An Engineered Cholesterol Oxidase Catalyses Enantioselective Oxidation of Non-steroidal Secondary Alcohols

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Experimental Procedures

1. General chemicals

Substrates and product standards were purchased from Sigma Aldrich in the highest grade possible unless otherwise stated. (R)-phenyl-2-propanol was from Fluorochem, and 4-phenyl-2-butanol, 3-methyl-2-butanol, and 2-pentanone were purchased from Alfa Aesar. The source of any other reagents that were used are referred to in the relevant section below.
Solvents were either analytical or HPLC grade - tert-butyl methyl ether, ethyl acetate, and cyclohexane were purchased from Fisher Scientific.

2. Biotransformations and analysis

2.1 Biotransformations

500 µl biotransformation reactions were set up (final concentration enzyme: 17µM, final concentration substrate: 10 mM or 50 mM or 100 mM). The biotransformations were made up in air-saturated 100 mM potassium phosphate buffer, pH 8.0. Biotransformations were typically carried out in a 2 mL eppendorf tube. Substrates were added either neat or from a 1 M solution made up in DMSO (Sigma). Reactions were incubated at 30 °C, 200 rpm for 24 hrs. Deracemisations additionally contained ammonia borane (Sigma) in a four-fold excess over substrate. Biotransformations were extracted by addition of 1 mL tert-butyl methyl ether (TBME), vortexing for 1 min followed by centrifugation at 13000 rpm for 2 min to separate the phases. The organic phase was analysed by GC-FID.

2.2 Analysis

2.2.1 GC-FID methods

GC-FID analysis was performed on an Agilent 6850 GC with a Gerstel Multipurposesampler MPS2L. The methods used are given in Table S1. Two columns were used for analysis: β-dex 325 (Supelco) with dimnsions 30 m x 0.25 mm x 0.25µm, and Chirasil β-dex CB (Agilent) with dimensions 25 m x 0.25 mm x 0.25µm with a detector temperature 250°C and injector temperature 250 °C. The methods used are described in Table S1 with Figure S1 showing which method was used for which substrate.

2.2.2 HPLC analysis method

HPLC analysis was performed on an Agilent system equipped with a G1379A degasser, G1312A binary pump, a G1329 autosampler unit, a G1315B diode array detector and a G1316A temperature controlled column compartment. The column used a CHIRALCEL® DAICEL OD-H (5 µm particle size, 4.6 mm diameter x 250 mm). HPLC analysis was used for compound 8 (Figure S1). The method used was: flowrate 0.8 mL / min; eluent = hexane/iPrOH 95:5; UV detection at 265 nm.

Table S1: Temperature gradients for GC-FID analyses

| Method name | Column            | Oven ramp | temp | hold |
|-------------|-------------------|-----------|------|------|
| A           | Supelco beta-dex 325 | 10 °C / min | 110 °C | 20 min |
| B           | Chirasil β-dex CB  | 5 °C / min | 80 °C | 5 min |
|             |                   | 10 °C / min | 120 °C | -    |
|             |                   |           | 200 °C | 5 mins |
| C           | Chirasil β-dex CB  | 5 °C / min | 50 °C | -    |
| D           | Chirasil β-dex CB  | 5 °C / min | 200 °C | 5 min |
|             |                   |           | 40 °C | 5 min |
|             |                   |           | 120 °C | 0 min |
|             |                   |           | 200 °C | 0 min |
Figure S1: Substrate and method number for GC and HPLC analysis

2.2.3 GC-FID and HPLC traces by substrate, alcohol to carbonyl conversions
Note: some biotransformations with WT enzyme were recorded on an older (shortened) GC column and thus have different retention times. They are thus shown as separate spectra with substrate and product standards. LHS denotes ‘left-hand side’, RHS denotes ‘right-hand side’ in the figure labelling. Traces for substrates 13 and 14 are not shown because there was no conversion.
2.2.3.1 Cyclopentanol (1)

Figure S2: LHS: Top: cyclopentanol (1) standard; middle: cyclopentanone standard; bottom: biotransformation with WT SHCO and 10 mM (1). RHS: Top: cyclopentanol (1) standard; 2nd: cyclopentanone standard; 3rd: biotransformation with ShCO and 10 mM (1); bottom: biotransformation with ShCO and 50 mM (1).
2.2.3.2 Cyclohexanol (2)

