N-Dealkylation of an *N*-Cyclopropylamine by Horseradish Peroxidase. Fate of the Cyclopropyl Group

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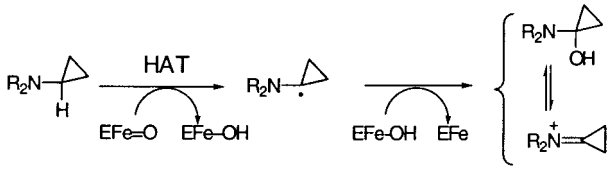
Abstract: Cyclopropylamines inactivate cytochrome P450 enzymes which catalyze their oxidative N-dealkylation. A key intermediate in both processes is postulated to be a highly reactive aminium cation radical formed by single electron transfer (SET) oxidation of the nitrogen center, but direct evidence for this has remained elusive. To address this deficiency and identify the fate of the cyclopropyl group lost upon N-dealkylation, we have investigated the oxidation of *N*-cyclopropyl-*N*-methylaniline (**3**) by horseradish peroxidase, a well-known SET enzyme. For comparison, similar studies were carried out in parallel with *N*-isopropyl-*N*-methylaniline (**9**) and *N*,*N*-dimethylaniline (**8**). Under standard peroxidatic conditions (HRP, H_2O_2, air), HRP oxidizes **8** completely to *N*-methylaniline (**4**) plus formaldehyde within 15−30 min, whereas **9** is oxidized more slowly (<10% in 60 min) to produce only *N*-isopropylaniline (**10**) and formaldehyde (acetone and **4** are not formed). In contrast to results with **9**, oxidation of **3** is complete in <60 min and affords **4** (20% yield) plus traces of aniline. By using [1′-^{14}C]-**3**, [1′-^{13}C]-**3**, and [2′,3′-^{13}C]-**3** as substrates, radiochemical and NMR analyses of incubation mixtures revealed that the complete oxidation of **3** by HRP yields **4** (0.2 mol), β-hydroxypropionic acid (**17**, 0.2 mol), and *N*-methylquinolinium (**16**, 0.8 mol). In buffer purged with pure O_2, the complete oxidation of **3** yields **4** (0.7 mol), **17** (0.7 mol), and **16** (0.3 mol), while under anaerobic conditions, **16** is formed quantitatively from **3**. These results indicate that the aminium ion formed by SET oxidation of **3** undergoes cyclopropyl ring fragmentation exclusively to generate a distonic cation radical (**14**$^{+\bullet}$) which then partitions between unimolecular cyclization (leading, after further oxidation, to **16**) and bimolecular reaction with dissolved oxygen (leading to **4** and **17** in a 1:1 ratio). Neither β-hydroxypropionaldehyde, acrolein, nor cyclopropanone hydrate are formed as SET metabolites of **3**. The synthetic and analytical methods developed in the course of these studies should facilitate the application of cyclopropylamine-containing probes to reactions catalyzed by cytochrome P450 enzymes.

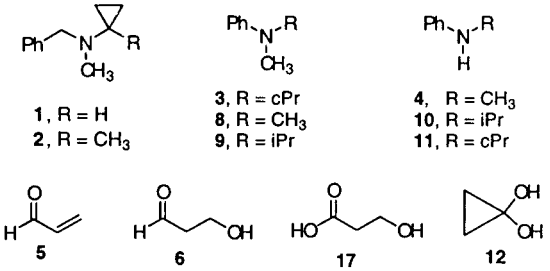
Introduction

The cyclopropylamine substructure is found in a number of drugs and drug candidates and is known to undergo biotransformation by cytochrome P450-catalyzed oxidative N-dealkylation reactions with loss of the cyclopropyl moiety. For ordinary amines not containing an *N*-cyclopropyl group, alkyl groups lost during N-dealkylation are converted to carbonyl metabolites formed by hydrolysis of a carbinolamine precursor. Thus, N-dealkylation was originally viewed as occurring via a hydrogen atom transfer (HAT) process analogous to ordinary aliphatic hydroxylation but α to the nitrogen atom.

