Supporting Information

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Oxygen-18 Labeling Reveals a Mixed Fe-O Mechanism in the Last Step of Cytochrome P450 51 Sterol 14α-Demethylation

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Abstract: The 14α-demethylation step is critical in eukaryotic sterol biosynthesis, catalyzed by cytochrome P450 (P450) Family 51 enzymes, e.g. with lanosterol in mammals. This conserved 3-step reaction terminates in a C-C cleavage step that generates formic acid, the nature of which has been controversial. Proposed mechanisms involve roles of P450 Compound 0 (ferric peroxide anion, FeO_2^-) or Compound I (perferryl oxygen, FeO^{3+}) reacting with either the aldehyde or its hydrate, respectively. Analysis of ^{18}O incorporation into formic acid from $^{18}O_2$ provides a means of distinguishing the two mechanisms. Human P450 51A1 incorporated 88% ^{18}O (one atom) into formic acid, consistent with a major but not exclusive FeO_2^- mechanism. Two P450 51 orthologs from amoeba and yeast showed similar results, while two orthologs from pathogenic trypanosomes showed roughly equal contributions of both mechanisms. An X-ray crystal structure of the human enzyme showed the aldehyde oxygen 3.5 Å away from the heme iron. Experiments with human P450 51A1 and $H_2{}^{18}O$ yielded primarily one ^{18}O atom but 14% of the formic acid product with two ^{18}O atoms, indicative of a minor contribution of a Compound I mechanism. LC-MS evidence for a Compound 0-derived Baeyer-Villiger reaction product (a 14α-formyl ester) was also found.

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Experimental Procedures

Reagents

SUPPORTING INFORMATION

Unless stated otherwise, reagents were purchased from Millipore-Sigma-Aldrich or Thermo Fisher, were of analytical grade, and were used without further purification. Solvents were of LC-MS grade and were filtered >5 times over basic alumina prior to use to remove contaminating acids.

Enzymes

P450 51 orthologs from human,[1] *N. fowleri*,[2] *C. albicans*,[3] *T. brucei*,[4] and *T. cruzi*[5] were expressed in *Escherichia coli* and purified as described. Rat NADPH-P450 reductase was also expressed in *E. coli* and purified as described.[6] P450 concentrations were measured by the reduced (ferrous) CO method (ferrous-CO vs. ferrous difference spectrum) using a molar extinction coefficient ($\varepsilon_{450\text{-}490}$) of 91,000 M^{-1} cm^{-1}.[7]

General

NMR spectra were recorded on Bruker AV-400 and AV-II-600 MHz instruments in the Vanderbilt Small Molecule NMR Facility Core. Tetramethylsilane (TMS, δ 0.00 ppm) was used as an internal standard for ^{1}H experiments, and the NMR solvent signal at δ 77.16 ppm (for $CDCl_3$) was used as a reference for ^{13}C experiments. Almost all mass spectra of sterols were collected (HRMS) using a Thermo Fisher Scientific LTQ XL Orbitrap mass spectrometer instrument operating in the APCI (positive ion) mode in the Vanderbilt Mass Spectrometry Research Core Facility, after introduction from UPLC using Waters ACQUITY ultra-performance liquid chromatography systems equipped with a ZORBAX Rx-C8 column (5 µm, 2.1 mm × 150 mm, Agilent), an ACQUITY UPLC® BEH C18 column (1.7 µm, 2.1 mm × 100 mm, Waters), or an Altima 2.1 mm × 100 mm silica column (3 µm). Most spectra (all sterols) were collected in the atmospheric pressure chemical ionization (APCI) mode. UV spectra were recorded either on-line from UPLC using a Waters Acquity instrument (diode array) or using an OLIS-Cary 14 spectrophotometer (On-Line Instrument Systems, Athens, GA) (in C_2H_5OH). Silicic acid chromatography was done using columns of SiliaFlash F60 (230-400 mesh). Analytical TLC was done with SilicaGel 60 F_{254} plates (Merck KGaA), all with mixtures of hexanes and ethyl acetate, with visualization by UV light (254 nm) and spraying with an ethanolic phosphomolybdic acid solution and heating on a hot plate.

X-Ray Crystallography

For crystallization purposes, the expression and purification of human P450 51A1 was performed as described previously with the membrane anchor sequence at the N-terminus (up to P61) being replaced with a MAKKTSSKGKL fragment.[8]

Initial screening of crystallization conditions was performed using Hampton Research crystallization kits. The protein sample (~6 µM in 20 mM potassium phosphate buffer, pH 7.4, containing 500 mM NaCl, 10% glycerol (v/v), and 5.6 mM *tris*(2-carboxyethyl)phosphine (TCEP)) was gradually saturated with the 14α-aldehyde derivative of 24,25-dihydrolanosterol (using a 1 mM stock solution in 45% (w/v) 2-hydroxypropyl-β-cyclodextrin (HPCD)), incubated for 30 min at room temperature, centrifuged to remove the precipitate, concentrated to ~700 µM, and then diluted 2-fold with 5 mM potassium phosphate buffer (pH 7.4) and mixed with 10% Anapoe 80 (w/v) Hampton Research), final concentration 2× the critical micelle concentration (CMC).

The crystals were grown using the hanging drop vapor diffusion technique after mixing equal volumes of the protein sample and the reservoir solution containing 0.20 M calcium acetate, pH 7.4, and 15% polyethylene glycol (PEG) 4000 (w/v). At 17 °C the crystals appeared after three days and were cryoprotected in mother liquor with 25% glycerol (v/v), and flash-cooled in liquid nitrogen.

X-ray diffraction data were collected at 100 K on beamline 21-ID-D at the Advanced Photon Source, Argonne National Laboratory, indexed and integrated with autoProc[9] and scaled with the program Aimless.[10] The structure was determined by molecular replacement using Phaser MR (CCP4 program suite[11]) and the coordinates of lanosterol-bound human CYP51 [PDB code 6uez][12] as a search model. The refinement and model building were performed with Refmac5 (CCP4 Suite) and Coot[13]). Details of the data collection and refinement statistics are shown in Table S1. Structure superimposition and root mean square deviation (RMSD) calculation were performed in lsqcab (CCP4 Suite). The structural figures were prepared in PyMOL (PyMOL Molecular Graphics System, Version 2.0 Schrödinger).

Human P450 51A1 was crystallized in the hexagonal $P6_222$ space group, and the 2.25 Å resolution structure was refined to an R-factor of 0.219 and R-free of 0.249. The asymmetric unit consisted of two polypeptide chains, with the root mean square deviation (rmsd) of all Cα atoms of chains A and B (molecular breathing) being 0.24 Å. Each protein chain contained one (Cys-449 coordinated) heme and one bound molecule of the 14α-aldehyde derivative of 24,25-dihydrolanosterol (100% occupancy). The binding mode of the sterol indicated that it was captured in what appears to be a catalytically productive state (Figure 1A). The 14α-aldehyde oxygen is positioned 3.5 Å from the heme iron, which is ~0.6 Å closer than the 14α-carbon atom of lanosterol (4.1 Å). The C3-OH group of the sterol maintains its H-bond with the main chain oxygen of the residue that precedes the β1-4 strand (substrate recognition site (SRS) 5[14]), as do the C3-OH groups of the sterol substrates in other P450 51 structures.[12, 15]

Preparation of Sterol Solutions

Sterol solutions were prepared as described earlier.[16] Briefly, sterol powder was dissolved in C_2H_5OH to a concentration of 10 mM. The solution was heated (37 °C), mixed with a vortex device, and sonicated. The ethanolic stock was diluted 10-fold in aqueous HPCD (45%, w/v), and the sterol solution (1 mM) was again heated (37 °C), mixed with a vortex device, and sonicated to ensure full incorporation into the cyclodextrin. Stock solutions were stored at 4 °C and were heated and sonicated immediately prior to use. The final volume of organic solvent from the sterol solutions to any P450 51 reaction was ≤ 0.5% (v/v).

