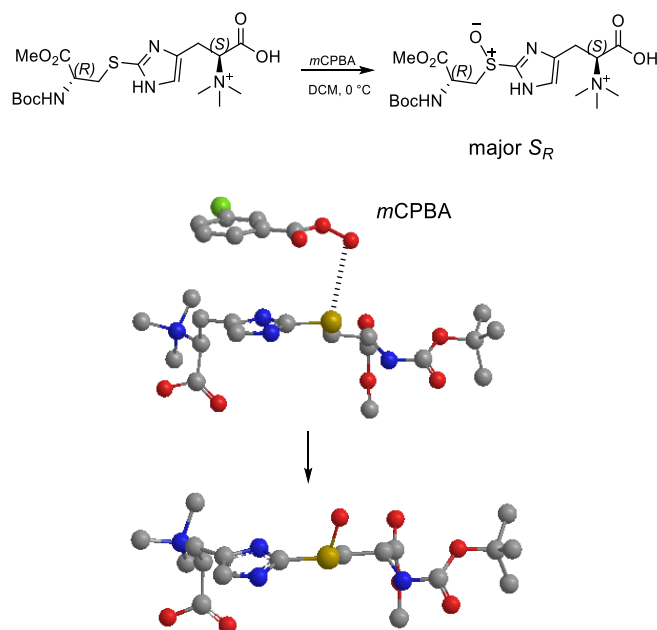


Figure (S1.3) 3D possible conformation of the sulfide (3a) indicates potential face selectivity (3D ChemBioDraw Ultra 11.0, Total energy 34.3722 Kcal/mol, Dipole/Dipole 3.0912, Steric minimise energy)

S1.4. Evidence of diastereoselectivity of sulfoxidation revealed by ^1H NMR spectrum of S-(β -amino- β -carboxyethyl)ergothioneine methyl ester sulfoxide (4b)

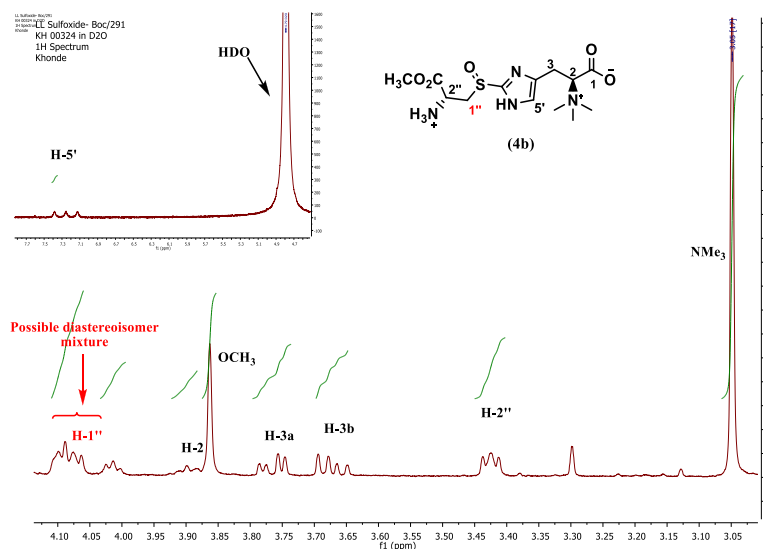


Figure (S1.4.1) ^1H NMR spectrum of S-(β -amino- β -carboxyethyl)ergothioneine methyl ester sulfoxide (4b) in D_2O at 400 MHz from 4.15 to 3.00 ppm

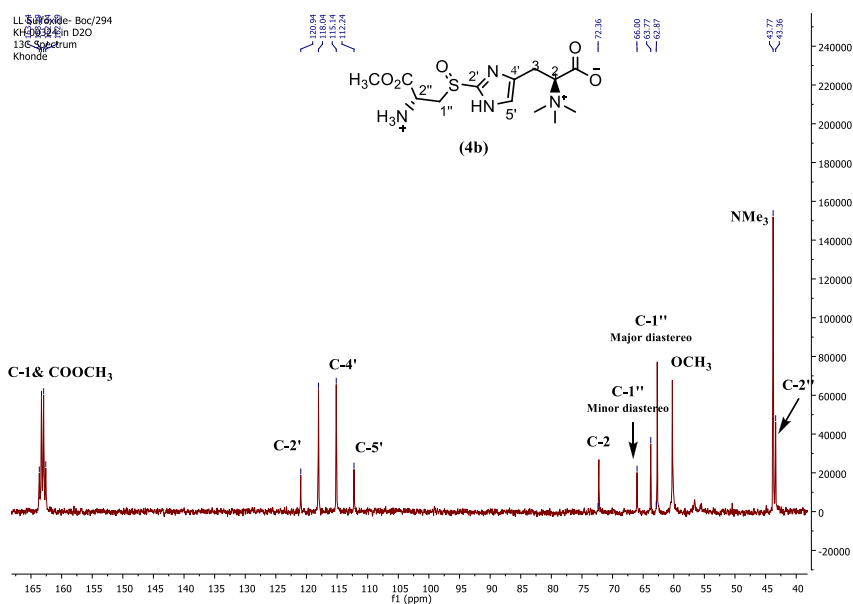


Figure (S1.4.2) ^{13}C NMR spectrum of *S*-(β -amino- β -carboxyethyl)ergothioneine methyl ester sulfoxide (**4b**) in D_2O at 101 MHz from 170.0 to 40.0 ppm

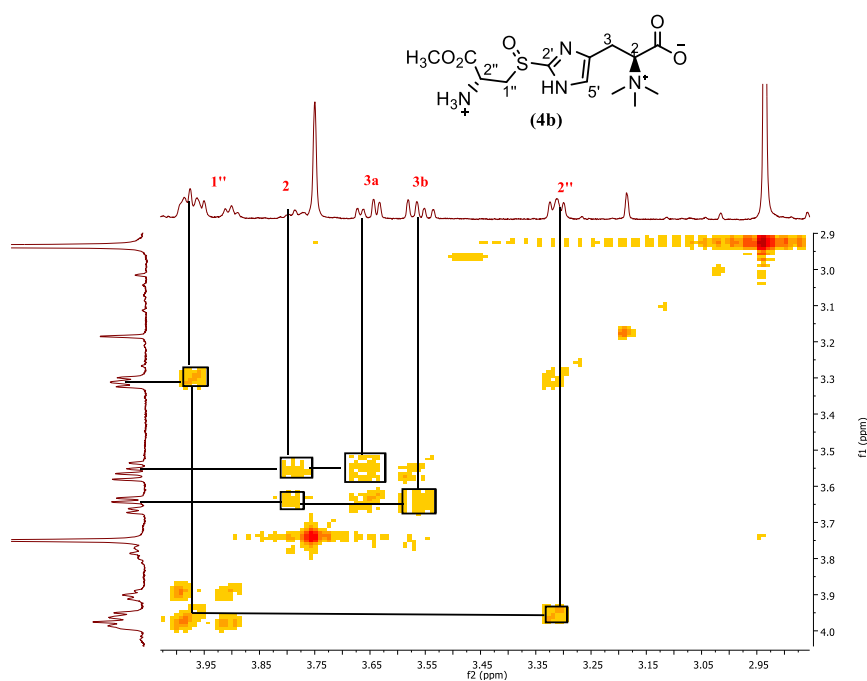


Figure (S1.4.3) COSY ^1H – ^1H NMR spectrum of *S*-(β -amino- β -carboxyethyl)ergothioneine methyl ester sulfoxide (**4b**) in D_2O at 400 MHz from 4.00 to 2.90 ppm

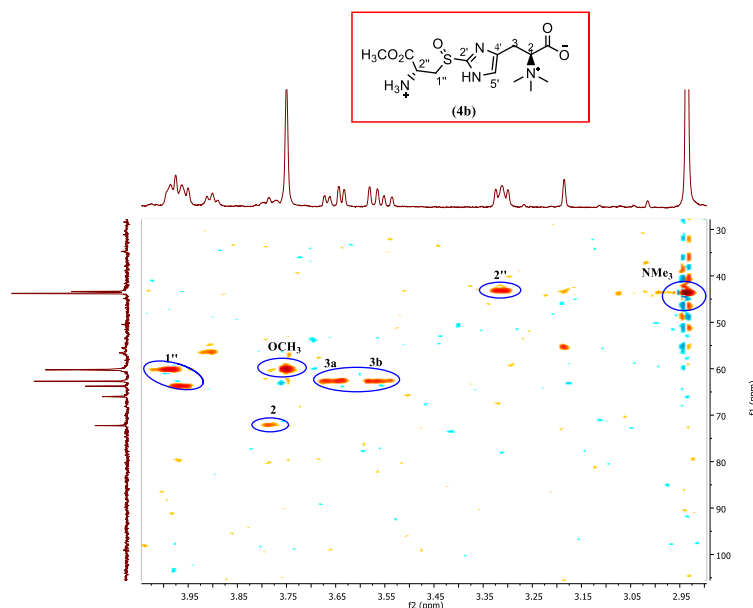
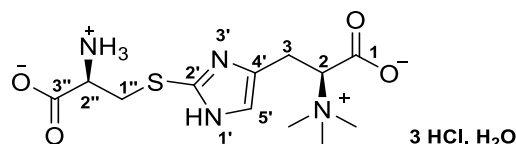


Figure (S1.4.3) HSQC $^1\text{H} - ^{13}\text{C}$ NMR spectrum of *S*-(β -amino- β -carboxyethyl)ergothioneine methyl ester sulfoxide (**4b**) in D_2O at 400 MHz from 4.00 to 2.90 ppm.

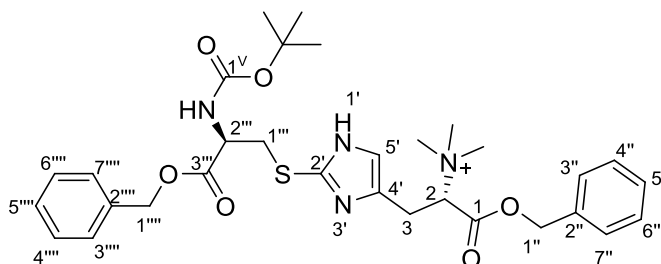
***S*-(β -amino- β -carboxyethyl)ergothioneine sulfide or (2*S*)-*N,N,N*-2-trimethylammonium-3-[2-((2*R*)-2-amino-2-hydroxycarbonyl)ethylthio)-1*H*-imidazol-4-yl] propanoic acid trihydrochloride salt (**5a**)**⁸



L-hercynine (100 mg, 0.50 mmol) was dissolved in distilled H_2O (5 ml), followed by the addition of concentrated HCl (37 %, 28 mg, 74 μl , 0.76 mmol). The solution was allowed to cool in an ice bath, thereafter Br_2 (104 mg, 0.65 mmol) was added drop wise, followed by the addition of cysteine monohydrate chloride (439 mg, 2.50 mmol) 5 min later. The reaction mixture was allowed to stir for 90 min at 0°C . Purification by Dowex chromatography (gradient 0.5-2N HCl, Dowex (50WX8-100) and reverse phase chromatography (C18) afforded (2*S*)-*N,N,N*-2-trimethylammonium-3-[2-((2*R*)-2-amino-2-hydroxycarbonyl) ethylthio)-1*H*-imidazol-4-yl] propanoic acid trihydrochloride salt (**5a**) as a pale yellow hygroscopic solid, in trihydrochloride hydrate salt form (193 mg, 47 %). Mp: 79°C (dec), Lit. 77°C (dec)²⁰; $[\alpha]_{\text{D}}^{20} = -86.6^\circ$ ($c = 0.5$, H_2O); ^1H NMR (600 MHz, D_2O) δ 8.73 (d, $J = 14.2$ Hz, 0.5 H, H-5'), 7.41 (dd, $J = 15.5$, 7.8 Hz, 0.5 H, H-5'), 4.49 (dd, $J = 7.9$, 4.4 Hz, 1H, H-2), 4.38 (dd, $J = 5.5$, 4.4 Hz, 1H, H-2''), 3.49 (dd, $J = 15.2$, 4.4 Hz, 1H, H-1a''), 3.38 (Overlapped, s, 9H, NMe_3), 3.34 (dd, $J = 15.2$, 7.9 Hz, 1H, H-1b''), 3.21 (dd, $J = 15.2$, 5.6 Hz, 1H, H-3a), 3.15 (dd, $J = 15.2$, 4.4

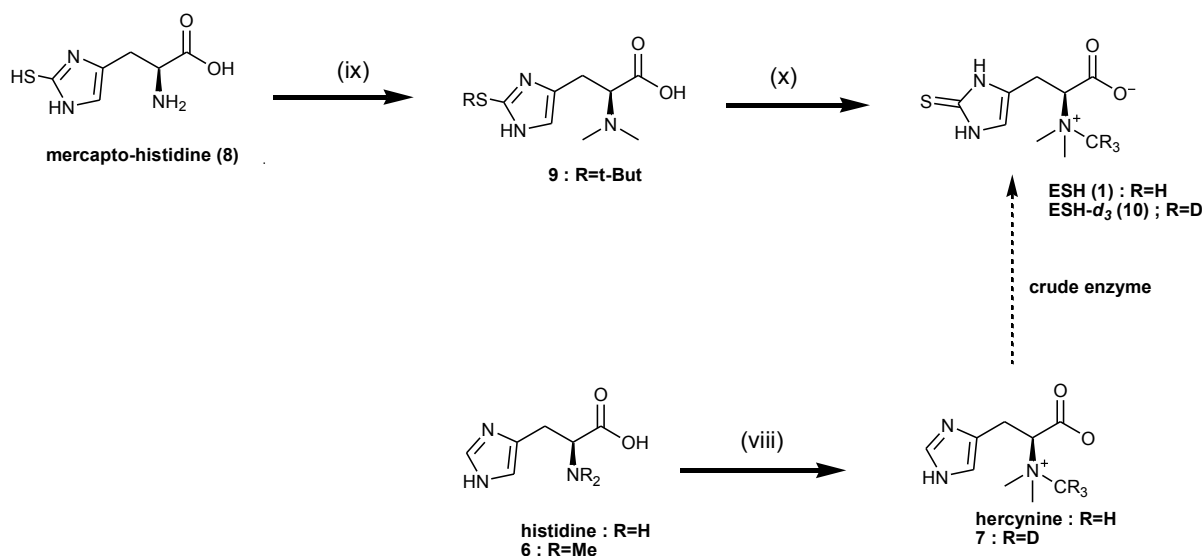
Hz, 1H, H-3b), ^{13}C NMR (151 MHz, D_2O) δ 171.3 (C-1), 170.9 (C-3'), 138.0 (C-2'), 129.25 (C-4'), 122.96 (C-5'), 75.29 (C-2), 55.18 (C-2''), 52.53 (NMe_3), 37.07 (C-3), 24.61 (C-1''); HRMS (ESI^+): m/z 317.1284 $[\text{M}]^+$. Calculated for $\text{C}_{12}\text{H}_{21}\text{N}_4\text{O}_4\text{S}^+$ found 317.1277 $[\text{M}]^+$.