For *cyclopropyl*amines, metabolic loss of the cyclopropyl group could, in principle, involve a similar mechanism (viz. Scheme 1), but the unique electronic structure of the cyclopropyl group compared to ordinary aliphatic groups,[1] and the unique ability of cyclopropylamines (e.g., **1**[2,3]) to act as suicide substrates for cytochrome P450 enzymes, suggest that other mechanisms may be involved. Indeed, the fact that cyclopropylamine **2** inactivates P450 as efficiently as **1**[2,3] shows that a C-1′ hydrogen is not required in the P450 inactivation process and rules out enzyme inactivation occurring as a side branch in the classical HAT pathway of N-dealkylation (Scheme 1).

Scheme 1





To account for the inactivation of P450 by cyclopropylamines, a mechanism involving single electron transfer (SET) as an

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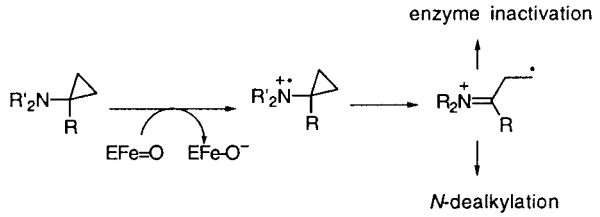
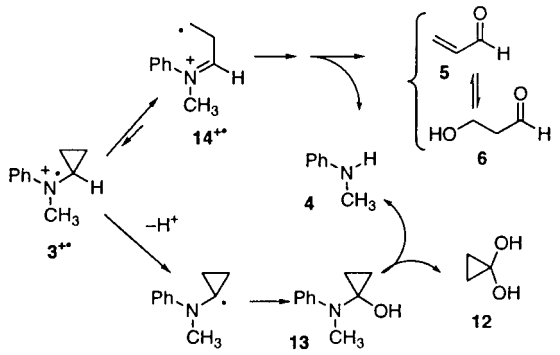
Scheme 2



initiating event has been proposed (Scheme 2).[2,3] Such a mechanism could also account for N-dealkylation of both cyclopropyl and ordinary aliphatic amines by cytochrome P450 enzymes and has been used to explain other features of the N-dealkylation process that are not well accounted for by a HAT mechanism like that shown in Scheme 1, such as the occurrence of very low KDIEs for N-dealkylation vs aliphatic hydroxylation,[4,5] negative Hammett ρ values for dealkylation of *N*,*N*-dialkylanilines,[6] and the release of alkyl radicals during oxidative aromatization of 4-alkyl-1,4-dihydropyridines.[7]

The one-electron oxidation of an *N*-cyclopropylamine leads to a highly unstable[8] cation radical that undergoes rapid cyclopropyl ring-opening.[9−11] This is consistent with the reported formation of *acyclic* metabolites during the oxidation of cyclopropylamines by non-P450 enzymes that are proposed utilize a SET mechanism. For example, oxidation of **1** by the flavoprotein monoamine oxidase (MAO) leads to formation of acrolein and acrolein-inactivated enzyme.[12] Oxidation of tranylcypromine (*trans*-2-phenylcyclopropylamine) by horseradish peroxidase (HRP), a hemoprotein whose redox cycle involves a ferryl intermediate similar to one postulated in P450 turnover[13] (cf. Scheme 2), leads to formation of cinnamaldehyde.[14] Unfortunately, in no case has the three-carbon metabolite arising from the P450-catalyzed N-dealkylation of a cyclopropylamine been determined.[15,16] Extensive data already support the hypothesis that P450, like MAO and HRP, can oxidize at least some of its substrates by an initial one-electron oxidation to generate a radical-cation.[13,17−20] However, to reach this conclusion for cytochrome P450-catalyzed N-dealkylations will require elucidating the fate of the cyclopropyl group that is lost.