Analysis of FF-MAS

Detection of the terminal product of P450 51 dihydrolanosterol 14α-demethylation ((4β,5α)-4,4-dimethyl-cholestra-8,14,24-trien-3-ol, dihydro FF-MAS) was carried out as described previously.[16] Briefly, samples (held at 4 °C) were injected (10 µL) on a Waters Acquity UPLC system and separated using an Acquity BEH octadecylsilane (C_{18}) UPLC column (2.1 mm × 100 mm, 1.7 µm) held at 25 °C with an isocratic mobile phase of CH_3CN (100%) at a flow rate of 0.20 mL min^{-1}. Dihydro FF-MAS was detected using a Waters Acquity photodiode array system set at 250 nm and was quantitated by comparison to a 12-point external standard curve.[16]

Preparation of Diazo Reagent

Caution! *This reagent and its nitroso precursor are strong alkylating agents and expected to be toxic, mutagenic, and possibly carcinogenic. Handle in a fume hood with adequate ventilation and appropriate skin protection!*

The analysis of the enzymatic byproduct of dihydrolanosterol demethylation/deformylization (DCO_2H) was facilitated by chemical derivatization to a pyridyl formyl ester with the diazo reagent described previously.[16] Briefly, the nitrosourea precursor (~3 mg, stored under N_2 at -20 °C) was dissolved in $(C_2H_5)_2O$ (2 mL) and treated with an equal volume of KOH (2 mL, 30% v/v). The solution was mixed, and the organic (top) layer was removed (~1.8 mL) and transferred to a fresh amber vial containing $MgSO_4$ (~200 mg). The sample was vortexed and filtered through a cotton-plugged Pasteur pipet to remove the drying reagent. This sample (~1.5 mL, ~14 mM) was capped and stored at 4 °C until use.

$^{18}O_2$ Incorporation Assays

The basic protocol used for the $^{18}O_2$ incorporation experiment was that described earlier[16] but with several modifications, which increased product recovery ~2,000-fold. Reaction mixtures of human P450 51A1 were performed by reconstituting P450s 51A1 (0.05 μM) and 17A1 (0.2 μM), NADPH-P450 reductase (1 μM), and 1,2-α-didodecanoyl (dilauroyl)-*sn*-glycero-3-phosphocholine (DLPC, added to 100 μM from a freshly sonicated 1 mg mL^{-1} aqueous stock) on ice (10 min) prior to addition of potassium phosphate buffer (50 mM, pH 7.4) and substrates 14α-CDO lanosterol (50 μM, in 45% w/v HPCD) and progesterone (50 μM, in C_2H_5OH). P450 17A1 17α-hydroxylation of progesterone was included as a control reaction in the P450 51 experiment to assess the actual atmospheric ^{18}O content in each reaction vessel (see data analysis below). The enzyme mixture (850 μL) was aliquoted into the vial component of a Thunberg tube (~15 mL), and an NADPH-generating system (0.5 mM $NADP^+$, 10 mM glucose 6-phosphate, and 2 μg mL^{-1} glucose 6-phosphate dehydrogenase)[17] was added (150 μL) to a greased cap. The tube was capped and sealed with parafilm, and the endogenous atmosphere was removed *in vacuo* and replaced with Ar (g), utilizing a gas train.[18] The removal and replenishment of an Ar (g) environment was repeated 10 times to ensure nearly complete removal of the endogenous $^{16}O_2$. The sample was again placed under vacuum, the sidearm of the tube was flushed with Ar (g) and capped, and $^{18}O_2$ (Aldrich, 97% atom ^{18}O) was injected via the sidearm at a rate of approximately ~2 bubbles s^{-1} for 2 min.

Samples were preincubated (37 °C, 3 min) prior to initiation of the reaction by inversion (mixing the contents of the cap and the vial). After 15 min, reactions were transferred to an ice bath to cool (5 min) prior to termination via extraction with *tert*-butyl methyl ether (^{t}BME) (3 mL). Quenched reactions were acidified to pH < 1 (with 250 μL of 3 M HCl) to extract the DCO_2H product, and the mixture was mixed with a vortex device and subjected to centrifugation ($10^3 \times$ g, 5 min, 23 °C) to separate the layers. The organic (top) layer (2.5 mL) was transferred to a fresh amber glass vial, and the extraction was repeated to maximize recovery of DCO_2H. The two extracts were pooled (5 mL), CH_3OH (0.5 mL, to 10% v/v) and diazo reagent (in $(C_2H_5)_2O$, 300 μL, ~4 μmol) were added (see above), and the solution was mixed and briefly allowed to react (23 °C, 10 min). The solvent was then removed under a stream of N_2 and the products of the derivatization were dissolved in CH_3OH (200 μL), transferred to autosampler vials, and analyzed by LC-HRMS.

Reaction conditions for all other P450 Family 51 orthologs were nearly identical to those used for the human enzyme, but with some modifications. The concentration of P450 was increased to 0.1 μM for *N. fowleri* (NF51) P450 51 and to 1 μM for the *T. brucei* and *T. cruzi* orthologs. The concentration of NADPH-P450 reductase was increased to 4 and 2 μM for the *T. brucei* and *T. cruzi* P450s, respectively, and the reaction time was increased to 60 min for both orthologs. Additionally, to increase analytical sensitivity,

the (LC-HRMS) sample volume was reduced to 25 µL for the *T. brucei* and *T. cruzi* P450s. The reaction conditions for *C. albicans* P450 51 were identical to those of the human enzyme.

LC-MS Analysis of Formate Esters

The detection of DCO_2H as a pyridyl formate ester was performed using the methods reported earlier.[16] Briefly, samples were injected (10 µL) using a Vanquish UHPLC system (Thermo Fisher Scientific) held at 4 °C on an Acquity BEH octadecylsilane (C_{18}) 2.1 mm × 100 mm UPLC column (1.7 µm, held at 23 °C) and were separated using a flow rate of 0.20 mL min^{-1} and a gradient mobile phase of (A) 5% aqueous CH_3CN (10 mM NH_4OAc) and (B) CH_3OH (10 mM NH_4OAc) as follows (all % v/v): 0 min, 2% B; 4 min, 100% B; 4.5 min, 100% B; 5.8 min, 2% B; 8 min, 2% B. The column eluate was then subjected to electrospray ionization using a Q Exactive HF Orbitrap Mass Spectrometer (Thermo Scientific) with a spray voltage of 5.0 kV. Sample analysis was performed in the positive ion mode, scanning from *m/z* 100 to 500 using a resolution setting of 120,000. Data were processed using Xcalibur QualBrowser (Thermo Fisher Scientific) software (version 2.0.7). Where indicated, ^{18}O incorporation into formate esters was normalized to the ^{18}O incorporated into 17α-OH progesterone via the P450 17A1 17α-hydroxylation reaction (to yield 17-^{18}OH-progesterone) to account for slight variability in atmospheric ^{18}O composition in each reaction (Figures S2, S3). P450 17A1 results were processed as the ratio of the ^{18}O product (*m/z* 333.2310) to the total product (^{18}O + ^{16}O, *m/z* 331.2268) formed in the reaction.

Preparation of 14α-CD^{18}O Dihydrolanosterol

14α-CD^{18}O dihydrolanosterol was prepared by exchanging the aldehyde oxygen of 14α-CD^{16}O dihydrolanosterol (50 µM, from a 1 mM stock in C_2H_5OH) with $H_2{}^{18}O$ (Cambridge Isotope Laboratories, 97% ^{18}O) in a solution (pH = ~2) of $H_2{}^{18}O$:tetrahydrofuran:C_2H_5OH (20:19:1, v/v/v). The inclusion of organic solvent (50% v/v) in the reaction was necessary to increase the solubility of the sterol and facilitate the exchange. The ^{18}O exchange reaction was performed at 37 °C with shaking for 72 h and was quenched by extraction into CH_2Cl_2 (3 mL). The organic (lower) layer (2.5 mL) was transferred to a clean glass test tube, and the extraction was repeated twice to maximize recovery of the substrate. The three extracts were pooled (~7.5 mL) and concentrated to dryness under a stream of N_2. The dried residue was dissolved in C_2H_5OH (100 µL), and ^{18}O incorporation into the substrate (79%, Figure S4) was assessed by LC-APCI-MS based on comparison of the relative abundance of the ^{16}O and ^{18}O ions (*m/z* $[MH - H_2O]^+$ of 426.3841 and 428.3883, respectively). The ethanolic stock of 14α-CD^{18}O dihydrolanosterol was then concentrated under a stream of N_2 until the volume was approximately 5 µL, at which point the sterol was diluted 20-fold in a solution of HPCD (45% w/v, 95 µL) prepared in $H_2{}^{18}O$. This stock (~500 µM) was heated (37 °C), mixed with a vortex device, and sonicated to ensure full incorporation into the cyclodextrin.