Figure S3: Top: cyclohexanol (2) standard; 2nd: cyclohexanone standard; 3rd: biotransformation with WT and 10 mM (2); 4th: biotransformation with ShCO<sub>b</sub> and 10 mM (2); 5th: biotransformation with ShCO<sub>b</sub> and 50 mM (2); bottom: biotransformation with ShCO<sub>b</sub> and 100 mM (2).
2.2.3.3 Cycloheptanol (3)

Figure S4: Top: cycloheptanol (3) standard; 2nd: cycloheptanone standard; 3rd: biotransformation with ShICO and 10 mM (5); bottom: biotransformation with ShICO and 50 mM (3).
Figure S5: Top: 2-cyclohex-2-en-1-ol (4) standard; 2nd: 2-cyclohex-2-en-1-one standard; 3rd: biotransformation with WT and 10 mM (4); 4th: biotransformation with ShCO and 10 mM (4); bottom: biotransformation with ShCO and 50 mM (4).
2.2.3.5 3-methylcyclohex-2-en-1-ol (5)

Figure S6: Top: 3-methylcyclohex-2-en-1-ol (5) standard; 2nd: 3-methylcyclohex-2-en-1-one standard; 3rd: biotransformation with WT and 10 mM (5); 4th: biotransformation with ShCO₂ and 10 mM (5); bottom: biotransformation with ShCO₂ and 50 mM (5).
2.2.3.6 1-phenylethan-1-ol (6)

Figure S7: LHS: Top: 1-phenylethan-1-ol (6) standard; 2nd: (S)-1-phenylethan-1-ol standard; 3rd: biotransformation with WT and 10 mM (6); bottom: acetophenone standard. RHS: Top: 1-phenylethan-1-ol (6) standard; 2nd: (S)-1-phenylethan-1-ol standard; 3rd: acetophenone standard; 4th: biotransformation with ShCO\(_b\) and 10 mM (6); bottom: biotransformation with ShCO\(_b\) and 50 mM (6).
2.2.3.7 1,2,3,4-tetrahydronaphthalen-1-ol (7)

Figure S8: LHS: Top: 1,2,3,4-tetrahydronaphthalen-1-ol (7) standard; middle: 1,2,3,4-tetrahydronaphthalen-1-one standard; bottom: biotransformation with WT ShCO and 10 mM (7). RHS: Top: 1,2,3,4-tetrahydronaphthalen-1-ol (7) standard; 2nd: (S)-1,2,3,4-tetrahydronaphthalen-1-ol standard; 3rd: 1,2,3,4-tetrahydronaphthalen-1-one standard; 4th: biotransformation with ShCO and 10 mM (7); 5th: biotransformation with ShCO, and 50 mM (7); bottom: deracemisation with ShCO, and 50 mM (7) and ammonia borane. Rt enantiomers = 19.29 min, 19.35 min. Rt (S)-enantiomer = 19.29 min, Rt deracemisation product = 19.35 min = (R) enantiomer.
2.2.3.8 1-indanol (8)

Figure S9: Top: 1-indanol (8) standard; 2nd: (R)-indanol standard; 3rd: indanone standard; 4th: biotransformation with ShCO\textsubscript{b} and 10 mM (8); 5th: biotransformation with ShCO\textsubscript{b} and 50 mM (8); bottom: deracemisation with ShCO\textsubscript{b} and 50 mM (8) and ammonia borane. Rt enantiomers = 11.9 min (S), 13.1 min (R). Rt deracemisation product = 13.1 min = (R) enantiomer.
2.2.3.9 Benzyl alcohol (9)

Figure S10: Top: benzyl alcohol (9) standard; 2nd: benzaldehyde standard; 3rd: biotransformation with ShCO₉ and 10 mM (9); bottom: biotransformation with ShCO₉ and 50 mM (9).
2.3.3.10 1-phenylpropan-2-ol (10)