(2*S*)-*N,N,N*-2-trimethylammonium-3-[2-((2*R*)-(tert-butoxycarbonylamino)-3-butoxycarbonyl)ethylthio)-1*H*-imidazol-4-yl] benzyl propanoate (5b).⁹



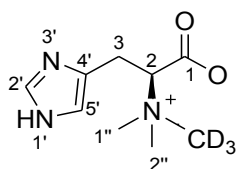
(2*S*)-*N,N,N*-2-trimethylammonium-3-[2-((2*R*)-2-amino-2-hydroxycarbonyl)ethylthio)-1*H*-imidazol-4-yl] propanoic acid trihydrochloride salt (**5a**) (765 mg, 2.51 mmol) was dissolved in a mixture of distilled $\text{H}_2\text{O}:\text{CH}_3\text{CN}$ (1:1, 10 ml), followed by the addition portion wise of NaOH (100 mg, 2.51 mmol). *Tert-butyl* dicarbonate (602 mg, 2.76 mmol) was added and the solution was allowed to stir overnight at room temperature. The solvent was evaporated under high vacuum to dryness, and the residue was triturated with Et_2O , dried under vacuum. The crude solid was dissolved in DMF (5ml) followed by the addition of benzyl bromide (943 mg, 5.52 mmol). The solution was allowed to stir overnight at room temperature. The solvent was removed under high vacuum to dryness. Purification by chromatography using silica gel column (CH_2Cl_2 : MeOH, 70:30) afforded (2*S*)-*N,N,N*-2-trimethylammonium-3-[2-((2*R*)-(tert-butoxycarbonylamino)-3-butoxycarbonyl)ethylthio)-1*H*-imidazol-4-yl]benzylpropanoate (**5b**) as a brown gum (563 mg, 38 %). $[\alpha]_D^{20} = +0.084^\circ$ ($c = 6.67$, MeOH), ^1H NMR (400 MHz, DMSO) δ 8.63 – 8.24 (m, 10H, 2Ph), 7.90 (s, 1H, H-5'), 4.72 (s, 2H, H-1''), 5.32 (brs, 2H, NH), 4.44 (s, 2H, H-1'''), 3.73 (d, $J = 3.1$ Hz, 1H, H-2), 3.66 (dd, $J = 4.1, 2.7$ Hz, 1H, H-2'''), 3.30 (dd, $J = 11.1, 3.1$ Hz, 1H, H-3a), 3.25 (dd, $J = 16.8, 4.1$ Hz, 1H, H-1'''a), 3.18 (dd, $J = 16.8, 4.1$ Hz, 1H, H-1'''b), 3.15 (dd, $J = 11.1, 3.1$ Hz, 1H, H-3b), 2.84 (s, 9H, NMe_3), 2.68 (s, 9H, Boc); ^{13}C NMR (101 MHz, DMSO) δ 169.4 (C=O), 169.2 (C=O), 168.7 (C=O), 138.3 (C-2'), 136.4 (C-4'), 129.7, 129.1, 128.8, 128.5, 128.0, 127.8, 127.1, 126.9 (8C, 2Ph), 125.57 (C-5'), 70.4 (C-1''), 69.5 (C-1'''), 68.0 (C(Boc)), 53.7 (C-2), 51.7 (NMe_3), 51.3 (C-2'''), 37.7 (C-3), 36.3 (3C(Boc)), 25.4 (C-1'''); LRMS (EI^+) m/z calculated for $\text{C}_{25}\text{H}_{31}\text{N}_4\text{O}_4\text{S}^+$ 483.2 $[\text{M} - \text{Boc} - \text{CH}_3]^+$ found 483.7 $[\text{M} - \text{Boc} - \text{CH}_3]^+$, 2 %).

S1.5. Synthesis of hercynine and ergothioneine deuterated compounds



Scheme S1. 2. 2. Synthesis and enzymatic production of hercynine-*d*₃ (7) and ESH-*d*₃ (10). Reagents and conditions: (viii) a) CH₂O, sodium triacetoxyborohydride / CH₃CN, 18-24 hrs at rt (6), b) NH₄OH, CH₃I or CD₃I / MeOH, 24 h at rt, (7); (ix) a) *t*-BuOH / HCl, refluxed for 3-4 hrs, *S-t*-butyl mercaptohistidine; b) CH₂O, sodium triacetoxyborohydride / THF, 6-8 hrs at 10 °C, (9); (x) a) NH₄OH, CD₃I / MeOH 24 h, rt; b) HCl, 2-mercaptopyruvic acid refluxed for 21 h (quantitative).

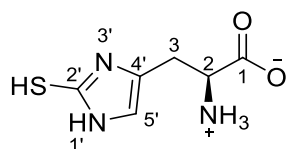
Deuterated hercynine or (2*S*)-*N,N,N*-2-trimethylamino-*d*₃-3-(1*H*-imidazol-4-yl)propanoic acid (7)



L-histidine (200 mg; 1.29 mmol) was dissolved in CH₃CN (10 ml), followed by the addition in one portion of formaldehyde (37 %; 157 mg; 145 μl; 1.93 mmol). The solution was allowed to equilibrate to room temperature, thereafter sodium triacetoxyborohydride (615 mg; 2.90 mmol) and acetic acid glacial (73 μl) were added at an internal temperature was maintained at 0-5°C. The resulting solution was allowed to stir at room temperature for 18-24 h period. The reaction was quenched with 5 ml of HCl 5 % to pH ≤ 1 and then extracted with EtOAc (5 x 20 ml), the remaining water layer was retained for recycling. The organic layer was separated, washed with brine, dried (anhydrous MgSO₄) and filtered. The filtrate was evaporated under vacuum to obtain crude *N,N*-dimethyl *L*-histidine (6) which was used without further purification.

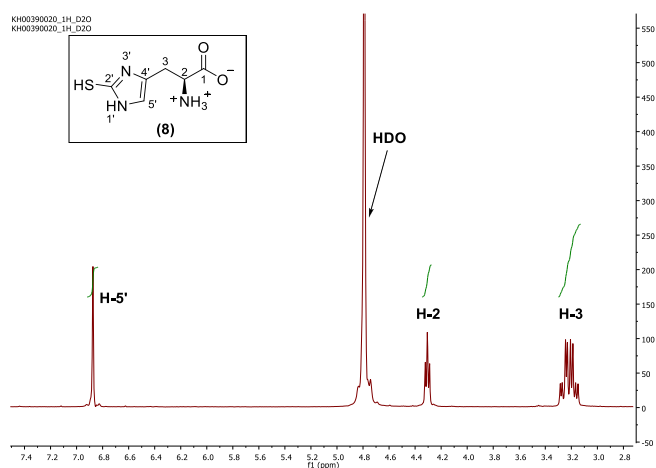
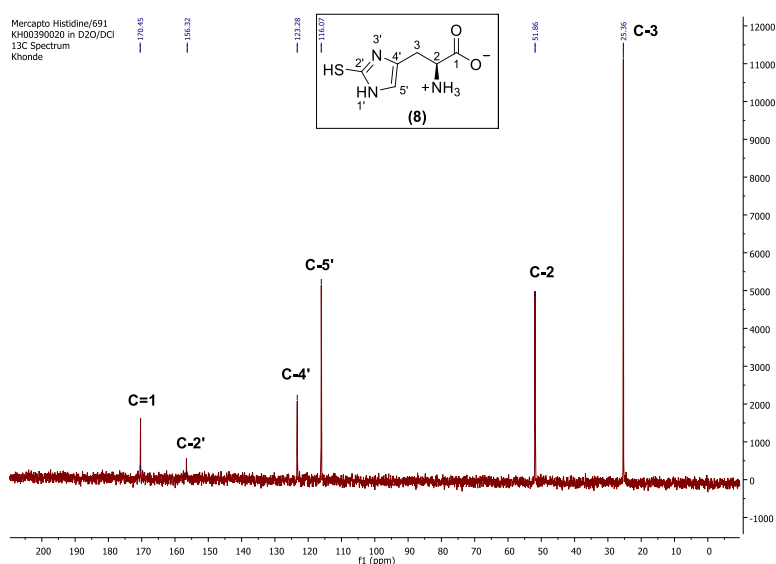
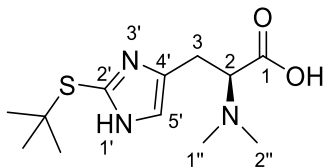
To the crude *N,N*-dimethyl *L*-histidine (**6**) (374 mg; assumed to be 2.02 mmol) dissolved in MeOH (5 ml) adjusted to pH 9-10 with a solution of concentrated ammonia (25%), followed by the addition of methyl iodate-*d*₃ (489 mg; 3.03 mmol). The solution was allowed to stir at room temperature for 24 hours. The solvent was evaporated under high vacuum to dryness. Reverse phase chromatography (C18) and recrystallization in mixture of warm EtOH/H₂O afforded deuterated hercynine (**7**) as a yellow solid (249 mg; 61 %); Mp: 142-145 °C (dec); ¹H NMR (400 MHz, D₂O) δ 8.70 (s, 1H, H-2'), 7.45 (s, 1H, H-5'), 4.44 (d, *J* = 7.7 Hz, 1H, H-2), 3.74 – 3.30 (m, 8H, H-1" H-2" & H-3); HRMS (ESI⁺): *m/z* 201.1431 [M]⁺. Calculated for C₉H₁₃D₃N₃O₂⁺ found 201.1414 [M]⁺.

Mecaptohistidine or (2*S*)-2-amino-3-(2-mercapto-1*H*-imidazol-4-yl) propanoic acid (**8**)³



The synthesis of mercaptohistidine (**8**) was carried out as reported by *Trampota et al.*³ Mercaptohistidine (**8**) was isolated as a white powder (2.01 g; 88 %). Mp: 206-208°C (with decomposition) Literature 204-206°C (with decomposition)³; ν_{\max} (KBr)/cm⁻¹ 3465s + 1634s + 1129w (RNH₂ Free) 2073s (N=C-S) 1383m (RCOO⁻), ¹H NMR (400 MHz, D₂O+DCl) δ 6.87 (s, 1H, H-5'), 4.31 (t, *J* = 6.6 Hz, 1H, H-2), 3.28 (dd, *J* = 16.1, 6.6 Hz, 1H, H-3a), 3.17 (dd, *J* = 16.1, 6.6 Hz, 1H, H-3b), Carboxylic acid and amine protons signals were exchanged with a D₂O. ¹³C NMR (101 MHz, D₂O) δ 170.5 (C-1), 156.6 (C-2'), 123.3 (C-4'), 116.1 (C-5'), 51.9 (C-2), 25.4 (C-3). LRMS (EI⁺) *m/z* calculated for C₆H₉N₃O₂S 187.0 found 187.0 ([M]⁺; 92.7 %).

S1.6. Characterisation of Mercaptohistidine (8) synthesized

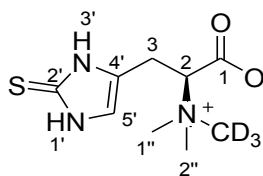
Figure (S1.6.1) ^1H NMR spectrum of Mercaptohistidine(8) in $\text{D}_2\text{O} + \text{DCl}$ at 400 MHzFigure (S1.6.2) ^{13}C NMR spectrum of mercaptohistidine(8) in $\text{D}_2\text{O} + \text{DCl}$ at 101 MHz.**(2S)-3-(2-(tert-butylthio)-1H-imidazol-4-yl)-2-(dimethylamino) propanoic acid (9)**

In distilled H_2O (18 ml) was added (2S)-2-amino-3-(2-mercapto-1H-imidazol-4-yl) propanoic acid (8) (2.27 g; 12.1 mmol), followed by the addition of *t*-butanol (2.34 g; 31.5 mmol) and concentrated hydrochloric acid (4 ml, 37 %). The resulting mixture was heated to 85-90°C and kept at this temperature for a 3 - 4 hr period. Subsequently, the reaction mixture was

concentrated by lyophilisation. The free amino acid was liberated by adjusting the pH of the solution to 5.0 with aqueous sodium acetate, followed by lyophilisation to dryness, where after the amino acid was extracted with warm 2-propanol. The product ***S-t-butyl mercaptohistidine*** was obtained as a yellow crystal (1.85 g; 62.7 %). $[\alpha]^{20}_{\text{D}} = +14.1^\circ$ ($c = 0.7$, H₂O) lit. $[\alpha]^{25}_{\text{D}} = +13^\circ$ ($c = 1$, H₂O)³; ν_{max} (KBr)/cm⁻¹ 3448s + 1561s + 1051w (RNH₂ Free) 2237w (N=C-S) 1703m (RCOOH) 1337m (CH₃) 643 (S-R), ¹H NMR (400 MHz, D₂O) δ 7.60 (brs, 1H, H-5'), 4.18 (t, $J = 3.0$ Hz, 1H, H-2), 3.39 (dd, $J = 11.0, 3.0$ Hz, 1H, H-3a), 3.30 (dd, $J = 11.0, 3.0$ Hz, 1H, H-3b), 1.44 (s, 9H, *t-butyl*); LRMS (EI⁺) m/z calculated for C₁₀H₁₇N₃O₂S 243.1 found 243.1 ([M]⁺; 4.2%), 199.1 ([M-CO₂]⁺; 7.1 %), 142.0 ([M-CO₂-C(CH₃)₃]⁺; 4.2%).

