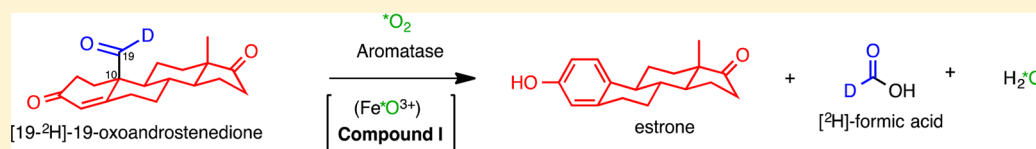# Mechanism of the Third Oxidative Step in the Conversion of Androgens to Estrogens by Cytochrome P450 19A1 Steroid Aromatase

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S *Supporting Information*

ABSTRACT: Aromatase is the cytochrome P450 enzyme that cleaves the C10−C19 carbon−carbon bond of androgens to form estrogens, in a three-step process. Compound I (FeO^{3+}) and ferric peroxide (FeO$_2^−$) have both been proposed in the literature as the active iron species in the third step, yielding an estrogen and formic acid. Incubation of purified aromatase with its 19-deutero-19-oxo androgen substrate was performed in the presence of $^{18}O_2$, and the products were derivatized using a novel diazo reagent. Analysis of the products by high-resolution mass spectrometry showed a lack of ^{18}O incorporation in the product formic acid, supporting only the Compound I pathway. Furthermore, a new androgen 19-carboxylic acid product was identified. The rates of nonenzymatic hydration of the 19-oxo androgen and dehydration of the 19,19-*gem*-diol were shown to be catalytically competent. Thus, the evidence supports Compound I and not ferric peroxide as the active iron species in the third step of the steroid aromatase reaction.

INTRODUCTION

Androgens are converted to estrogens by the steroid aromatase, cytochrome P450 (P450 or CYP) 19A1. This reaction is essential in maintenance of hormone balance.[1,2] P450 19A1 is also an important target for drugs used in treating estrogen-dependent cancers.[3] The conversion of an androgen (androstenedione or testosterone) to an estrogen is a three-step process (Scheme 1, **I** to **IV**). The first two steps are relatively straightforward and can both be rationalized in the context of a perferryl "Compound I" (FeO^{3+}) P450 intermediate (**M7**, Scheme 2).

There has been considerable controversy regarding the mechanism of the third step, however, and at least five proposals have been made, including 1β- and 2β-hydroxylation, 4,5-epoxidation, a concerted Compound I mechanism not involving a stable hydroxyl product, and the use of a preceding ferric peroxide form of the enzyme in the catalytic cycle (**M5**, Scheme 2).[4−15] Computational,[7,8] atom-labeling,[9,12,15] spectroscopic,[6,16] biomimetic model,[17,18] synthesis of proposed intermediates,[10,11] and other approaches[19] have been applied, and the most popular view today is that the FeO$_2^−$ (ferric peroxide) form of the enzyme reacts with the 19-aldehyde (CHO) of the androgen in a nucleophilic attack.[12,20] Crystal structures of human P450 19A1 are now available,[21,22] but these do not resolve the catalytic controversy. The most compelling evidence for the FeO$_2^−$ nucleophilic attack mechanism comes from $^{18}O_2$ labeling studies.[9,12,15,20]

From an incubation of purified P450 19A1 with its third substrate, 19-oxo androgen (Scheme 3, **III-o** or **III-g**), in the presence of ^{18}O-labeled molecular oxygen ($^{18}O_2$), an FeO^{3+} (**M7**, Compound I) mechanism (Scheme 3, step 3b) should not lead to the recovery of an ^{18}O atom in the product formic acid (**Vb**), but an FeO$_2^−$ (**M5**) mechanism (Scheme 3, step 3a) will (**Va**).[12,15] Akhtar et al.[12,15] reported 60% and 90% incorporation of one ^{18}O atom into formic acid, and Caspi et al.[9] reported 70% incorporation, all in studies with human placental microsomes. These results have provided the major evidence for a FeO$_2^−$ mechanism or possibly a mixed mechanism with the FeO$_2^−$ pathway being dominant (Scheme 3, step 3a).

EXPERIMENTAL SECTION

The experimental procedures are provided in detail in the Supporting Information.

RESULTS

Overall Strategy. Several experimental results would clarify the mechanism of the final aromatization step of P450 19A1 (Scheme 3, steps 3a and 3b). The ferric peroxide (Scheme 3, step 3a) mechanism is supported if: (i) one ^{18}O atom is incorporated into the formic acid product (Scheme 3, **Va**) in the incubation of 19-oxo androgen in atmospheric $^{18}O_2$ with P450 19A1 and (ii) the aldehyde (Scheme 3, **III-o**) is required as the substrate for the carbon−carbon bond cleavage step.

Scheme 1. Three-Step Oxidation of Androgens to Estrogens Catalyzed by P450 19A1 (I to IV)[a]
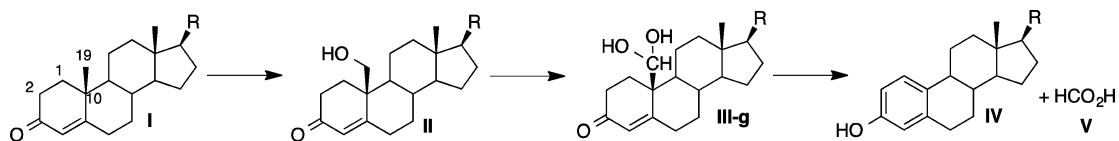
[a]Testosterone → 17β-estradiol, R: −OH; androstenedione → estrone, R: =O. **III-g**: III-*gem*-diol. **III-o**: III-oxo.

