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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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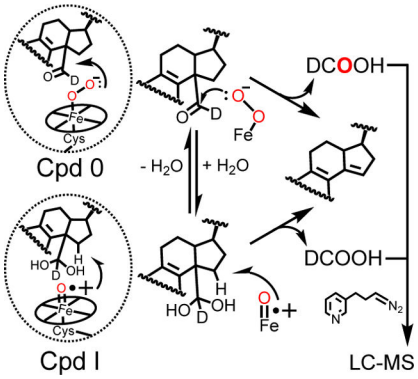
Abstract

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.

Graphical Abstract



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Supporting Information

The authors have cited references within the Supporting Information.[1–14]

P450 51 enzymes demethylate sterols, releasing formic acid (DCOOH). Analysis of the DCOOH byproduct provides for the mechanistic discrimination of the contribution(s) of Compound 0 (Cpd 0) and Compound I (Cpd I) to catalysis. When enzyme reactions were run under $^{18}O_2$ (red), >50% of the DCOOH yielded contained one atom of ^{18}O (for all P450 51 enzymes tested), indicative of the major contribution of Cpd 0 in 24,25-dihydrolanosterol C-C cleavage.

Keywords

cytochrome P450; sterol biosynthesis; enzyme mechanism; mass spectrometry; oxidation mechanism

Introduction

Cytochrome P450 (P450 or CYP) enzymes catalyze oxidation and reductions of many molecules,[1] particularly drugs, steroids, pesticides, fatty acids, fat-soluble vitamins, and chemical carcinogens.[2] In addition to the extensive involvement of P450s in intermediary steroid metabolism,[3] these enzymes also play a critical role in the biosynthesis of cholesterol in mammals and in the synthesis of similar essential sterols in microorganisms and plants – a role fulfilled by the Family 51 enzymes.[4] Accordingly, inhibition of the Family 51 P450s is a common means of treating a number of diseases caused by fungi and parasites.[5]

The general P450 mechanism (Scheme 1) includes both Compound 0 (ferric peroxide anion, FeO_2^-) and its product Compound I (perferryl oxygen, FeO^{3+}) in the catalytic cycle, and the question of which catalytic oxygen species is operative in P450 has long been a source of scientific interest.[6] Two seminal advances towards the rather ubiquitous role of the FeO^{3+} entity termed Compound I were made by Groves in 1978[7] and Green in 2010,[8] and many P450 oxidations can be rationalized in the context of Compound I chemistry.[9] In fact, some of the alternative explanations for hydroxylation and epoxidation reactions[10] have not held up to further mechanistic scrutiny.[11] However, there are still a number of observed P450 reactions that are difficult to explain with only Compound I mechanisms, and the literature contains some credible evidence for so-called Compound 0 (FeO_2^-)-based reactions (Scheme 1).[12]

We have recently reviewed the Compound 0 vs. Compound I question within the major P450 enzymes involved in C-C bond cleavage of steroids.[9, 13] With P450 11A1 (cholesterol side chain cleavage enzyme), Compound I has been prepared and shown to be catalytically competent in the reaction.[14] The lyase reaction of P450 17A1 is controversial, with evidence supporting both Compound I and Compound 0 mechanisms.[13, 15] Our own work with P450 19A1 (steroid aromatase) favors a Compound I mechanism.[16]

A variety of approaches have been employed in attempts to distinguish Compound I and Compound 0 (including protonated Compound 0) mechanisms. The list includes the use of biomimetic models,[17] theoretical considerations,[18] oxygen surrogates,[19] site-directed mutagenesis,[20] diagnostic substrates,[21] kinetic solvent isotope effects (KSIE),[22] synthetic formation of Compound 0 or Compound I and use in a reaction,[8, 14] and ^{18}O

labeling.[16, 23] Each of these approaches has deficiencies, as well as some attractions. One of the shortcomings of almost all of these approaches is that the answers are interpreted as one-or-the-other mechanism, with no provision for considering a mixed mechanism.

P450 Family 51 enzymes all catalyze a 3-step 14α-demethylation reaction with sterols (Scheme 2). The first two steps involve a carbon hydroxylation and a formal 2-electron oxidation of the carbinol to an aldehyde, both of which are common P450 reactions, generally attributed to a Compound I (FeO^{3+}) mechanism.[24] The third step is more unusual, involving C-C bond cleavage and release of the 14α-carbon atom (C32) as formic acid, where mechanisms involving both P450 Compound 0 and Compound I as the oxidant have been proposed.[12c, 18b, 20b, 25] Fischer et al.[12c] proposed a Compound 0 mechanism involving a Baeyer-Villiger intermediate. Shyadehi et al.[25a] later proposed a Compound 0 mechanism for a yeast (*Candida albicans*) P450 51 enzyme but without a Baeyer-Villiger rearrangement. At least one theoretical study has been published in favor of a Compound 0 mechanism[25b] but a more recent one is strongly in favor of a Compound I mechanism.[18b]

On the basis of some mutagenesis and structural information, we proposed that human P450 51A1 uses a Compound I mechanism in catalysis.[20b] A major tenet of the Shyadehi et al.[25a] conclusion about the role of Compound 0 is work on the incorporation of ^{18}O from $^{18}O_2$ into formic acid in an incubation of a 14α-formyl analogue (Δ^7) of 24,25-dihydrolanosterol. However, there are a number of caveats about the results of Shyadehi et al.,[25a] including the utilization of a crude yeast P450 51 microsomal preparation with low enzymatic activity (rate of 0.25 nmol product formed min^{-1} (nmol enzyme)$^{-1}$), the use of an artificial Δ^7 sterol instead of the natural Δ^8 isomer, and analysis using only low resolution MS (which cannot distinguish enzymatically produced DCO_2H from the ^{13}C component of HCO_2H ($H^{13}CO_2H$)). Although only 62% incorporation of ^{18}O (from $^{18}O_2$) into formic acid was reported (and only 5–7% of the total formic acid was derived from the substrate), the authors concluded that an exclusive Compound 0 mechanism was operative.[25a]

In considering the significance of this $^{18}O_2$ incorporation study to the catalytic mechanism of P450 51 enzymes, we synthesized the requisite deuterated 14α-aldehyde derivative of (24,25-dihydro) lanosterol (14α-CDO dihydrolanosterol) and utilized it in ^{18}O incorporation studies using (five) different purified Family 51 P450 enzymes, employed a sensitive derivatization procedure with a formate ester suitable for UPLC-electrospray MS analysis of formic acid, and analyzed the products with HRMS to resolve issues of ^{13}C contribution that we had developed in a similar study with P450 19A1.[16] We also performed complementary labeling studies with $H_2{}^{18}O$, labeling the carbonyl atom of the aldehyde substrate and analyzing the formic acid for ^{18}O content. We conclude that P450 51 enzymes catalyze the last step of sterol 14α-demethylation reactions using both Compound I and Compound 0 mechanisms, the latter also including a Baeyer-Villiger intermediate. Thus, an individual P450 enzyme can catalyze a single reaction via multiple chemical mechanisms.

Results and Discussion

Strategy: ^{18}O Labeling.

The approach to discerning the role of Compound I vs. Compound 0 is shown in Scheme 3. A Compound 0 mechanism incorporates one ^{18}O atom from $^{18}O_2$ into the product formic acid, and a Compound I mechanism does not. The background level of formic acid in the laboratory environment is problematic for these measurements, necessitating (i) the use of substrate with a deuterated formyl group (DC=O), (ii) chemical measures to reduce the contamination of formic acid in solvents, and (iii) the use of high resolution mass spctrometery (HRMS) (resolution > 56000) for analysis. HRMS can readily distinguish between (derivatized) $H^{13}CO_2H$ (*m/z* 167.0896) and $D^{12}CO_2H$ (*m/z* 167.0925),[16] which is necessary because of the natural abundance of ^{13}C in the endogenous formic acid and its derivatives. HRMS traces of the esters of $H^{13}CO_2H$ and $D^{12}CO_2H$ in a typical experiment are shown in Figures S3, S4, and S6.

The synthesis of deuterated 14α-formyl 24,25-dihydrolanosterol (14α-CDO dihydrolanosterol) was based on work of Morisaki and associates,[26] which began with commercial 7-dehydrocholesterol and generated the protiated product in 14 steps to give the desired 14α-aldehyde.[27] The partially deuterated aldehyde was generated in three more steps by sequential Dess-Martin periodinane oxidation,[28] reduction with $NaBD_4$, Dess-Martin periodinane oxidation, and hydrolytic cleavage of the 3-*O*-acetyl group (Figure S1). The deuterium content in the product was ~75% (not 50%), presumably due to a kinetic isotope effect in the Dess-Martin oxidation. The lack of complete substitution is not a requirement in the experimental protocol, in that the formate ester derived from the protium is not used in the mechanistic analysis (i.e., it is identical to the ester derived from endogenous formate contaminant (HCO_2H)).