Scheme 3



Toward this end, we have investigated the oxidation of *N*-cyclopropyl-*N*-methylaniline (**3**) by HRP, a well-known SET enzyme.[20−22] We used **3** rather than **1** as a probe because HRP has a lower oxidation potential than P450 (~0.75 V[23] vs 1.7−2.0 V,[24] respectively) and is therefore unable to oxidize amines such as **1** because of their higher $E_{1/2}$[25] and pK_a values[14] compared to anilines such as **3**. We previously reported[26] that the HRP oxidation of [1′-^{14}C]-**3** yields *N*-methylaniline (**4**), but the anticipated three-carbon metabolites acrolein (**5**) and 3-hydroxypropanal (**6**) were *not* formed (see Scheme 3). Instead, a polar unreactive ^{14}C-containing metabolite eluted in the solvent front on reverse phase HPLC, but was refractory to isolation and characterization. In an attempt to trap an intermediate in the formation of this solvent front metabolite, we added cyanide ion to the incubations. This substantially decreased the amount of ^{14}C in the solvent front peak and resulted in the formation of a new extractable metabolite, which we identified as cyanotetrahydroquinoline **7**.[26]

We have now extended this work and report herein the complete fate of the cyclopropyl moiety during HRP-catalyzed SET oxidation of **3** in the presence and absence of cyanide. The results demonstrate that after one-electron oxidation the cyclopropyl group in **3** undergoes rapid quantitative ring-opening to an acyclic, distonic radical cation, and that the latter partitions between (a) reacting with molecular oxygen leading to **4** and 3-hydroxypropionic acid in a 1:1 ratio, and (b) intramolecular cyclization leading to quinoline-derived products. This work also demonstrates and validates methodology appropriate for elucidating the fate of the cyclopropyl moiety of cyclopropylamines undergoing P450-catalyzed N-dealkylation reactions; such studies are currently under way in our laboratory.

Results and Discussion

Product Identification. To provide context for our study of the SET oxidation of cyclopropylaniline **3** by HRP, we first investigated the HRP oxidation of the related compounds *N*,*N*-dimethylaniline (**8**) and *N*-isopropyl-*N*-methylaniline (**9**). HRP oxidizes **8** completely within 15−30 min producing **4** plus formaldehyde. Substrate **9** is oxidized very slowly (<10% consumption after 60 min) to *N*-isopropylaniline (**10**) and

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formaldehyde; neither **4** nor acetone are formed. The slow, minimal oxidation of **9** by HRP may be due to unfavorable steric interactions between the isopropyl group and the HRP apoprotein,[13,27] but the preference for HRP-catalyzed N-demethylation over N-dealkylation has been reported previously for other *N*-alkyl-*N*-methylanilines.[28]

Incubation of **3** with HRP results in complete oxidation of the substrate within 30−60 min. However, in contrast to the oxidation of **9** to **10**, the only observed metabolite of **3** is **4**; neither *N*-cyclopropylaniline (**11**) nor formaldehyde are formed, nor is there any inactivation of HRP. Control experiments demonstrated that **11** is susceptible to oxidation by HRP, but not so fast as to preclude its detection if it had actually formed in the incubation. On the other hand, mass balance studies by HPLC (A_{254}) showed that the amount of **4** detected accounts for only 20−25% of the amount of substrate **3** consumed. Treatment of quenched incubations with DNP reagent[29] and subsequent HPLC and GC/EIMS analysis revealed the absence of DNP derivatives of **5** and **6**, the acyclic carbonyl metabolites expected to arise from the N-dealkylation of **3** (viz. Scheme 3), although control experiments showed these compounds to be readily detectable at levels corresponding to as little as 5% conversion of substrate. Unfortunately, cyclopropanone hydrate (**12**), which could conceivably arise via carbinolamine **13** if C-1′ deprotonation of $\mathbf{3}^{+\bullet}$ occurred instead of ring-opening to $\mathbf{14}^{+\bullet}$, does not react with DNPH.[26] Because **12** is a well-known irreversible inactivator of HRP,[30] it would be expected to inactivate HRP at least partially if it were formed during the oxidation of **3**, causing deviations from the strict first-order kinetics observed for loss of **3** under standard incubation conditions. To explore this possiblity we synthesized **12** and added it to incubations with HRP to observe its effect on enzyme activity. In this control experiment, HRP was incubated with **12** (250 μM, half the normal concentration used for **3** and related aniline-type substrates) under otherwise standard conditions for 5 min, after which dimethylaniline (**8**, 500 μM) was added and the incubation monitored visually and by HPLC. Visually, the reaction remained colorless for 60 min, and HPLC showed that *no* loss of **8** took place. Under identical conditions without addition of **12**, HRP incubations of **8** become quite colorful as cation-radical intermediates and quinoid reaction products are generated, and HPLC shows complete consumption of **8** within ca. 15 min. Since incubations with **3** follow strict first-order kinetics up to >90% consumption of **3**, no enzyme inactivation is occurring, which suggests that **12** is not forming as a significant metabolite from **3**.

