Supplementary Information:

The bacterial cytochrome P450 (CYP) CYP125 enzymes can competitively oxidise sitosterol in the presence of cholesterol.

Experimental

General

Unless otherwise stated, chemicals were purchased from Sigma-Aldrich. Kanamycin, detergents, DTT, and IPTG were from Astral Scientific. NADPH was from Applichem, and glucose-6-phosphate and glucose-6-phosphate dehydrogenase from Roche. Steroid substrates were purchased from Carbosynth (UK). The media for cell growth and maintenance (LB, SOC, and trace elements) were prepared as reported previously[1].

Recombinant protein expression and purification

MmarCYP125A6 and MtbCYP125A1 were cloned into the pET26 vector, expressed in BL21(DE3) cells and purified as previously described[1]. The codon optimised MulcCYP125A7 gene, along with a C-terminal 6xHis-tag, two stop codons and a HindIII restriction site were cloned in a pET29 vector obtained from Twist Biosciences. The sequence of the amino acid sequence encoded by the gene is shown below.

MAPCPNLPPGFDFTDPDIYAERLPVEEFAELRSSEPIWWDEQFPGQGGGFHDGGFWAITKLKDVKEVSRRSDVFSSYENG
VIPRFKNDIAREDIDVQRFVMLNMDAPHHTRLRKIISRGFTPRAIGRLHDELNDRAQNIAKAAAAAGSGDFVEQVSCELPL
QAIAGLLGIPQEDRGKLFDWSNEMTGTEDPEFAHIDAKASSVELIGYAMKMAEEKAKNPGDDIVTQLIQADIDGEKLSDDE
FGFFVVMLAVAGNETTRNSITQGMMAFADNPEQWELYKRERPGTAADEIVRWATPVTSFQRTALEDYELSGVQIKKGQR
VLMFYRSANFDEEVFEDPFSFNILRNPNPHVGFGGTGAHYCIGANLARMTINLIFNAVADHMPDLKPIAAPERLRSGWLN
GIKHWQVDYTGKCPVSHHHHHHH

The codon optimised RglobCYP125-04087 gene was cloned into the pET28 vector, expressed in BL21(DE3) cells and purified as previously described[1]. The sequence of the amino acid sequence encoded by the gene is shown below.

MTISDTTRTPDLPAGFDVTDPAILGERIPFEEFAELRRSAPVWWCEQPPTVGGFQDEGYWVVSRHADVKEVSQRSDIFSS
WENTAIARFADDMPREAVEMLRHLLLNKDAPEHTKLRKLISKLFTPRAINGMRDELDRRARSIVDTAVGEGPGDFVKQIAS
ELPLQAIADLIGVPQDDRDKLFKWSNEMMGYDDPEYVGDPAVASTEVLGYAYQMADARRSCPADDIVTTLVQADIDGD
ALSPEEFGFFVLILAVAGNETTRNAITHGMIAFLENPEQWELYKKERPKTTADEIVRWATPVTAFQRTALEDTELAGVSIKKG
QRVVMLYSSANFDEDVFEDPMTFDITRNPNPHLGFGGTGAHFCIGANLARMEIDLMFNALADNVPDITKIGDPRRLRSG
WINGIKEFQVDYKSTGCPVAH

Chemically competent BL21(DE3) *E. coli* (C2527 strain from New England Biolabs) cells were then transformed with the MulcCYP125A7-pET29 complex. The transformed cells underwent selection by plating onto LB_{kan}. Single colonies were used to inoculate 500 mL of LB media in 2L flasks (for a total growth volume of 3L). The cells were grown at 37°C and 110 rpm for approximately 8 hrs at which point they were cooled to 18 °C. Once cooled, chaperone proteins were induced by the addition of benzyl alcohol (2 mM) and ethanol (2% v/v). Trace elements (0.4% v/v) were also added at this time. After 30 min, MulcCYP125A7 protein production was induced by the addition of IPTG (50mM). The cultures were shaken for 48 hours at 18 °C and 90 rpm.

The cells were harvested by centrifugation (5000 rpm for 10 min at 4 °C). The cells were re-suspended in 200 mL of 50 mM Tris pH 7.4 and lysed by sonication (40 cycles of 10:50 s on/off, 70% amplitude, 19 mm probe, Sonics Vibra-Cell). Cell debris was removed by centrifugation (18,000 rpm for 20 min at 4 °C). The supernatant was pooled and loaded directly onto a Nickel affinity column (Protino Ni-NTA Agarose 5 mL,