$H_2{}^{18}O$ Incorporation Assays

Human P450 51A1 (0.2 µM), NADPH-P450 reductase (0.8 µM), and DLPC (100 µM, freshly sonicated) were reconstituted on ice (10 min) prior to addition of the following assay components (all prepared in $H_2{}^{18}O$): potassium phosphate (pH 7.4, 50 mM), $H_2{}^{18}O$, and 14α-CD^{18}O dihydrolanosterol (25 µM). Samples (170 µL) were preincubated (37 °C, 5 min) with shaking prior to initiation with NADPH (3.5 mM, 30 µL from a 24 mM stock in $H_2{}^{18}O$). The final reaction nominal composition (200 µL) was 92.3% (v/v) $H_2{}^{18}O$. Reactions (20

min) were terminated, extracted, and derivatized as described for the $^{18}O_2$ incorporation assays and analyzed using the LC-HRMS conditions described above. Data were normalized to the ^{18}O content present in the substrate at the time of reaction.

KSIE Assays (D_2O)

To perform the kinetic solvent isotope effect experiment, a 20× enzyme premix was prepared by reconstituting human P450 51A1 (0.4 μM), NADPH-P450 reductase (1.6 μM), and DLPC (200 μM, added from a freshly sonicated 2 mg mL^{-1} stock) on ice (10 min). The premix was then combined with the following assay components: glycerol (10% v/v), potassium phosphate buffer (50 mM, pH or pD 7.4), D_2O (or H_2O), and 14α-CDO dihydrolanosterol (15 μM). Reactions were prepared in a gradient of D_2O compositions (0 to 80% (v/v)) and each treatment was tested in triplicate. To achieve higher D_2O reaction compositions, all assay components except for proteins and lipids were prepared in the solvent. Samples (900 μL) were preincubated (37 °C, 2 min) prior to initiation with NADPH (1 mM, added from 10 mM stock in either D_2O or H_2O). Initiation (100 μL) brought the reaction to its desired final volume (1.0 mL), where the concentrations of P450, NADPH-P450 reductase, and lipid were 0.02, 0.08, and 100 μM, respectively. Reactions (1 min, 37 °C) were terminated by the addition of CH_2Cl_2 (5 mL), and the mixture was vortexed and centrifuged (10^3 × g, 5 min, 23 °C) to separate layers. The bottom (organic) layer was transferred to a fresh vial and the solvent was removed under a stream of N_2. The dried residue was dissolved in 100 μL CH_3OH, transferred to autosampler vials, and analyzed for FF-MAS formation as described above.[16]

Baeyer-Villiger Intermediate

A reaction of human P450 51A1 was prepared according to the steady-state conditions reported earlier,[16] with minor modifications. Briefly, a reconstitution mixture of P450 51A1 (0.01 μM), NADPH P450 reductase (0.04 μM), and DLPC (100 μM) was allowed to incubate on ice (10 min) before addition of potassium phosphate buffer (50 mM, pH 7.4) and 14α-CDO dihydrolanosterol substrate (25 μM). The solution was divided into test tubes (4.25 mL) and reactions were preincubated (5 min, 37 °C) prior to initiation with an NADPH regenerating system (750 μL, *vide supra*). Reactions (5 mL) were allowed to proceed over a variable time course (2.5, 5, and 7.5 min) before quenching by extraction into CH_2Cl_2 (5 mL). Each mixture was mixed with a vortex device and centrifuged (10^3 × g, 5 min, 23 °C) to separate the layers and the organic (bottom) layers (4 mL) were transferred to fresh vials. The extraction was repeated to maximize product recovery, and the two extracts were pooled (~8 mL) and brought to dryness under a stream of N_2. The dried residue was dissolved in CH_3OH (50 μL) for LC-MS analysis.

For normal phase analysis, samples (2 μL) were injected using a Vanquish normal phase HPLC system (Thermo) on an Altima 2.1 mm × 100 mm silica column (3 μm) and separated using an isocratic mobile phase of 2% 2-propanol in hexane (0.1% formic acid, all v/v) at a flow rate of 0.4 mL min^{-1}. The column eluate was subjected to atmospheric pressure chemical ionization (APCI) and analyzed on a TSQ Vantage triple quadrupole mass spectrometer (Thermo). Sample analysis was performed in the Q1MS positive ion mode scanning from *m/z* 100 to 500 with a Q1 peak width setting of 0.70 and a declustering voltage of 10 V. For reverse phase analysis, samples (10 μL) were injected as described for the LC-ESI-MS analysis of formate esters (above), except the mobile phase was 100% Buffer B (10 mM NH_4OAc in CH_3OH) and the run time was extended to 10 min. Data were processed using Xcalibur QualBrowser (Thermo Fisher Scientific) software (version 2.0.7).

SUPPORTING INFORMATION

Oxygen Surrogates

Iodosylbenzene diacetate (Millipore-Sigma-Aldrich) was treated with NaOH as described by Saltzman and Sharefkin.[19] The product iodosylbenzene (PhI=O) was stored at -20 °C. Cumene hydroperoxide (CuOOH) was dissolved in CH_2Cl_2, washed with 1 M NaOH, dried with anhydrous Na_2SO_4, filtered, concentrated in vacuo, and stored at 4 °C.

Reaction conditions for the use of H_2O_2, CuOOH, and PhI=O as oxygen surrogates in P450 catalysis were adapted from Yoshimoto et al.[20] Briefly, a P450 reaction mixture was prepared by reconstituting human P450 51A1 (0.2 μM) in DLPC (100 μM) on ice (10 min) prior to the addition of the following assay components: glycerol (10% (v/v)), potassium phosphate buffer (50 mM, pH 7.4), and 14α-CDO dihydrolanosterol (50 μM, added from a 1 mM stock in 45% (w/v) HPCD). Samples were preincubated (37 °C, 5 min) prior to initiation with either H_2O_2 (3 mM), CuOOH (0.1 or 1 mM), or PhI=O (0.75 mM). Reactions (0.5 mL) were terminated (1 to 5 min) by extraction with CH_2Cl_2 (5 mL), and each sample was mixed with a vortex device and submitted to centrifugation ($10^3 \times$ g, 5 min, 23 °C) to separate the layers. The bottom (organic) layer was transferred to a fresh vial and the solvent was removed under a stream of N_2. The dried residue was dissolved in 100 μL CH_3OH, transferred to autosampler vials, and analyzed for FF-MAS formation as described above.[16]

Synthesis of [32-^{2}H]-24,25-Dihydrolanosterol 14α-Aldehyde (5) [16, 21]