In the course of our studies, we also made several changes to improve the extraction and derivatization of formic acid since our earlier report.[16] The major changes (Figure S2) were (1) the use of *tert*-butyl methyl ether to extract formic acid instead of CH_2Cl_2, (2) omitting the $MgSO_4$ drying step, and (3) the inclusion of 10% CH_3OH (v/v) in the diazotization step.[29] Extraction of formic acid from the aqueous reaction mixture was found to be inefficient with CH_2Cl_2. While drying the organic extract with $MgSO_4$ was performed previously based on the premise that the derivatization reaction was sensitive to H_2O, the derivatization of acid with the diazo reagent was shown to be improved by the addition of both H_2O and CH_3OH at 10% (v/v). The drying step routinely reduced formic acid recovery, so the step was omitted. Finally, 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, and accordingly this was subsequently included in all derivatizations. Collectively these modifications increased the recovery by three orders of magnitude.

The sensitive analysis of DCO_2H to discriminate the involvement of Compound 0 and Compound I requires nearly complete exchange of the endogenous environment with $^{18}O_2$. To monitor our reaction vessels for leaks (and ensure the accuracy of our ^{18}O labeling results) we included in each reaction mixture a control incubation of P450 17A1 and progesterone, done in the absence of cytochrome b_5 for nearly exclusive formation of its

17α-hydroxy product. Because the environment is replaced with $^{18}O_2$ and the P450 51 system is mixed with NADPH to start the reaction, the P450 17A1 control reaction is also initiated. The incorporation of ^{18}O into both progesterone to yield 17α−^{18}OH-progesterone by P450 17A1 and DCO_2H by P450 51A1 is monitored simultaneously by LC-MS. The P450 17A1 ^{18}O incorporation results report on the actual $^{18}O_2$ content in each reaction vessel and act as a normalization factor for the P450 51 labeling results, allowing us to assess the exact contribution of each mechanism to dihydrolanosterol deformylation. Our previous work with P450 19A1 established minimal (< 6%) DCO_2H oxygen exchange with the medium under these conditions.[16]

The ^{18}O labeling approach is limited in its scope, in that only some reactions such as deformylations are amenable. With an α-ketol substrate (α-hydroxyketone), the results are not unambiguous for the interpretation, e.g., P450 17A1 lyase.[9, 13] This approach cannot be applied to many P450 reactions, but it is realistic for the last reaction steps of P450s 19A1 and 51. The approach is technically challenging, due to the need to synthesize deuterated aldehydes (because of background formic acid)[23b] and the requirement to generate an $^{18}O_2$ atmosphere. However, it is an approach that, when carefully applied, can identify a mixed mechanism, as observed here.

X-ray Crystallography of Human P450 51A1.

The first two oxidations in the demethylation of lanosterol (hydroxylation of C32 and oxidation to an aldehyde) are understood to be mediated by a P450 Compound I mechanism.[24] We had previously reported that the overall three-step reaction was highly kinetically processive, in that lanosterol and reaction intermediates are bound tightly to the enzyme and driven to final product formation.[27] Accordingly, we investigated whether the binding mode of the aldehyde (for C-C cleavage) was similar to the binding mode of lanosterol (for hydroxylation) and crystallized the enzyme in complex with our synthetic aldehyde intermediate for comparison.

Human P450 51A1 was crystallized and the 2.25 Å resolution structure was refined. The overall binding mode of the aldehyde is essentially the same as the binding of lanosterol (Fig. 1A). The protein conformation is also essentially the same (with a closed substrate entry and only minor fluctuations in the positions of some loops, GH in particular, which is known to be the most flexible region in P450 51 structures) (Figure 1B,C). This is in line with the finding that the three-step reaction of sterol 14α-demethylation is a highly kinetically processive process.[27]

$^{18}O_2$ Incorporation into Formic Acid Reveals a Dominant Compound 0 Mechanism.

With an optimized protocol for the extraction and derivatization of formic acid in-hand (see Strategy section), we began by assaying the 24,25-dihydrolanosterol C-C cleavage reaction catalyzed by human P450 51A1. When an incubation of the enzyme and 14α-CDO dihydrolanosterol was performed under an atmosphere of $^{18}O_2$, >80% of the recovered pyridyl ester of the formate product (DCO_2H) contained one atom (*m/z* 169.0968) of the isotopic label (Figure 2, S3), with the remaining < 20% containing zero atoms (*m/z* 167.0925). The concomitant P450 17A1 control reaction (included in the P450 51A1

reaction mixture) incorporated >92% of the label (one atom) in the 17α-hydroxylation of progesterone, indicating high fidelity in the replacement of the endogenous atmosphere with $^{18}O_2$ (see Strategy section, vide supra). When normalized to the control reaction (i.e., P450 17A1), the deformylation of dihydrolanosterol by human P450 51A1 yielded primarily the recovery of one atom of $^{18}O_2$ in DCO_2H (88 ± 3%, n= 5, Figure 2), suggestive of a dominant but not exclusive contribution of the ferric peroxide nucleophile (Compound 0) in facilitating 24,25-dihydrolanosterol C-C cleavage. Because our control experiments established that the chromatographic peaks of interest (Figure 2) are not formed non-enzymatically (e.g., in the absence of NADPH) we were confident in this result, which was reproducible in both technical and biological replicates.

In light of the dominance of Compound 0 observed in incubations with the human enzyme, we examined whether this extends to other well-studied P450 51 orthologs. Interestingly, we found that P450 51 enzymes from *Naegleria fowleri* and *Candida albicans* (Figure 3) showed nearly identical results to the human ortholog, where incubations with 14α-CDO dihydrolanosterol yielded DCO_2H that incorporated >82% of the label (one atom), on average, from $^{18}O_2$ (87 ± 2 and 82 ± 3%, respectively, after normalization, n = 5) (Table 1, Figure 4). In these experiments, the mean label incorporation in the control reaction (P450 17A1) was >91% for both reactions, again ensuring high technical accuracy. As we found with human P450 51A1, the ^{18}O labeling results with P450 51 enzymes from *N. fowleri* and *C. albicans* were also suggestive of Compound 0 dominance in 24,25-dihydrolanosterol C-C cleavage.

We then applied the analysis to P450 51 enzymes from *Trypanosoma cruzi* and *Trypanosoma brucei* (Figure 3). As lanosterol is not a natural substrate of either enzyme, both orthologs show low rates of 24,25-dihydrolanosterol 14α-demethylation. Accordingly, incubations of trypanosomal P450 51 enzymes were performed with an elevated P450 concentration (1 μM), an extended reaction time (60 min), and modified analysis conditions, all to increase sensitivity (see Experimental Procedures). When we performed the incubations of these orthologs with 14α-CDO dihydrolanosterol, the DCO_2H recovered showed lower label incorporation from $^{18}O_2$ than the other P450 51 enzymes tested (59 ± 2% and 51 ± 1%, (Figure S4), respectively, after normalization, n = 3) (Table 1, Figure 4). Despite this (significant and reproducible) difference, the mean label incorporation in the control reaction (progesterone 17α-hydroxylation by P450 17A1) was >93% for both reactions, and residual substrate (progesterone) was detected after reaction termination, indicating that reduced label incorporation (into DCO_2H) could not be attributed to loss of $^{18}O_2$ in the reaction (e.g., due to gas leaks). For both trypanosomal P450 51 orthologs tested, the ^{18}O labeling results were suggestive of roughly equal contributions of both Compound 0 and Compound I mechanisms in dihydrolanosterol C-C cleavage.

$H_2{}^{18}O$ Labeling with Human P450 51A1 –a Minor Contribution of Compound I.

While the incorporation of ^{18}O (from $^{18}O_2$) into DCO_2H (~85%) is unambiguous evidence for the contribution of a P450 Compound 0 mechanism, the lack of complete incorporation (~15%) is only indirect evidence of the role of Compound I. To directly measure the contribution of Compound I to dihydrolanosterol C-C cleavage by human P450 51A1,

we employed an alternative approach to the $^{18}O_2$ experiment by running the enzymatic incubation in $H_2{}^{18}O$. In this experiment, the starting aldehyde substrate is equilibrated with the medium ($H_2{}^{18}O$) prior to the enzymatic incubation (to yield 14α-CD^{18}O dihydrolanosterol, with the carbonyl oxygen exchanged). The action of P450 Compound 0 (in an $^{16}O_2$ atmosphere) yields one atom of ^{16}O incorporated into DCO_2H upon C-C cleavage and recovers the ^{18}O label from the (aldehyde) starting substrate (Scheme 3A). Conversely, P450 Compound I acts on the substrate only after hydration of the 14α-CD^{18}O aldehyde to 14α-CD($^{18}OH)_2$ (i.e., *gem*-diol). Upon C-C cleavage, both atoms of ^{18}O present in the *gem*-diol (derived from the medium) will be recovered in DCO_2H. Accordingly, the $H_2{}^{18}O$ experiment discriminates between the relative contributions of Compound 0 and Compound I on the basis of incorporation of either one or two atoms of label into DCO_2H, respectively (Scheme 3).