Machery-Nagel) pre-equilibrated with binding buffer (0.5 M NaCl, 20 mM Phosphate Buffer, 20 mM Imidazole, pH 7.4) at 4 mL/min. After loading, impurities were removed by washing the column with 25 mL (5 column volumes) of binding buffer at 2 mL/min, followed by elution of the protein with elution buffer (0.5 M NaCl, 20 mM Phosphate Buffer, 200 mM Imidazole, pH 7.4). The protein was desalted by gel-filtration (Sephadex G-25 medium grain) and the relative purity was measured by the 412/280 nm peak absorbance ratio, which was 0.5 at this stage. The protein was then concentrated to approximately 10 mL (Vivacell 100 10kDa membrane, Sartorius), followed by further anion-exchange purification using a 5 mL Hi-Trap Q-HP anion exchange column (Cytiva) and AKTA protein purification system. The column was pre-equilibrated in 50 mM Tris (pH 7.4) and the protein was eluted in a continuous salt gradient from 0-400 mM KCl (in 50 mM Tris pH 7.4). Red coloured fractions (2mL) were collected and the purity of each was measured by the 412/280 nm peak ratio. Fractions with 412/280 nm > 0.8 were pooled and concentrated to approximately 10 mL. The concentrated protein was desalted by gel-filtration (Sephadex G-25 medium grain) and concentrated again to 5 mL. Finally, the protein was filtered, mixed with 80% glycerol (50% v/v) and stored at -20 °C.

Extinction coefficient determination

Extinction coefficients for MulcCYP125A7 (97 $mM^{-1}cm^{-1}$) was determined by methods previously described[2], using the known extinction coefficient for CO-bound P450 absorbance at 450 nm of 91 $mM^{-1}cm^{-1}$. Extinction coefficients were determined at 412 nm, the isosbestic point between the low-spin and high-spin P450 heme states.

Substrate binding analysis

Initial screening of substrate binding for all enzymes was undertaken by measuring the UV-Vis absorbance spectrum, where addition of substrate shifts the absorption maximum from the low-spin ~418 nm, 6-coordinate heme to the ~390 nm high-spin 5-coordinate heme, was measured[3]. Stored enzyme was eluted through a PD-10 size exclusion column (GE Healthcare) to remove glycerol and diluted to approx. 2-3 µM. Substrate stocks were made up in 40% hydroxypropyl-β-cyclodextrin in 50 mM Tris (pH 7.4) to 5 mM and 10 mM for sitosterol and cholesterol respectively. A Cary 60 UV-Vis Spectrophotometer (Agilent) was used to measure, after subtracting the baseline spectrum of 50 mM Tris (pH 7.4), the substrate-free P450 (600 µL) UV-Vis spectrum from 250-700 nm using a quartz cuvette. Substrate was then titrated, 1 µL at a time into the P450 solution (substrate was not added to more than 5% v/v to avoid unwanted solvent effects), each time measuring the UV-Vis spectrum of the enzyme-substrate mixture until no further changes in the 418/390 nm peak ratio were observed.

GC-MS activity assay

In vitro enzymatic oxidation reactions using a reconstituted NADPH/spinach ferredoxin/ferredoxin reductase redox system were conducted on a 600 µL scale in duplicate. P450 (2 µM) was incubated with either cholesterol or sitosterol (100 µM) or a mixture of cholesterol/sitosterol (75 µM of each) and oxygen saturated Tris buffer for 2 min at room temperature. Spinach ferredoxin (4 µM), spinach ferredoxin reductase (0.2 units/mL), glucose 6-phoshpate (5 mM), glucose 6-phosphate dehyrogenase (0.7 units/mL) and catalase (0.2 mg/mL) were then added. The reaction was initiated by the addition of NADPH (1 mM). The reaction was allowed to continue in the dark for 24 hrs. Octanoic acid (100 µM) was added as an internal standard. The analytes were extracted three times with ethyl acetate and dried with $MgSO_4$. Ethyl acetate was evaporated over N_2 after which the sample was dissolved in acetonitrile (150 µL). The sample was then derivatised for GC-MS analysis with BSFTA + TMCS (99:1, 15 µL) and heating at 37°C for 2 hrs. The derivatised samples were analysed by GC-MS using a Shimadzu GC-2010 equipped with a QP2010S GC-MS detector. The injection port temperature was 300 °C. The column (DB5 ms, 30 mm × 0.25 mm × 0.25 µm) was held at 70

°C for 1 min. The temperature was then increased to 280 °C at a rate of 15 °C min^{-1} and held at 280 °C for 1 min. Finally, the temperature was increased to 300 °C at a rate of 15 °C min^{-1} and held for 10 min.