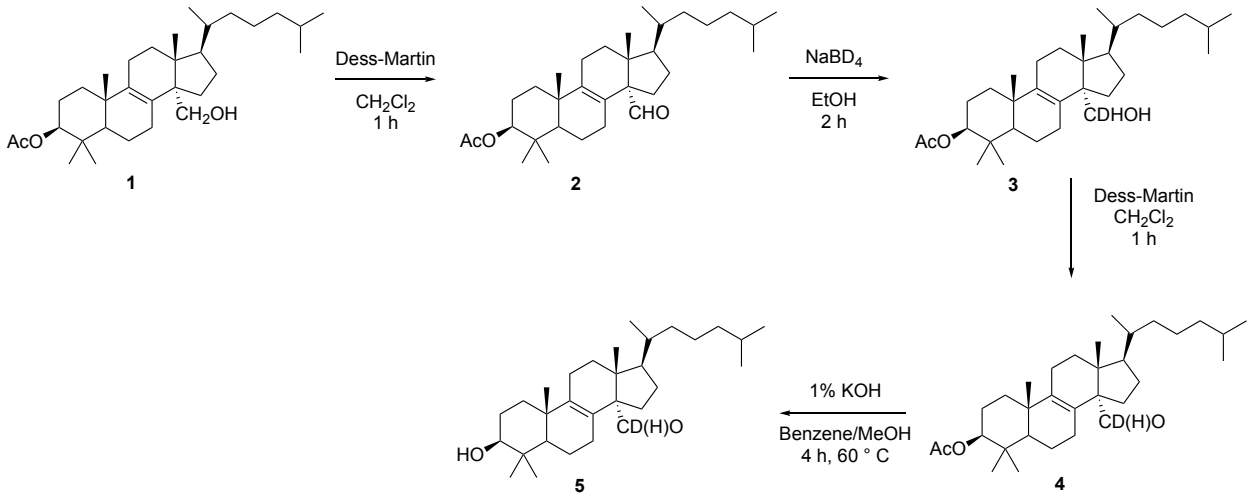


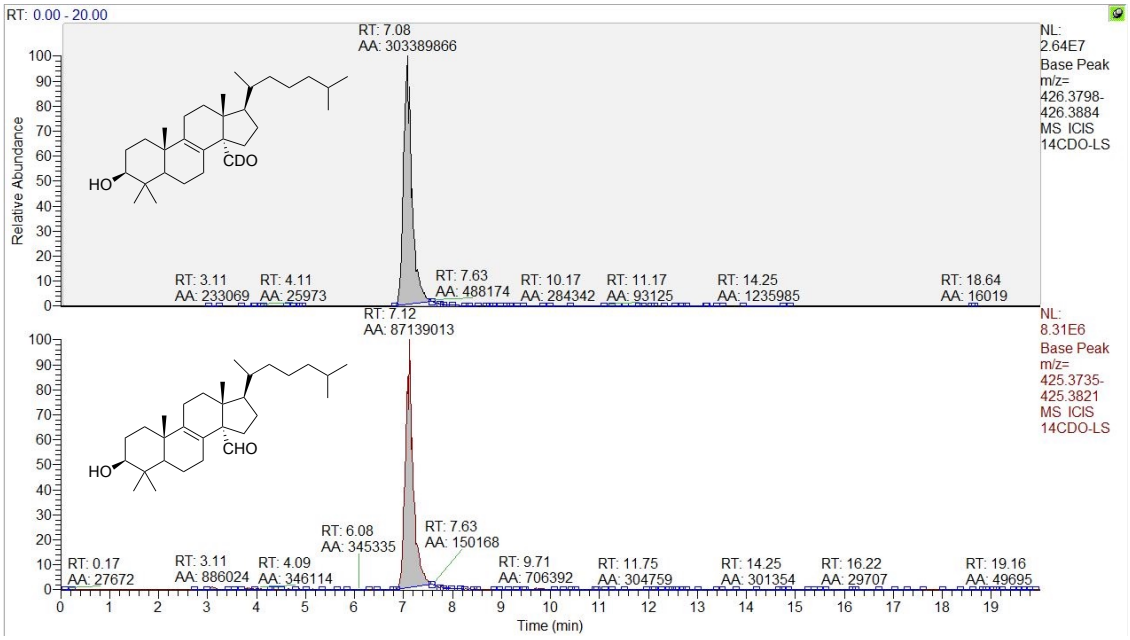
Figure S1. Synthesis of [32-^{2}H]-24,25-dihydrolanosterol 14α-aldehyde (**5**)

Step 1. 3-Acetoxy lanostene-32-ol (**1**) (93 mg, 0.19 mmol)[16] was dissolved in 2 mL of CH_2Cl_2 and stirred with 85 mg of Dess-Martin periodinane (3-oxo-1λ^5,2-benziodoxole-1,1,1(3*H*)-triyl triacetate)[22] (0.20 mmol) for 1 h at 23 °C, at which time TLC indicated that the reaction was complete (silica, hexanes-ethyl acetate, 9-1, v/v). The reaction was diluted with 10 mL of $(C_2H_5)_2O$ and added to 10 mL of saturated aq. $NaHCO_3$ containing 220 mg (1.4 mmol) $Na_2S_2O_3$. The $(C_2H_5)_2O$ layer was washed 3× with H_2O and dried *in vacuo*, to yield **1**.

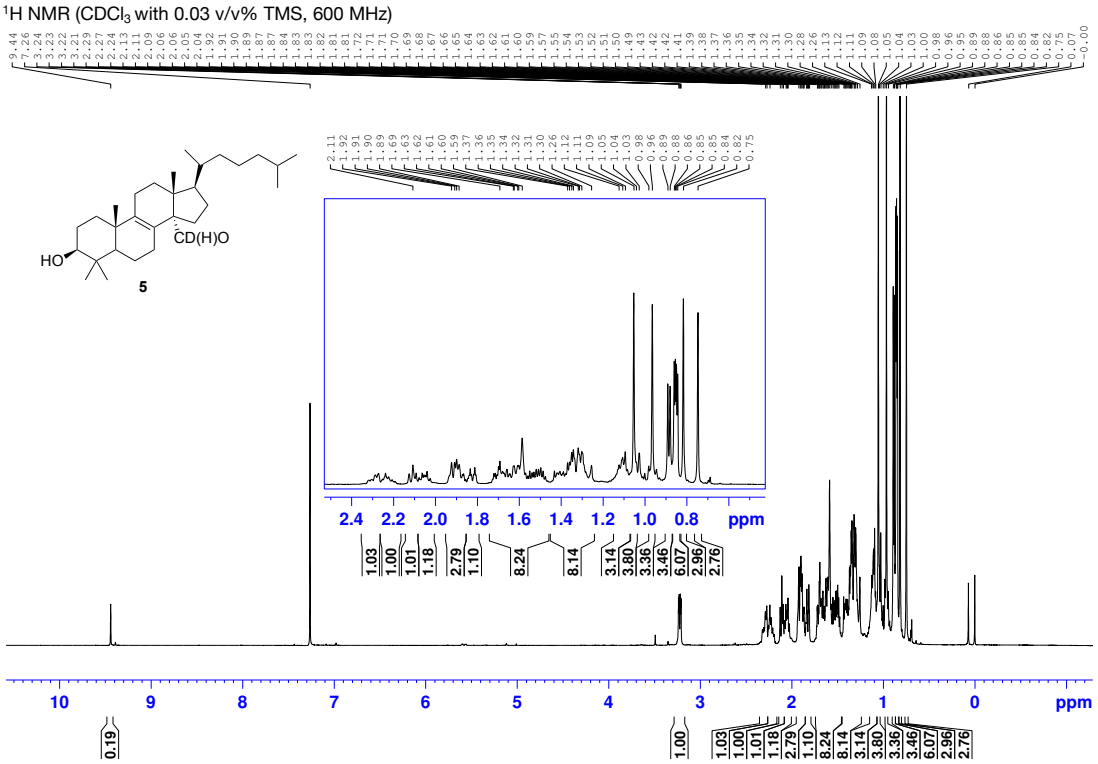
Step 2. Compound **2** from above was dissolved in 10 mL of C_2H_5OH. $NaBD_4$ (19.5 mg, 0.46 mmol) was added, and the mixture was stirred for 2 h at 23 °C, when TLC indicated that the reaction was complete (silica, hexanes-ethyl acetate, 3-1, v/v). The solvent was removed *in vacuo* and the residue was dissolved in $(C_2H_5)_2O$ and washed 3× with H_2O. The residue (**2**) was concentrated to dryness *in vacuo*. HRMS-APCI (*m/z*): $[M+H–H_2O]^+$ calcd for $C_{32}H_{52}DO$, 470.4103; found, 470.4091 (–2.5 ppm), $[M+H–HOAc]^+$ calcd for $C_{30}H_{50}DO$, 428.3997; found, 428.3990 (–1.8 ppm), $[M+H–H_2O–HOAc]^+$ calcd for $C_{30}H_{48}D$, 410.3892; found, 410.3880 (–2.8 ppm), (base peak).

Step 3. Compound **3** (from above) was treated with Dess-Martin periodinane as in step 1, yielding the aldehyde **4**. This product was used for the next step without further purification.