Figure S11: LHS: Top: 1-phenylpropan-2-ol (10) standard, 2nd: (R)-1-phenylpropan-2-ol standard, 3rd: biotransformation with ShCO₃ and 10 mM (10), bottom: biotransformation with ShCO₃ and 50 mM (10). RHS: Top: 1-phenylpropan-2-ol (10) standard, 2nd: (R)-1-phenylpropan-2-ol standard, 3rd: deracemisation with ShCO₃ and 50 mM (10) and ammonia borane.
2.2.3.11 4-phenylbutan-2-ol (11)

Figure S12: Top: 4-phenyl-2-butanol (11) standard; 2nd: 4-phenyl-2-butanone standard; 3rd: (R)-4-phenyl-2-butanol standard; 4th: (S)-4-phenyl-2-butanol standard; 5th: biotransformation with WT and 10 mM (11); 6th: biotransformation with ShCO<sub>b</sub> and 10 mM (11); 7th: biotransformation with ShCO<sub>b</sub> and 50 mM (11); bottom: deracemisation with ShCO<sub>b</sub> and 50 mM (11) and ammonia borane. Rt enantiomers = 23.4 min (S), 23.6 min (R). Rt deracemisation product = 23.6min – (R) enantiomer
2.3.3.12 α-ionol (12)

Figure S13: Top: α-ionol (12) standard; 2nd: α-ionone standard; 3rd: biotransformation with ShCO\textsubscript{b} and 10 mM (12); bottom: biotransformation with ShCO\textsubscript{b} and 50 mM (12).
Figure S14: Top: 3-methylbutan-2-ol (15) standard; 2nd: 3-methylbutan-2-one standard; 3rd: biotransformation with WT and 10 mM (15); 4th: biotransformation with ShCO and 10 mM (15); 5th: biotransformation with ShCO and 50 mM (15); bottom: deracemisation with ShCO and 50 mM (15) and ammonia borane. Rt enantiomers = 12.25 min, 12.45 min. Rt deracemisation product = 12.2 min.
2.2.3.14 2-pentanol (16)

Figure S15: Top: 2-pentanol (16) standard; 2nd: 2-pentanone standard; 3rd: biotransformation with WT and 10 mM (16); 4th: biotransformation with ShCO\textsubscript{b} and 10 mM (16); 5th: biotransformation with ShCO\textsubscript{b} and 50 mM (16); bottom: deracemisation with ShCO\textsubscript{b} and 50 mM (16) and ammonia borane. Rt enantiomers = 12.1 min, 12.3 min, Rt deracemisation product = 12.1 min (dominant isomer)
2.2.3.15 1-hexanol (17)

2.2.4 100 mg scaled reactions
To 20 mg of purified ShCO variant enzyme in 20 mL air-saturated 100 mM potassium phosphate buffer pH 8.0, in a 200 mL Duran bottle, was added 2 mg catalase (Sigma) and 100 mg of substrate, followed by 20 mL of TBME. The reaction was shaken at 150 rpm, 30 °C overnight. When GC-FID analysis showed complete conversion the product was extracted with 3-fold excess of TBME and dried over magnesium sulphate. TBME was removed under reduced pressure and no further purification was carried out.

2.2.5 NMR and optical rotation (α)

$^1$H NMR spectra were recorded on a Bruker Advance 400 ($^1$H at 400.1 MHz) without additional internal standard. Chemical shifts are reported in δ values (ppm) and are calibrated against residual solvent signal.

Optical rotation was recorded on a ADP440 polarimeter (Bellingham + Stanley) against the sodium D-line at 30 °C.

Cyclohex-2-en-1-one

$^1$H NMR (400 MHz, CDCl$_3$) δ = 6.98 (d, $J$=18.3, 1H), 6.42 (td, $J$=31.6, 17.5, 1H), 1.20 (m, 3H), 0.78 (dq, $J$=10.3, 7.5, 7.0, 3H).
OH
(R)-1,2,3,4-tetrahydronaphthalen-1-ol

[α]D20 ^1^ = +33 ± 1°, c = 2.5% in chloroform^1^ Observed [α]D20^2^ = -33.2°, c = 1.25% in chloroform, thus product is (R) enantiomer.

^1^H NMR (400 MHz, CDCl^3^) δ = 7.36 (m, 1H), 7.13 (m, 2H), 7.03 (m, 1H), 4.72 (dd, J = 5.7, 3.8, 1H), 2.71 (m, 2H), 1.89 (m, 3H), 1.71 (m, 1H), 1.61 (s, 1H).