Scheme 2. Classic P450 Catalytic Cycle[a]
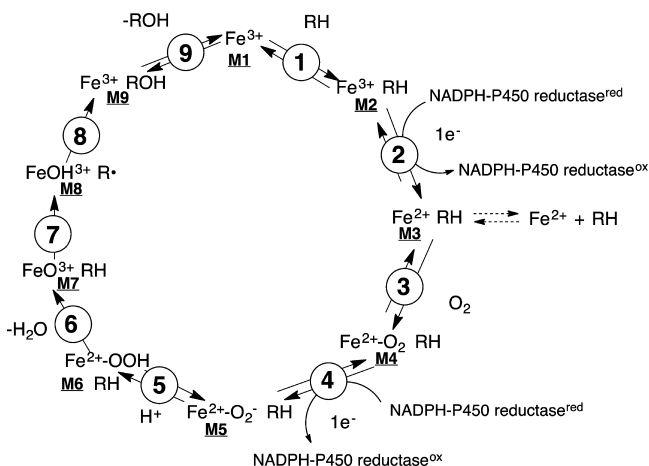
[a]Note the FeO_2^- (**M5**, ferric peroxide) and FeO^{3+} (**M7**, Compound I) forms discussed in the text. Note to reader: in the literature there exists different nomenclature for the same iron intermediates in this P450 catalytic cycle (i.e., **M5**: $Fe^{III}O_2^-$, **M6**: $Fe^{III}O_2H$, **M7**: $(Fe^{IV}O)^{\bullet+}$, **M8**: $Fe^{IV}OH$).[23] For clarity, throughout the text of this manuscript Compound I (**M7**) is referred to interchangeably with FeO^{3+}, and ferric peroxide (**M5**) is referred to interchangeably with FeO_2^-.

In light of the critical nature of the previously reported $^{18}O_2$ incorporation experiments, we re-examined the findings with purified recombinant human P450 19A1 and newer analytical methods. The analysis of trace formic acid is difficult due to the presence of endogenous levels of the compound in laboratory reagents, and (as did Akhtar et al.)[12] we prepared [19-^{2}H]-labeled 19-oxo androstenedione (Figure 2, **4-o**) and testosterone substrates (i.e., 19-CDO androgens) to improve the MS analysis, with a shift of +1 amu (Figure S1, Supporting Information). In addition, a new diazo reagent bearing a pyridine moiety was designed for the mass spectrometric detection of the formic acid enzymatic product using electrospray ionization in the positive mode. However, the analysis is still complicated by the ^{13}C natural abundance contribution (1.109% of ^{12}C) from endogenous formic acid. Accordingly, high-resolution mass spectrometry (HRMS) was used (at a resolution of 100 000) to distinguish $^2HCO_2H$ from the $H^{13}CO_2H$ natural abundance peak present in endogenous formic acid.

Second, the enzymatic and nonenzymatic rates of oxygen exchange between the 19-aldehyde (Scheme 3, **III-o**) and water would indicate whether or not the aldehyde is required as the substrate for the third step. Several case scenarios can arise from the oxygen exchange rate measurements. One is if the nonenzymatic *gem*-diol dehydration rate to the aldehyde (Scheme 3, nonenzymatic $k_{dehydration}$, **III-g** to **III-o**) is faster than the enzymatic rate of 19-hydroxy androgen to estrogen (Scheme 3, **II** to **IV**), then either the aldehyde or the *gem*-diol is a possible substrate for the final step. The method to determine the *gem*-diol dehydration rate ($k_{dehydration}$) requires two experimental measurements: the distribution of the *gem*-diol

Scheme 3. Mechanisms of P450 19A1 Oxidation of Androgens (Rings A and B Shown)[a]
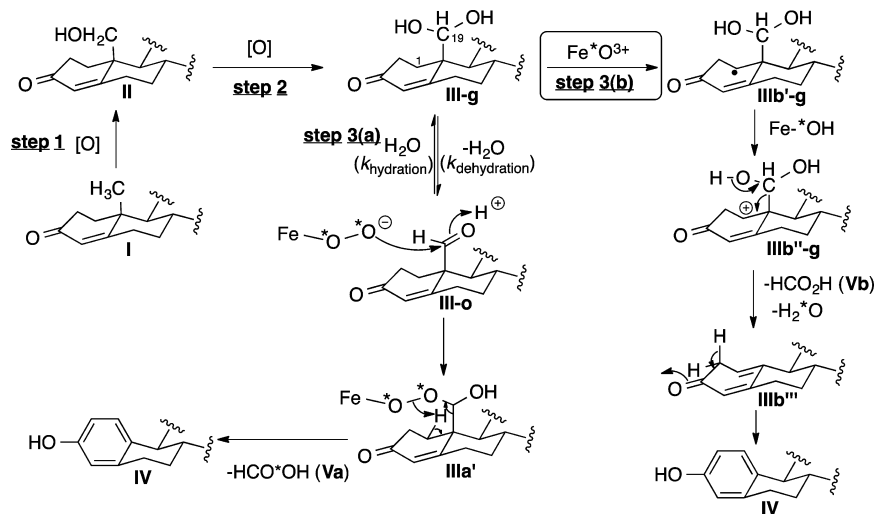
[a]Steps 1 and 2 are generally agreed to involve the P450 FeO^{3+} entity and hydrogen atom abstraction/oxygen rebound.[19] Two possibilities are shown for Step 3 in the presence of $^{18}O_2$. In Step 3a, the FeO_2^- entity participates in a nucleophilic attack on the 19-aldehyde **III-o** (**III-o**: III-oxo). In Step 3b, the FeO^{3+} form of the P450 19A1 abstracts the 1β-hydrogen atom of *gem*-diol **III-g** (**III-g**: III-*gem*-diol). Electron transfer yields the carbocation **IIIb″-g**, which collapses to yield the estrogen product **IV**. In Step 3a, the formic acid must contain label (^{18}O) but not in Step 3b. "*O" = "^{18}O". The step 3(b) pathway is supported by the current study.