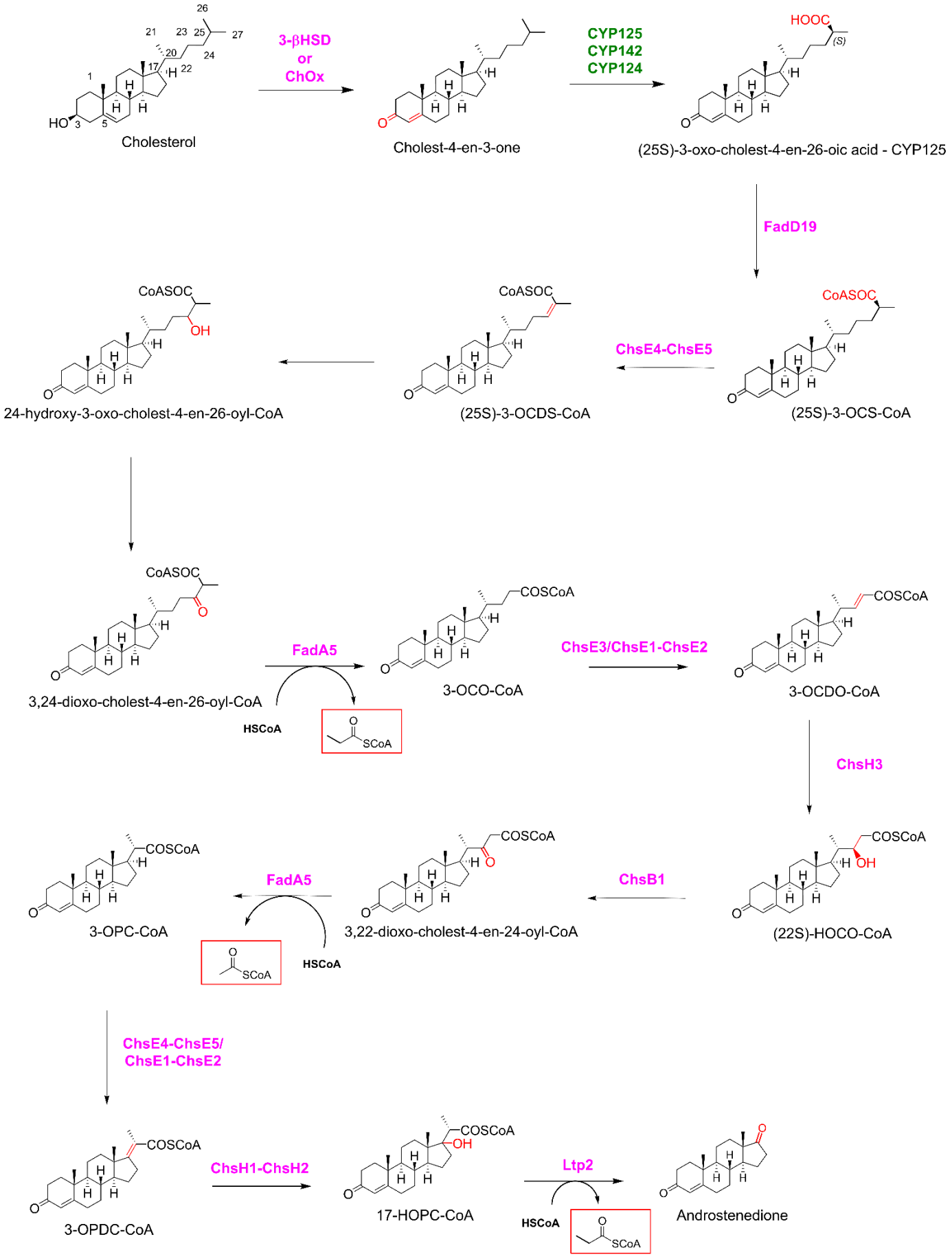
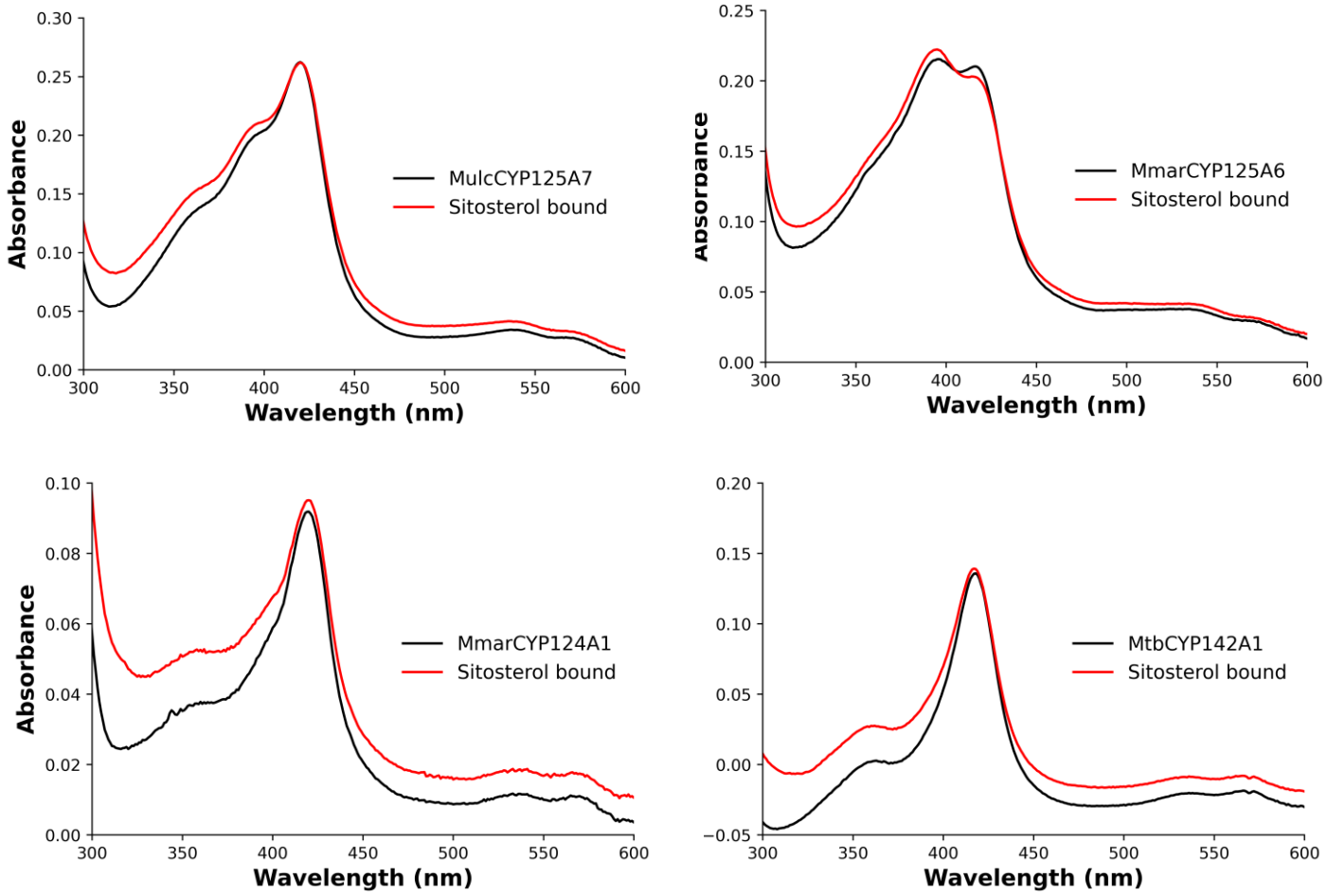