In a solution of THF (19 ml) was dissolved ***S-t-butyl mercaptohistidine*** (1.57 g, 6.45 mmol) followed by the addition of formalin (37 %, 2.04 g, 25.13 mmol) portion wise. The resulting mixture was allowed to equilibrate to room temperature and then sodium triacetoxyborohydride (3.76 g, 17.7 mmol) was added while the reaction temperature was maintained at 0-5°C. The resulting suspension was allowed to stir at 10°C for 6-8 hr. The reaction mixture was cooled to -10°C, and acidified with 2 N HCl (pH ≤ 1). The resulting solution was lyophilised and the residue was mixed with a 13 ml methanol followed by filtration of the undesired inorganic salts. The filtrate was lyophilised to yield the dihydrochloride salt. The free amine was liberated by triturating with aqueous sodium acetate to pH 5.0 evaporated to dryness, and extraction into 2-propanol, from where it was crystallized. The product (**9**) was obtained as colourless crystals (590 mg; 33.7 %). ¹H NMR (300 MHz, D₂O) δ 6.30 (s, 1H, H-5'), 3.45 (d, $J = 10.7$ Hz, 1H, H-2), 3.08 (m, 2H, H-3), 3.01 (s, 6H, H-1" H-2"), 1.50 (s, 9H, *t-butyl*).

Deuterated ergothioneine or [(2*S*)-*N,N,N*-2-trimethylamino-*d*₃-3-(2-mercapto-1*H*-imidazol-4-yl)propanoic acid (10**)**



(2*S*)-3-(2-(*tert*-butylthio)-1*H*-imidazol-4-yl)-2-(dimethylamino)propanoic acid (**9**) (110 mg; 0.405 mmol) was dissolved in MeOH (3 ml) and the pH was adjusted to 8.8-9.0 with ammonium hydroxide, followed by the addition of iodomethane deuterated (70 mg, 0.61 mmol) and the solution was allowed to stir at room temperature for 24 h. The mixture was concentrated under vacuum and the resulting white solid (ammonium chloride) was filtered and the cake

was washed with methanol. The combined filtrates were evaporated to dryness to afford a crude, deuterated *S*-(*tert*-butyl) ergothioneine. In the presence of 2-mercaptopropionic acid (1.70 g; 16.1 mmol), crude deuterated *S*-(*tert*-butyl) ergothioneine was dissolved in 2 ml H₂O followed by the addition of HCl (1 ml, 32%). The resulting mixture was refluxed for 21 hours. After cooling the reaction mixture was extracted with EtOAc (3 x 15 ml) and then the aqueous layer was adjusted to pH 7 with a solution of ammonia (25 % v/v) followed by lyophilisation. The residue was again extracted with ethyl acetate (3 x 20 ml) followed by partitioning in a mixture of distilled water: ethyl acetate 50:50 (v/v). The aqueous layer was retained and the organic phase discarded. The aqueous phase was lyophilised to dryness. Purification by reverse chromatography (C18) and recrystallization afforded the product (**10**) as a yellow solid (93 mg; quantitative). Mp: 158-160 °C (dec); ¹H NMR (400 MHz, D₂O) δ 6.95 (s, 1H, H-5'), 4.34 – 4.13 (m, 1H, H-2), 3.73 – 3.43 (m, 6H, H-1" H-2"), 3.41 (dd, *J* = 11.3, 8.5 Hz, 2H, H-3); ¹³C NMR (101 MHz, D₂O) δ 180.3 (C-1), 134.9 (C-2'), 119.1 (C-4'), 115.8 (C-5'), 75.5 (C-2), 52.5 (C-1" C-2"), 22.7 (C-3); HRMS (ESI⁺): *m/z* 233.1152 [M]⁺. Calculated for C₉H₁₃D₃N₃O₂S⁺, found 233.1161 [M]⁺.

S1.7. ¹H NMR of ergothioneine-*d*₃ (**10**) synthesized

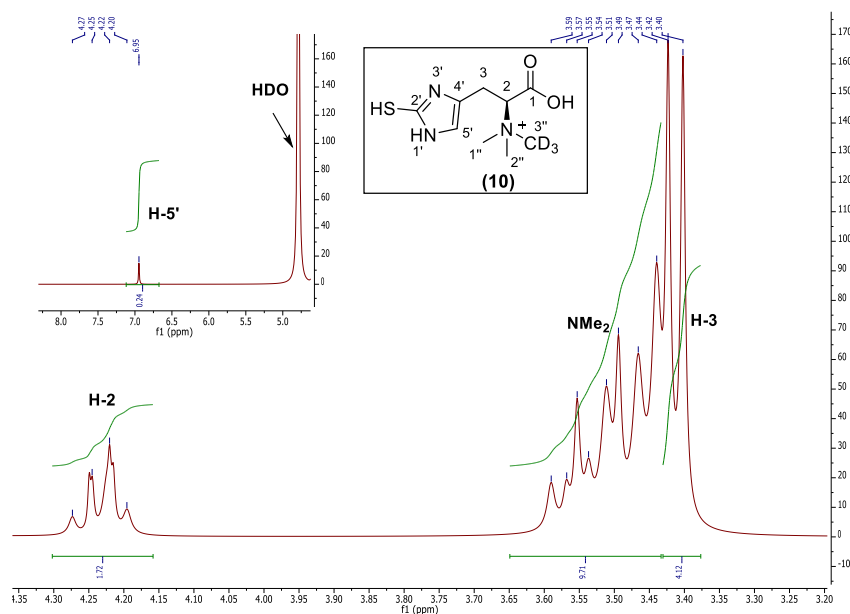
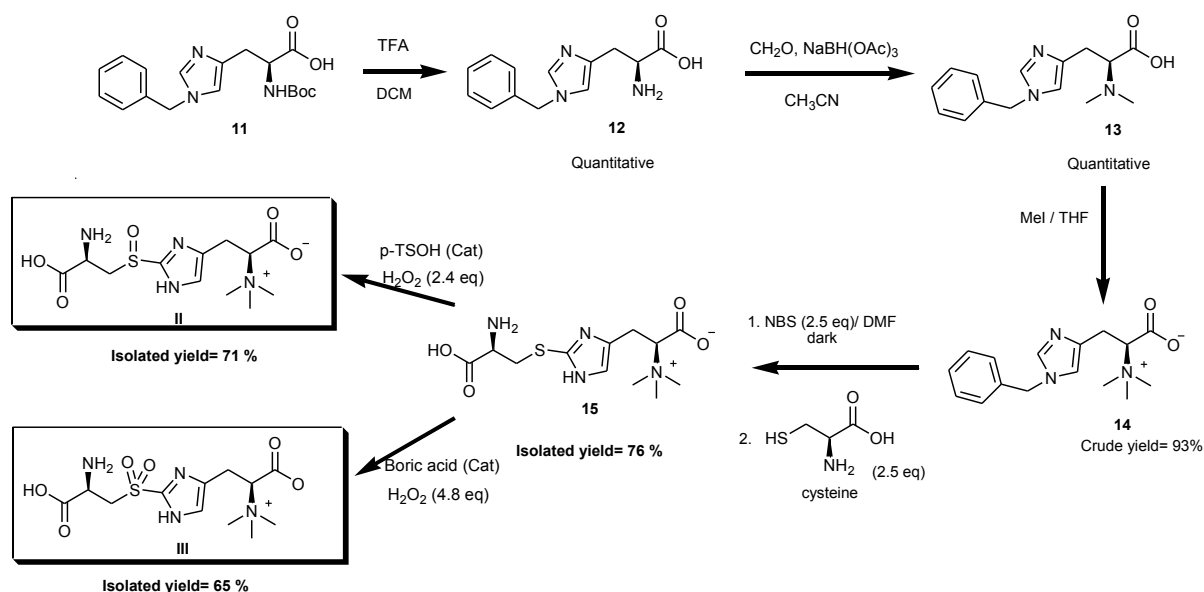
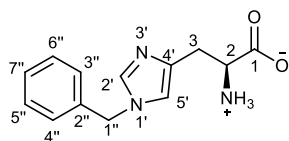


Figure (S1.7.1) ¹HNMR of ergothioneine-*d*₃ (**10**) in D₂O at 400 MHz

S1.8. Synthesis of Ergothioneine substrates and inhibitor



Scheme S8. Synthesis of sulfoxide (II) and sulfone (III).

***N*-benzyl-*L*-histidine (12)**

In dichloromethane (10 mL) was suspended **11** (750 mg, 2.17 mmol) followed by the addition of trifluoroacetic acid (1 mL) with cooling in an ice bath. The resulting homogenous solution was allowed to stir at room temperature until complete deprotection as showed by thin layer chromatography. Solvent was removed and triturated with Et₂O (15 mL) and dried to afford the TFA salt product **12** as white crystal (700 mg, 90 %). Mp: 230-233 °C, (Lit. 240 °C)¹⁰; ¹H NMR (400 MHz, DMSO) δ 9.01 (s, 1H, H-2'), 7.50 (s, 1H, H-5'), 7.39 (m, 5H, Phenyl), 5.37 (s, 2H, H-1''), 4.22 (t, *J* = 7.0 Hz, 1H, H-2), 3.22 (dd, *J* = 15.6, 7.0 Hz, 1H, H-3a), 3.14 (dd, *J* = 15.6, 7.0 Hz, 1H, H-3b); ¹³C NMR (101 MHz, DMSO) δ 170.1 (C-1), 136.3 (C-2''), 135.8 (C-2'), 130.0 (C-4'), 129.4 (C-5' C-6''), 129.0 (C-7''), 128.6 (C-3''C-4''), 120.6 (C-5'), 51.7 (C-1''), 51.6 (C-2), 26.3 (C-3).

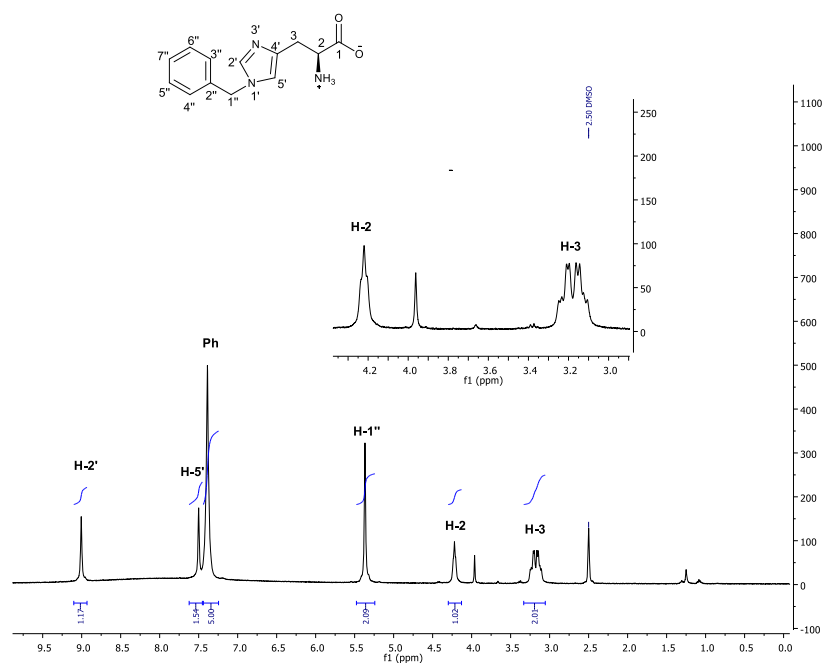


Figure S1. 8. 1. ¹H NMR spectrum of (12) in DMSO at 400 MHz.

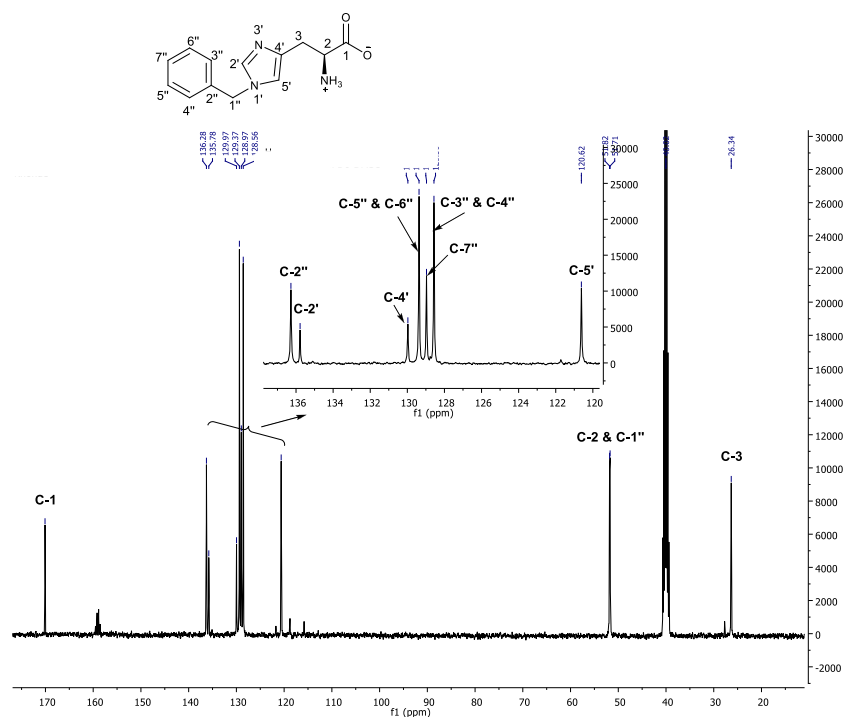
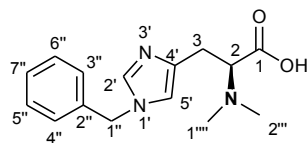


Figure S1. 8. 2. ¹³C NMR spectrum of (12) in DMSO at 101 MHz.

(S)-3-(1-benzyl-1*H*-imidazol-4-yl)-2-(dimethylamino)propanoic acid (13)

In CH₃CN (20 mL) was suspended **12** (1.30 g, 5.30 mmol) followed by the addition of formaldehyde (1.2 mL, 15.5 mmol, 37 %). To the resulting homogenous solution was added NaBH(OAc)₃ (3.2 g, 15.5 mmol) and the solution was allowed to stir at room temperature for 24 hours. Undesirable salts were filtered through celite and the solvent evaporated to dryness to afford the crude dimethyl product **13** as yellow oil (1.44 g, quantitative). Reverse C18 column chromatography afforded a product as a colourless solid (1.4 g, quantitative). Mp: 70-73 °C (dec); ¹H NMR (300 MHz, D₂O) δ 8.73 (s, 1H, H-2'), 7.51 – 7.37 (m, 5H, Ph), 7.34 (m, 1H, H-5'), 5.35 (s, 2H, H-1''), 4.31 (dd, *J* = 9.5, 4.7 Hz, 1H, H-2), 3.48 (dd, *J* = 15.4, 4.7 Hz, 1H, H-3a), 3.39 (dd, *J* = 15.4, 9.5 Hz, 1H, H-3b), 2.96 (s, 6H, H-1''' H-2'''); ¹³C NMR (101 MHz, D₂O) δ 168.7 (C-1), 135.2 (C-2'), 135.1 (C-2''), 133.6 (C-4'), 129.4 (C-3'' C-4''), 128.5 (C-5'' C-6''), 127.6 (C-7''), 121.0 (C-5'), 66.0 (C-2), 65.9 (C-1''), 52.9 (C-1''' C-2'''), 21.9 (C-3); LRMS (EI⁺) *m/z* calculated for C₁₅H₁₉N₃O₂ 273.1 [M]⁺ found 273.1 ([M]⁺, 7 %), calculated for C₁₄H₁₉N₃ 229.2 [M-CO₂]⁺ found 229.1 ([M-CO₂]⁺, 58 %), calculated for C₁₂H₁₃N₂ [M-H-CO₂-N(CH₃)₂]⁺ 185.1 found 185.1 ([M-H-CO₂-N(CH₃)₂]⁺, 69 %).