Scheme 4. Hydration of the Radical Aldehyde Intermediate (IIIb′-o to IIIb′-g) in the Compound I Mechanism (Step 3b) Pathway
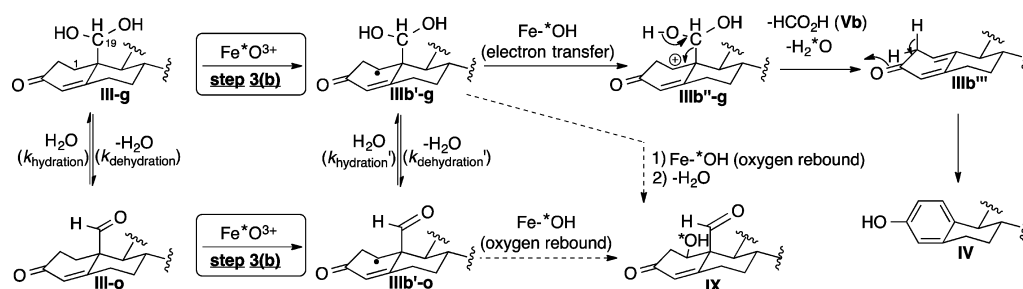
and aldehyde in water through proton NMR (K_{eq}) and the exchange rate of the aldehyde oxygen in water over time (k_{obs}). The latter measurement (k_{obs}) is more challenging in that the synthesis of a 19-[^{18}O]-labeled 19-oxo androgen compound is required. The ^{18}O-labeled 19-oxo androgen was exposed to unlabeled water ($H_2{}^{16}O$) and extracted at time intervals with methyl *tert*-butyl ether (MTBE), followed by subsequent measurement of the ^{18}O-abundance by mass spectrometry. (Our preliminary studies with unlabeled 19-oxoandrostene-dione in ^{18}O-labeled water ($H_2{}^{18}O$) resulted in the incorporation of ^{18}O atoms into the 3- and 17-ketone groups, which complicated the measurements for detecting the aldehyde oxygen exchange.)

If the nonenzymatic *gem*-diol to aldehyde dehydration rate is slower than the enzymatic estrogen formation rate from 19-hydroxy androgen (**II** to **IV**), then the comparison to the enzymatic dehydration rate is necessary to show whether or not the enzyme catalyzes the dehydration of the *gem*-diol to the aldehyde. The ferric peroxide mechanism would be supported in the case where the enzyme catalyzes the dehydration of the *gem*-diol (Scheme 3, enzymatic $k_{dehydration}$: **III-g** to **III-o**), while the Compound I mechanism could potentially use either the aldehyde (**III-o**) or the *gem*-diol (**III-g**) as the substrate (water may hydrate the aldehyde to the *gem*-diol at the radical intermediate stage if Compound I initially abstracts the 1β-hydrogen atom of aldehyde **III-o**, Scheme 4: **IIIb′-o** to **IIIb′-g**). In order to simplify our results, we present the data obtained with the androstenedione series in this report; however, the results from the testosterone series are consistent with the androstendione work and are included in the Supporting Information.

New Diazo Reagent for Formic Acid Detection by MS (ESI-Positive Mode). We designed and synthesized a new diazoalkane reagent (1-diazo-3-(3-pyridinyl)propane, **2**) for formic acid derivatization and analysis, in order to utilize liquid chromatography (LC)−mass spectrometry (MS) for increased sensitivity. The diazo reagent (**2**) was accessed by treating a nitrosourea precursor with base. Freshly prepared diazo reagent (**2**) was added directly to an extract of the incubation that was treated with HCl at 0 °C. Control experiments established that significant oxygen exchange of the formic acid (<6%) did not occur with the medium ($H_2{}^{18}O$) under these conditions (Figure 1).

When [19-^{2}H$_1$]-19-oxo-androstenedione (**4-o**) or -testoster-one was incubated with P450 19A1, no label (≤2%, limit of detection) derived from $^{18}O_2$ was recovered in the formic acid product (detected as formate ester **3b**) in three separate experiments with each substrate (Figures S4 and S5, Supporting Information, one data set shown for each). These results are in contrast with those reported previously by Akhtar et al.[12,15] and

Caspi et al.[9] The isotopic labeling patterns can clearly be seen in the LC−MS traces (**3b** vs **3c**) and in the full spectra generated in the analyses (Figure 2 and Figures S4 and S5, Supporting Information). In control experiments, P450 3A4 routinely incorporated >98% of the label (one atom) from $^{18}O_2$ in the oxidation of testosterone to 6β-hydroxytestosterone, as expected[24] (this control experiment was done along with each set of incubations with P450 19A1) (Figure S3, Supporting Information).

19-Carboxylic Acid Product Supports Compound I Formation. Our previous work on the kinetics and processivity of androstenedione oxidation by purified P450 19A1 had shown slightly less estrone produced than androstenedione oxidized in single turnover assays,[25] suggestive of additional products. Careful analysis of the oxidation of 19-oxo-androstenedione and -testosterone revealed the presence of two new peaks in each case, either using LC−MS (Figure S6, Supporting Information) or ^{14}C-HPLC (Figure S7, Supporting Information). One product is the 19-CO$_2$H (19-oic acid) derivative, and this assignment was confirmed by coincident chromatography with an authentic sample in the case of androstenedione 19-oic acid, as well as by MS analysis of the propylpyridine ester derivatives (**6a** and **6b**, Figure 3 and Figure S6 of Supporting Information).