When 14α-CD^{18}O dihydrolanosterol was prepared (Figure S5) and incubated with human P450 51A1 (in $H_2{}^{18}O$), the major DCO_2H product contained just one atom of ^{18}O label (from the starting substrate) (86 ± 1%, *m/z* 169.0968 (for the ester derivative), with the minor product incorporating an additional atom from the solvent ($H_2{}^{18}O$, 14 ± 1%, *m/z* 171.1010) (Figures 5, S6). The finding that the Compound I product (two ^{18}O atoms recovered in DCO_2H) was a minor product in the $H_2{}^{18}O$ experiment (~15%) is in excellent agreement with our finding that the Compound 0 product (one atom of ^{18}O (from $^{18}O_2$) recovered in DCO_2H) was the major product (~85%, Figure 2, Table 1) in the $^{18}O_2$ experiment. Together, the $^{18}O_2$ and $H_2{}^{18}O$ labeling experiments track the relative contributions of Compound 0 and Compound I in the mechanism of dihydrolanosterol C-C cleavage by human P450 51A1, independently demonstrating that the enzyme predominantly (≥ 85%) incorporates one atom of O_2 from the atmosphere in DCO_2H to achieve deformylation of 14α-CDO dihydrolanosterol, i.e. the Compound 0 mechanism predominates but the Compound I mechanism is also operative.

Given the consistency of results for a dominant (~85%) role of Compound 0 with a minor (15%) contribution of Compound I (Figures 4 and 5, Table 1) in human P450 51A1, we questioned if structural elements can be identified that govern the mechanistic balance.[30] We had previously mutated salt-bridged residues (Asp-231 and His-314) to alanine in P450 51A1 and analyzed the effects of the resulting double-mutant on sterol demethylation.[20b] These results had been interpreted to support a Compound I mechanism due to a significant (~160-fold) reduction in aldehyde demethylation (providing evidence for a requirement for solvent accessible protons, i.e. in the formation of Compound I).

When we incubated the human P450 51A1 double mutant (D213A/H314A, 1 μM) with 14α-CDO dihydrolanosterol in more $H_2{}^{18}O$ experiments, we were unable to reproducibly recover significant amounts of DCOOH from the reaction (as a pyridyl formate ester), likely due to the low rates of sterol demethylation (even from 60-min incubations). We then incubated the single-mutant P450 51A1 D213A, which had shown higher reaction rates (roughly ¼ that of the WT enzyme, starting with lanosterol [31]) with 14α-CDO dihydrolanosterol (50 μM) we observed that 8.1 ± 0.2% (n = 3) of the ^{18}O-labeled DCOOH product was derived from Compound I (2 ^{18}O atoms incorporated), representing a reduction of the Compound I product by ~½ compared to the WT enzyme (i.e.,14% (Figure 5) to 8%). However, a ~50%

reduction in the Compound I contribution to sterol deformylation does not account for the >4-fold reduction in enzymatic activity observed for the enzyme, [31] and it is likely that the catalytic consequence of this mutation may be the result of unknown structural changes in the enzyme.

Oxygen Surrogates were not Catalytically Competent with P450 51A1 Deformylation.

Historically, one approach commonly employed to discern the roles of Compound 0 and Compound I in P450 reactions has been the use of chemical reagents acting as "oxygen surrogates" (iodosylbenzene (PhI=O), H_2O_2, cumene hydroperoxide (CuOOH)), in efforts to gauge the dependence of catalysis on either species.[12a, 19] In theory, a reaction that is mediated via a ferric peroxide (Compound 0) mechanism might be supported by the addition of H_2O_2 (or CuOOH) to ferric P450. Similarly, a reaction mediated by the oxyferryl species Compound I might be supported by the addition of the single-oxygen donor PhI=O to the enzyme. In these experiments, reactions supported by single oxygen donors can rule out a Compound 0 pathway, but reactions supported by 2-oxygen donors (hydroperoxides) cannot definitively rule out a Compound I pathway (i.e., Compound I forms naturally from Compound 0, Scheme 1). (All of these reagents are often (eventually) destructive to the P450 heme, however.)

We examined whether such oxygen surrogates could be used to gauge the dependence of P450 51 catalysis on Compound I. We tested the ability of PhI=O, CuOOH, and H_2O_2 to support demethylation of 14α-CDO dihydrolanosterol by human P450 51A1. We did not detect any product formation from any of these surrogates at any time point (≤ 5 min) tested but detected typical enzymatic activity (> 20 nmol product min^{-1} (nmol P450)$^{-1}$) from the control reaction (supported by NADPH), as expected.[27] Interestingly, we did observe some product formation with both PhI=O and H_2O_2 (but not CuOOH) when the reaction mixture was reconstituted with NADPH-P450 reductase, although the activity was still < 1% of the control (NADPH) reaction in both cases. Overall, experiments with oxygen surrogates were largely unable to support P450 51A1 deformylation and did not provide a means of addressing the P450 Compound 0 vs. Compound I question.

Kinetic Solvent Isotope Effect (KSIE) Analyses are Unreliable.

Previous cases for discerning the involvement of Compound I vs. Compound 0 in P450 catalysis have also been proposed through the analysis of KSIE data.[22, 32] In the general P450 catalytic cycle (Scheme 1), protonation of Compound 0 (step 5) to yield Compound I ultimately relies on the availability of protons (from the solvent). Substitution of the solvent (H_2O) with D_2O may – in theory – slow the formation of Compound I and either (i) increase the rate of a Compound 0-driven process (inverse KSIE), or (ii) decrease the rate of a Compound I-driven process (normal KSIE).

While curious as to whether such type of analysis would support our ^{18}O labeling results (e.g. the dominant but not exclulsive role of P450 Compound 0), we also recognize that the use of D_2O in enzyme incubations is controversial due to the accompanying (and unknown) structural changes arising from the exchange of labile backbone protons in enzymes,[33] in that a protein prepared in D_2O may be chemically, structurally, and functionally distinct

from the native enzyme (in H_2O). Accordingly, results obtained utilizing each solvent system may not be directly comparable, as is done in the KSIE experiment.

For the sake of comparison to existing studies addressing the P450 Compound 0 vs. Compound I question, we conducted the experiment with human P450 51A1 (and 14α-CDO dihydrolanosterol). Our results indicated a decrease in enzymatic activity from 72 ± 7 min^{-1} (in 100% H_2O, v/v) to 42 ± 6 min^{-1} (in 80% D_2O, v/v) corresponding to a (normal) KSIE (k_H/k_D) of 1.7 (Figure S7). A normal KSIE has been historically interpreted as a dominant contribution of P450 Compound I, although our result is not a significant KSIE (< 2) and we are inherently skeptical of the use of KSIE data to assess protein function due to the unknown enzyme structural (and potentially functional) changes that occur with deuterium exchange, as noted earlier by others.[33]

A P450 51A1 Baeyer-Villiger Intermediate.

Baeyer-Villiger rearrangements are well-known in chemistry, and a number of flavoproteins use 4a-hydroperoxide intermediates to catalyze such reactions,[9, 34] including mammalian flavin-containing monooxygenase (FMO) enzymes.[35] There has been limited evidence for these reactions in P450 catalysis, likely due in part to the preponderance of Compound I (FeO^{3+}) mechanisms.[9, 24a] Mak and Swinney[22a, 36] proposed Baeyer-Villiger chemistry in the oxidation of progesterone to 17-*O*-acetyltestosterone by P450 17A1 in hog testicular microsomes, but we were unable to detect this compound in our own work with recombinant human P450 17A1.[15d]

In plants, Arabidopsis P450 85A2 and 85A3 oxidize a 6-keto steroid to brassinolide, a secosteroid with a 7-membered B-ring lactone, suggestive of Baeyer-Villiger chemistry.[37] However, even this transformation can be rationalized in the context of a FeO^{3+} reaction (Compound I) in the absence of ^{18}O labeling studies.[9] Ortiz de Montellano and associates have identified several oxidations of cholesterol by *Mycobacterium tuberculosis* P450 125A1, including five deformylation products derived from the C26-aldehyde.[38] Several of these can be rationalized by Compound 0 (FeO_2^-) chemistry, and one (M2) was proposed to be generated by 1-electron oxidation of the C25 radical generated by homolytic decomposition of a peroxyhemiacetal intermediate, followed by trapping of a C25 carbocation by the released formic acid.[38] However, the authors indicate that a Baeyer-Villiger rearrangement could also explain their results. Another possibility of a P450 Baeyer-Villiger reaction is that of *Sordaria araneosa* SdnB.[39]