Since DNP-trapping and HPLC with UV detection failed to reveal any metabolites derived from the cyclopropyl moiety of **3**, we resorted to radiolabeling it with ^{14}C. HPLC analyses of incubations showed that HRP oxidation of [1′-^{14}C]-**3** generates one weakly UV-absorbing radioactive metabolite that elutes within the solvent front (Figure 1, Panel A). Throughout the entire incubation period, ^{14}C eluted only in the solvent front peak and under the peak corresponding to unmetabolized **3**, and total recovery of injected radioactivity was quantitative. As shown by HPLC with both UV and ^{14}C detection, the solvent front metabolite is inert to DNPH reagent, not volatile upon lyophilization, not extractable from aqueous solution by organic solvents, and unaffected by refluxing HCl (1.0 M). The latter two observations in particular rule out the possibility that this material was *N*-methylpropionanilide, formed by acid catalyzed rearrangement of carbinolamine **13**.[31]

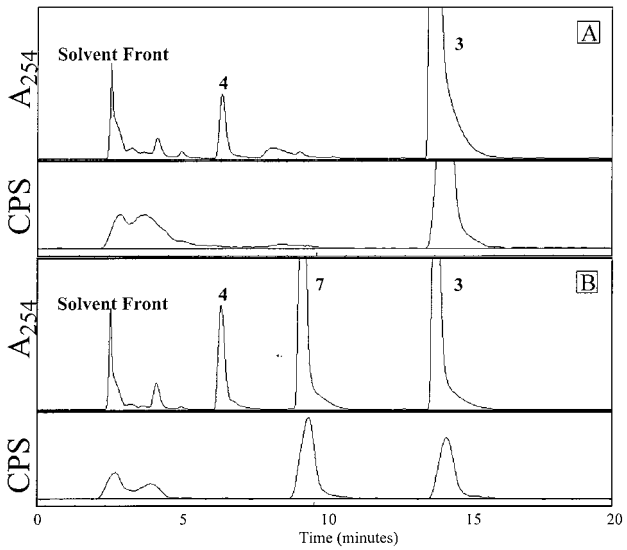

Figure 1. HPLC chromatograms (A_{254} and radioactivity) of a 15 min incubation of **3** with HRP/H_2O_2 in the absence of cyanide (Panel A) and a 45 min incubation of **3** with HRP/H_2O_2 in the presence of 1 mM cyanide (Panel B).

Because oxidative N-dealkylation reactions often involve iminium ion intermediates that can be trapped by cyanide,[32−34] HRP incubations with **3** (0.5 mM) were conducted with 1 mM KCN added. The presence of dilute cyanide slows the oxidation of **3** slightly (~60−90 min to attain complete oxidation) and gives rise to a major new UV-absorbing radioactive peak which was extracted and identified as the tetrahydroquinoline derivative **7** (Figure 1, Panel B).[26] Throughout the incubation, ^{14}C only eluted in the solvent front peak and under the peaks corresponding to unmetabolized **3** and cyanide adduct **7**, the ratio of **7** to the solvent front metabolite was constant at 60:40, and the total recovery of injected radioactivity was quantitative. The radioactive material in the solvent front displayed the same physical and chemical properties as that formed in the cyanide-free incubations.

That the cyanide-trapped intermediate formed in the HRP oxidation of **3** is a tetrahydroquinoline derivative suggests that the ring-opening of $\mathbf{3}^{+\bullet}$ is substantially faster[9,11] than its deprotonation[35] on either the methyl group or the C-1′ position of the cyclopropyl group (Scheme 3). Once the cyclopropyl ring of $\mathbf{3}^{+\bullet}$ opens to form $\mathbf{14}^{+\bullet}$, intramolecular cyclization followed by deprotonation and a second one-electron oxidation would form enamine **15**, a two-electron oxidation product of **3**, whose conjugate acid form $\mathbf{15H}^{+}$ could readily be trapped by cyanide[36] to form **7** (Scheme 4). The fact that cyanide substantially decreases the amount of ^{14}C in the solvent front peak suggests that cyanide is indeed trapping and stabilizing a precursor to the solvent front metabolite. In the presence of cyanide, **4** is