Figure S1: Proposed cholesterol oxidation pathway in mycobacteria. Characterised enzymes are shown in **pink**. P450 enzymes mentioned in this study are shown in **green**. Functional changes at each stage are

highlighted in **red**. This mechanism is based off of the initial findings of Sih *et. al* [4]. See main text references for individual enzyme characterizations.

A



B

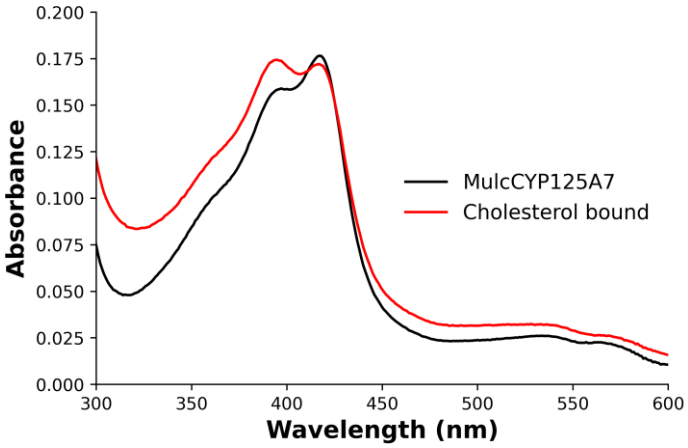


Figure S2. A: Spin state shift induced by the addition of sitosterol to MulcCYP125A7 (top left), MmarCYP125A6 (top right), MmarCYP124A1 (bottom left) and MtbCYP142A1 (bottom right). **B:** Spin state shift induced by the addition of cholesterol to MulcCYP125A7.

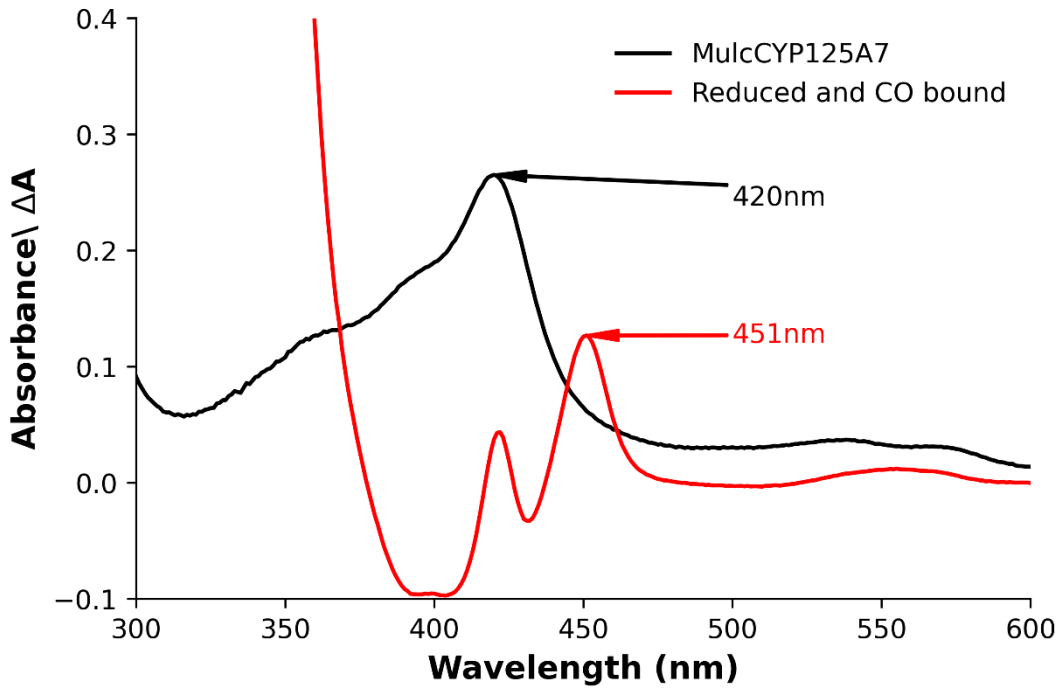


Figure S3: **Black:** Absolute UV-is absorbance spectrum of MulcCYP125A7. **Red**: Difference spectrum (with absolute protein spectrum as the baseline) after reduction with dithionite and addition of carbon monoxide, forming the characteristic 450 nm peak indicating functional enzyme.