The best case for Baeyer-Villiger P450 chemistry may be the study of Fischer et al.[12c] on lanosterol 14α-demethylation in rat liver microsomes. While it is generally accepted that the P450 51 reaction culminates in cleavage of the C14-C32 bond of 14α-CHO lanosterol, their group identified a competing reaction facilitated by Baeyer-Villiger type chemistry (Scheme 3C) that yielded a third oxidative intermediate in the reaction series.[12c] The intermediate (a 14α-formyl derivative of lanosterol) was initially identified by radiochromatography as an NADPH-dependent product of lanosterol metabolism in rat liver microsomes, based on chromatography as an early shoulder on the substrate peak when separated using reversed-phase (C_{18}) HPLC. The group found that the chromatographic separation (of substrate and intermediate) was substantially improved when performed with a silica column (normal

phase HPLC), and after isolation and some spectroscopic analyses assigned the molecule as 14α-formyloxy-lanost-8-en-3β-ol. To our knowledge, this remains the only report of an additional intermediate of lanosterol metabolism, in that further kinetic establishment of the intermediacy of the Baeyer-Villiger compound and catalytic competence has not been described. Our recent analysis of the kinetics of dihydrolanosterol 14α-demethylation by human P450 51A1[27] did not include this as an intermediate. The compound is reportedly formed in lower abundance relative to the final product[12c] and may have been below the limit of detection in our previous analyses. Additionally, it might not be expected to survive the acidic conditions used in quenching with HCl in single-turnover analysis.[27]

As our ^{18}O labelling data clearly indicated the dominance of P450 Compound 0 (Figure 4), we were interested as to whether the proposed 14α-formyl lanosterol derivative (Baeyer-Villiger intermediate) was also a product under our experimental conditions, namely, utilizing a purified recombinant enzyme system (devoid of esterases in microsomes, which could degrade such an ester intermediate) and on-line LC-MS (which was not available to Fischer et al.[12c]). We performed a 14α-CDO dihydrolanosterol deformylation assay under steady-state conditions[27] and subjected the products of the reaction to reversed-phase (C_{18}) HPLC coupled to electrospray ionization (ESI) MS analysis. Our analysis revealed an NADPH-dependent chromatographic peak (t_R 3.05 min) eluting as an early shoulder on the substrate peak (t_R 3.51 min), corresponding to the chromatographic migration of the putative Baeyer-Villiger intermediate in the earlier report (Figures 6, S8).[12c] HRMS analysis of this product was also consistent with that of a Baeyer-Villiger intermediate, which was characterized by the fragment ions of *m/z* 413.3783 due to the in-source loss of DCO_2 from the 14α-formyl group and a hydrogen atom from C15, and *m/z* 395.3672 due to the additional loss of H_2O from the 3β-hydroxyl in the ESI source. All detected *m/z* values were within 5 ppm of theoretical values, giving high confidence in the assignment.

We then applied the sample to a normal phase HPLC system coupled to atmospheric pressure chemical ionization (APCI) MS, adapting the conditions of Fischer et. al.[12c] to determine if the chromatographic retention of our suspected intermediate–like that described in their report–would subsequently elute later than the substrate and product. We observed a chromatographic peak (Figure S9; t_R 5.38 min) with ions of *m/z* 395.3672 and 413.3783 now eluting significantly after both the final product (dihydro FF-MAS, t_R 1.41 min) and substrate (14α-CDO dihydrolanosterol, t_R 2.22 min). The formation of this peak was NADPH-dependent, reproducible, and appeared to increase with increasing reaction time.

We believe that this intermediate compound is the same one observed by Fischer et al.,[12c] who reported IR and ^1H NMR spectra consistent with the proposed structure (i.e., IR 1717 cm^{-1}, NMR δ 7.86) but were also unable to obtain mass spectra of the intact molecule. Despite numerous attempts utilizing both normal and reversed phase chromatography systems coupled to either APCI or ESI HRMS, under various ionization conditions, we were also unable to obtain the parent ion. Fischer et al.[12c] noted that this product (which was acid-labile) decomposed when isolated and added back to rat liver microsomes, but the roles of microsomal esterases[40] cannot be dismissed. Based on the chromatographic behavior on both normal and reversed phase columns (in comparison to the original report) and HRMS

analysis of the molecule, we assign the product as the 14α-formyloxy-lanost-8-en-3β-ol intermediate proposed by Fischer et al.[12c]

Conclusion

Our $^{18}O_2$ analyses indicate that all of the P450 51 reactions examined operate with a mixed reaction mechanism with participation of both Compound 0 (FeO_2^{-}) and Compound I (FeO^{3+}) mechanisms, the former accounting for ~85% of the reaction with all enzymes tested (Figure 4), with the exception of the *T. cruzi* and *T. brucei* enzymes. The ~85% values (Figure 4) are based on internal standard P450 17A1 progesterone 17α-hydroxylation, and the lack of complete incorporation is not due to leaks in the glassware or other artifacts. The contribution (14%) of the FeO^{3+} pathway is supported by our results with reactions done in $H_2{}^{18}O$, labeling the carbonyl oxygen atom (Figure 5, Table 1). Although we provide evidence for the involvement of a Baeyer-Villiger rearrangement in the Compound 0 pathway (Figure 6), we cannot conclude what fraction of the reaction proceeds through this pathway as opposed to a direct elimination (Scheme 3B).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

[1]. Rendic S, Guengerich FP, Chem. Res. Toxicol 2015, 28, 38–42. [PubMed: 25485457]

[2]. Ortiz de Montellano PR, Cytochrome P450: Structure, Mechanism, and Biochemistry, 4th ed., Springer, New York, 2015.

[3]. Auchus RJ, Miller WL, in Cytochrome P450: Structure, Mechanism, and Biochemistry, Vol. 2, 4th ed. (Ed.: Ortiz de Montellano PR), Springer, New York, 2015, pp. 851–879.

[4]. McLean KJ, Leys D, Munro AW, in Cytochrome P450:Structure, Mechanism, and Biochemistry, 4th ed. (Ed.: Ortiz de Montellano PR), Springer, New York, 2015, pp. 261–407.

[5]. Hargrove TY, Wawrzak Z, Lamb DC, Guengerich FP, Lepesheva GI, J. Biol. Chem 2015, 290, 23916–23934. [PubMed: 26269599]

[6]. Ullrich V, Angew. Chem., Int. Ed 1972, 11, 701–712.

[7]. Groves JT, McClusky GA, White RE, Coon MJ, Biochem. Biophys. Res. Commun 1978, 81, 154–160. [PubMed: 656092]

[8]. Rittle J, Green MT, Science 2010, 330, 933–937. [PubMed: 21071661]

[9]. Guengerich FP, Yoshimoto FK, Chem. Rev 2018, 118, 6573–6655. [PubMed: 29932643]

[10]. a)Newcomb M, Le Tadic-Biadatti MH, Chestney DL, Roberts ES, Hollenberg PF, J. Am. Chem. Soc 1995, 117, 12085–12091;b)Newcomb M, Shen R, Choi S, Toy PH, Hollenberg PF, Vaz

ADN, Coon MJ, J. Am. Chem. Soc 2000, 122, 2677–2686;c)Jin S, Makris TM, Bryson TA, Sligar SG, Dawson JH, J. Am. Chem. Soc 2003, 125, 3406–3407. [PubMed: 12643683]

[11]. a)Auclair K, Hu Z, Little DM, Ortiz de Montellano PR, Groves JT, J. Am. Chem. Soc 2002, 124, 6020–6027; [PubMed: 12022835] b)Krest CM, Onderko EL, Yosca TH, Calixto JC, Karp RF, Livada J, Rittle J, Green MT, J. Biol. Chem 2013, 288, 17074–17081. [PubMed: 23632017]

[12]. a)Vaz ADN, Roberts ES, Coon MJ, J. Am. Chem. Soc 1991, 113, 5886–5887;b)Roberts ES, Vaz ADN, Coon MJ, Proc. Natl. Acad. Sci. USA 1991, 88, 8963–8966; [PubMed: 1924356] c)Fischer RT, Trzaskos JM, Magolda RL, Ko SS, Brosz CS, Larsen B, J. Biol. Chem 1991, 266, 6124–6132. [PubMed: 2007571]

[13]. Guengerich FP, Tateishi Y, McCarty KD, Med. Chem. Res 2023, 32, 1263–1677.

[14]. Davydov R, Strushkevich N, Smil D, Yantsevich A, Gilep A, Usanov S, Hoffman BM, Biochemistry 2015, 54, 7089–7097. [PubMed: 26603348]

[15]. a)Miller SL, Wright JN, Corina DL, Akhtar M, J. Chem. Soc., Chem. Commun 1991, 157–159;b)Mak PJ, Gregory MC, Denisov IG, Sligar SG, Kincaid JR, Proc. Natl. Acad. Sci. USA 2015, 112, 15856–15861; [PubMed: 26668369] c)Gregory M, Mak PJ, Sligar SG, Kincaid JR, Angew. Chem., Int. Ed 2013, 52, 5342–5345;d)Yoshimoto FK, Gonzalez E, Auchus RJ, Guengerich FP, J. Biol. Chem 2016, 291, 17143–17164. [PubMed: 27339894]

[16]. Yoshimoto FK, Guengerich FP, J. Am. Chem. Soc 2014, 136, 15016–15025. [PubMed: 25252141]

[17]. a)Cole PA, Robinson CH, J. Am. Chem. Soc 1988, 110, 1284–1285;b)Watanabe Y, Ishimura Y, J. Am. Chem. Soc 1989, 111, 8047–8049.