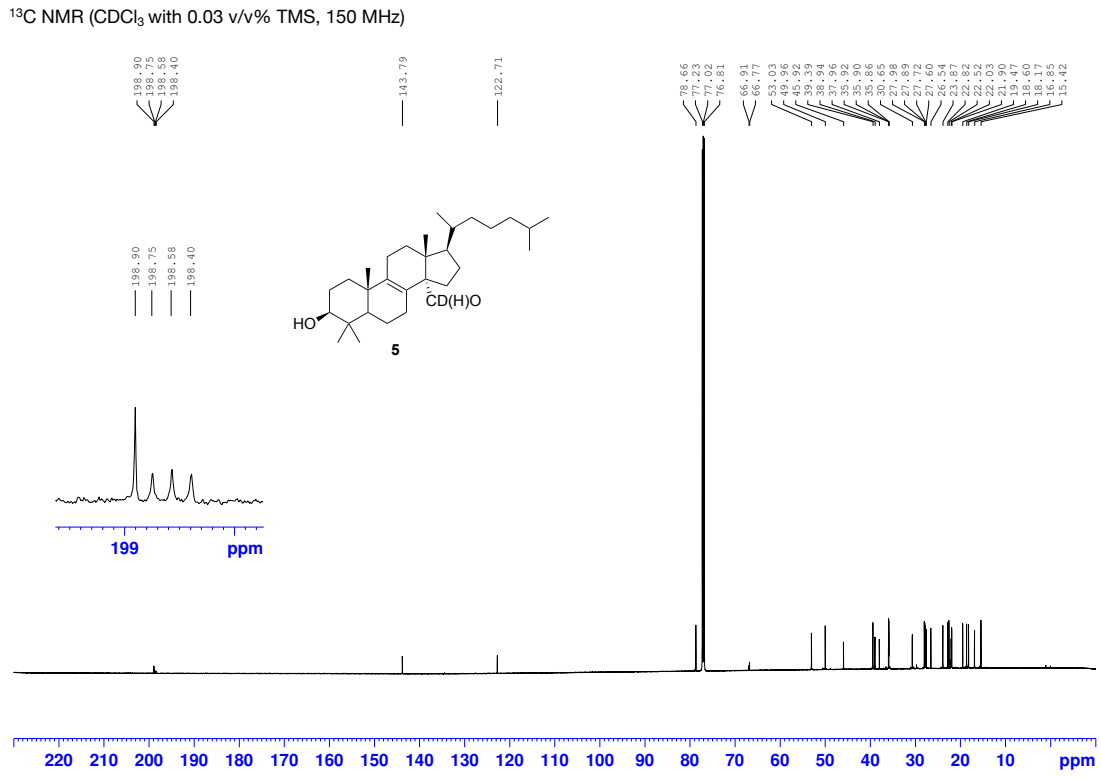
Step 4. Compound **4** (from step 3) was dissolved in 2 mL of benzene. To this was added 2 mL of CH_3OH containing 2% (w/v) KOH. The mixture was stirred and heated at 60 °C for 4 h. After cooling, the mixture was added to 15 mL of $(C_2H_5)_2O$, which was washed with brine and H_2O. The product was concentrated *in vacuo* to yield the final product **5**, a mixture of the protiated (~25%) and deuterated (~75%) aldehydes (calculated from peak areas of each compound, see below), which was purified by preparative TLC (hexanes-ethyl acetate, 3-1, v/v, elution with $(C_2H_5)_2O$, followed by acetone, filtration through paper, and concentration to dryness in vacuo. The overall yield (steps 1-4) was ~ 40% after purification. Portions were dissolved and quantified for preparation of substrate by HPLC comparisons with a standard curve based on dihydrolanosterol UV absorbance. HRMS (APCI): *m/z* calcd for $C_{30}H_{50}DO_2^+$: 444.3946 $[M+H]^+$; found: 444.3902; calcd for $C_{32}H_{48}DO^+$, 426.3841 $[M+H–H_2O]^+$; found: 426.3797.



LC-HRMS analysis of compound 5.



^{1}H NMR spectra of compound 5.



^{13}C NMR spectra of compound 5.

SUPPORTING INFORMATION

Supplementary Figures and Tables

Table S1. Crystallographic data collection and refinement statistics (See Figure 1)

PDB ID	8SS0
Data collection	
Beamline	ID-D
Wavelength, Å	1.12713
Space group	$P6_222$
Cell dimensions	
a, b, c, Å	145.8 145.8 261.2
α, β, γ, °	90.0 90.0 120.0
Molecules per asymmetric unit	2
Resolution (upper shell), Å	19.9-2.25 (2.5-2.25)
Solvent content, %	67
R_{merge} (upper shell)	0.085 (0.602)
CC (1/2) (upper shell)	0.997 (0.848)
I/σ(I) (upper shell)	5.8 (1.9)
Completeness (upper shell), %	94.9 (83.6)
Redundancy (upper shell)	4.4 (3.6)
Refinement	
No. of unique reflections	36891
R_{work}/R_{free}	0.219/0.249
R.m.s deviations	
Bond lengths, Å	0.007
Bond angles, °	1.4
Ramachandran plot	
Favorable/allowed, %	94.4/99.9
Outliers, %	0.1
Wilson B factor, $Å^2$	33.0
Model	
No. of atoms (mean B factor, $Å^2$)	7379 (37.13)
No. of residues per molecule	A/B
Protein (B factor, $Å^2$)	446(40.1)/441(35.5)
Heme (B factor, $Å^2$)	1 (25.2/24.8)
Sterol [WQR] (B factor, $Å^2$)	1 (26.6/23.2)
Water (B factor, $Å^2$)	45 (29)/62(29)

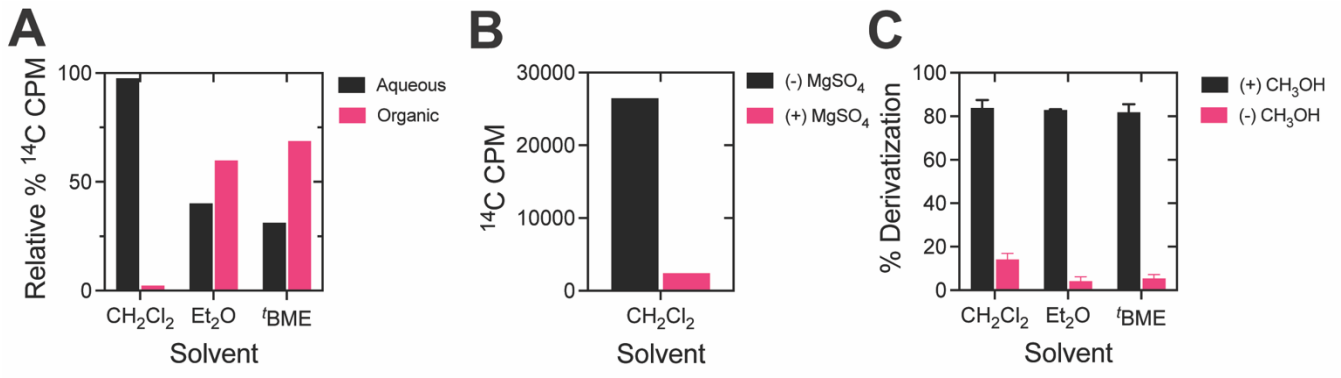


Figure S2. Optimization of reaction conditions for the derivatization of formic acid to pyridyl formate esters. (A) H^{14}COOH (10 µCi) was added to H_2O (4 mL) and extracted into organic solvent using a volume (5 mL) of either methylene chloride (CH_2Cl_2), diethyl ether (Et_2O), or *tert*-butyl methyl ether (^{t}BME). The organic layer (4.5 mL) and aqueous layer (~4 mL) of each sample was removed (separately) into vials containing 15 mL scintillation cocktail, the mixture was vortexed and samples were analyzed on a Beckman LS 6500 Multi-Purpose Scintillation Counter for ^{14}C CPM. The data (single replicate) are reported as relative %^{14}C CPM, or the ^{14}C CPM of the aqueous (black) or organic (red) layers relative to the sum of ^{14}C CPM of both layers for each sample. In summary, extraction of formic acid from the aqueous reaction mixture was found to be inefficient with CH_2Cl_2, and the formic acid recovery was increased from < 3% (in CH_2Cl_2) to ~70% (in ^{t}BME) (B) H^{14}COOH (10 µCi) was added directly to CH_2Cl_2 (5 mL). The organic layer was either (i) derivatized directly (in black), or (ii) added to ~200 mg $MgSO_4$, vortexed, filtered over a cotton-plugged glass pipette, and then derivatized (in red). Derivatization was performed with the diazo reagent as described in the $^{18}O_2$ incorporation assays above and analyzed on radio-HPLC for the formation of ^{14}C-pyridyl formate esters. The data (single replicate) are reported as the integration of the ^{14}C-pyridyl formate ester peak with or without initial treatment of the sample with$MgSO_4$. Drying the organic layer with $MgSO_4$ reduced formic acid recovery to <10%. (C) H^{14}COOH (300 nCi) was added to 5 mL of CH_2Cl_2, Et_2O, or ^{t}BME. Samples were treated with or without 10% (v/v) CH_3OH (0.5 mL). Samples were vortexed and derivatized as described in the $^{18}O_2$ incorporation assays above and analyzed on radio-HPLC for the formation of ^{14}C-pyridyl formate esters. The data (in duplicate) are reported as % Derivatization, or the % conversion of H^{14}COOH to a ^{14}C-pyridyl formate ester. The peaks corresponding to each analyte were analyzed, and the relative ^{14}C CPM of each peak was used to calculate % derivatization. The inclusion of CH_3OH (10% (v/v)) in the derivatization mixture was found to increase the yield of formic acid recovered as the pyridyl ester from ~15% to ~85% (in all solvents tested).