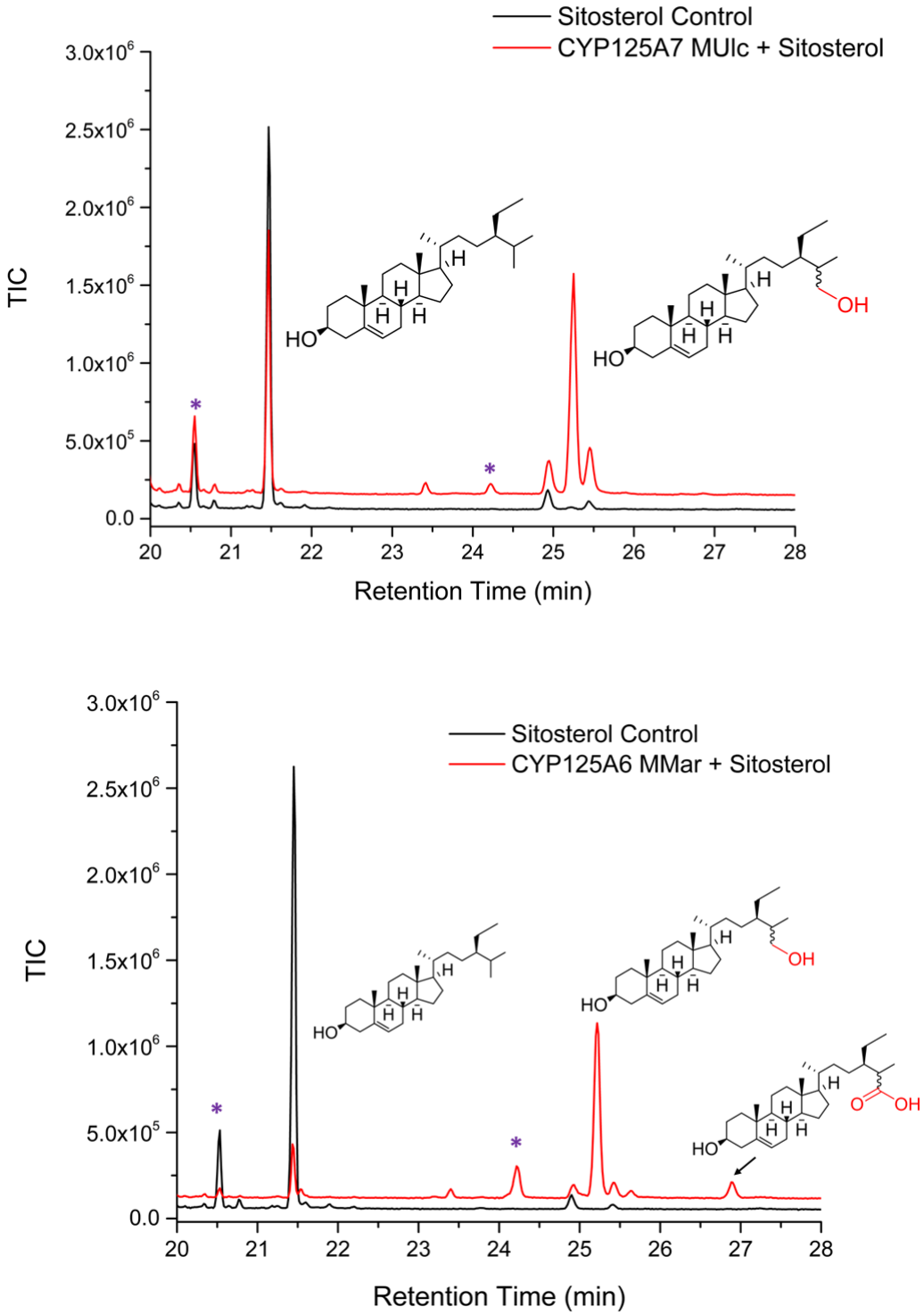


Figure S4: GC chromatograms of MulcCYP125A7 (left) and MmarCYP125A6 (right) *in vitro* oxidation of sitosterol using a reconstituted NADPH/Spinach Ferredoxin/Spinach Ferredoxin Reductase electron transfer system. Sitosterol control GC traces are shown in **black** and *in vitro* enzyme driven oxidation traces in **red**. A * indicates peaks arising from campesterol impurity in the sitosterol stocks. Product peaks were identified by MS.