[18]. a)Shaik S, Cohen S, Wang Y, Chen H, Kumar D, Thiel W, Chem. Rev 2010, 110, 949–1017; [PubMed: 19813749] b)Kalita S, Shaik S, Dubey KD, ACS Catal. 2022, 12, 5673–5683.

[19]. a)Lichtenberger F, Nastainczyk W, Ullrich V, Biochem. Biophys. Res. Commun 1976, 70, 939–946; [PubMed: 7255] b)White RE, Coon MJ, Annu. Rev. Biochem 1980, 49, 315–356; [PubMed: 6996566] c)Guengerich FP, Vaz AD, Raner GN, Pernecky SJ, Coon MJ, Mol. Pharmacol 1997, 51, 147–151. [PubMed: 9016357]

[20]. a)Mak PJ, Duggal R, Denisov IG, Gregory MC, Sligar SG, Kincaid JR, J. Am. Chem. Soc 2018, 140, 7324–7331; [PubMed: 29758981] b)Hargrove TY, Wawrzak Z, Guengerich FP, Lepesheva GI, J. Biol. Chem 2020, 295, 9998–10007. [PubMed: 32493730]

[21]. a)Cryle MJ, De Voss JJ, Angew. Chem., Int. Ed 2006, 45, 8221–8223;b)Podgorski MN, Coleman T, Churchman LR, Bruning JB, De Voss JJ, Bell SG, Chem. Eur. J 2022, 28, e202202428. [PubMed: 36169207]

[22]. a)Swinney DC, Mak AY, Biochem. 1994, 33, 2185–2190; [PubMed: 8117675] b)Gregory MC, Denisov IG, Grinkova YV, Khatri Y, Sligar SG, J. Am. Chem. Soc 2013, 135, 16245–16247. [PubMed: 24160919]

[23]. a)Akhtar M, Corina D, Pratt J, Smith T, J. Chem. Soc., Chem. Commun 1976, 854–856;b)Akhtar M, Calder MR, Corina DL, Wright JN, Biochem. J 1982, 201, 569–580. [PubMed: 7092812]

[24]. a)Ortiz de Montellano PR, in Cytochrome P450: Structure, Mechanism, and Biochemistry, 4th ed. (Ed.: Ortiz de Montellano PR), Springer, New York, 2015, pp. 111–176;b)Guengerich FP, ACS Catal. 2018, 8, 10964–10976. [PubMed: 31105987]

[25]. a)Shyadehi AZ, Lamb DC, Kelly SL, Kelly DE, Schunck WH, Wright JN, Corina D, Akhtar M, J. Biol. Chem 1996, 271, 12445–12450; [PubMed: 8647850] b)Sen K, Hackett JC, J. Am. Chem. Soc 2010, 132, 10293–10305. [PubMed: 20662512]

[26]. a)Morisaki M, Igata T, Yamamoto S, Chem. Pharm. Bull 2000, 48, 1474–1479;b)Araki S, Eguchi S, Morisaki M, Chem. Pharm. Bull 1990, 38, 1796–1797;c)Takano Y, Morisaki M, Chem. Pharm. Bull 1991, 39, 1647–1648.

[27]. McCarty KD, Sullivan ME, Tateishi Y, Hargrove TY, Lepesheva GI, Guengerich FP, J. Biol. Chem 2023, 299, 104841. [PubMed: 37209823]

[28]. Dess DB, Martin JC, J. Org. Chem 1983, 48, 4155–4156.

[29]. a)Knapp DR, Handbook of Analytical Derivatization Reactions, Wiley New York, 1979;b)Schlenk H, Gellerman JL, Anal. Chem1960, 32, 1412–1414;c)Vorbeck ML, Mattick LR, Lee FA, Pederson CS, Anal. Chem 1961, 33, 1512–1514.

[30]. Gotoh O, J. Biol. Chem 1992, 267, 83–90. [PubMed: 1730627]

[31]. Hargrove TY, Lamb DC, Smith JA, Wawrzak Z, Kelly SL, Lepesheva GI, Sci. Rep 2022, 12, 16232. [PubMed: 36171457]

[32]. Khatri Y, Luthra A, Duggal R, Sligar SG, FEBS Lett. 2014, 588, 3117–3122. [PubMed: 24997347]

[33]. a)Jencks WP, Catalysis in Chemistry and Enzymology, McGraw-Hill, New York, 1969, p. 276;b)Kresge AJ, J. Am. Chem. Soc 1973, 95, 3065–3067;c)Walsh C, Enzymatic Reaction Mechanisms, W. H. Freeman, San Francisco, 1979, pp. 121–122;d)Abeles RH, Frey PA, Jencks WP, Biochemistry, Jones and Bartlett, New York, 1992, pp. 113–115;e)Fersht A, Structure and Mechanism in Protein Science, Freeman, New York, 1999, p. 99;f)Hermans J Jr., Scheraga HA, Biochim. Biophys. Acta 1959, 36, 534–535. [PubMed: 14401269]

[34]. Walsh CT, Chen YCJ, Angew. Chem., Int. Ed. Engl 1988, 27, 333–343.

[35]. Fiorentini F, Geier M, Binda C, Winkler M, Faber K, Hall M, Mattevi A, ACS Chem. Biol 2016, 11, 1039–1048. [PubMed: 26771671]

[36]. Mak AY, Swinney DC, J. Am. Chem. Soc 1992, 114, 8309–8310.

[37]. a)Nomura T, Kushiro T, Yokota T, Kamiya Y, Bishop GJ, Yamaguchi S, J. Biol. Chem 2005, 280, 17873–17879; [PubMed: 15710611] b)Kim TW, Hwang JY, Kim YS, Joo SH, Chang SC, Lee JS, Takatsuto S, Kim SK, Plant Cell 2005, 17, 2397–2412; [PubMed: 16024588] c)Mizutani M, Sato F, Arch. Biochem. Biophys 2011, 507, 194–203. [PubMed: 20920462]

[38]. Sivaramakrishnan S, Ouellet H, Matsumura H, Guan S, Moenne-Loccoz P, Burlingame AL, Ortiz de Montellano PR, J. Am. Chem. Soc 2012, 134, 6673–6684. [PubMed: 22444582]

[39]. Chen QB, Yuan GY, Yuan T, Zeng HT, Zou ZR, Tu ZC, Gao J, Zou Y, J. Am. Chem. Soc 2022, 144, 3580–3589. [PubMed: 35170947]

[40]. a)Hosokawa M, Maki T, Satoh T, Mol. Pharmacol 1987, 31, 579–584; [PubMed: 3600603] b)Satoh T, Hosokawa M, Annu. Rev. Pharmacol 1998, 38, 257–288.

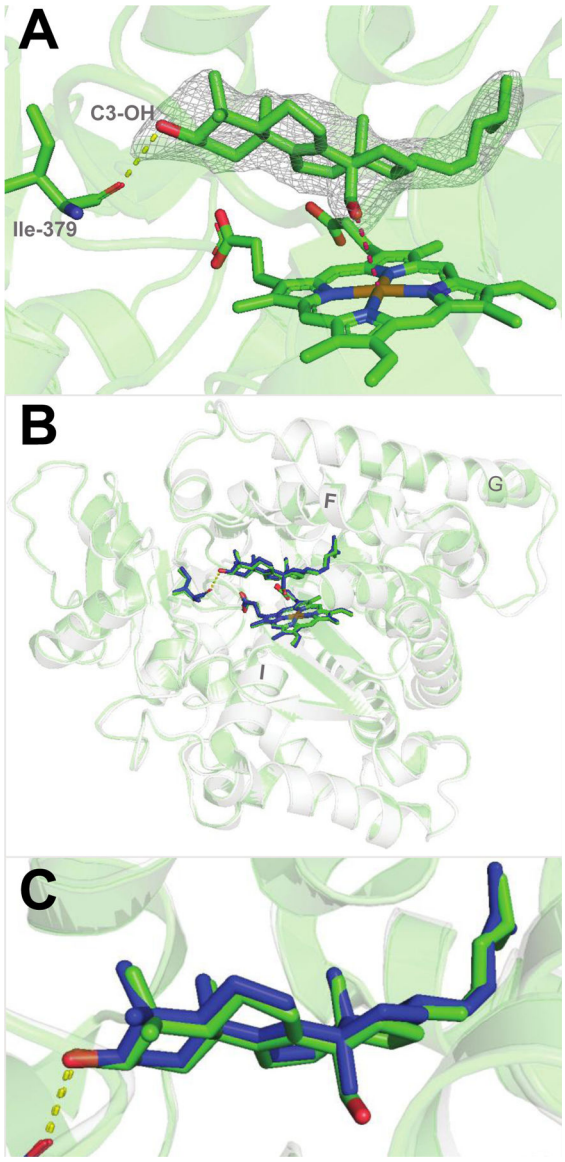


Figure 1.