3. DNA and protein methods

3.1 Protein sequences

**Rhodococcus erythropolis**

MSGVKRRSFLGTAAVGVMAMAPRTAKAATGIQRRRSDGRTTALAEAGVPTTLERGRIQWQTPGNAETTPHPS PDKRILWQHPQNTFLGPRFAFEPYAGLLETVGTENMAICAHVGGVGGVGLYQGMLPEEAVNFTHPEELEYDARDHRYVPVRAMLLOQVETAPDLSIPNYEAAPVRFAHRNRSQPLVSPVKMPIDWNALREGRPMPSY YogNDGALVNGKHSVDTIIYAAAEATGLVNVETLHQVTDERAMGRWRYVQRTDIDSGKLIKLTDLMAAGSMNTTTLKVRRAATGIRDLPDELAGWGTNADRIYYWSDL AENFGATQGGPVPVYGRSWDDPHNATVQASFPPVAFDAHSTVLVGVSGSGRDFYVSDAREGEVLRWERPDGDSIAQQHIGPA AORIAAGGLLTDTNAPLSTWHPPLGACMNVSVCDDLRGVRGLGRLVLDGALLPGNSAACPNTMIAAVERAELNVIKDDGTFL Brevisbacterium sterolicum

MSTGPVAPLPTPNFDALSFAQYYQNYWSKEIMLDATWVCSPTPDVVRMLANWAEHDYKIRPRGMAMHGWTPLTEVKANGKEVKN LAADMTHLHINTVGTPGVTAGAASEAITELOKQHDGLWANLPAPGVLIGSAGALVNAHAALPAGQOTLPHTYGSLNLVT ELTAVVWNTTYALEYQTSQRTPLTPTLNLGRCFLTSVMTQAPGFNRQRQCYTDIPWRELAPKGDGRTFKEXAFEESGAEEAIYWP FTETPKMKVWTYTSPKPDSSNEGSLASGASLGKPPAREVSGPYNYIFSNDNLPEPITDMIGAINNGPAPIAPLFGAMYETKGL AATANIDWGWSDKQVFYKATTLRLTEGGLAVTSRANIAITVRDFTEWHERIEYRAKGEFLNOEPVEIRCGLDQAAKVPVSFG PPTISATRPPRPDPDWDVAIWLNVLVGPGTPGMEFEMREMEQWMRSHYNNDTDRTFREPWSKGWAFGPDYPYTDINTVKNMATYIE GYPPTENNATARYNOIPDRHFVRFTNGFDKLPL

**Streptomyces hygrospinosus**

MFENQONQLHSLRRLLGLAALGGAAGTVLTSITSAAPAAADKRSPRADSGSVFVAPVITGYGAAVSALRLEAGVETLMEMGQL WNKPAGDQNVFGCMLTDPDDRSWFKSRTEAPLSLWDLVINDIEYPAGVLDRHFQMSYVVRGGMVQVNLNGMMAPVKRA YFEEVLPQVDAAQAANALPRANAAKHNWFQETFKEWTNYNARFSREQAGKAGLSTFFTPVNYVDHMOQREAAGTAPKSALEG EVGYGNHHGKOSLQDKYLAALGTKQKTVIELHRVTAIRQQADGQSYLVSQDVSDANGTVIAHEIACRHLFLQAGSGSTELLVARADTG ALPHELNQMDQGVGQGPNQNMTRGNHVVNPTJAHOSSSIPALGIDWDWPNDPAPVFAJMPAPLGFTELWSLYALTIKNPRGRSFYVDK ATRDAMLRKWTRQONAPAASSKLFLDRININKNTTMYRDFYLGPQLKINNADFCYHPLGGCVCGLJATDDYGRVAYHNLYVTDGALIP GSGVNPFVTITALAIERNIERTIAEVADVRTA

3.2 Cloning

The gene for cholesterol oxidases from **Streptomyces hygrospinosus**, **Brevibacterium sterolicum** and **Rhodococcus erythropolis** were codon optimised for expression in **E. coli** and synthesised by GeneArt® (Life Technologies). The gene was sub-cloned into the vector **pET28b** (also digested with NdeI/XhoI and then CIP (New England Biolabs (NEB)) to remove phosphates) following the protocol in the Quick ligation kit (NEB) and transformed into **E. coli** NEB10β (NEB). Clones were sequenced at MWG Eurofins using T7 and T7term primers to verify the insert and then transformed into **E. coli** BL21 (DE3) (NEB) for expression.