From the knowledge of this novel 19-oic acid aromatase product, a Compound I mechanism suggests a distribution of products arising from a 1β-hydrogen atom abstraction or a 19-hydrogen atom abstraction of the 19-oxo androgen substrate to yield either the estrogen or carboxylic acid, respectively, in the incubation with P450 19A1. Although the 19-oic acid product is minor based on the [4-^{14}C]-androstenedione substrate incubation with P450 19A1 (~95:5, estrogen:carboxylic acid, Figure S8 of Supporting Information), the derivatization of products with the diazo reagent (**2**) allowed for simultaneous detection of the derivatized carboxylic acid and estrogen products in one LC−MS run in the ESI-positive mode. The diazo reagent (**2**) had reacted with the carboxylic acid group of 19-oic androstenedione (**5b**) to afford the ester (**6b**) and also reacted with the phenol moiety of estrone (**4A**) to furnish a phenolic ether (Figure S11, Supporting Information). The use of a deuterium-labeled substrate such as [19-^{2}H]-19-oxoan-drostenedione (**4-o**) as the substrate suggests a possible metabolic switching[26] to favor the formation of estrone over 19-oic androstenedione when compared to the use of 19-oxoandrostenedione (nondeuterated, **7b−o**) as the substrate. Indeed, the product partition changed to yield an increased formation of estrogen relative to carboxylic acid when [19-^{2}H]-19-oxoandrostenedione (**4-o**) was used in comparison to 19-oxoandrostenedione (**7b−o**) as the substrate (Figure S11, Supporting Information). This observed metabolic switching
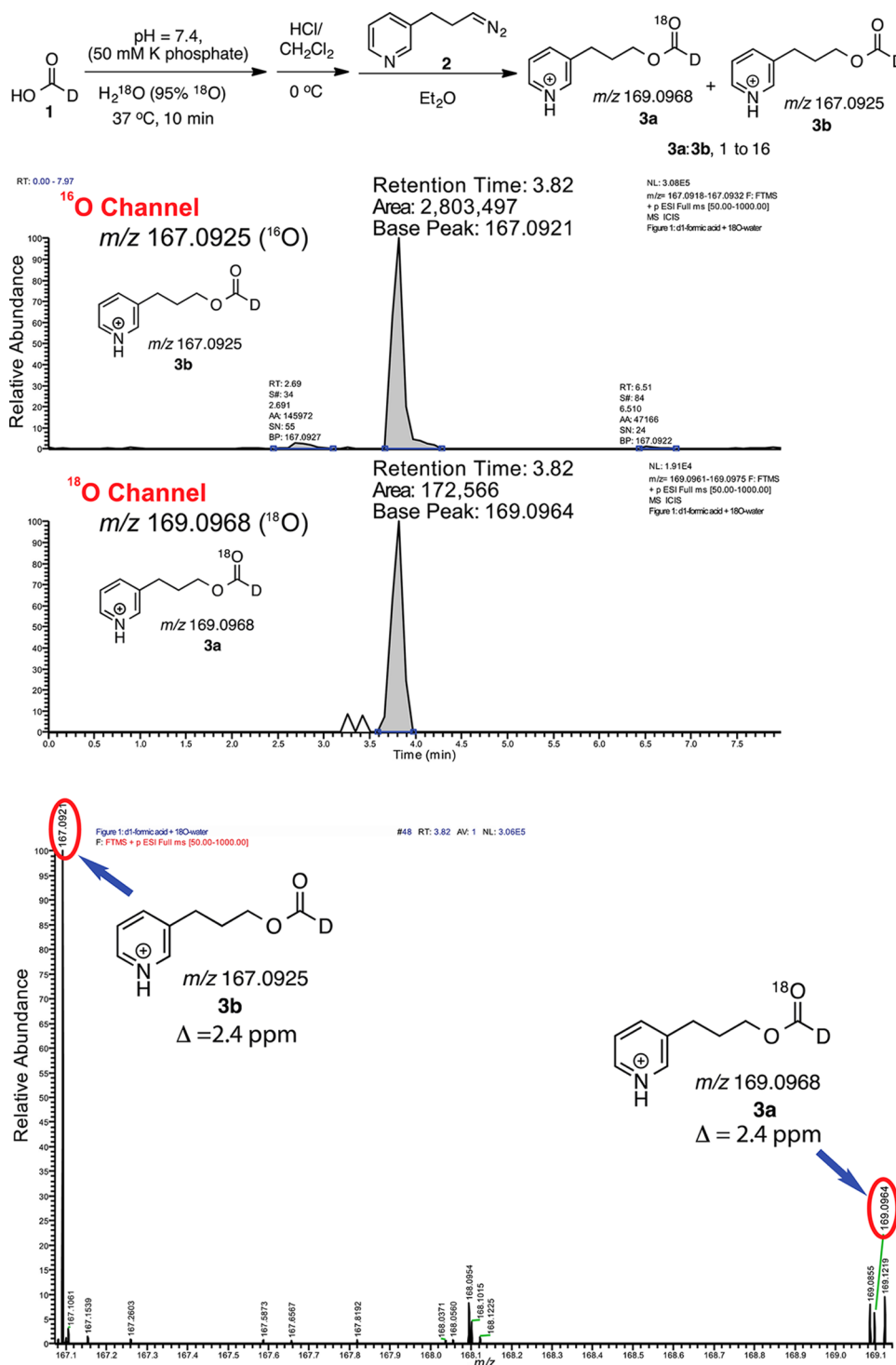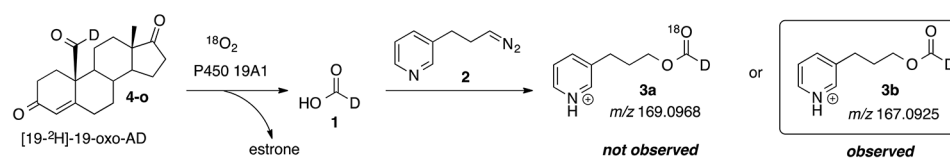
Figure 1. Control experiment confirming minimal exchange between oxygen of [²H]-formic acid (**1**) and medium during the derivatization process (<6% ¹⁸O exchange). (A) Ion chromatogram scanning for exact masses with a 4 ppm mass tolerance. (B) Mass spectrum (*m/z* 167.0721–169.1413).

supports the Compound I pathway as the active iron species in the reaction of P450 19A1 with its 19-oxo androgen substrate.
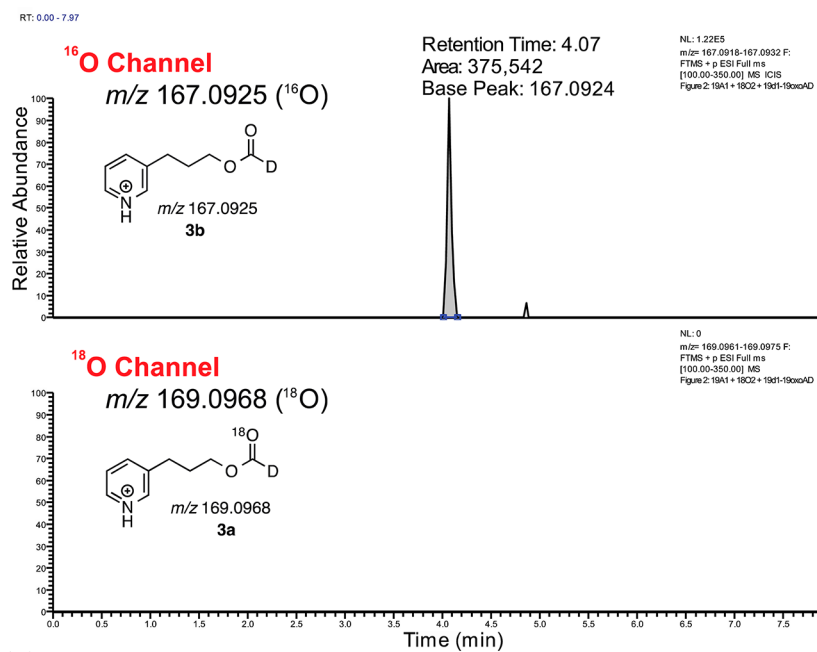
In addition, another product (M + 18) was formed from both 19-oxo androgen substrates (from ¹⁸O₂) (Figures S6, Supporting Information), corresponding to the addition of a single oxygen atom, but the site of oxidation has not been established. The 19-oic acid had been reported to be present in hog follicles[27] but had not actually been demonstrated to be an