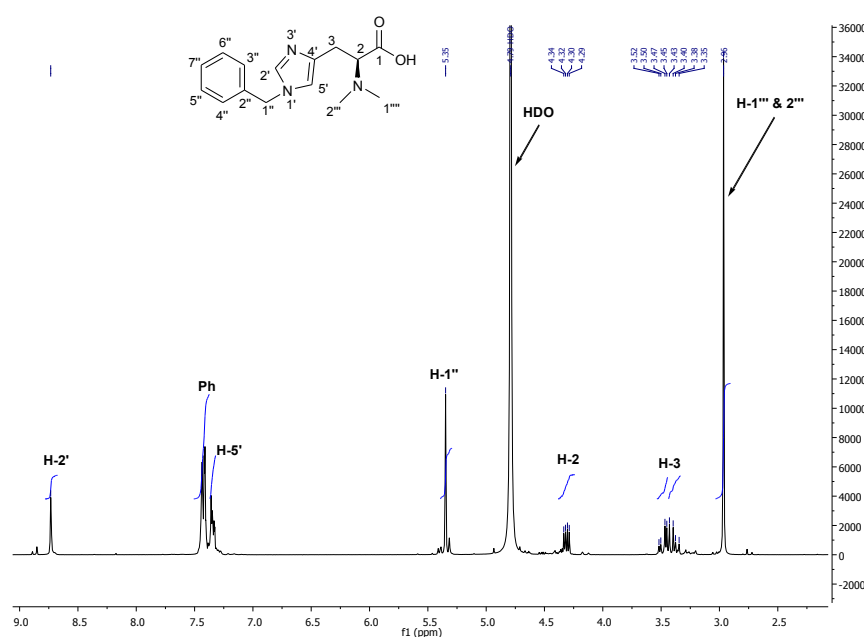


Figure S1. 8. 3. ¹H NMR spectrum of (**13**) in D₂O at 300 MHz.

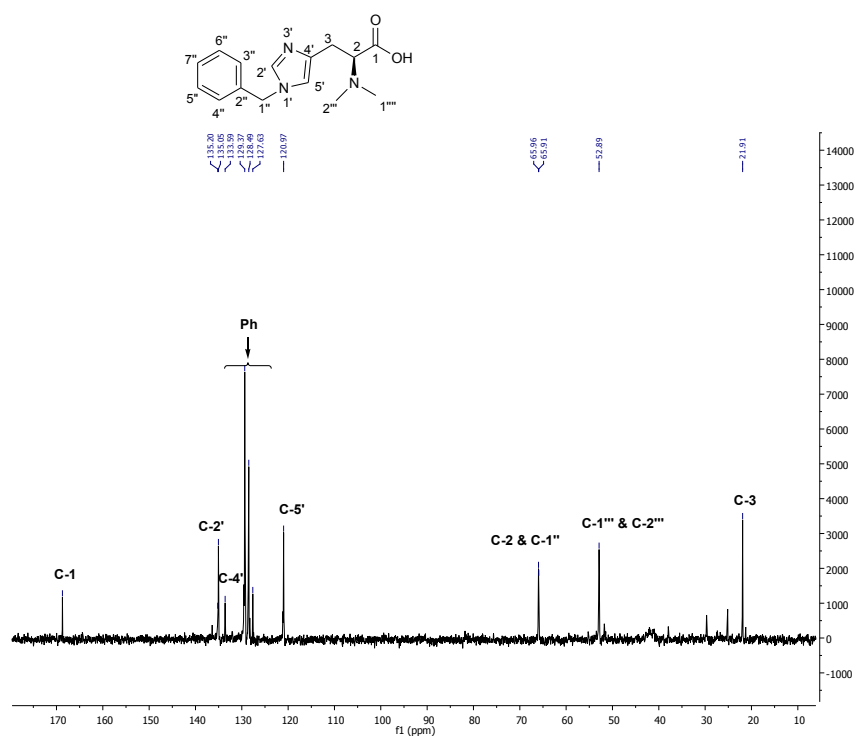
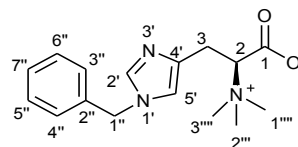


Figure S1. 8. 4. ^{13}C NMR spectrum of (**13**) in D_2O at 101 MHz.

(2*S*)-*N,N,N*-2-trimethylethanaminium-3-(1-benzyl-1*H*-imidazol-4-yl)propanoic acid (14**)**



In dry tetrahydrofuran (10 mL) was dissolved the crude dimethyl product **13** (200 mg, 0.732 mmol) followed by the addition of MeI (50 μL , 125 mg, 0.878 mmol). The resulting solution was allowed to stir at room temperature in the dark for 1-2 days. The solvent was removed to afford the product **14** as a yellow oil (197 mg, 93 %). Crystallization in the absolute ethanol afforded product **14** as yellow solid. Mp: 90-93 $^{\circ}\text{C}$; ^1H NMR (300 MHz, D_2O) δ 8.32 (s, 1H, H-2'), 7.63 – 7.34 (m, 5H, Ph), 7.25 (s, 1H, H-5'), 5.34 (s, 2H, H-1''), 3.99 – 3.87 (m, 1H, H-2), 3.50 – 3.20 (m, 2H, H-3), 3.33 (s, 9H, H-1'''H-2'''H-3'''); ^{13}C NMR (101 MHz, D_2O) δ 170.4 (C-1), 137.1 (C-2'), 135.7 (C-2''), 132.0 (C-4'), 129.2 (C-3''C-4''), 128.7 (C-5''C-6''), 128.0 (C-7''), 119.4 (C-5'), 78.0 (C-2), 52.3 (C-1''' C-2''' C-3'''), 51.4 (C-1''), 24.8 (C-3); LRMS (EI^+) m/z calculated for $\text{C}_{16}\text{H}_{22}\text{N}_3\text{O}_2^+$ 288.2 $[\text{M}]^+$ found 288.2 ($[\text{M}]^+$, 11 %), calculated for $\text{C}_{15}\text{H}_{22}\text{N}_3^+$ 244.2 $[\text{M}-\text{CO}_2 \text{ and } -\text{H}]^+$ found 244.1 ($[\text{M}-\text{CO}_2 \text{ and } -\text{H}]^+$, 64 %), calculated for $\text{C}_{14}\text{H}_{18}\text{N}_3^+$ 228.2

$[\text{M-CO}_2\text{-H-CH}_3]^+$ found 228.1 ($[\text{M-CO}_2\text{-H-CH}_3]^+$, 100 %), calculated for $\text{C}_{12}\text{H}_{13}\text{N}_2^+$ 185.1
 $[\text{M-CO}_2\text{-2H-N(CH}_3)_3]^+$ found 185.1 ($[\text{M-CO}_2\text{-2H-N(CH}_3)_3]^+$, 62 %).

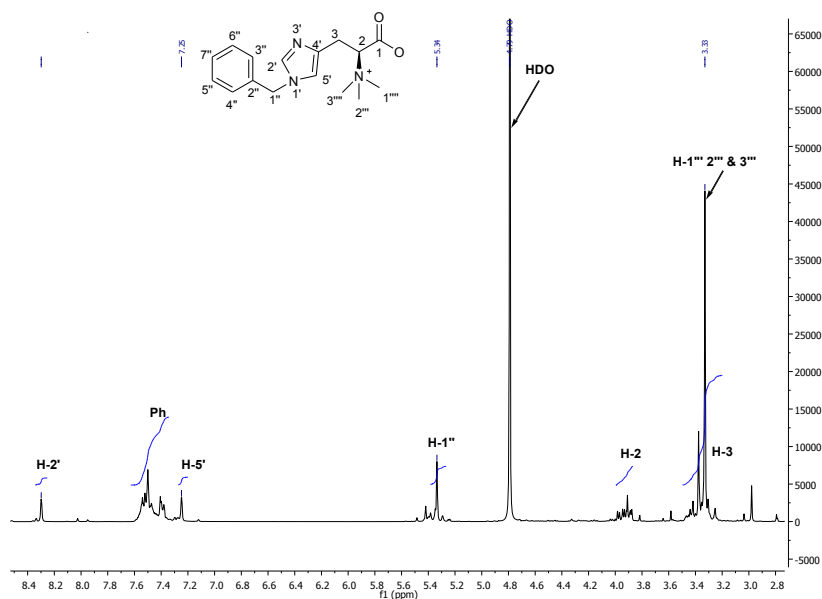


Figure S1. 8. 5. ^1H NMR spectrum of (14) in D_2O at 300 MHz.

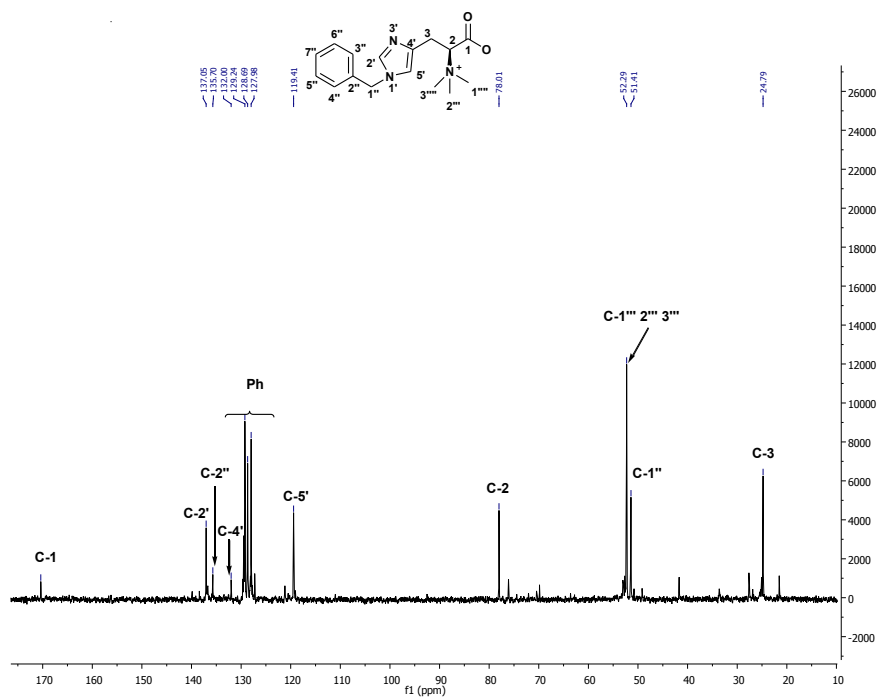
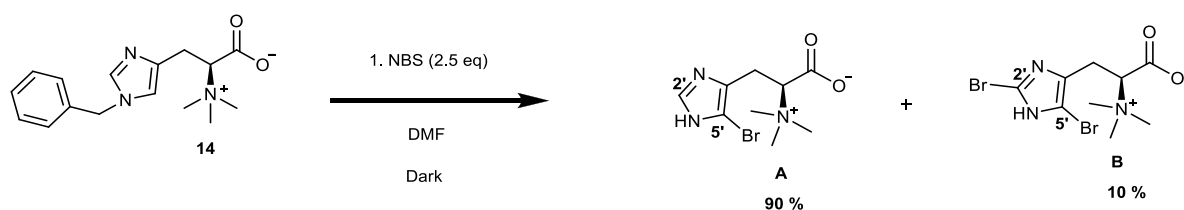


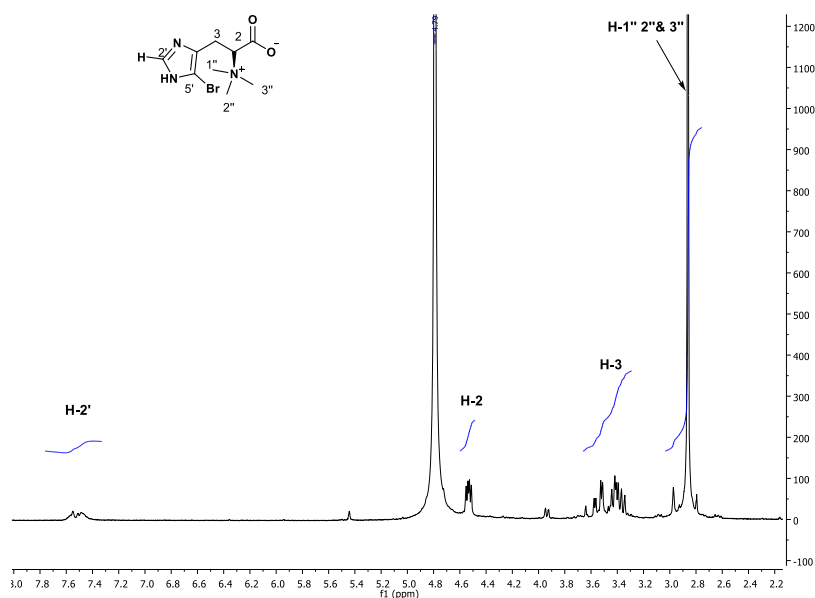
Figure S1. 8. 6. ^{13}C NMR spectrum of (14) in D_2O at 101 MHz.

S1.9. Selective and mild bromination of the imidazole ring



Scheme S1.9. 1. Selective and mild bromination of the imidazole ring.

The bromination conditions were optimised to be very selective. Two brominated intermediates have been found stable enough to be isolated by reverse phase C18 chromatography. The mono brominated intermediate, 5-bromo hercynine (**A**) was isolated in very high yield (90 %), while the 2, -5 dibromo hercynine intermediate (**B**) was isolated in a low yield of 10 %.