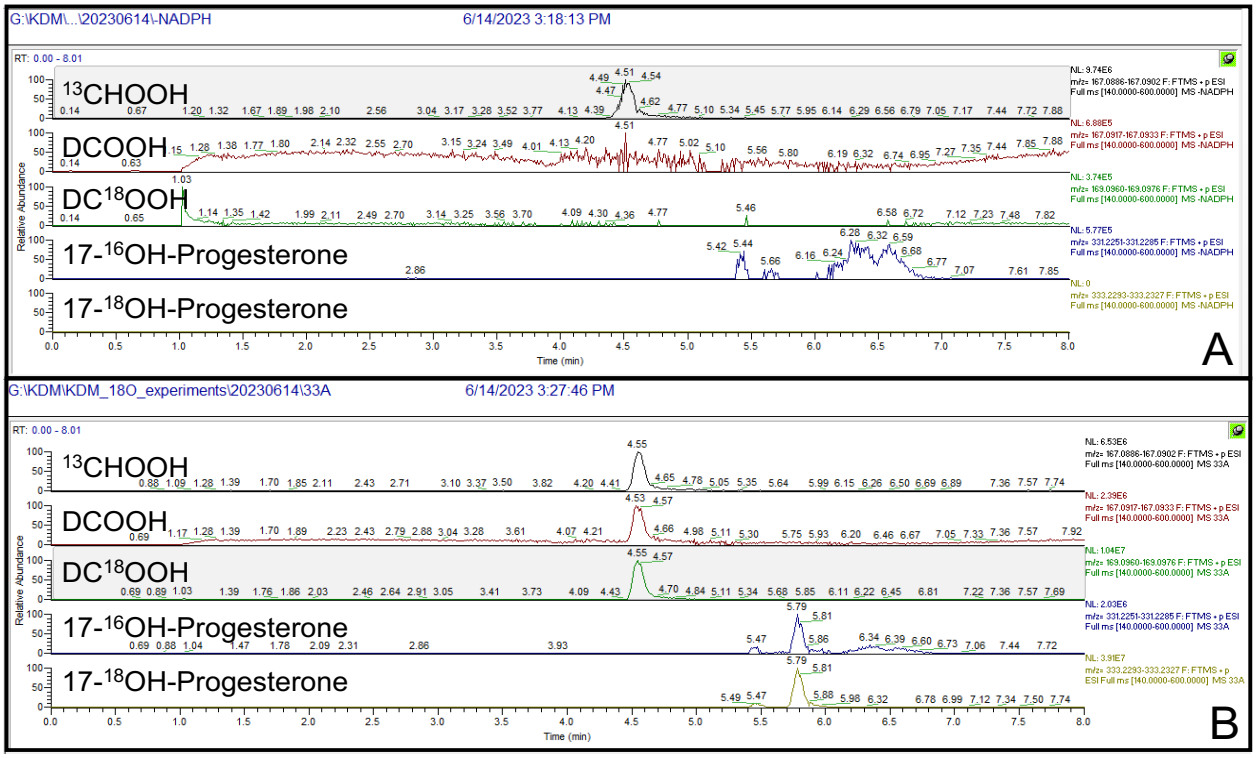


Figure S3. $^{18}O_2$ results with human P450 51A1 (from Figure 2). Raw data (chromatograms) are shown for the negative (-NADPH) control (Panel A) and of the initiated reaction (Panel B). Channels (*m/z* windows) for the ^{13}C contribution of the endogenous HCO_2H contaminant (*m/z* 167.0896), the P450 51A1 products DCO_2H (*m/z* 167.0925) and $DC^{18}OOH$ (*m/z* 169.0968), and the P450 17A1 products 17-^{16}OH- (*m/z* 331.2268) and 17-^{18}OH-progesterone (*m/z* 333.2310) are shown. The mass window (5 ppm mass tolerance) is indicated along with ion intensity to the right of each chromatogram.

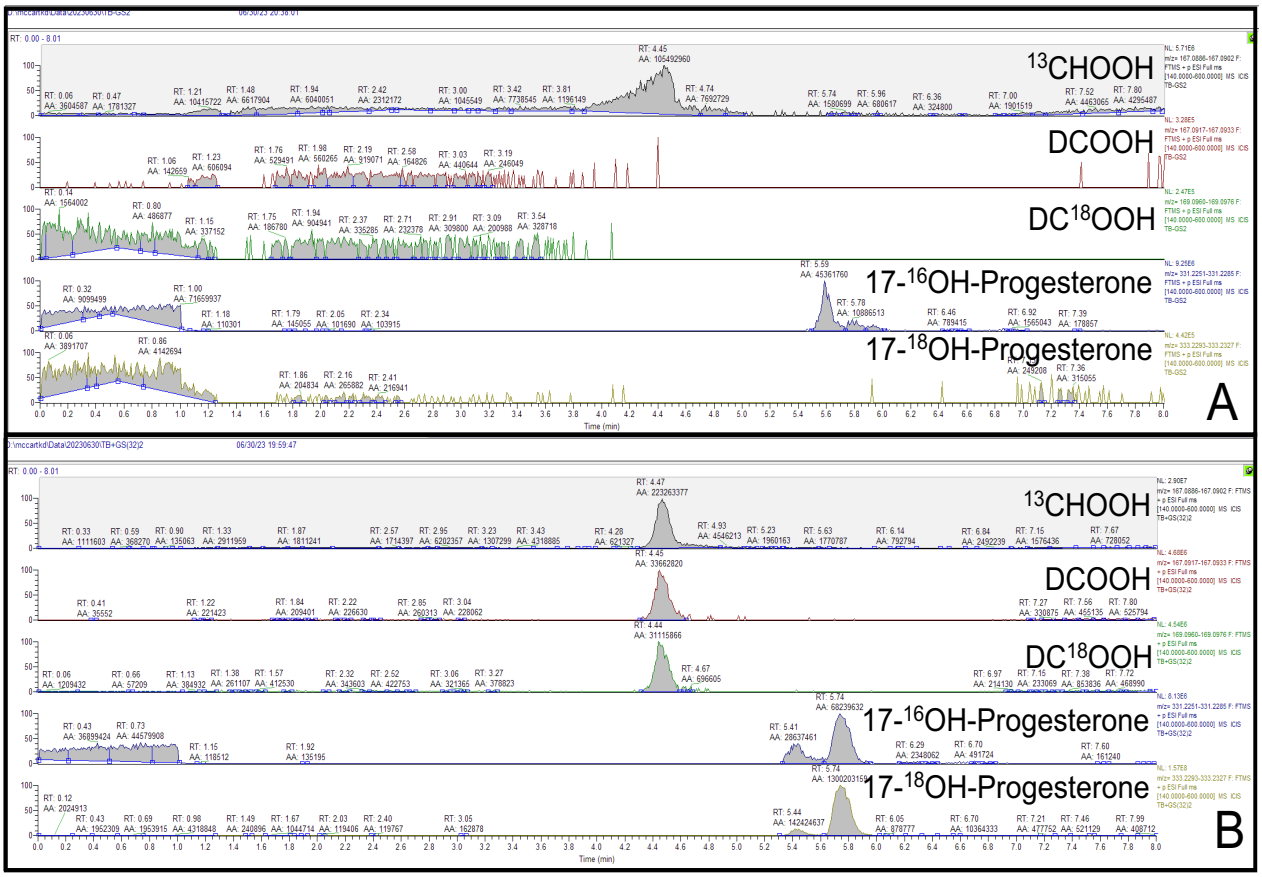


Figure S4. $^{18}O_2$ results with P450 51 from *T. brucei* (from Figure 3). Raw data (chromatograms) are shown for the negative (-NADPH) control (Panel A) and of the initiated reaction (Panel B). Channels (*m/z* windows) for the ^{13}C contribution of the endogenous HCO_2H contaminant (*m/z* 167.0896), the P450 51A1 products DCO_2H (*m/z* 167.0925) and $DC^{18}OOH$ (*m/z* 169.0968), and the P450 17A1 products 17-^{16}OH- (*m/z* 331.2268) and 17-^{18}OH-progesterone (*m/z* 333.2310) are shown. The mass window (5 ppm mass tolerance) is indicated along with ion intensity to the right of each chromatogram.