aromatase product. This product (the 19-oic acid of either androstenedione or testosterone) appears to be stable and was not converted to an estrogen (or 19-norandrogen) in P450 19A1 incubations. The identity of the mono-oxygenated aldehyde product is unknown; like the 19-oic acid, it appears to be an end product (Figure S8, Supporting Information). Moreover, the 19-oic androstenedione derivative contained an ¹⁸O atom, which confirms the retention of the oxygen atoms in
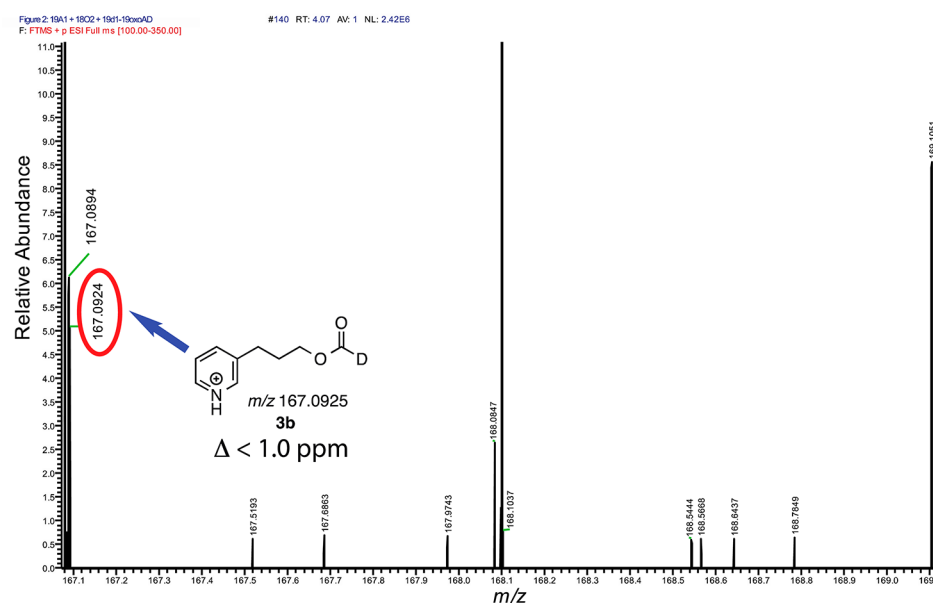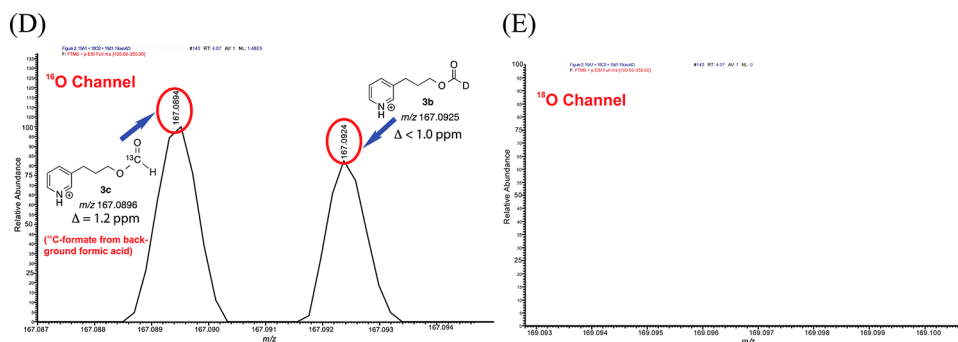
(A)

(B)

(C)

Figure 2. continued

15020

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Figure 2. (A) Schematic depicting purified P450 19A1 incubation with [19-^{2}H]-19-oxoandrostenedione **4-o** ([19-^{2}H]-19oxoAD) in the presence of $^{18}O_2$ followed by derivatization with diazo reagent **2**. (B) LC−MS analysis of the deuterated formic acid incubation product, which was derivatized to the formate ester **3b**. The masses of the formates with one ^{18}O atom (**3a**, m/z 169.0968) and no ^{18}O atom (**3b**, m/z 167.0925) were scanned within a window of 4 ppm. No incorporation of the ^{18}O atom was detected. (C) Mass spectrum (m/z 167.0721−169.1413) corresponding to the 4.07 min retention time. An unknown impurity with a mass of m/z 169.1051 was also detected, which is 25 ppm different from the mass of **3a** (m/z 169.0968). (D) Mass spectrum of the formate product (m/z 167.0870−167.0940 range) corresponding to the 4.07 min retention time. Background formate contribution was detected as [^{13}C]-formate ester **3c** (m/z 167.0896). (E) Mass spectrum in the range of the ^{18}O-incorporated formate (m/z 169.0960−169.0980 range) corresponding to the 4.07 min retention time.

carboxylic acid functional groups during the workup conditions of the incubation (cf. Figure 1 control experiment and also with the detection of 19-oic testosterone derivative by LC−MS). Hahn and Fishman had previously identified 2β-hydroxy-19-oxoandrostenedione in incubations with human placental microsomes.[10,11]

19-Aldehyde Oxygen Exchange with Water. One of the differences between the (FeO)$^{3+}$ and FeO$_2^-$ mechanisms is that the former species is an electrophile and the latter a nucleophile (Scheme 3). The nucleophilic FeO$_2^-$ attack mechanism requires an aldehyde and not the *gem*-diol (Scheme 3, step 3a). If a *gem*-diol is the product of the hydroxylation of the 19-carbinol in the second reaction of the sequence (Scheme 1, **III-g**), then a finite time is required for dehydration to form the aldehyde (**III-o**).[28]

19-CH^{18}O-labeled androstenedione (**7a−o**) was synthesized (Figure S2, Supporting Information) to measure the exchange rate with H$_2^{(16)}$O using LC−MS. The strategy involved subjecting the ^{18}O-labeled steroid to unlabeled water followed by extraction and derivatization with NaBH$_4$ at low temperature, to chemoselectively reduce the aldehyde to an alcohol (Figure 4A, **8a** or **8b**). This reduction to 19-hydroxyandrostenedione (**8a** and **8b**) would prevent any further exchange of the 19-oxygen atom with the aqueous mobile phase during LC−MS analysis (Figure 4A).