3.3 Expression and purification

Overnight cultures were prepared by inoculating 6 mL of LB (complemented with 50 µg / mL kanamycin) with a single colony and grown for 16 hours at 37 °C at 200 rpm. The overnight culture was then added to a 2 L flask containing 600 mL autoinduction media (LB based with trace elements (Formedium)) and 50 µg / mL kanamycin and grown at 14 °C with shaking at 200 rpm for 96 hours. Cells were harvested by centrifugation for 40 minutes at 200 rpm and stored as pellets at -20 °C. Purification was carried out as in reference 2.\[^2\]

3.4 Libraries

3.4.1 Synthesis of libraries

Saturated libraries were made by designing primers at the appropriate regions in the **Streptomyces hygrospinosus** cholesterol oxidase gene and following the protocol from the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies). Pfu polymerase was replaced with Phusion polymerase and dNTPs replaced with dNTPs from NEB. Primers were synthesised by MWG Eurofins. Primer sequences are shown below in Table S2. N means any base and S = C/G.
For the screen, filter paper containing 0.1 mg mL\(^{-1}\) Horse radish peroxidase (HRP) (Sigma) in pH 8.0, 100 mM phosphate buffer was prepared. The membranes were freeze-thawed before use. The assay was carried out in a 96 well assay plate (containing 50 µg mL\(^{-1}\) kanamycin and 1 mM IPTG) for induction of protein expression. After six hours at 20 °C the membranes were transferred to petri dishes and stored at -20 °C until use. To perform the screen, filter paper containing 0.1 mg mL\(^{-1}\) Horse radish peroxidase (HRP) (Sigma) in pH 8.0, 100 mM phosphate buffer was prepared. The membranes were freeze-thawed three times (using liquid N\(_2\)) in order to partially lyse the colonies and were then placed on the filter paper. After one hour at room temperature (to ensure removal of any cellular H\(_2\)O\(_2\)) the membrane was transferred to another filter paper which had been soaked in assay solution. (The assay solution consisted of 0.1 mg mL\(^{-1}\) HRP, 3,3'-Diaminobenzidine (DAB) (1 tablet per 15 mL, Sigma), and 100 mM substrate in 100 mM potassium phosphate buffer, pH 8.0.). A positive hit was indicated by colonies that changed to a dark red/brown colour. These colonies were culture and the plasmid DNA extracted and sequenced using T7 or T7 terminator primers (MWG Eurofins).

### 3.5 Kinetics

Cholesterol oxidase produces hydrogen peroxide as it turns over. Hydrogen peroxide production can be detected by horse-radish peroxidase (Type VI, Sigma) and the dye ABTS (Sigma) (ε = 36000 L mol\(^{-1}\) cm\(^{-1}\)). The assay was carried out in a 96 well assay plate to which was added 50 µL substrate (dissolved in DMSO to 1 M then diluted in buffer), 50 µL ABTS (0.7 mg mL\(^{-1}\)) and 50 µL HRP (0.4 mg mL\(^{-1}\)). The assay was started by adding 50 µL purified enzyme (typically 0.1 – 0.3 mg mL\(^{-1}\)). Initial rates were calculated by following the absorbance at 420 nm over time on a TECAN Infinite M200 spectrophotometer at 30 °C. Typically, eight different substrates concentrations were examined (typically 250 mM down to 0.5 mM). A plot of rate vs substrate concentration (Figure S17) allowed the extraction of V\(_{\text{max}}\) and K\(_{\text{M}}\) values using a non-linear curve fit analysis using the Hill model (OriginPro 9.1). k\(_{\text{cat}}\) and K\(_{\text{M}}\) error values represent a 95 % confidence interval based on the standard error of the regression.