Substrate-bound human P450 51A1. (A) Binding mode of the 14α-aldehyde reaction intermediate inside the enzyme active site (PDB 8SS0, 2.25 Å.). The 2Fo-Fc electron density map within 1.6 Å of the sterol atoms is shown as gray mesh and contoured at 1.5σ. The H-bond between the C3-OH of the sterol molecule and the main chain oxygen of Ile-379 is depicted as yellow dashes. The distance between the aldehyde oxygen and the heme iron is 3.5Å (pink dashes). (B) Superimposition with the lanosterol-bound structure (PDB 6UEZ): the carbon atoms of lanosterol are colored in blue, the ribbon is transparent light-gray, and the rmsd of all Cα-atoms is 0.47 Å. (C) Enlarged view of the superimposed molecules of lanosterol and the 14α-aldehyde intermediate of 24,25-dihydrolanosterol.

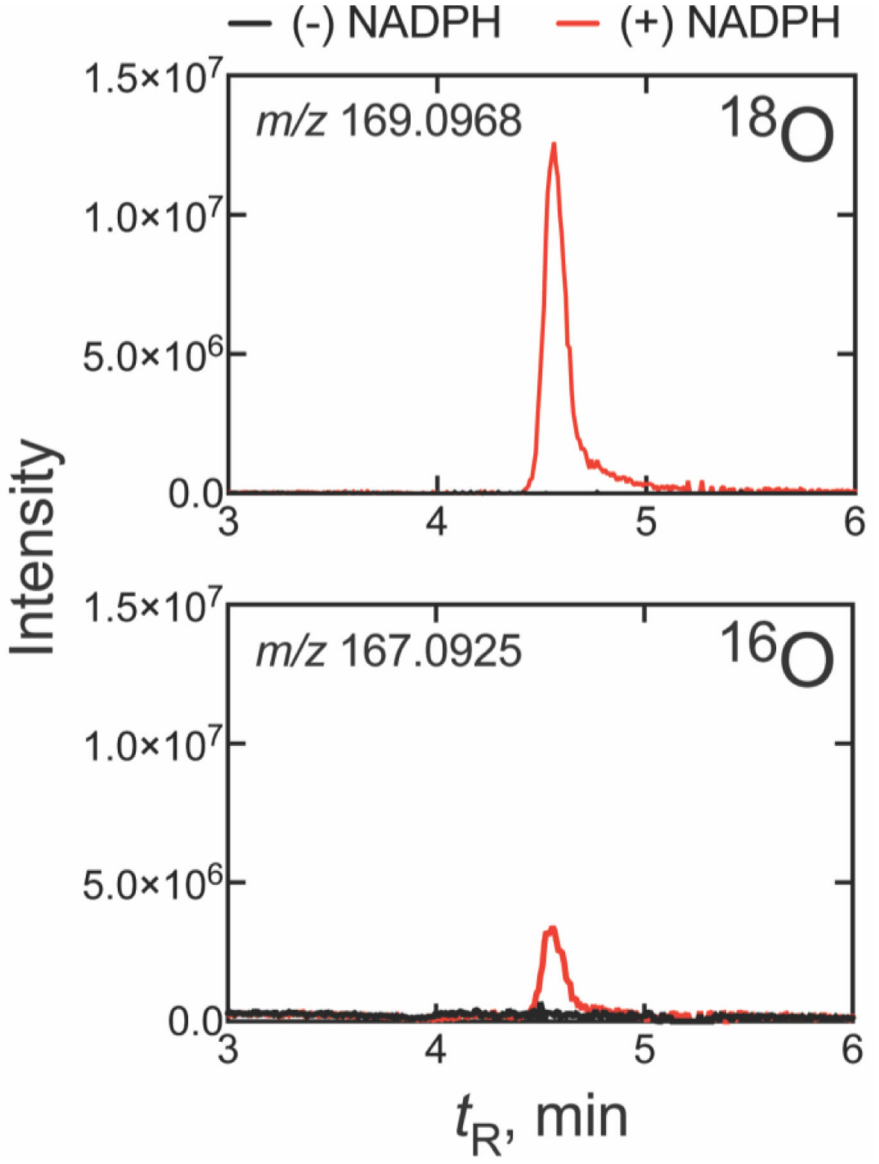


Figure 2.

UPLC-HRMS analysis of formic acid formed from 14α-CDO dihydrolanosterol by human P450 51A1 in an atmosphere of $^{18}O_2$. The two frames show the ^{18}O (*m/z* 169.0968) and ^{16}O (*m/z* 167.0925) channels. All *m/z* values are calculated for the derivatized pyridyl formate esters. (The corresponding raw chromatographic traces are presented in Figure S3).

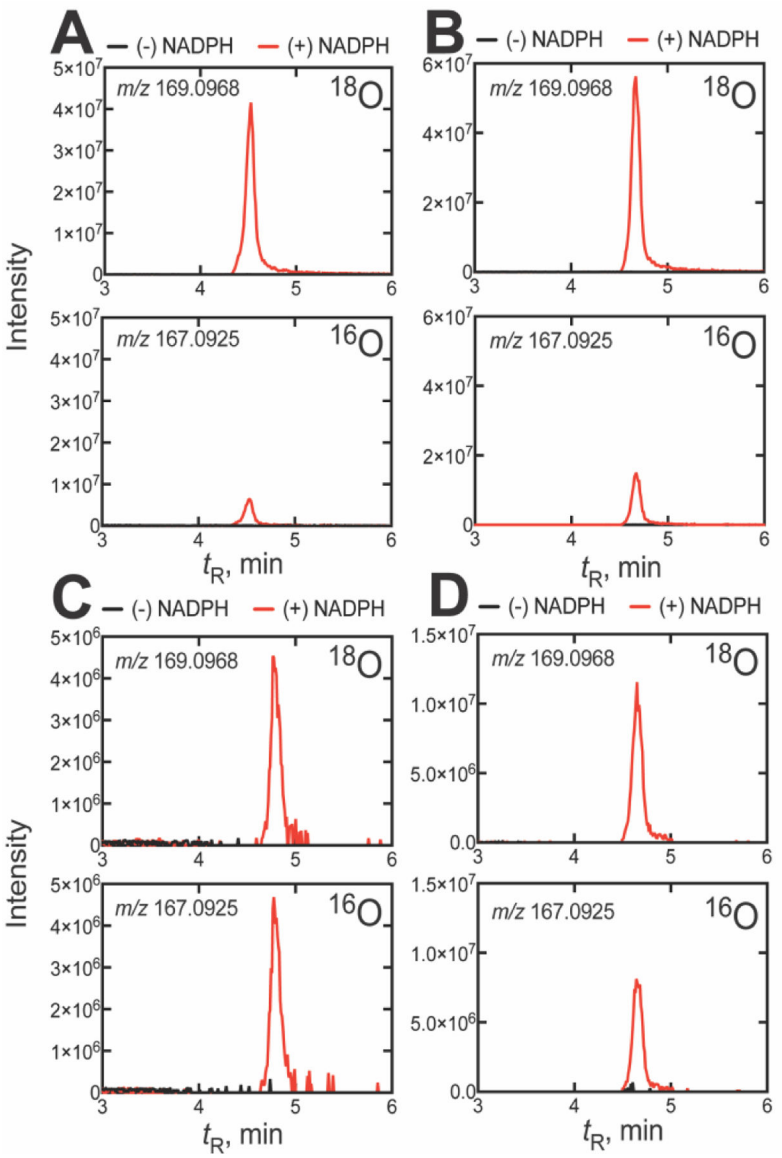


Figure 3.

UPLC-HRMS analysis of formic acid formed from 14α-CDO dihydrolanosterol by other P450 51A enzymes in an atmosphere of $^{18}O_2$. The two frames show the ^{18}O (*m/z* 169.0968) and ^{16}O (*m/z* 167.0925) channels for the formic acid derivative formed from the P450 51A enzymes of (A) *N. fowleri*, (B) *C. albicans*, (C) *T. brucei*, and (D) *T. cruzi*. All *m/z* values are calculated for the derivatized pyridyl formate esters. (The corresponding raw chromatographic traces for panel C are presented in Figure S4).