The observed rate k_{obs} is the sum of $k_{hydration}$ plus $k_{dehydration}$ (i.e., forward and reverse rates),[29] and from measurement of the equilibrium constants (*gem*-diol/aldehyde) by ^{1}H NMR (Figure 4B and Figure S9, Supporting Information) the individual rates can be estimated (Figure S10, Supporting Information). The ratio of the *gem*-diol to the aldehyde (**7b−g** to **7b−o**) was determined to be 1.5:1.0 in D$_2$O at a pH of 7.8 (adjusted with potassium phosphate) by ^{1}H NMR. The apparent rates of hydration of the aldehyde and dehydration of the *gem*-diol, both ≥0.5 s^{-1} for 19-oxoandrostenedione, were greater than k_{cat} for conversion of the androstenedione 19-alcohol to estrone (0.13 s^{-1}, Scheme 1, **II** to **IV**) and therefore are catalytically competent steps.[25] Hence, either the *gem*-diol (**7b−g**) or the aldehyde (**7b−o**) could be a substrate for oxidation by the FeO^{3+} intermediate, and possible mechanisms of oxidations of both to the carboxylic acid have been presented.[4] These results contradict the lack of exchange of

the aldehyde oxygen reported by Akhtar et al.,[12] most likely due to insolubility problems resulting from the higher concentration of steroid in water (current report: <10 μM, Akhtar report:[12] 300 μM), which we also noted in our NMR and MS work. Specifically, in regard to the ^{1}H NMR experiments, at higher concentrations of 19-oxoandrostenedione in D$_2$O (>100 μM or saturated concentration), the 19,19-*gem*-diol proton (8.46 ppm) was not detectable. Moreover, during the time course measurements of the aldehyde oxygen exchange with [19-^{18}O]-19-oxoandrostenedione (**7a−o**) in unlabeled water, the ^{18}O atom remained intact even after 3 h (when the concentration was increased to >100 μM).

■ DISCUSSION

A scheme consistent with all of the results is shown (Scheme 5), based on a proposal by Covey et al.[30] and modified by Hackett et al.[8] to include a subsequent internal electron transfer (Scheme 5, **3Ab′** to **3Ab″**) to facilitate the final rearrangement. The FeO$_2^-$ intermediate is excluded, based on the formic acid ^{18}O labeling results. The production of the androgen 19-oic acid (**5b**) provides strong evidence that the FeO^{3+} intermediate can form, through an alternate H atom abstraction (C-19) initiating this reaction (Scheme 5, **7b−g** to **5-r**). Although a mechanism involving the (unhydrated) aldehyde cannot be ruled out, a scheme involving the *gem*-diol (**7b−g**) is more straightforward with regard to formic acid formation (Scheme 5). Additionally, the tautomerization step of the 3-keto group to the enol (Scheme 5, **3Ab‴** to **4A**) may occur before the hydrogen abstraction step (Scheme 5, **7b−g** to **3Ab′**) as proposed in the Hackett report on the basis of density functional theory.[8]

Comparison of our differences in results with those of Akhtar et al.[12,15] and Caspi et al.[9] is difficult due to several major improvements in technology. For example, the previous methods to detect formic acid used a diazo toluene reagent to detect formic acid as benzyl formate (i.e., a minimum of 250 ng of benzyl formate was required for its analysis by the reported method).[31] However, in this current study, we designed a new pyridine-containing diazo reagent (**2**) for the sensitive detection of formic acid by mass spectrometry (14 ng of the derivatized formate was readily detected by our method,
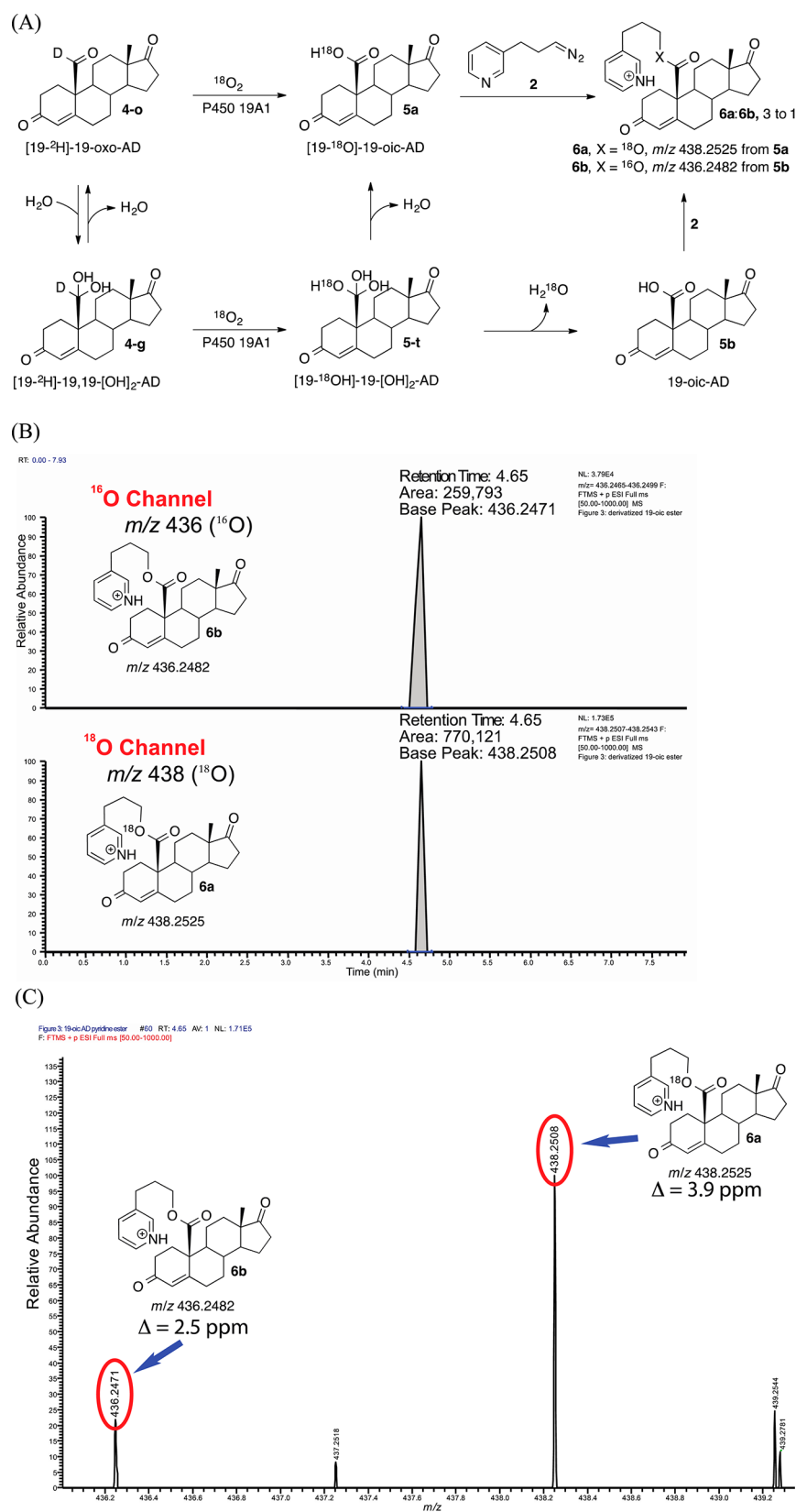
(A)