### Table S2: Primer sequences

| Name       | Sequence                                                                 |
|------------|--------------------------------------------------------------------------|
| L418L420_F | ACCCTGGGTAGCNSNATSNSSGCAATTACCAAAATCC                                     |
| L418L420_R | GGATTTTTGATTTGCTGGSNNSATNGCTAACCAGGT                                      |
| P387_F     | CAGAGCAGCAGTNSGCACTGGATTTG                                              |
| P387_R     | CAACTAGCCAGTGSNSAATGCTGTCTGT                                             |
| F122_F     | CGCTGGGTAGCNSNCTGTCGTGTGAT                                              |
| F122_R     | CATCCAGCAGSNSNCTGACTACCAACG                                             |
| F476_F     | CGCTAGCCAGTNSGCACTGGATTTG                                               |
| F476_R     | CGAGCTGGGCAGCSNSNCACTGGGTCATACCA                                         |
| M165_F     | GTGTAGTGTTGGSNNSGCCTGTTGAT                                              |
| M165_R     | CGGAAGACGCGSNNSACACCTTACAC                                               |
| E404P409_F | CGGGTTTTTGCNCNSSATTGTGCACTGGATGGTCTG                                    |
| E404P409_R | CCCAGCTGCSNNSACTGGTGGATGGATGACTG                                        |
| Y489H490_F | GCAGATTTTGTNNSNCCGCCTGTTGTTG                                            |
| Y489H490_R | CACACCACCCAGCAGGSNNSNCACAAAATCATCTG                                      |
| N528_F     | GGTAGCTTTGTGTTNSCCGGTTGTTG                                              |
| N528_R     | GGTACAAACCCAGSNAAACCCAATGCTACC                                           |

3.4.2 Solid phase screen

Libraries were transformed into E. coli BL21 (DE3) (NEB) according to the manufactureres instructions, except that the whole transformation reaction was plated on top of a membrane (HyBond) on LB agar containing 50 µg mL\(^{-1}\) kanamycin and grown overnight at 30 °C. The membrane was then transferred to a second LB agar plate (containing 50 µg mL\(^{-1}\) kanamycin and 1 mM IPTG) for induction of protein expression. After six hours at 20 °C the membranes were transferred to petri dishes and stored at -20 °C until use. The assay was started by adding 50 µL purified enzyme (typically 0.1 μM substrate (dissolved in DMSO to 1 M then diluted in buffer), 50 μL ABTS (0.7 mg mL\(^{-1}\)) and 50 μL HRP (0.4 mg mL\(^{-1}\)). The assay was started by adding 50 µL purified enzyme (typically 0.1 – 0.3 mg mL\(^{-1}\)). Initial rates were calculated by following the absorbance at 420 nm over time on a TECAN Infinite M200 spectrophotometer at 30 °C. Typically, eight different substrate concentrations were examined (typically 250 mM down to 0.5 mM). A plot of rate vs substrate concentration (Figure S17) allowed the extraction of V\(_{\text{max}}\) and K\(_{\text{M}}\) values using a non-linear curve fit analysis using the Hill model (OriginPro 9.1). k\(_{\text{cat}}\) and K\(_{\text{M}}\) error values represent a 95 % confidence interval based on the standard error of the regression.

![Figure S17: Rate vs [S] plots for ShCO with cyclohexanol (top left), ShCO with cyclohexanol (top right), ShCO with hexanol (bottom left), ShCO with cyclohexanol (bottom right)](image-url)
3.6 Temperature and solvent stability

3.6.1 Biotransformations using solvent overlays

500 µl biotransformation reactions were set up (final concentration enzyme: 1 mg ml\(^{-1}\), final concentration 2-cyclohexen-1-ol: 90 mM) and 500 µl of solvent was overlayed. Biotransformations were incubated at 30°C, 180 rpm for 24 hrs. Extractions were carried out by addition of a further 500 µl of the same solvent and extraction and analysis continued as described in 2.1 and 2.2.1 using GC-FID method B.

![Figure S18: Solvent tolerance](image)

Figure S18: Solvent tolerance: Top: 2-cyclohex-2-en-1-ol (4) standard, 2nd: 2-cyclohex-2-en-1-one standard, 3rd: biotransformation with no solvent (4), 4th: biotransformation with tert-butyl methyl ether overlay, 5th: biotransformation with cyclohexane overlay, bottom: biotransformation with ethyl acetate overlay.