Figure S1. 9. 1. ^1H NMR spectrum of (**A**) in D_2O at 300 MHz.

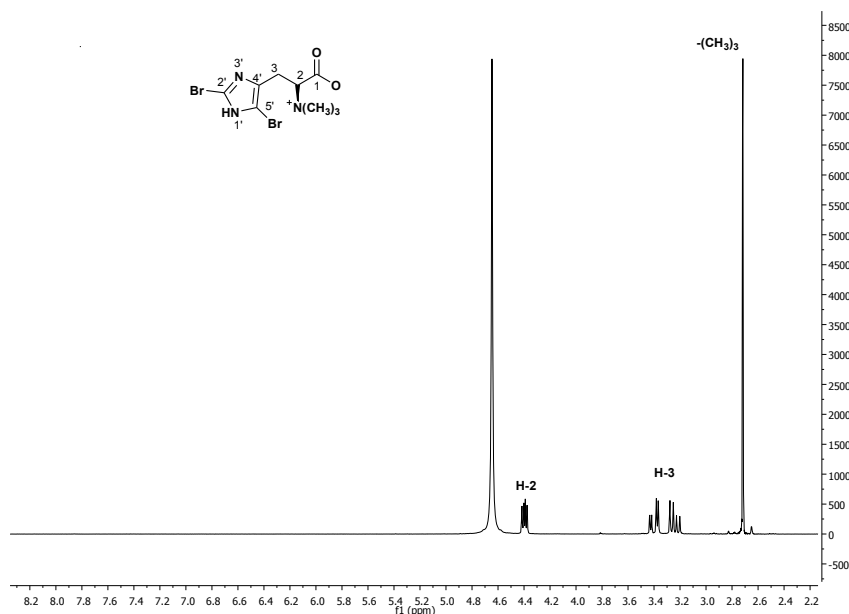
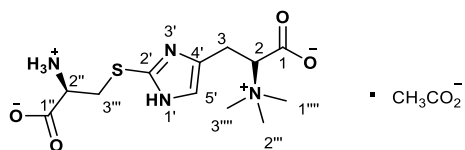


Figure S1. 9. 2. ^1H NMR spectrum of (**B**) in D_2O at 300 MHz.

S1.10. Hercynyl cysteine thioether (**15**) (One pot synthesis)



In dimethylformamide (8 mL) was dissolved **14** (595 mg, 2.43 mmol) followed by the addition of *N*-bromosuccinimide (1.8 g, 6.08 mmol). The resulting solution was allowed to stir at room temperature until complete disappearance of the starting material (thin layer chromatography monitoring), the solution became red-orange indicating the successful bromination.

After successful bromination, cysteine $\text{HCl} \cdot \text{H}_2\text{O}$ (1.07 g, 6.08 mmol) was added in one portion and the resulting solution was allowed to stir at room temperature for 24 hours. Reverse phase chromatography C18 afforded the product **15** isolated as the yellow hygroscopic solid acetate salt form (695 mg, 76 %). ^1H NMR (400 MHz, D_2O) δ 7.41 (m, 1H), 4.54 (dd, $J = 7.7, 4.4$ Hz, 1H, H-2"), 4.42 (t, $J = 5.0$ Hz, 1H, H-2), 3.50 (dd, $J = 15.2, 4.4$ Hz, 1H, H-3a"), 3.36 (dd, $J = 15.2, 7.7$ Hz, 1H, H-3b"), 3.19 (m, 2H, H-3), 2.80 (s, 9H, H-1"H-2"H-3"), 2.75 (s, 3H, acetate); ^{13}C NMR (101 MHz, D_2O) δ 170.3 (C-1"), 170.0 (C-1), 129.4 (C-2'), 128.9 (C-4'), 120.9 (C-5'), 61.0 (C-2), 54.4 (C-2"), 51.7 (C-1"C-2"C-3"), 36.3 (C-3"), 23.9 (C-3); HRMS (ESI⁺): m/z 317.1284 [M]⁺. Calculated for $\text{C}_{12}\text{H}_{21}\text{N}_4\text{O}_4\text{S}^+$ found 317.1277 [M]⁺.

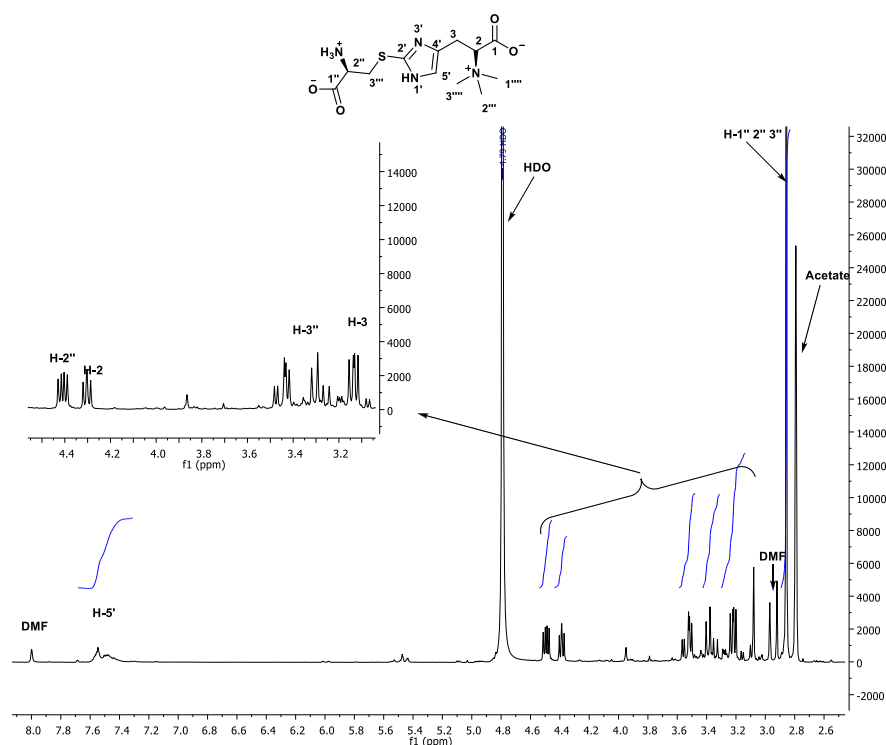
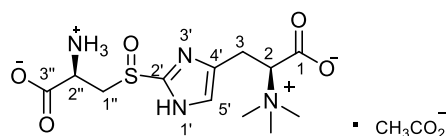


Figure S1. 10. 1. ^1H NMR spectrum of (**15**) in D_2O at 400 MHz.

S1.11. Synthesis of hercynyl cysteine sulfoxide (**I**) and sulfone (**II**).

S.11.1. *S*-(β -amino- β -carboxyethyl)ergothioneine sulfoxide or (2*S*)-*N,N,N*-2-trimethylammonium-3-[2-((2*R*)-2-amino-2-hydroxycarbonyl) ethylsulfinyl)-1*H*-imidazol-4-yl] propanoic acid (**II**)¹¹



To a solution of H_2O_2 (30 %, 224 mg, 6.58 mmol, 2.4 eq) were added **15** (870 mg, 2.32 mmol) and para toluene sulfonic acid (15 mg, 0.08 mmol). The resulting reaction mixture was allowed to stir at room temperature for 24 hours. At the end the reaction was quenched by the addition of H_2O (10 mL) and evaporated under high vacuum to afford crude product, which was purified by C18 reverse phase to afford product **II** as a yellow solid (640 mg, 71 %). ^1H NMR (300 MHz, D_2O) δ 8.01 (s, 1H, H-5'), 4.49 (dd, J = 8.6, 3.3 Hz, 1H, H-2''), 3.90 (dd, J = 16.1, 9.3 Hz, 1H, H-2), 3.65 (dd, J = 15.0, 3.3 Hz, 2H, H-1''), 3.52 (dd, J = 9.3, 4.9 Hz, 1H, H-3a), 3.44 (dd, J = 9.3, 4.9 Hz, 1H, H-3b), 2.86 (s, 9H, NMe_3), 2.79 (s, 3H, acetate); ^{13}C NMR (101 MHz, D_2O) δ 171.8 (C-3''), 170.1 (C-1), 156.6 (C-2'), 129.5 (C-4'), 125.5 (C-5'), 72.5 (C-2), 49.5

(NMe₃), 49.1 (C-1''), 43.5 (C-2''), 20.8 (C-3); HRMS (ESI⁺): *m/z* 334.1306 [MH]⁺. Calculated for C₁₂H₂₂N₄O₅S²⁺, found 334.1321 [MH]⁺.

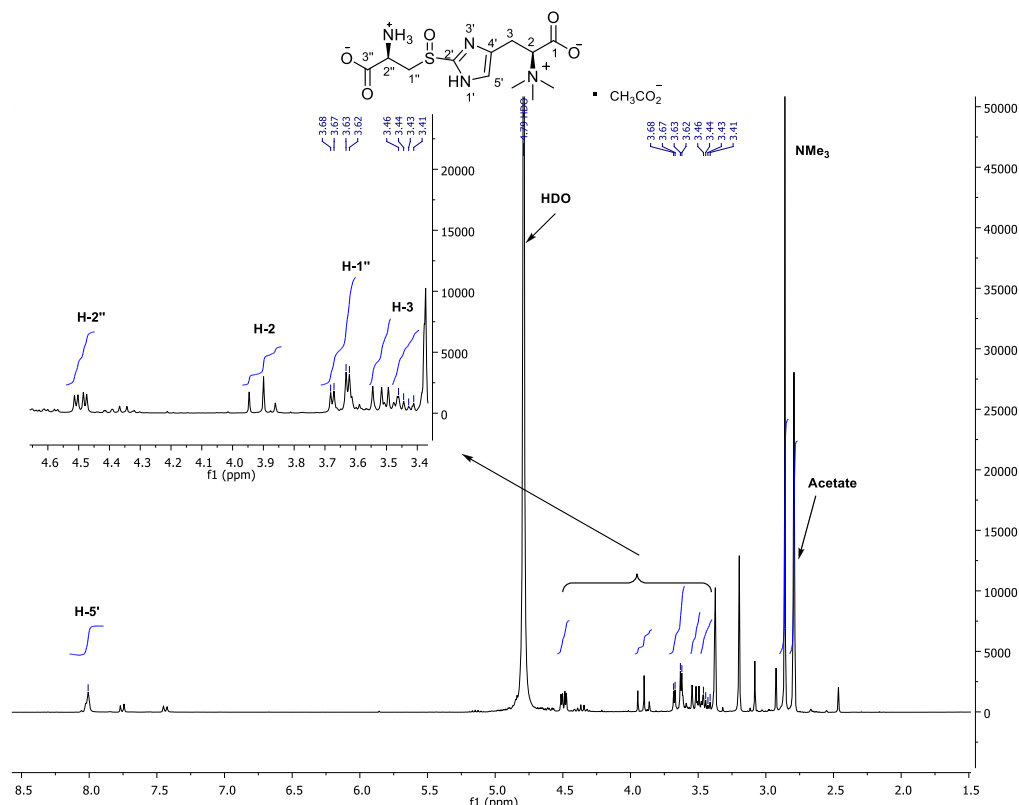
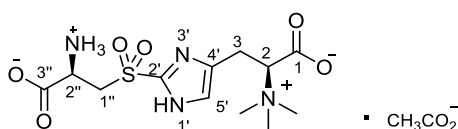


Figure S1. 11. 1. ¹H NMR spectrum of (I) in D₂O at 300 MHz.

S1.11.2. *S*-(β-amino-β-carboxyethyl)ergothioneine sulfone or (2*S*)-*N,N,N*-2-trimethylammonium-3-[2-((2*R*)-2-amino-2-hydroxycarbonyl) ethylsulfonyl)-1*H*-imidazol-4-yl] propanoic acid (III)¹²



15 (810 mg, 2.07 mmol) was added to a solution of H₂O₂ (30 %, 416 mg, 12.24 mmol, 4.8 eq) and boric acid (5 mg, 0.08 mmol), and the reaction mixture was allowed to stir at room temperature for 24 hours. At the end the reaction was quenched by the addition of H₂O (10 mL) and evaporated under high vacuum to afford crude product, which was purified by C18 reverse phase to afford product **III** as a yellow solid (545 mg, 65 %). ¹H NMR (300 MHz, D₂O) δ 8.01 (s, 1H, H-5'), 4.52 (dd, *J* = 8.3, 3.1 Hz, 1H, H-2''), 3.89 (dd, *J* = 16.1, 9.3 Hz, 1H, H-2), 3.65 (dd, *J* = 15.0, 2.8 Hz, 2H, H-1''), 3.59 – 3.43 (m, 2H, H-3), 2.85 (s, 9H, NMe₃), 2.79 (s, 3H, acetate); ¹³C NMR (101 MHz, D₂O) δ 171.7 (C-3''), 170.0 (C-1), 159.8 (C-2'), 156.6 (C-

4'), 132.9 (C-5'), 64.3 (C-2), 56.7 (C-1'), 49.4 (NMe₃), 49.1 (C-2''), 34.7 (C-3); HRMS (ESI⁺): m/z 349.1177 [M]⁺. Calculated for C₁₂H₂₁N₄O₆S⁺, found 349.1192 [M]⁺.

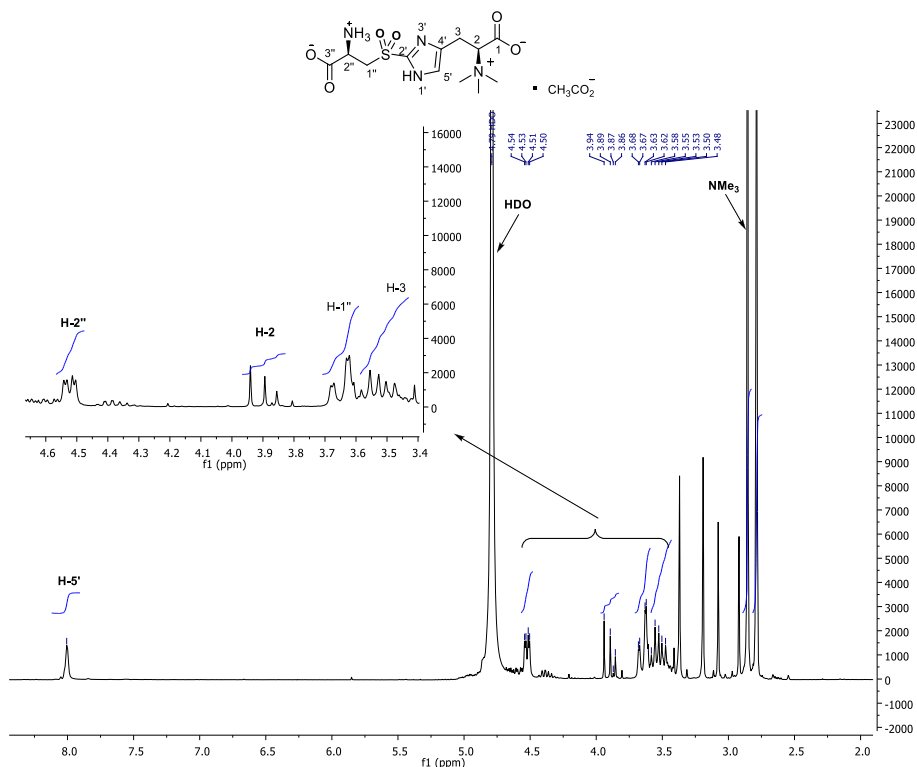


Figure S1. 11. 2. ¹H NMR spectrum of (II) in D₂O at 300 MHz.