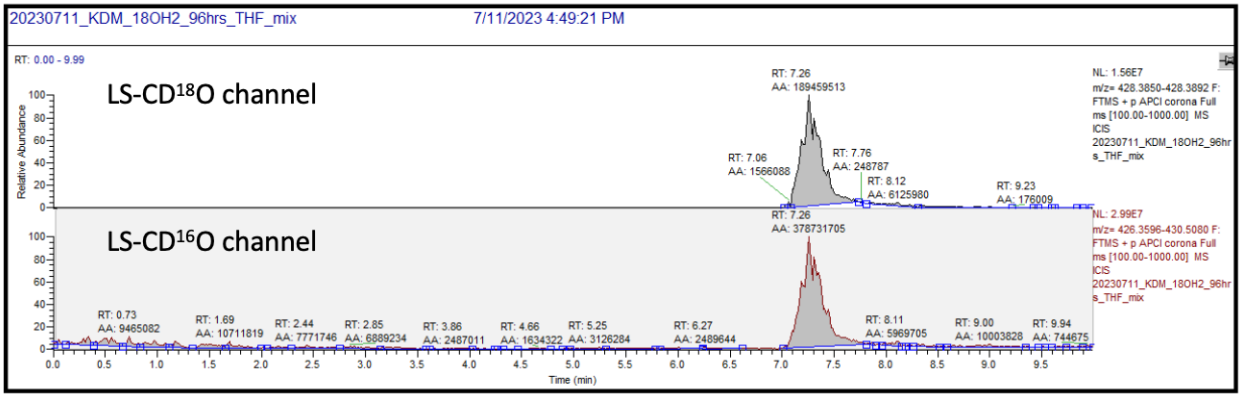


Figure S5. Preparation of 14α-CD^{18}O dihydrolanosterol (from Experimental Methods). The chemical was prepared by exchange of aldehyde oxygen of 14α-CDO dihydrolanosterol (*m/z* 426.3841) with $H_2{}^{18}O$ 14α-CD^{18}O dihydrolanosterol (*m/z* 428.3883). The mass window (5 ppm) is indicated along with ion intensity to the right of each chromatogram.

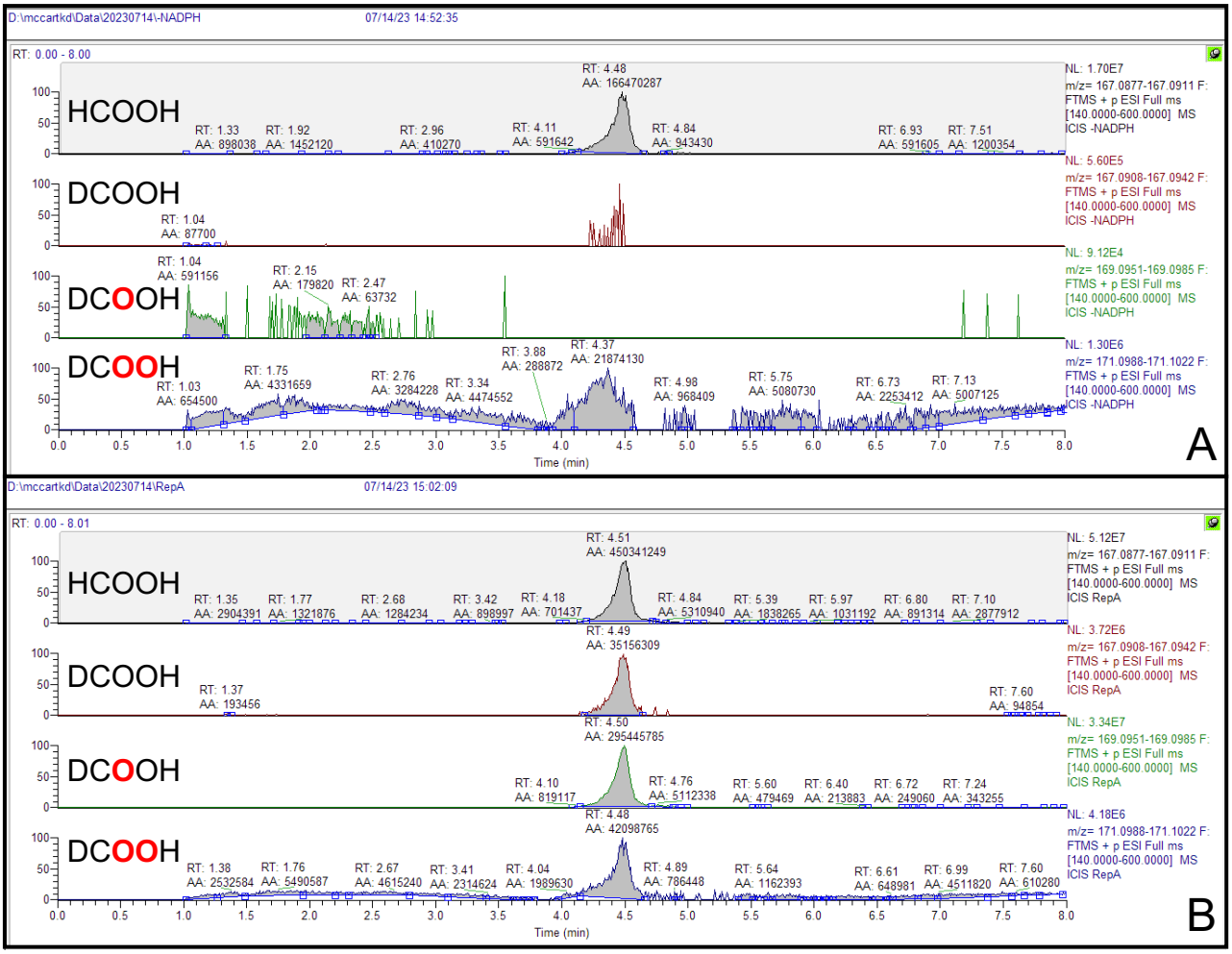


Figure S6. $H_2{}^{18}O$ labeling results with human P450 51A1 (from Figure 5). Chromatograms are shown of the negative (-NADPH) control (Panel A) and of the initiated reaction (Panel B). Channels (*m/z* windows) for the ^{13}C contribution of the endogenous HCO_2H contaminant (*m/z* 167.0896) and the P450 51A1 products DCO_2H (*m/z* 167.0925), $DC^{18}OOH$ (*m/z* 169.0968), and $DC^{18}O^{18}OH$ (*m/z* 171.1010). ^{18}O atoms are indicated in red. The mass window (5 ppm mass tolerance) is indicated along with ion intensity to the right of each chromatogram.

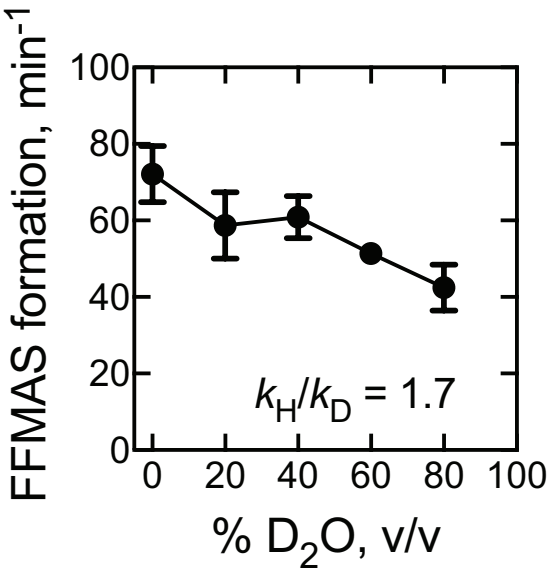


Figure S7. Solvent KIE (KSIE) for oxidation of 14α-CHO dihydrolanosterol to dihydro FF-MAS by human P450 51A1. Data are reported as nmol dihydro FF-MAS formed min^{-1} (nmol P450)$^{-1}$ and each data point was performed in triplicate. Error bars indicate means ± standard deviation. % D_2O reflects the final solvent composition (v/v) for each reaction.