3.6.2 \(T_{50}\) calculations

Purified enzyme was incubated at the given temperature for 15 minutes. An initial rates experiment was then conducted with 100 mM hexanol (see 3.5 for assay components) and the highest initial rate for each variant was set to 100%. Other rates were then compared to this to give a relative activity. A sigmoidal dose-response (variable slope) non-linear fit was applied in GraphPad Prism.

3.7 Modelling

A homology model of Streptomyces hygrospinosus cholesterol oxidase was prepared using YASARA (www.yasara.org).[^3]
Results and Discussion

Hits from screening libraries
The hits from each library are described in Table S3 with the most active variant confirmed in the right-hand column.

| Screening substrate | Library screened | Hits | Most active variant |
|---------------------|------------------|------|---------------------|
| E404/P409           | E404C/P409S, E404C/P409L |      | E404C/P409S |
| P387                | P387W            |      |                     |
| L418/L420           | L418A/L420F      |      |                     |
| F122                | No hits          |      |                     |
| Y489/H490           | No hits          |      |                     |
| N528                | No hits          |      |                     |
| M165                | No hits          |      |                     |
| F476                | No hits          |      |                     |
| E404C/P409S/L418A/L420F/P387 | No hits |      |                     |
| E404C/P409S/P387    | No hits          |      |                     |
| L418A/L420F/P387    | No hits          |      |                     |
| E404/P409           | E404N/P409L, E404N/P409C, E404A/P409I |      | E404A/P409I |
| L418/L420           | No hits          |      |                     |
| P387                | No hits          |      |                     |
| F122                | F122C            |      |                     |
| M165                | No hits          |      |                     |
| N528                | No hits          |      |                     |
| E404N/P409L/F122    | No hits          |      |                     |
| F476                | No hits          |      |                     |
| Y489/H490           | No hits          |      |                     |

*a* These libraries were constructed because we found hits in the initial libraries and proposed that we could gain better activity by taking an iterative approach where one library is made in the background of a variant that is already active.

Additionally we combined E404C/P409S and P387W to make a three point variant but activity with 2-cyclohexen-1-ol went from full conversion (with E404C/P409S) to 25% conversion. A four point variant E404C/P409S/L418A/L420F was constructed and conversion went from full conversion (E404C/P409S) to 65% with 2-cyclohexen-1-ol although it was apparent that the enzyme was selective. F122C was combined with E404N/P409L but activity remained the same. Activities were comparable between E404N/P409L and E404A/P409I on cyclohexanol but E404A/P409I showed better conversions with the other substrates.

Kinetics of variants with hexanol and cyclohexanol
The kinetics of the variants were established with hexanol and cyclohexanol (Table S4)

| Substrate | Enzyme | $k_{cat}$ s$^{-1}$ | $K_{M}$ / mM |
|-----------|--------|------------------|--------------|
| wt        | ShCO$_a$ | 0.69±0.06        | 46.8±17.1    |
| ShCO$_b$  | 1.86±0.12 | 9.7±2.6          |
| wt        | ShCO$_a$ | 0.14±0.03        | 175.7±71.5   |
| ShCO$_b$  | 0.54±0.06 | 95.8±19.6        |

For comparison, a homologous *Streptomyces* cholesterol oxidase has a $k_{cat}$ of 44±2 s$^{-1}$ and a $K_{M}$ of 3±0.4 μM with the natural substrate cholesterol.$^{[4]}$
Modelling of inactive substrate
Modelling of substrate 13 (Figure S19) in the active site shows a potential clash with the FAD cofactor, suggesting why 13 is not a good substrate.

Figure S19: Model of 13 in the active site of ShCOx, FAD cofactor shown in yellow.

Temperature stability
The LogEC_{50} values returned (in this case the temperature at which 50% activity remained) were defined as the T_{50} for the enzyme of 50.9 ± 0.5 °C. (Figure S20).

Figure S20: Non-linear fit to data from temperature stability experiments using GraphPad Prism

References
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Author Contributions
RSH designed and performed the experiments and analysis and wrote the manuscript, JJS performed analytical experiments and NJT wrote the manuscript and acquired the funding.