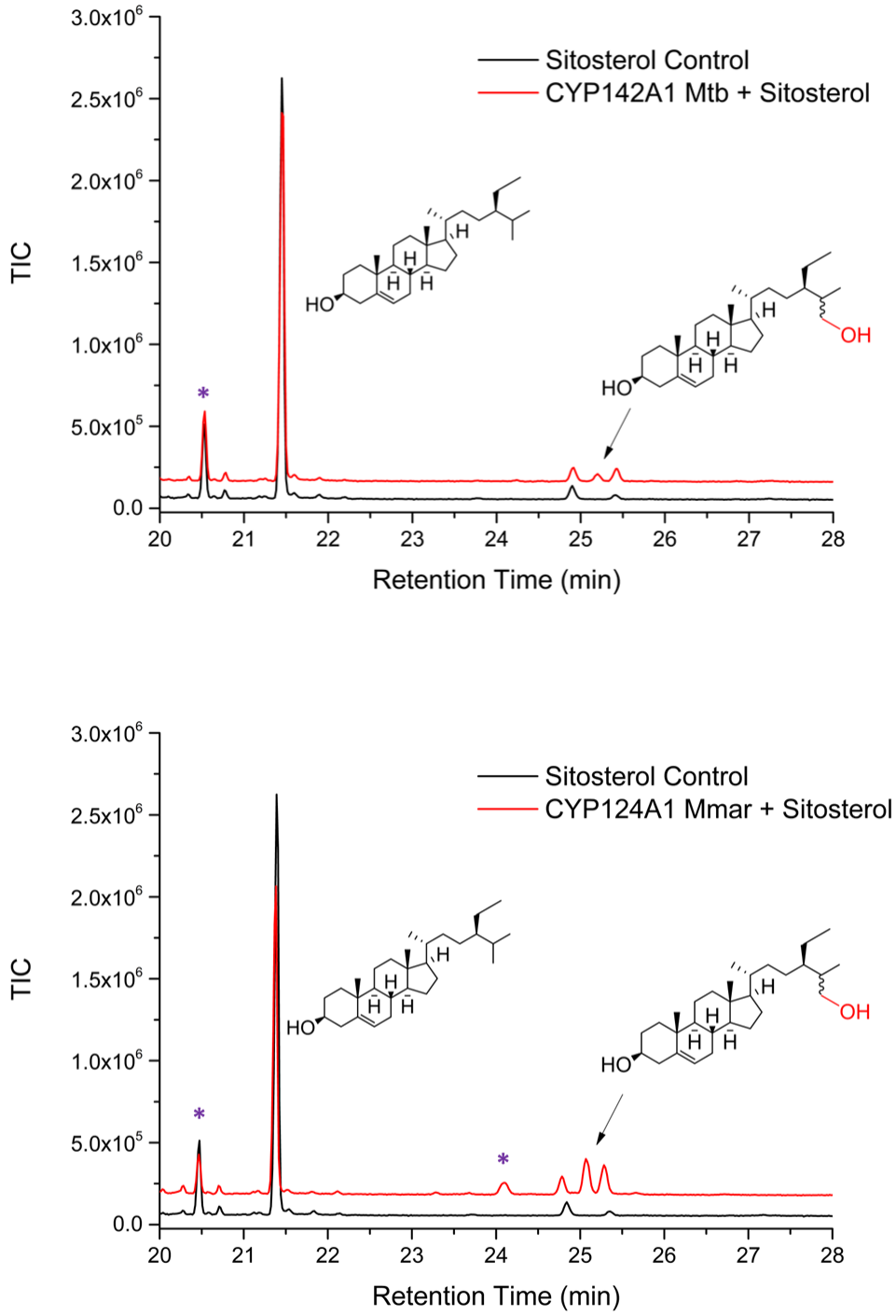
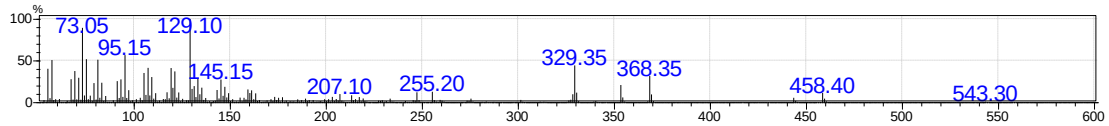
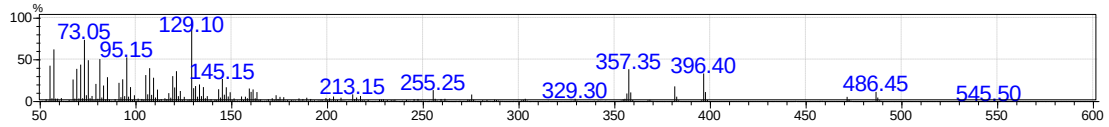


Figure S5: GC chromatograms of MtbCYP142A1 (top) and MmarCYP124A1 (bottom) *in vitro* oxidation of sitosterol using a reconstituted NADPH/Spinach Ferredoxin/Spinach Ferredoxin Reductase electron transfer system.