(B)

(C)

Figure 3. (A) Detection of 19-oic androstenedione as the propylpyridine ester (**6a** or **6b**). (B) High-resolution mass spectrometry trace of the masses scanned with a mass tolerance of 4 ppm for the derivatized carboxylic acid products **6b** and **6a** (m/z 436.2471 and m/z 438.2508) from purified P450 19A1 incubation with [19-²H]-19-oxoandrostenedione ([19-²H]-19oxoAD, **4-o**) in the presence of ¹⁸O₂ followed by derivatization.

Supporting Information) in the positive electrospray ionization mode.

As we have noted previously in this laboratory[32] and known since the earlier aromatase studies,[12] MS analysis of formic acid
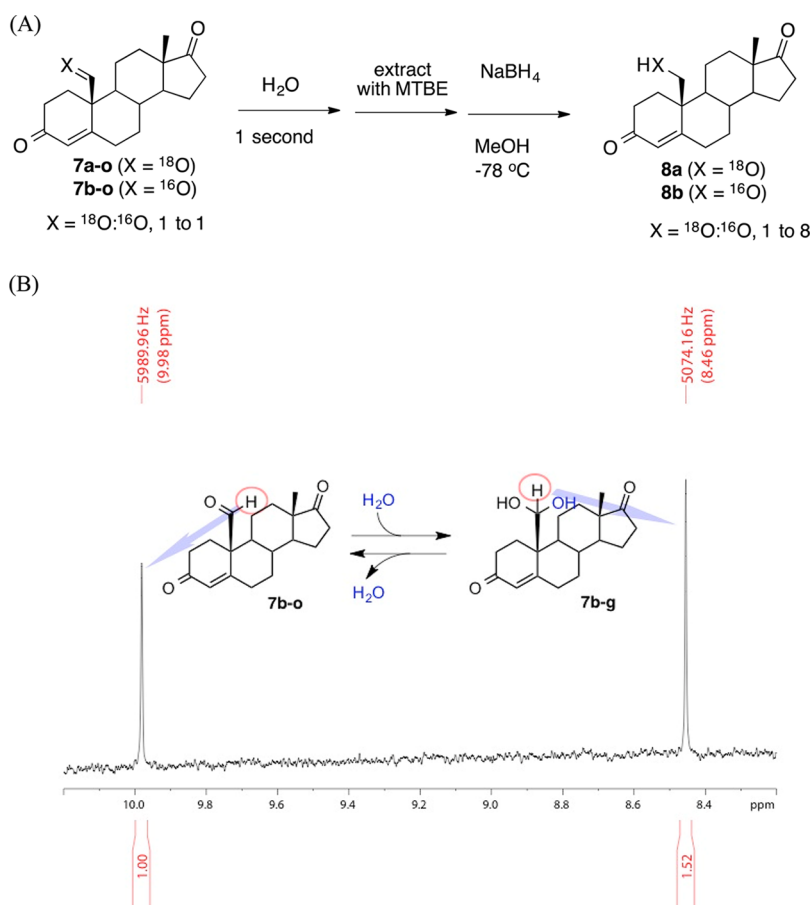
Figure 4. (A) Near complete exchange of the ^{18}O atom after 1 s exposure of [19-^{18}O]-19-oxoandrostenedione to water (50%−11%, ^{18}O abundance from $t = 0$ s to $t = 1$ s in water). LC−MS was used to detect isotopic abundance (^{18}O vs ^{16}O). (B) ^1H NMR (600 MHz spectrometer) of 19-oxoandrostenedione (**7b−o**) in D_2O (pH 7.8) expanded to show the 10.2−8.2 ppm chemical shift region of the aldehyde (**7b−o**, 9.98 ppm) and *gem*-diol (**7b−g**, 8.46 ppm) C-19 methine protons. MTBE: methyl *tert*-butyl ether.

is not trivial due to the background contamination problem; HRMS clearly reveals the presence of interfering materials (Figure 1 and Figures 2C, S4, and S5, Supporting Information), even with high-resolution LC separation. Prior to use, the laboratory reagents were filtered with basic alumina in order to remove endogenous formic acid; however, the contaminant (**3c**) was still present in the analyses of the incubation products (Figure 2C). In order to completely resolve the [^2H]-formate (**3b**, *m/z* 167.0925) from [^{13}C]-formate (**3c**, *m/z* 167.0896), a minimum resolution setting of 60 000 on the LTQ Orbitrap mass spectrometer was required (17.4 ppm mass difference between **3b** and **3c**, Supporting Information). The use of low-resolution mass spectrometers such as the one used in the Akhtar report (i.e., AEI MS 30 mass spectrometer with 1000 resolution setting)[31] would definitely not be able to distinguish between the two benzyl formate isotopomers with a 21.9 ppm mass difference (i.e., [^2H]-benzyl formate with *m/z* 137.0582 vs [^{13}C]-benzyl formate with *m/z* 137.0552). This observation is based solely on our data because there are no mass spectra for the benzyl formates previously published for comparison.[9,12,15] Altogether, our work differs from previous studies in that we used a purified P450 19A1 enzyme, a sensitive new reagent to derivatize formic acid for LC−MS (1-diazo-3-(3-pyridinyl)-propane **2**), and, in particular, HRMS analysis to resolve the formic acid derived from the enzyme incubation (**3b**) and from background contamination (**3c**). Additionally, the use of the new diazo reagent made it possible to detect the novel 19-oic

androgen product as the derivatized ester containing an ^{18}O atom (Figure 3, **6a**).