S2. Total Protein extraction and Purification from *Mycobacterium smegmatis*.

S2.1. Mc²155 (*M. smegmatis*) growth conditions.

M. smeg culture (800 ml) were grown to exponential phase, and then dried to obtain 10 g of dry cells. The obtained pellets of *M. smeg* cells were thereafter stored at -80°C until it was required.

S2.2. Total protein extraction.

M. smeg cells was sonicated for 35 minutes at 4°C (25 pulsars), followed by the addition of potassium phosphate buffer (60 ml; pH 7). The solution was allowed to stir at 4°C for 10 minutes and thereafter centrifuged at 3000 rpm for 20 min. The supernatant was collected, measured and then the appropriate amount of ammonium sulphate gradually added while stirring at 4 °C overnight to obtain 60-70 % saturation.¹³

After precipitation of total protein the suspension was centrifuged at 4°C at 3000 rpm for 20 min and stored at -20°C.

S2.3. Total protein purification.

The complex total protein ammonium salts precipitate was resuspended in buffer mixture (20 ml; pH 7) containing pyridoxal phosphate (10 ml; 20 μ M), potassium phosphate buffer (8 ml; 50 mM; pH 7) and (2 ml; 1mM EDTA).

S2.4. Protein calibration curve

In order to determine the total protein concentration the protein Dc assay and the Bradford assays were used, here we report the Bradford calibration curve which was found to be more accurate than the protein Dc in our case.

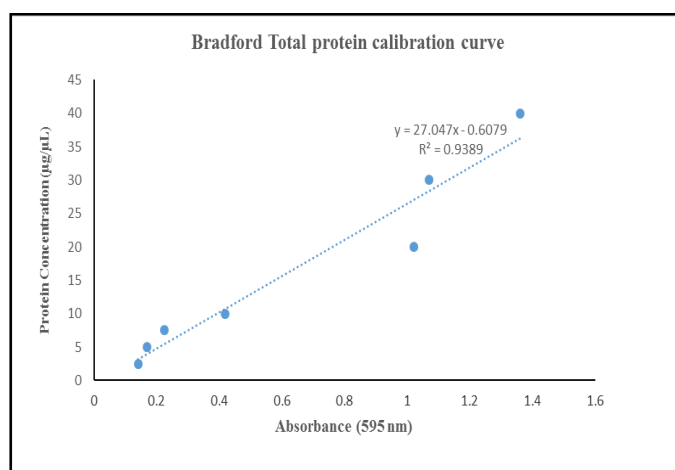


Figure (S2.4) Bradford protein concentration calibration curve.

The calculated *M. smeg* total protein concentration was found to be 10.33 μ g/ μ l

S3. HPLC –ESI/MS (QTOF) analysis.

S3.1. Materials and methods

Analyses were carried out with a UHPLC Agilent 1290 Infinity Series (Germany), accurate mass spectrometer Agilent 6530 Qrupole Time Of Flight (QTOF) equipped with an Agilent jet stream ionization source (positive ionization mode) (ESI⁺) and column (Polaris 3 C₁₈ Ether 100 X 2 mm, particle size 3 μ m, Agilent, Germany).

15 μ L of concentrate samples were injected into the LCMS. Analyte separation was attempted in 0.1 % formic acid in milli-Q water (solvent A) and mixture of 90 % acetonitrile, 0.1 %

formic acid, 10 % milli-Q water (solvent B) as mobile phase in an isocratic flow rate of 0.3 mL/min.

The system was controlled with the software packages Mass Hunter workstation software (Qualitative and Quantitative version B.05.00; Build 5.0.519.0, Agilent 2011, Germany)

S3.2. Experimental LCMS

Due to the polarity and charge on the quaternary ammonium group present in all metabolites, poor retention on the UHPLC (Eclipse + C18 RRHD 1.8 μ m.2.1 X 50) column was observed for ESH (RT= 0.8 min). All later analysis were performed with an improved column as described in the section S3.1 (RT = 1.5 min)

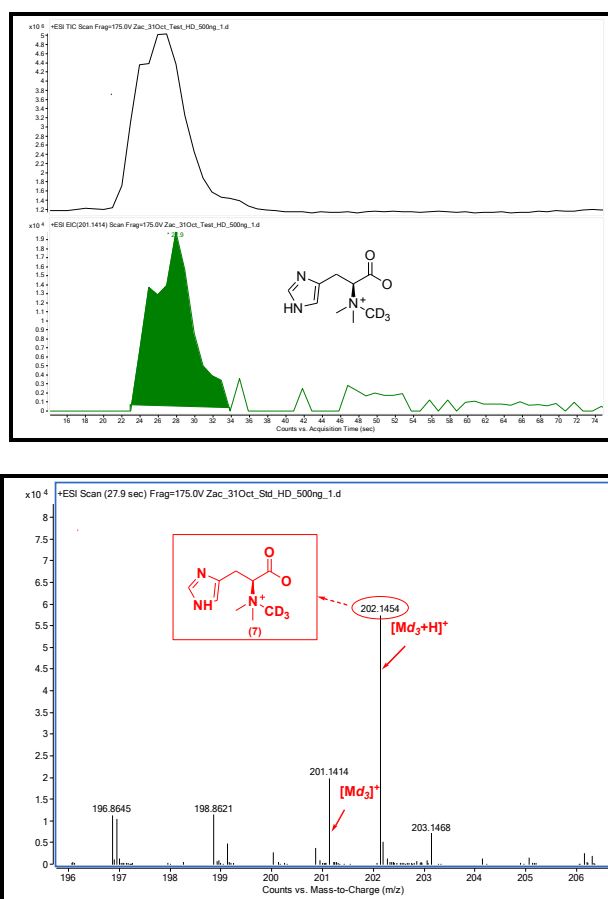


Figure (S3.2.1) TIC and ESI/QTOF mass spectra of Hercynine- d_3 (**7**) in positive ion mode

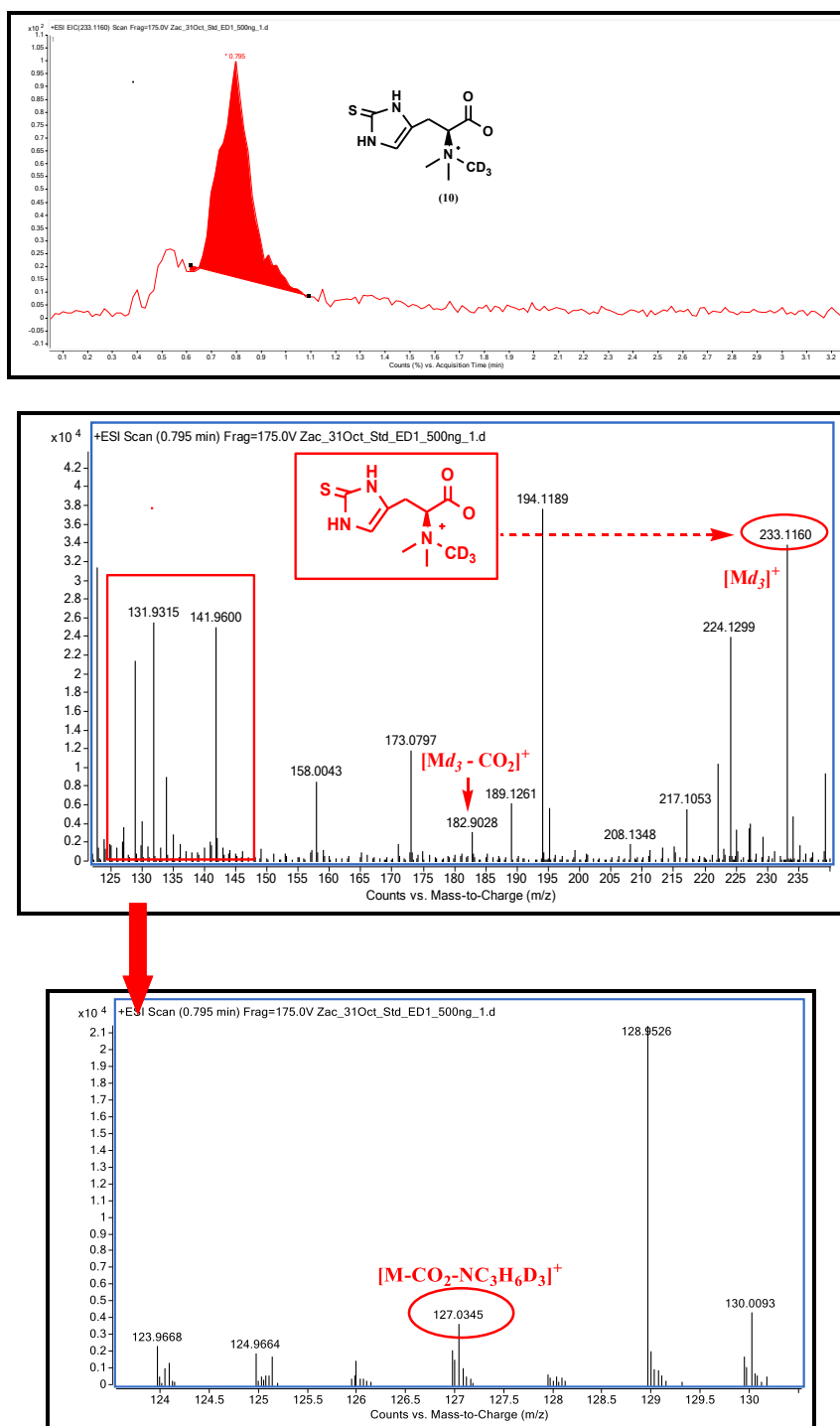


Figure (S3.2.2) TIC and ESI/QTOF mass spectra of Ergothioneine- d_3 (**10**) in positive ion mode

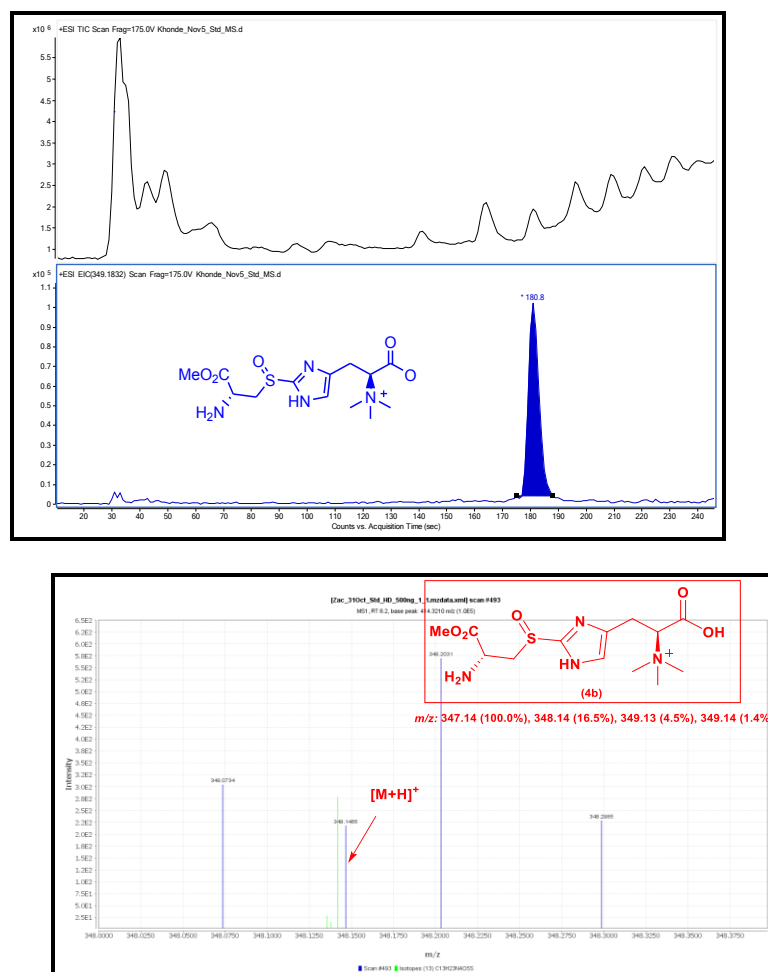
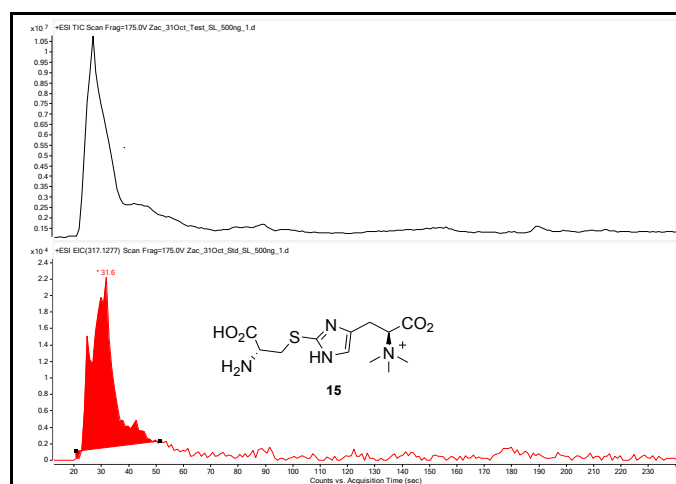


Figure (S3.2.3) ESI/QTOF mass spectra of *S*-(β -amino- β -carboxyethyl)ergothioneine methyl ester sulfoxide (**4b**) in positive ion mode.