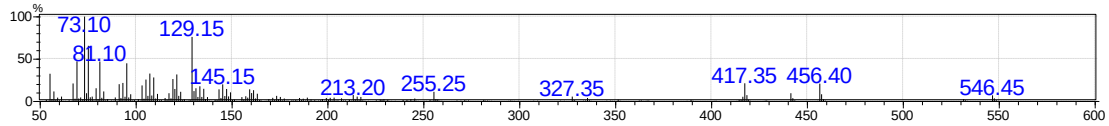
Cholesterol control (RT: 19.5 – 19.6 min, Parent ion mass: 458)



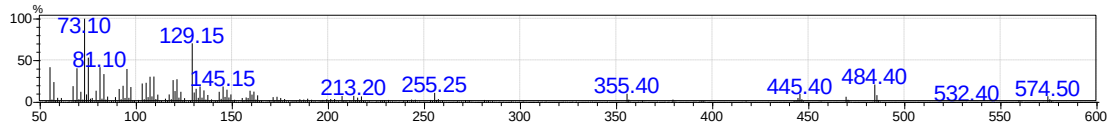
Sitosterol control (RT: 21.3 – 21.5 min, Parent ion mass: 486)



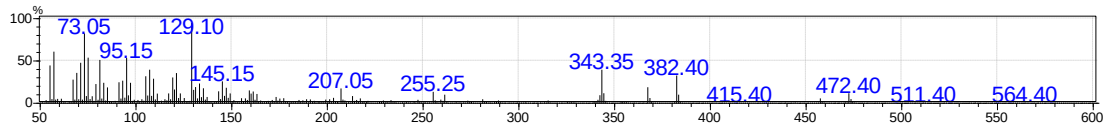
26-hydroxycholesterol control (RT: 23.1 – 23.2 min, Parent ion mass: 546)



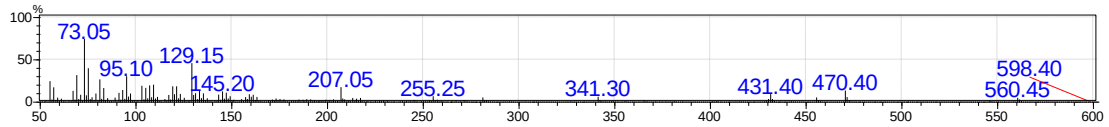
26-hydroxysitosterol (RT: 25.1 – 25.2 min, Parent ion mass: 574)



Campesterol (RT: 20.4 – 20.5 min, Parent ion mass: 472)



26-hydroxycampesterol (RT: 24.1 min – 24.2 min, present in all turnovers, Parent ion mass: 560)



26-sitostenoic acid (RT: 26.8 – 27.0 min, Parent ion mass: 588)

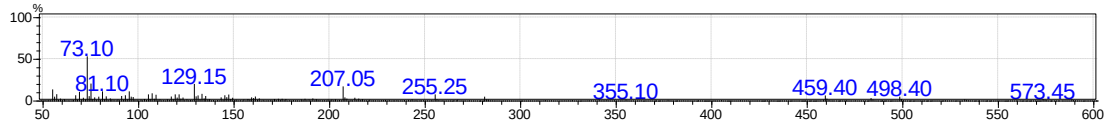


Figure S6: Mass spectra of major product peaks in the GC chromatograms for MtbCYP125A1 cholesterol/sitosterol oxidation reactions. These mass peak fragmentation patterns are representative of all hydroxylated and substrate peaks for CYP125 mediated TMS derivatised cholesterol and/or sitosterol turnovers.