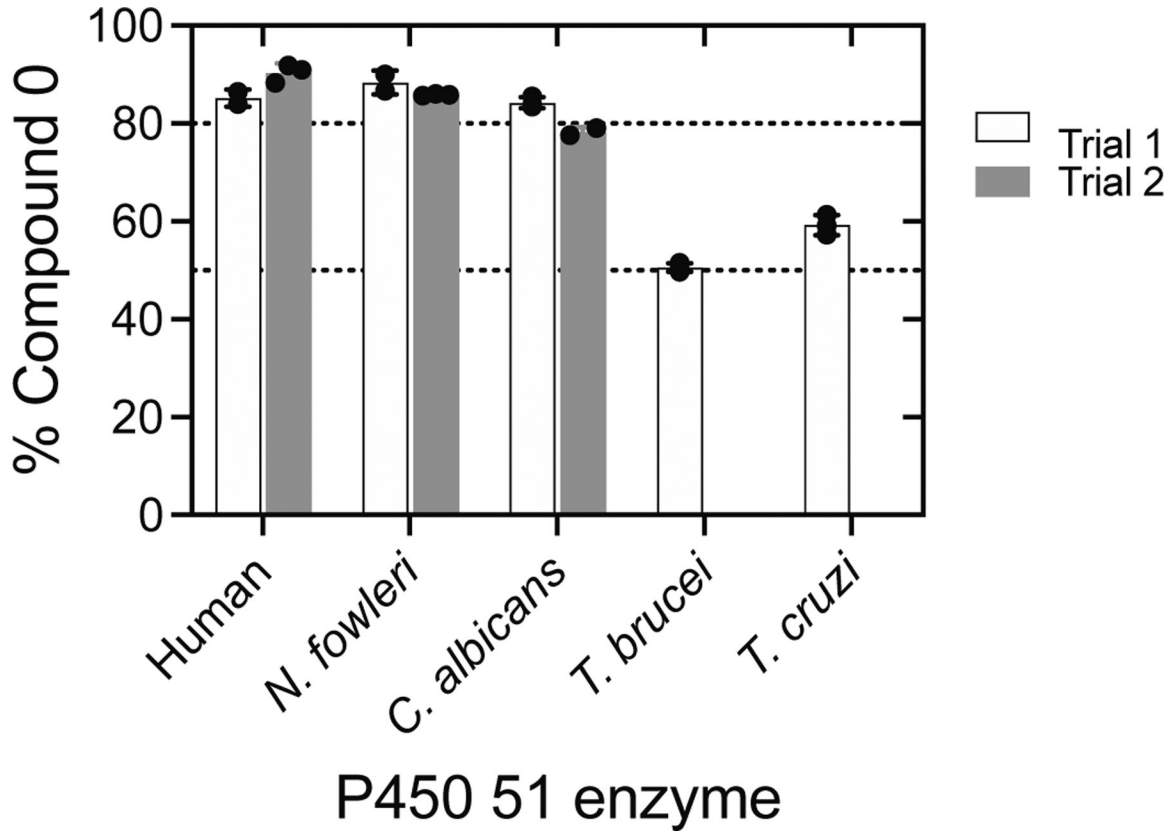


Figure 4.

Summary of ^{18}O incorporation from $^{18}O_2$ into formic acid with five P450 51A enzymes. Results from Figures 2 and 3. All values were normalized for the content of ^{18}O (from $^{18}O_2$) incorporated into 17α-OH progesterone by P450 17A1. The individual points indicate the results of individual incubations (3), with the means ± SD. Separate trials were run on different days, each with a set of three more enzyme reactions. The stippled lines are set at 50 and 80% for reference.

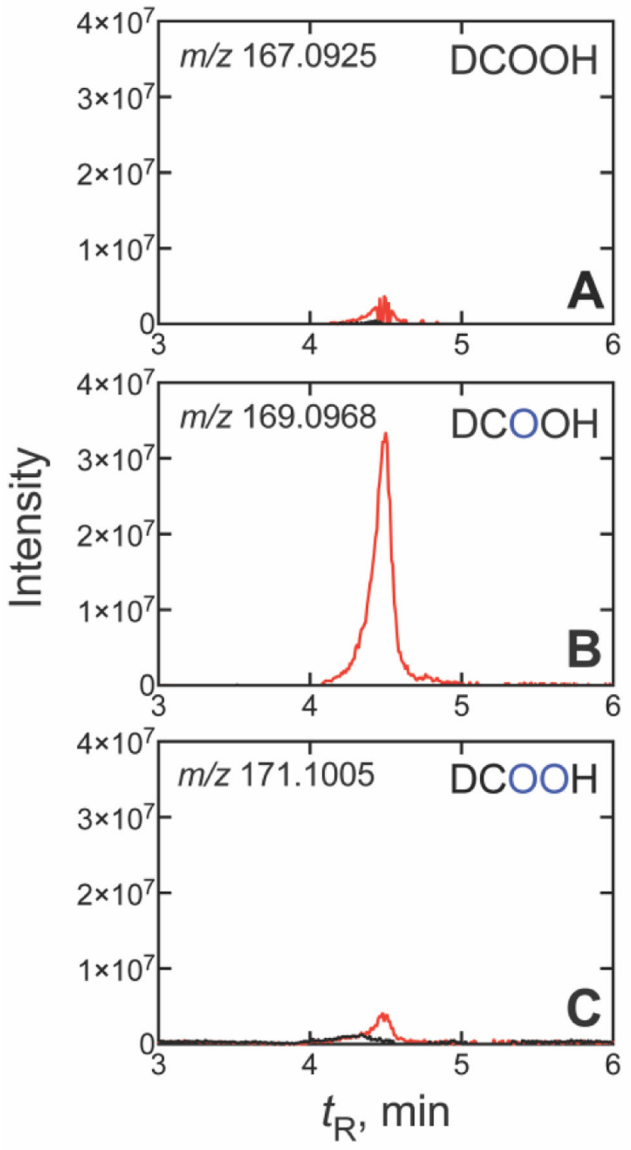


Figure 5.

UPLC-HRMS analysis of formic acid formed from 14α-CD^{18}O dihydrolanosterol by human P450 51A1 in $H_2{}^{18}O$. Traces are shown for (A) no ^{18}O (only ^{16}O, *m/z* 167.0925), (B) one ^{18}O atom (*m/z* 169.0968), and (C) two ^{18}O atoms (*m/z* 171.1010). Isotopically labelled oxygen atoms (^{18}O) are indicated in blue, and all *m/z* values are calculated for the derivatized pyridyl formate esters. (The corresponding raw chromatographic traces are presented in Figure S6).