■ CONCLUSIONS

We conclude that the FeO_2^- mechanism is not operative in the oxidations of either of the 19-oxo androgens to an estrogen and formic acid. Specifically, the supporting evidence includes: (i) the formic acid product did not contain any labeled oxygen atom (^{18}O) from molecular oxygen ($^{18}O_2$), (ii) a 19-oic androgen product was detected, suggesting the utilization of Compound I in the third step, which can abstract either the 1β- or 19-hydrogen atom of the 19-oxo androgen substrate to form either the estrogen or carboxylic acid, respectively, and (iii) the rate of oxygen exchange of the [19-^{18}O]-19-oxoandrostenedione compound in unlabeled water was measured (k_{obs}), and the nonenzymatic rates of $k_{hydration}$ and $k_{dehydration}$ were determined to be catalytically competent and thus support the likelihood that the 19,19-*gem*-diol intermediate can be the substrate for the third step.

The FeO^{3+} mechanism best explains the results, with a semiconcerted reaction without stable intermediates (Scheme 5). These findings are consistent with recently reported results from resonance Raman spectroscopy[6] and kinetic solvent isotope effects[19] from the laboratories of Sligar and Kincaid. The possibilities of 1β- and 2β-hydroxy intermediates that decompose to estrogens[10,11,33] cannot be unambiguously ruled out, in that the formic acid labeling evidence of Caspi et al.[9]

Scheme 5. Proposed Catalytic Mechanism of the Third Step of Androgen Oxidation by P450 19A1[a]
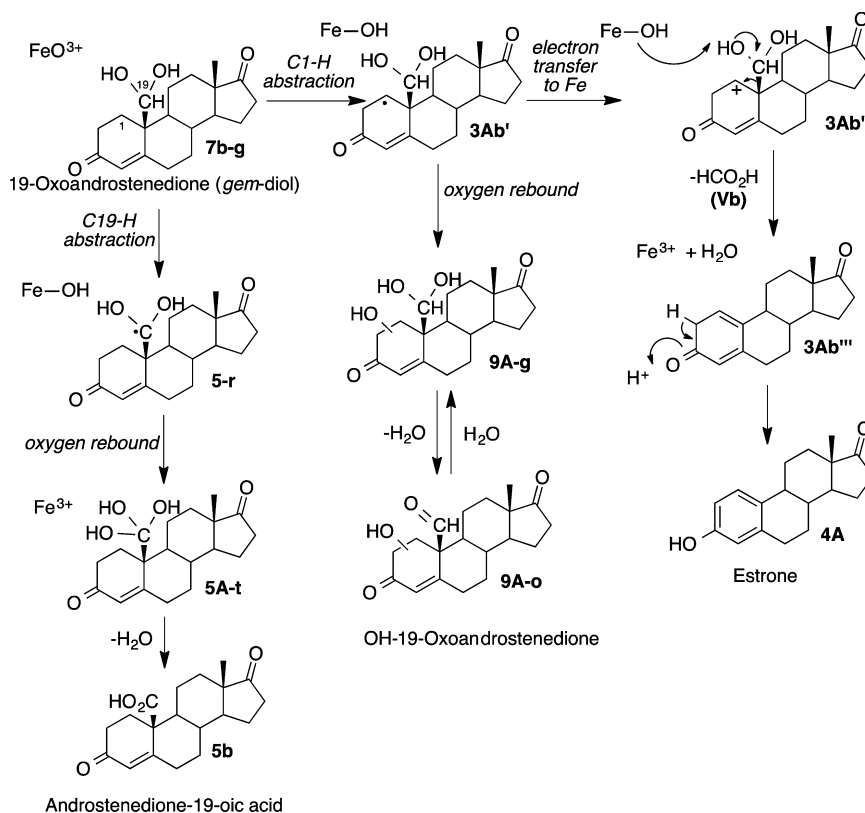
[a]The reaction begins with abstraction of the 1β-hydrogen atom by FeO^{3+}. Further electron transfer, as proposed by Hackett et al.,[8] yields the C-1 carbocation **3Ab″**, and proton abstraction from the *gem*-diol by Fe—OH and rearrangement yields formic acid (**Vb**) and subsequently estrone **4A**. Alternatively oxygen rebound can occur to the A ring to yield the hydroxyl 19-aldehyde (**9A-o**) seen by LC—MS; this may be the product reported to be the 2β-hydroxy 19-aldehyde by Fishman and associates.[10,11] In an alternative initial reaction, FeO^{3+} abstracts the C-19 hydrogen atom. Oxygen rebound yields a *gem*-triol **5A-t**, which degrades to the 19-carboxylic acid, identified here. **5-r**: 5-radical. **5A-t**: 5A-triol.

against a role for a 2β-hydroxy intermediate may be suspect in light of the issues with the ^{18}O formic acid analysis. However, the rate of nonenzymatic decomposition of 2β-hydroxy 19-oxoandrostenedione ($0.0013 \ s^{-1}$ at pH 7.4)[34] is too slow to be catalytically competent (cf. $k_{cat} \ 0.42 \ s^{-1}$ for the conversion of the 19-oxoandrostenoedione to estrone)[25] (the rate has not been measured in the presence of enzyme).

Some other P450 enzymes have also been proposed to utilize FeO_2^- chemistry.[20] We do not know if our conclusions with P450 19A1 apply to these, although our approach may be employed. However, the steroid aromatase reaction is best explained by classic FeO^{3+} chemistry (Scheme 5).

■ ASSOCIATED CONTENT

ⓢ Supporting Information

Experimental methods, syntheses, and figures as described in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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