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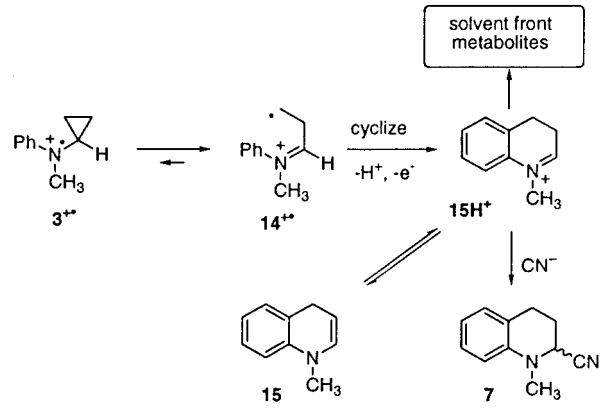
Scheme 4



also formed in lower amounts; however, with or without cyanide, much more ^{14}C is found in the solvent front than would be expected if the solvent front metabolite were only being formed in a process stoichiometric with the formation of **4**.

In an attempt to identify the highly polar, nonderivatizable solvent front metabolite, we next resorted to ^{13}C-labeling of the cyclopropyl moiety of **3**, which we hoped would allow us to structurally elucidate the metabolite by NMR in situ. Thus, we synthesized two different ^{13}C-enriched forms of **3**, namely [1′-^{13}C]-**3** and [2′,3′-$^{13}C_2$]-**3**. Each ^{13}C-enriched probe (with [1′-^{14}C]-**3** added as a tracer for quantitation) was incubated separately with HRP under standard peroxidatic conditions. After complete oxidation of **3**, the incubations were quenched, and the solvent front metabolite peaks were isolated by preparative HPLC and, following concentration in vacuo, analyzed by ^1H-decoupled ^{13}C NMR. To our surprise, *two* metabolites were detected in the same ratio in *each* isolated solvent front fraction. The ^{13}C spectrum of the solvent front fraction from [1′-^{13}C]-**3** (Figure 2, Spectrum A) showed a major metabolite as a singlet at δ149.1 and a minor metabolite as a singlet at δ178.7. In the ^{13}C spectrum of the solvent front fraction from [2′,3′-^{13}C]-**3** (Figure 2, Spectrum B), the major metabolite appeared as two doublets at δ147.1 ($^1J_{C-C}$ = 56 Hz) and δ121.4 ($^1J_{C-C}$ = 56 Hz) while the minor metabolite appeared as two doublets at δ 58.5 ($^1J_{C-C}$ = 37 Hz) and δ 39.5 ($^1J_{C-C}$ = 37 Hz). The observation of carbon−carbon couplings in both of the solvent front metabolites from [2′,3′-^{13}C]-**3** shows that the two enriched carbons remain contiguous in both of the solvent front metabolites. Extensive ^1H, ^{13}C, $^1H-^1H$ COSY, HMQC, and HMBC studies (see Supporting Information) identified the major metabolite of **3** as 1-methylquinolinium (**16**) and the minor metabolite as 3-hydroxypropionic acid (**17**). Structural assignments of these metabolites were confirmed by spiking incubation samples with authentic standards and re-measuring the NMR spectra; this approach avoided small pH-dependent variations in the chemical shifts of various signals, which were otherwise often observed. Thus, complete oxidation of **3** by HRP under standard peroxidatic conditions yields **4** and **17** (0.2 mol each) and **16** (0.8 mol). Complete oxidation of **3** by HRP in the presence of 1 mM cyanide yields **4** and **17** (0.08 mol each), **16** (0.32 mol), and **7** (0.6 mol). With the elucidation of **16** and **17** as solvent front metabolites of **3**, it became obvious why our earlier HPLC-based mass balance study accounted for only 20−25% of starting material consumed; compound **17** has no UV activity at 254 nm, and, ironically, compound **16** has a UV absorption *minimun* at 254 nm.[37]