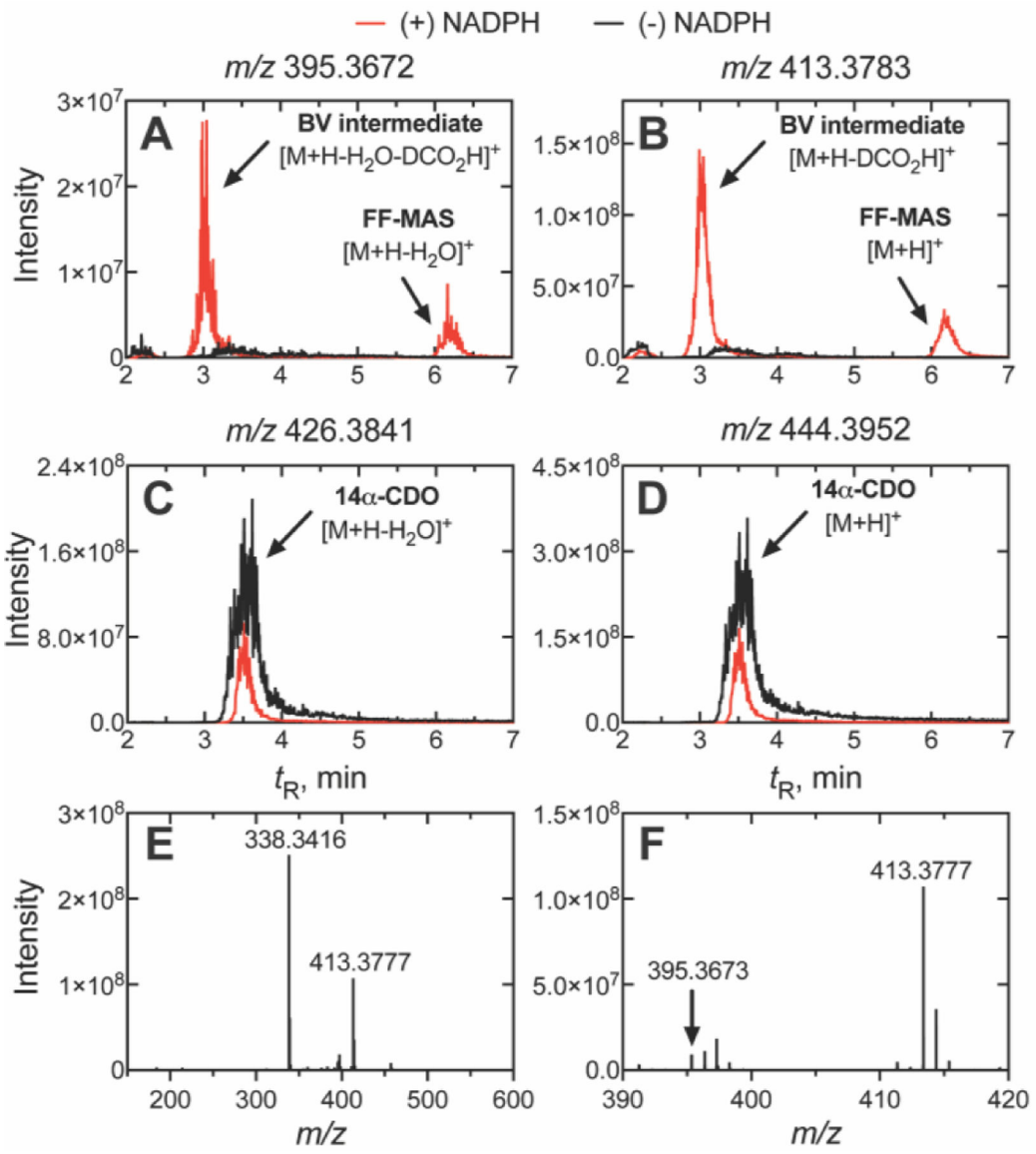
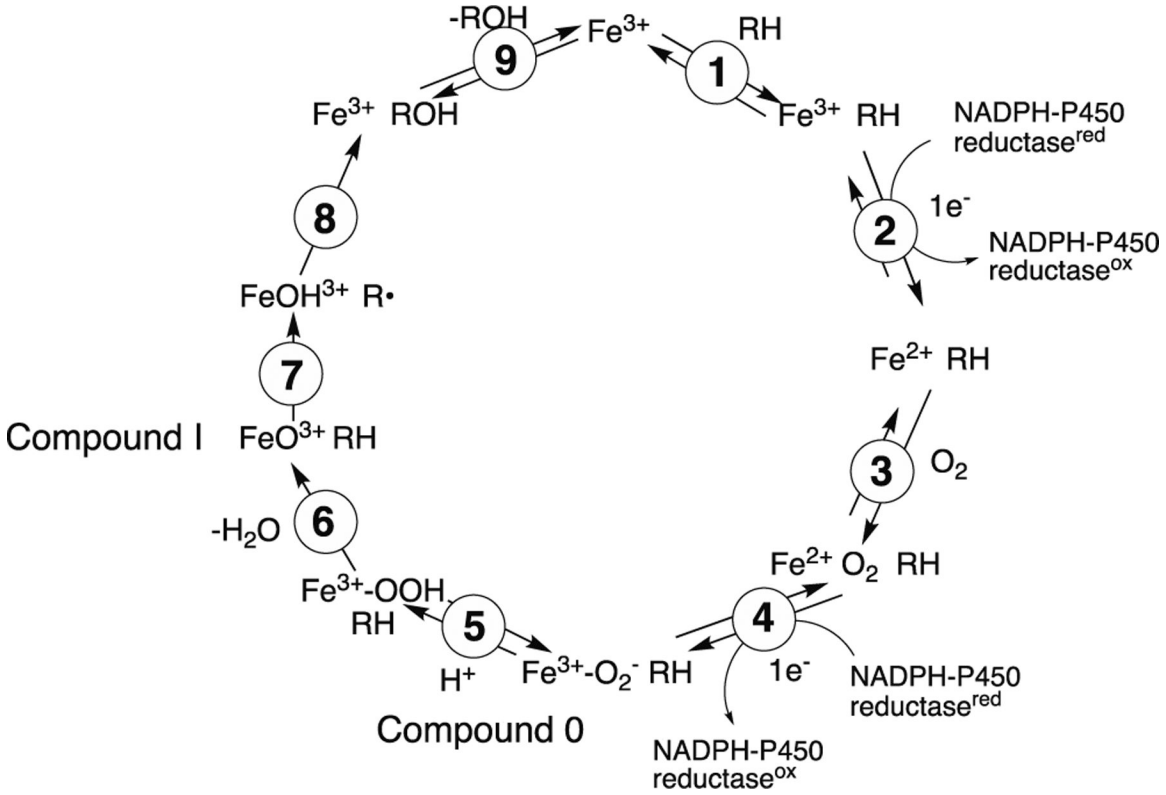


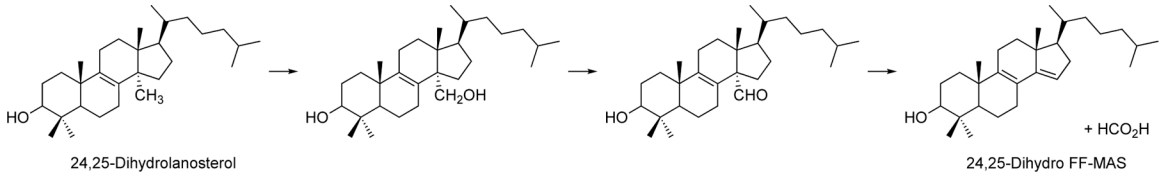
Figure 6. Reversed phase (C_{18}) UPLC-HRMS analysis of Baeyer-Villiger intermediate.

Steady-state incubations of P450 51A1 and 14α-CDO dihydrolanosterol were run for 2.5 min and the products were extracted and analyzed via (reversed phase) LC-ESI-HRMS. The 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 $[M+H-H_2O-DCO_2-H]^+$ and $[M+H-DCO_2-H]^+$ ions, respectively. 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. The full mass spectrum (*m/z* 150–600) of the intermediate (t_R 3.05 min, Panels A and B) showed the major $[M+H-DCO_2-H]^+$ fragment ion (*m/z* 413.3777) of the intermediate (Panel E), while the minor $[M+H-H_2O-DCO_2-H]^+$ fragment ion (*m/z* 395.3673) was observable when the trace was magnified (*m/z* 390–420, Panel F). The Baeyer-Villiger (BV) intermediate (t_R 3.05 min) eluted as an early shoulder of the substrate peak (14α-CDO

dihydrolanosterol, t_R 3.51 min) while FF-MAS eluted later (t_R 6.17 min). similar to the results reported by Fischer et al.[12c] (The corresponding raw chromatographic traces are presented in Figure S8). (The ion *m/z* 338.3416 in Panel E is from a closely eluting peak (Δt_R 0.1 min) and could not be identified; it is not a fragment of *m/z* 413.3777).



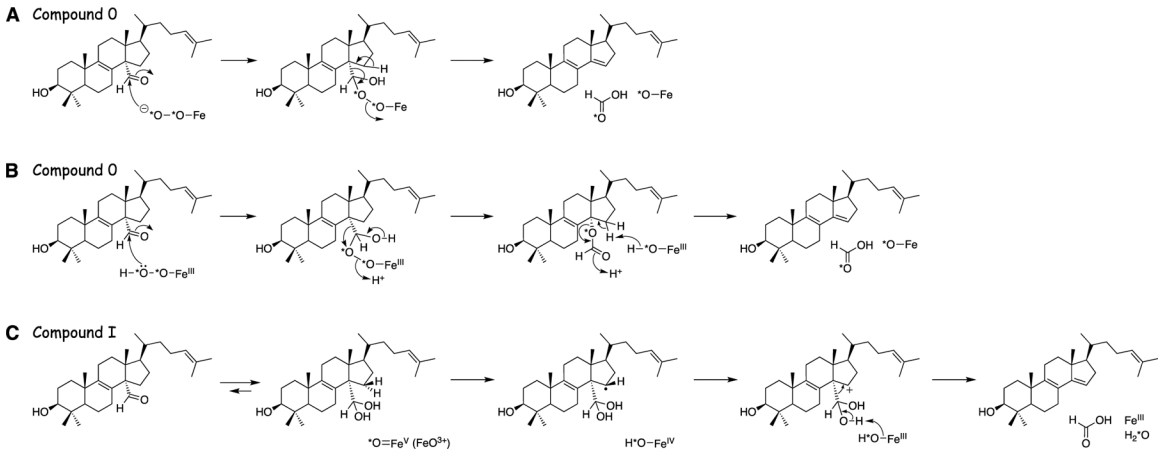
Scheme 1.
P450 catalytic cycle.



24,25-Dihydrolanosterol 24,25-Dihydro FF-MAS

Scheme 2.

Oxidation of 24,25-dihydrolanosterol to 24,25-dihydro-4.4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol (24,25-dihydro FF-MAS) and formic acid.



Scheme 3.

Three proposed mechanisms of oxidation of 14α-CHO dihydrolanosterol to 24,25-dihydro FF-MAS and formic acid.[12c, 18b, 20b, 25] (A) Compound 0. (B) Compound 0 plus Baeyer-Villiger rearrangement. (C) Compound I.

Table 1.

Summary of $^{18}O_2$ incorporation results

P450 51 enzyme	DCO_2H (% ^{18}O)	17-OH progesterone (% ^{18}O)	% Compound 0
Human	82.6 ± 2.0	93.6 ± 1.5	88.3 ± 3.2
N. fowleri	79.4 ± 4.0	91.4 ± 3.0	86.9 ± 1.8
C. albicans	76.8 ± 3.9	93.8 ± 1.2	81.9 ± 3.4
T. brucei	48.0 ± 0.8	94.8 ± 0.2	50.6 ± 0.9
T. cruzi	55.3 ± 1.8	93.2 ± 0.3	59.3 ± 2.0