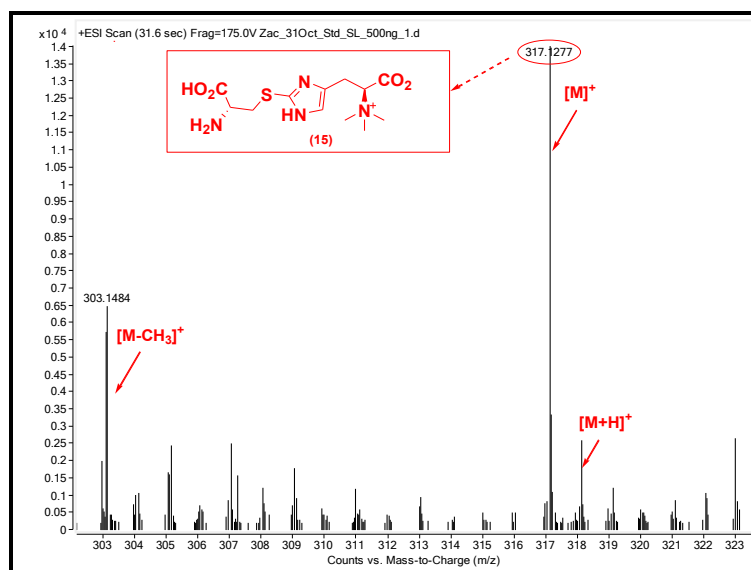


Figure (S3.2.4) TIC and ESI/QTOF mass spectra of *S*-(β -amino- β -carboxyethyl)ergothioneine sulfide (15) in positive ion mode

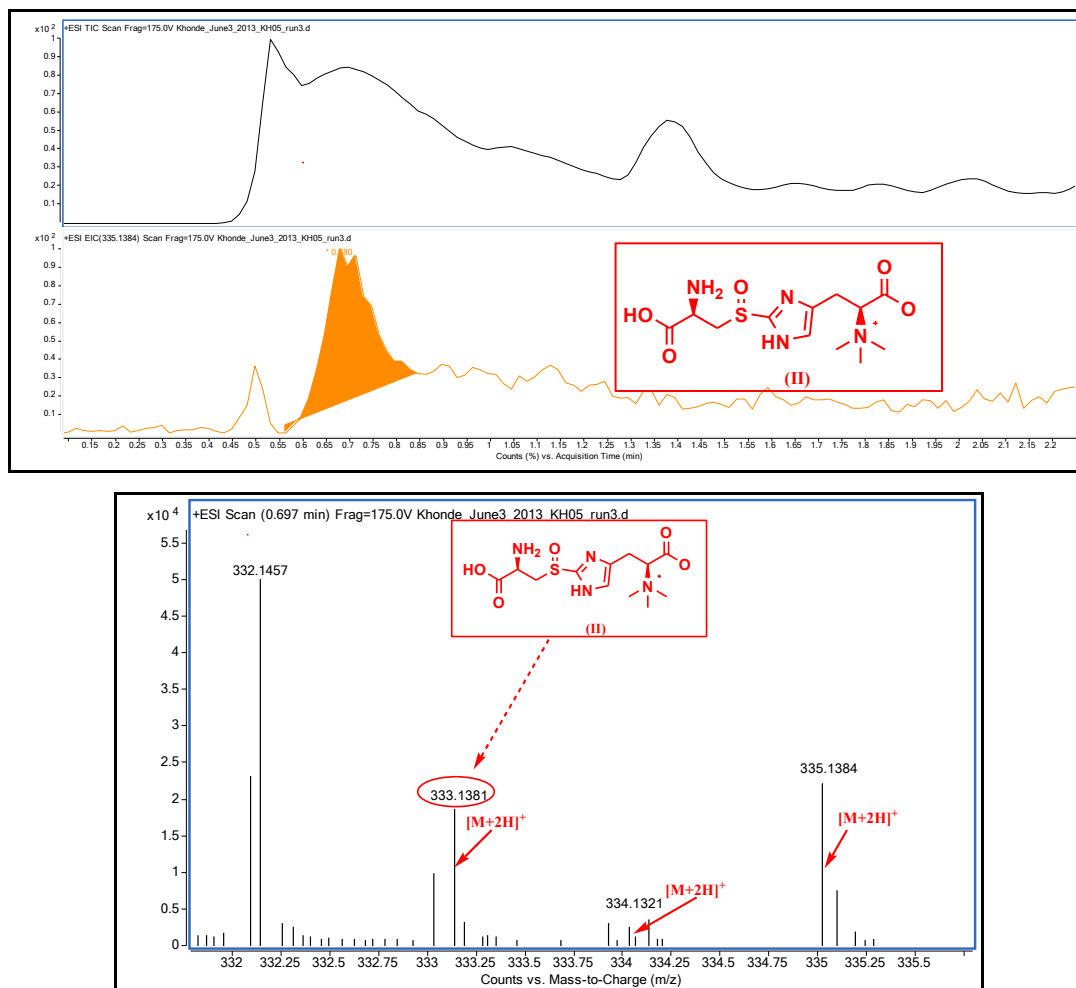


Figure (S3.2.5) TIC and ESI/QTOF mass spectra of *S*-(β -amino- β -carboxyethyl)ergothioneine sulfoxide (II) in positive ion mode.

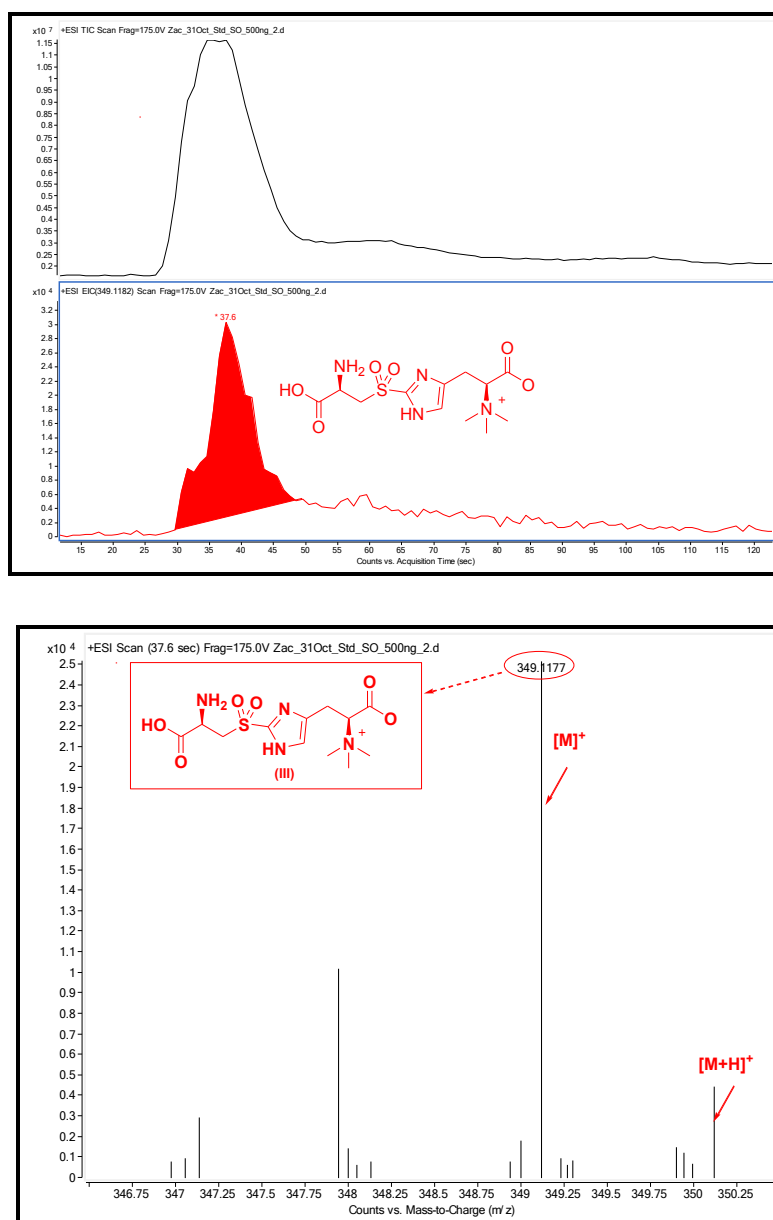


Figure (S3.2.6) TIC and ESI/QTOF mass spectra of *S*-(β-amino-β-carboxyethyl)ergothioneine sulfone (III) in positive ion mode

S4. *In vitro* reconstituted biosynthesis of ergothioneine in *Mycobacteria smegmatis*.¹⁴

The experiments were performed in triplicate, repeated several times (more than three times) and these results were reproducible.

S4.1. ESH Calibration curve

In order to establish a calibration curve for the quantification of ESH, eight different concentrations (0.78, 1.56, 3.125, 6.25, 12.5, 25, 50, 100 ng/ml) of ESH were prepared in triplicate, giving a limit of quantification (LOQ) 0.78 ng/ml for ESH which was similar to the one found by *L-Z Wang et al.*¹⁵ The limit of detection (LOD) for ESH was 9 pg/ mL. The retention time for ESH was 1.5 minute. Excellent symmetric peaks were achieved for both ESH standard and reactions samples analysed.

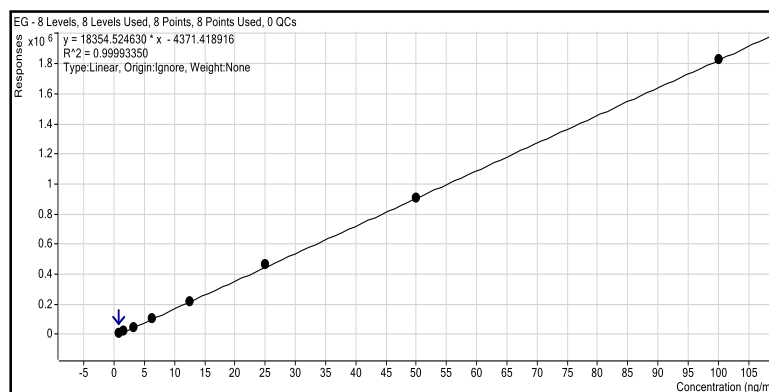


Figure (S4.1.1) Calibration curve of ESH.

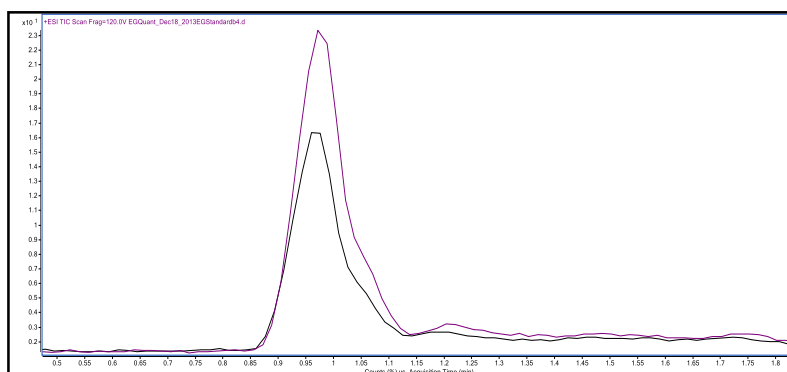


Figure (S4.1.2) Overlaid TIC of Ergothioneine. Retention time of 1.5 min

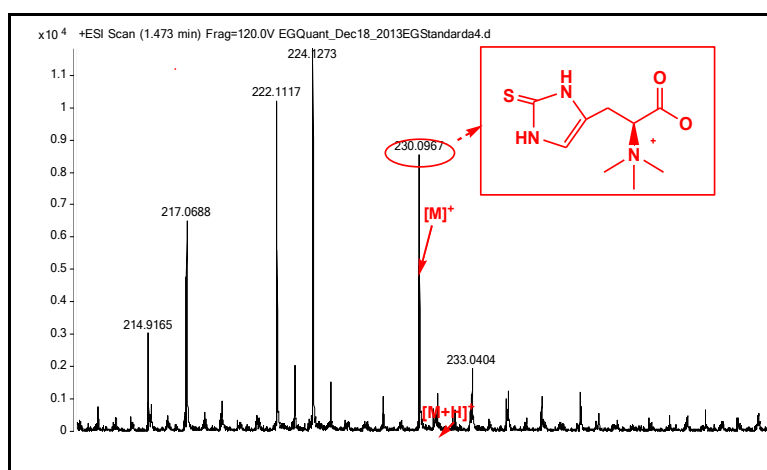
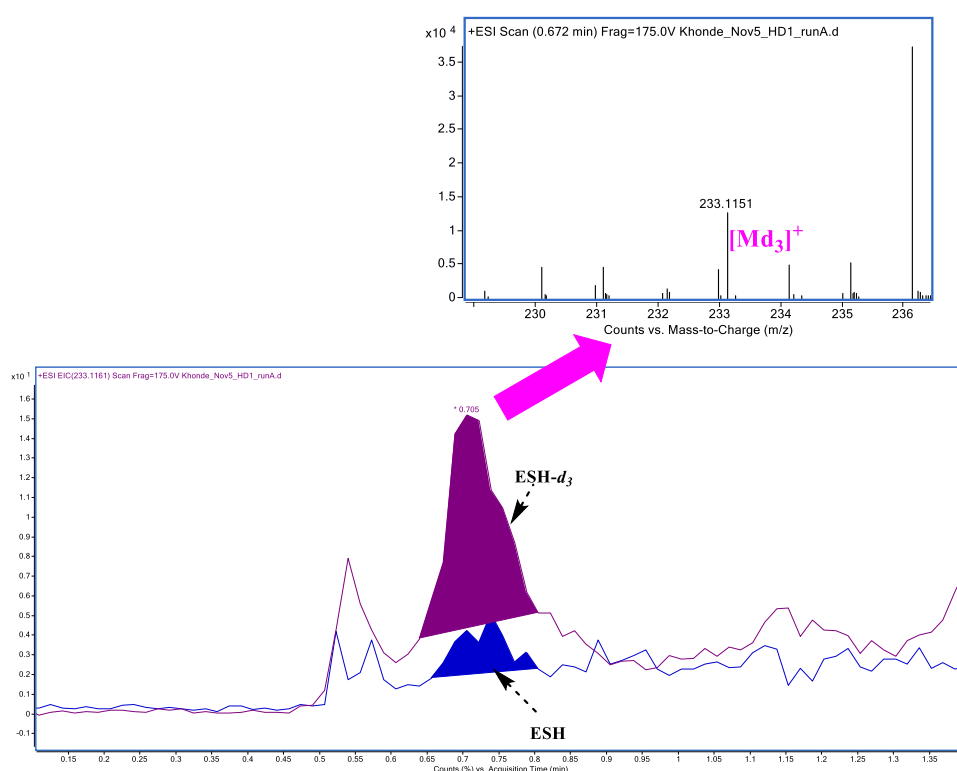


Figure (S4.1.3) ESI/QTOF mass spectra of ESH standard in positive ion mode**S4.2. Enzymatic synthesis of ergothioneine- d_3 (10) using Hercynine- d_3 (7) as a substrate.**

One set of 100 μ l reactions (1) containing 20 mM Tris HCl pH= 7.4, 20 mM NaCl , 0.2 Mm FeSO₄.7 H₂O, 0.5 mM mercaptoethanol, 83 μ l of crude enzymes and 50 mM of either (1) *S*-hercynine- d_3 (7). The crude enzyme reactions were incubated for 1 day at 37° C. The reaction was stopped by heating the mixture at 90°C for 2 min followed by lyophilisation and subsequent reconstitution in LC buffer before analysis by LC/MS.