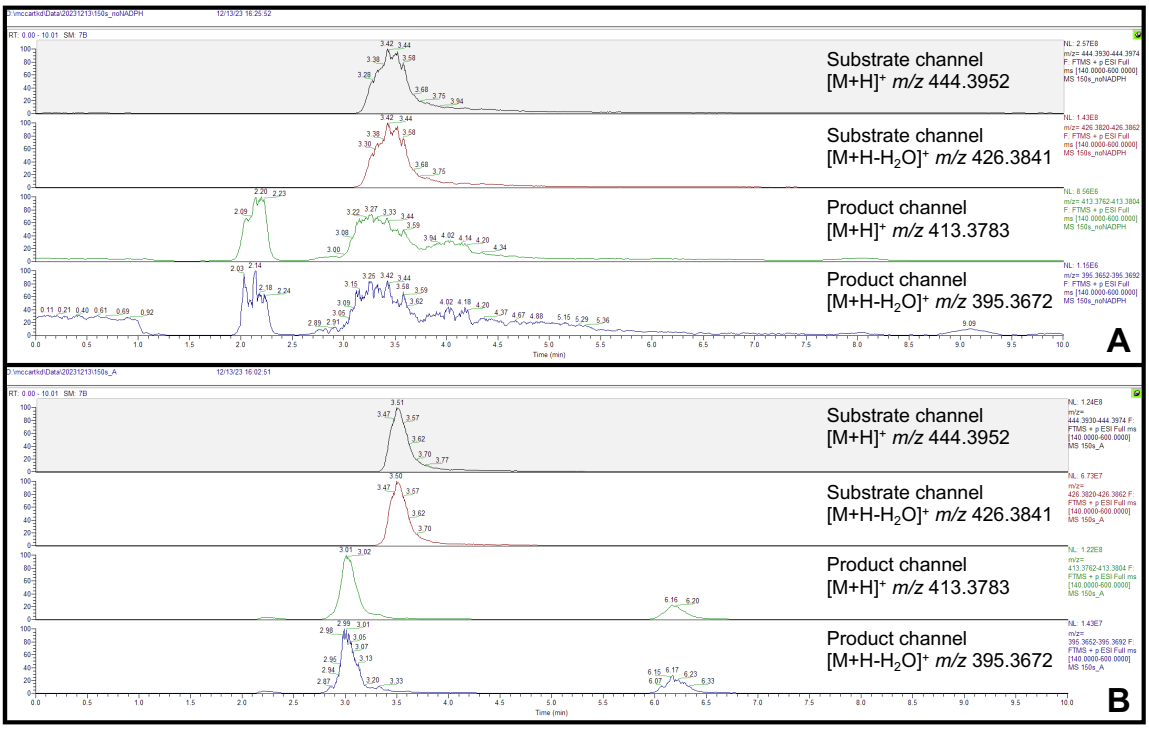


Figure S8. Characterization of Baeyer-Villiger intermediate on reversed-phase (C_{18}) UPLC-HRMS. Chromatograms are shown of the negative (-NADPH) control (Panel A) and of the initiated reaction (Panel B). The Baeyer-Villiger (BV) intermediate (*m/z* 395.3672, t_R 3.01 min) was identified by reversed-phase HPLC as a peak eluting just before the substrate (14α-CDO dihydrolanosterol, *m/z* 426.3841, t_R 7.53 min), similar to the results reported by Fischer et al.[23] Extracted *m/z* values of 395.3672 and 413.3783 are fragment ions of the suspected Baeyer-Villiger (BV) intermediate corresponding to the $[M+H-H_2O-DCO_2H]^+$ and $[M+H-DCO_2H]^+$ ions, respectively, and to the $[M+H]^+$ and $[M+H-H_2O]^+$ ions (respectively) of the final product, FF-MAS. The extracted *m/z* values of 426.3841 and 444.3952 correspond to the intact $[M+H]^+$ and $[M+H-H_2O]^+$ ions of the starting substrate, 14α-CDO dihydrolanosterol. The presence of the BV intermediate peak was NADPH-dependent (Panel B) and reproducible over technical and biological replicates. The mass window (± 5 ppm) is indicated along with ion intensity to the right of each chromatogram.

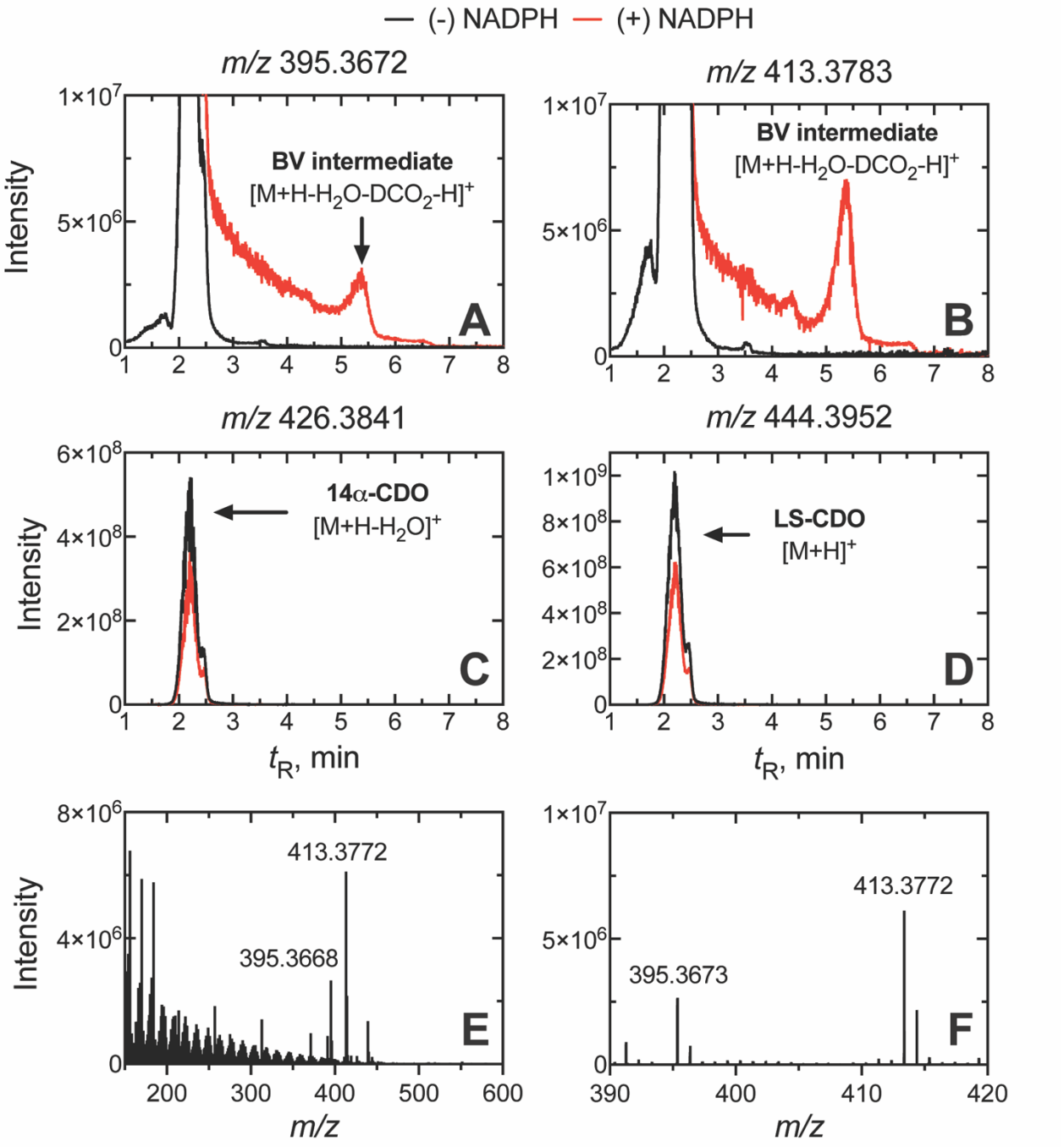


Figure S9. Characterization of Baeyer-Villiger intermediate on normal-phase (silica) UPLC-APCI-HRMS. Chromatographic traces of enzyme incubations (7.5 minutes) with extracted *m/z* values of (A) 395.3672 and (B) 413.3783 (utilizing a ± 5 ppm mass window) are fragment ions of the suspected Baeyer-Villiger (BV) intermediate corresponding to the $[M+H-H_2O-HCO_2]^+$ and $[M+H-HCO_2]^+$ states, respectively. Uninitiated reactions (controls) are in black (-NADPH) while initiated reactions (+NADPH) are in red. Depletion of the aldehyde starting substrate (14α-CDO dihydrolanosterol) was observed with extracted *m/z* values of (C) 426.3841 and (D) 444.3952 corresponding to the intact $[M+H]^+$ and $[M+H-H_2O]^+$ ions of the molecule. The full mass spectrum (*m/z* 150-600) of the intermediate (t_R 5.38 min) showed the major fragment ions of the intermediate (E), which are magnified (*m/z* 390-420) in (F). The intermediate was significantly retained on the column relative to the aldehyde starting substrate (t_R 2.22 min) and the final product, FF-MAS (t_R 1.41 min), similar to the results reported by Fischer et al.[23] (Only the downslope of the dihydro FF-MAS peak is visible in panels A & B).

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Author Contributions

F. P. G., G. I. L., and K. D. M. participated in the study design. K. D. M. and Y. T., conducted the experiments and contributed to the data curation; Y. T., G. I. L., and T. Y. H. contributed resources (chemicals and enzymes). F. P. G. and K. D. M. contributed to visualization and writing-original draft; F. P. G., K. D. M., and Y. T. contributed to writing-review, and editing. All the authors have read and approved the final manuscript.