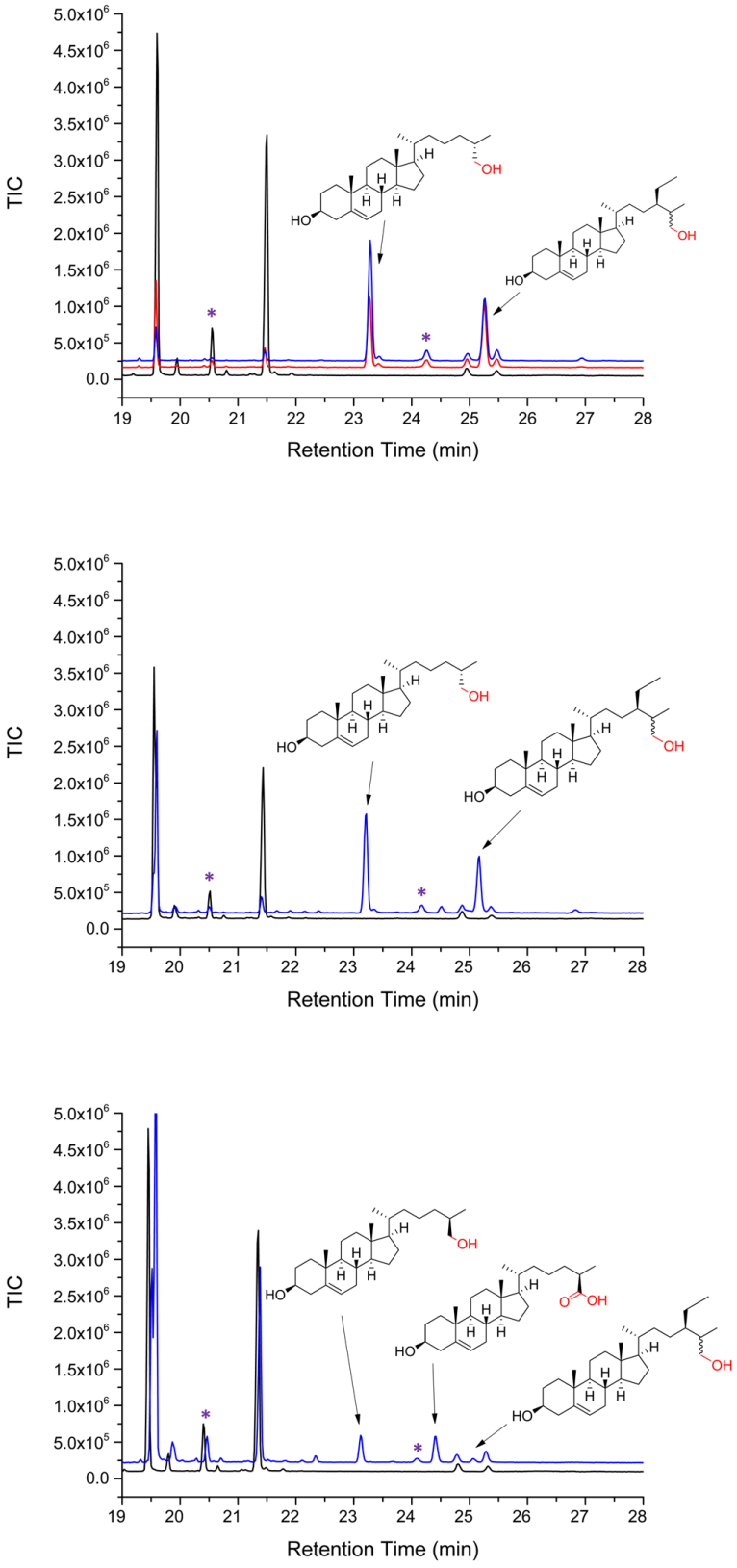


Figure S7A: GC chromatograms of MmarCYP125A6 (top) and MtbCYP125A1 (middle) and MmarCYP124A1 (bottom) *in vitro* competitive oxidation of sitosterol vs cholesterol using a reconstituted NADPH/Spinach Ferredoxin/Spinach Ferredoxin Reductase electron transfer system. 1:1 cholesterol/sitosterol control GC traces are shown in **black** and *in vitro* enzyme driven oxidation traces in **red** (6 hrs) and **blue** (24 hrs). A * indicates peaks arising from campesterol impurity in the sitosterol stocks. Product peaks were identified by MS.

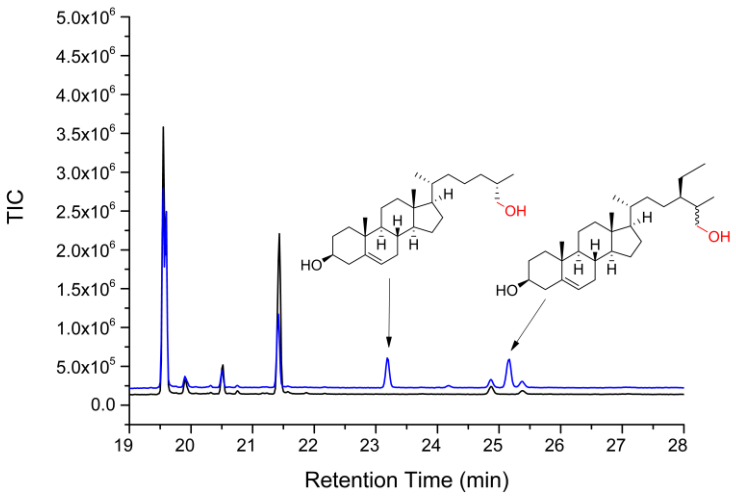


Figure S7B: GC chromatogram of RglobCYP125-04087 *in vitro* competitive oxidation of sitosterol vs cholesterol using a reconstituted NADPH/Spinach Ferredoxin/Spinach Ferredoxin Reductase electron transfer system. The 1:1 cholesterol/sitosterol control GC trace is shown in **black** and the *in vitro* enzyme driven oxidation trace in **blue** (24 hrs). A * indicates peaks arising from campesterol impurity in the sitosterol stocks. Product peaks were identified by MS.

References

1. Child, S. A.; Ghith, A.; Bruning, J. B.; Bell, S. G., A comparison of steroid and lipid binding cytochrome P450s from Mycobacterium marinum and Mycobacterium tuberculosis. *J Inorg Biochem* **2020,** *209*, 111116.
2. Omura, T.; Sato, R., The Carbon Monoxide-Binding Pigment of Liver Microsomes. I. Evidence for Its Hemoprotein Nature. *J Biol Chem* **1964,** *239*, 2370-8.
3. Isin, E. M.; Guengerich, F. P., Substrate binding to cytochromes P450. *Anal Bioanal Chem* **2008,** *392* (6), 1019-1030.
4. Sih, C. J.; Tai, H. H.; Tsong, Y. Y.; Lee, S. S.; Coombe, R. G., Mechanisms of steroid oxidation by microorganisms. XIV. Pathway of cholesterol side-chain degradation. *Biochemistry* **1968,** *7* (2), 808-18.