**Figure (S4.2.1).** LCMS spectrum of ESH- d_3 *in-vitro* reconstituted experiment using hercynine- d_3 (7) as substrate.**S4.3. Biotransformation of substrates to ergothioneine using crude *M. smegmatis* enzymes preparation**

Three sets of 100 μ l reactions (1- 4) containing 20 mM Tris HCl pH= 7.4, 20 mM NaCl , 0.2 Mm FeSO₄.7 H₂O, 0.5 mM mercaptoethanol, 83 μ l of crude enzymes and 50 mM of either (1) *S*-(β -amino- β -carboxyethyl)ergothioneine sulfide (15) (2) *S*-(β -amino- β -

carboxyethyl)ergothioneine sulfoxide (**II**) (3) *S*-(β -amino- β -carboxyethyl)ergothioneine sulfone (**III**) or (4) control (only crude enzymes no substrates). The crude enzyme reactions were incubated for 1 day at 37° C. The reaction was stopped by heating the mixture at 90°C for 2 min followed by lyophilisation and subsequent reconstitution in LC buffer before analysis by LC/MS.

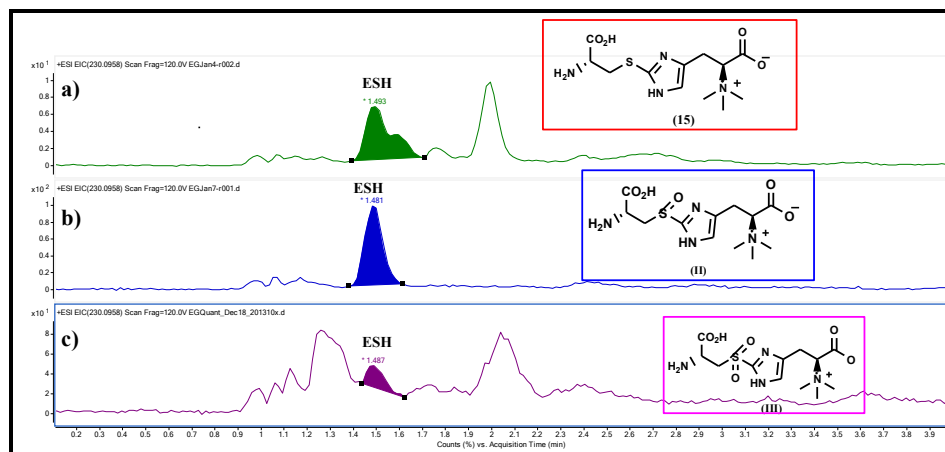


Figure (S4.3.1). TIC extracted for ESH *in-vitro* reconstituted experiment using substrate: (a) *S*-(β -amino- β -carboxyethyl)ergothioneine sulfide (**15**) as substrate, (b) *S*-(β -amino- β -carboxyethyl)ergothioneine sulfoxide (**II**) as substrate and (c) *S*-(β -amino- β -carboxyethyl)ergothioneine sulfone (**III**) as substrate

S4.4. Non enzymatic cleavage of C-S bond catalysed by PLP

Three sets of 100 μ l reactions (1-3) containing 20 mM Tris HCl pH= 7.4, and 50 mM of either (1) *S*-(β -amino- β -carboxyethyl)ergothioneine sulfide (**15**) and PLP (2) *S*-(β -amino- β -carboxyethyl)ergothioneine sulfoxide (**II**) and PLP or (3) *S*-(β -amino- β -carboxyethyl)ergothioneine sulfone (**III**) and PLP. The non-enzymatic reactions were incubated for 1 day at 37° C, followed by lyophilisation and subsequent reconstitution in LC buffer before analysis by LC/MS.

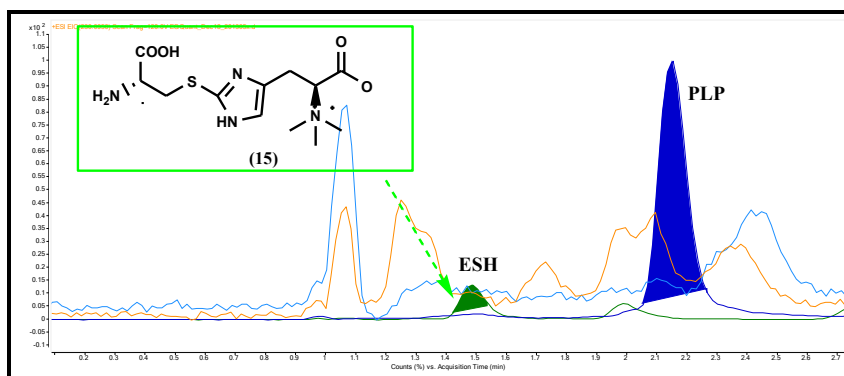


Figure (S4.5.1). Non enzymatic production of ESH catalysed by PLP. TIC extracted for ESH and PLP using *S*-(β -amino- β -carboxyethyl)ergothioneine sulfide (**15**), *S*-(β -amino- β -carboxyethyl)ergothioneine sulfoxide (**II**) and *S*-(β -amino- β -carboxyethyl)ergothioneine sulfone (**III**). Only *S*-(β -amino- β -carboxyethyl)ergothioneine sulfide (**15**) produced significant amount of ESH (96.34 ng/ mL) while sulfoxide (**II**) and sulfone (**III**) did not produce ESH at all.

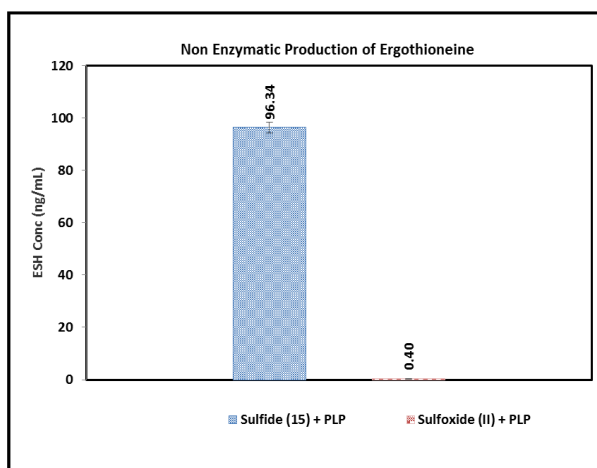
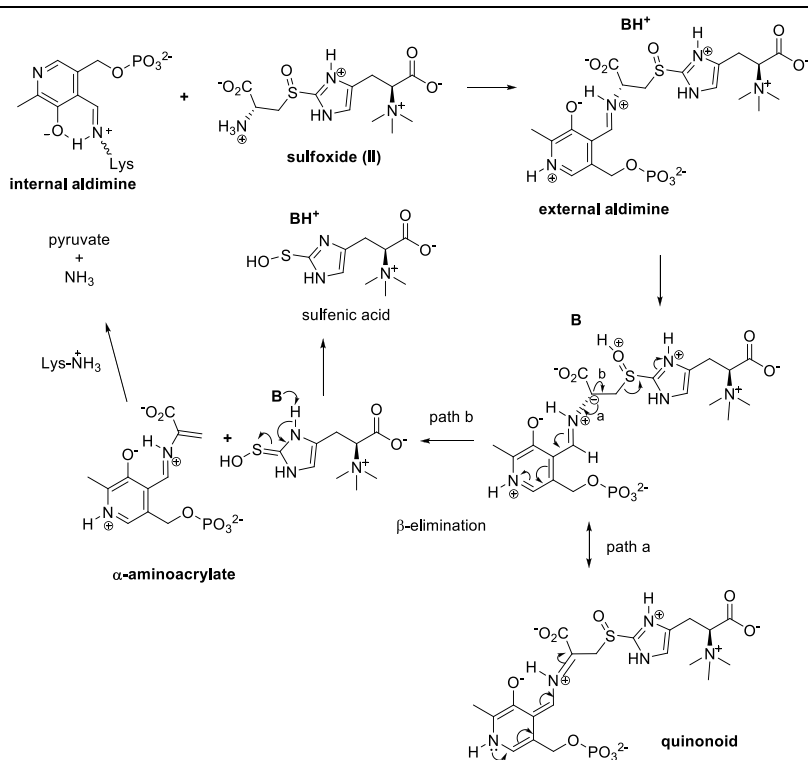


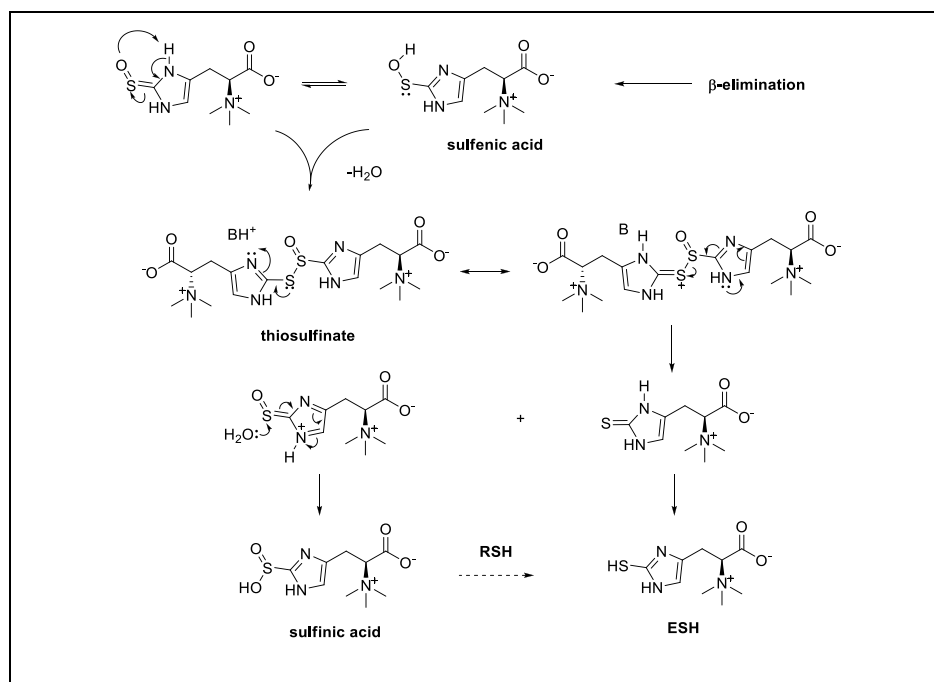
Figure (S4.5.2) Non enzymatic production of ESH graph contain; 100 μ l reactions containing 20 mM Tris HCl pH= 7.4, 20 mM NaCl, 50 mM of either (1) sulfide (**15**) plus PLP, (2) sulfoxide (**II**) plus PLP, (3) sulfone (**III**) plus PLP respectively. **Reaction time: 24 h.**

S5. Proposed mechanism of reaction of C-S of sulfide (15), sulfoxide (II) and sulfone (III) catalysed by PLP.

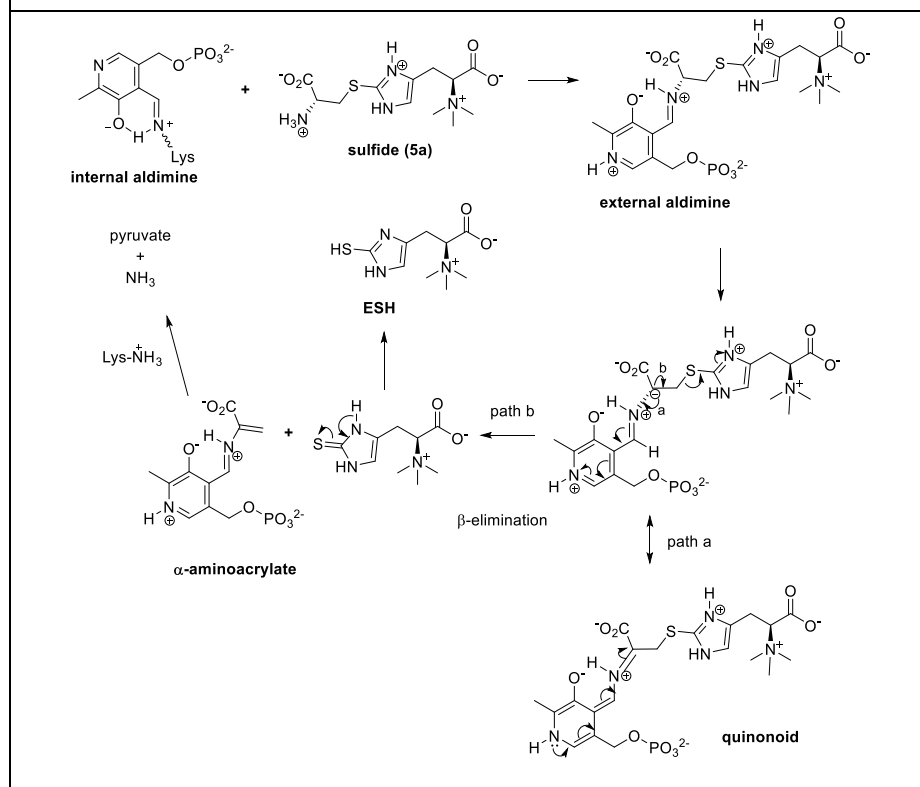
S5.1 β -Elimination of the sulfoxide (II)



S5.2 Sulfenic acid conversion to ESH



S5.3 β -Elimination of the sulfide (15)



S6. References

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