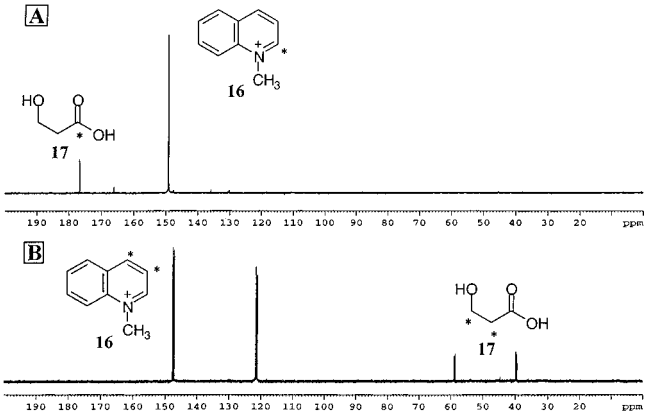


Figure 2. ^1H-Decoupled ^{13}C NMR spectra (125.8 MHz, NS = 57730) of the HPLC-isolated solvent front metabolites from [1′-^{13}C]-**3** (Spectrum A) and [2′,3′-^{13}C]-**3** (Spectrum B). Asterisks denote the sites of ^{13}C-enrichment.

Mechanistic Considerations. The identification of **16** as the major HRP metabolite of **3** is consistent with our previous suggestion[1] that enamine **15**, a 2-electron oxidation product from **3**, is an intermediate en route to cyanide adduct **7**. However, formation of **17** from **3** requires a 4-electron oxidation process. To determine if **17** arose via oxidation of either **5** or **6**, which were anticipated but never observed as metabolites of **3**, each of these compounds was incubated separately with HRP and H_2O_2 but neither was affected. Carbon-centered radicals generated from amines and other substrates undergoing HRP-oxidation have been reported to react avidly with molecular oxygen.[38−40] We therefore conducted incubations of **3** with HRP/H_2O_2 under *anaerobic* conditions to determine if molecular oxygen was involved in the formation of **17** (and **4**). Under these conditions we found that **3** was converted quantitatively to **16** and that this conversion was twice as fast under nitrogen as under air. Anaerobic incubation of **3** in the presence of cyanide yields both **16** (65%) and **7** (35%). In neither anaerobic reaction is either **4** or **17** formed. Incubations were next conducted under an atmosphere of 100% O_2. Upon complete consumption of **3**, the products formed included **16** (0.3 mol) and **4** and **17** (0.7 mol each). These observations show that mechanistic branching occurs during the HRP oxidation of **3** and that the metabolites formed are dependent on the interaction of $\mathbf{14}^{+\bullet}$ with O_2. In other words, if $\mathbf{14}^{+\bullet}$ is not trapped by molecular oxygen it undergoes intramolecular cyclization leading to **16** (Scheme 5, Path A), while if $\mathbf{14}^{+\bullet}$ is trapped by oxygen, intramolecular cyclization is prevented and **4** and **17** are formed in equimolar amounts (Scheme 5, Path B).

During the aerobic oxidation of **3** by HRP we observed by HPLC a minor transient peak at early times in the oxidation reaction. This material is formed in larger amounts in anaerobic oxidations and is not seen in incubations conducted under a pure oxygen atmosphere. By means of GC/EIMS examination of extracts of reaction mixtures, and GC/EIMS and HPLC comparisons to an authentic standard, this transient intermediate was identified as *N*-methyl-1,2,3,4-tetrahydroquinoline (**18**). The transient appearance of **18** during the oxidation of **3** by HRP is somewhat surprising, because it is an *isomer* of **3** rather than an oxidation product. Nevertheless, control studies demonstrated that HRP and H_2O_2 are *both* required for the conversion of **3** to

(37) For **16**, λ_{max} = 315 nm, ϵ_{315} = 5,990 M^{-1} cm^{-1} (ϵ_{254} = 306 M^{-1} cm^{-1}, ϵ_{320} = 5,390 M^{-1} cm^{-1}); for **4**, λ_{max} = 246 nm, ϵ_{246} = 12,720 M^{-1} cm^{-1} (ϵ_{254} = 9,093 M^{-1} cm^{-1}, ϵ_{320} = 258 M^{-1} cm^{-1}).

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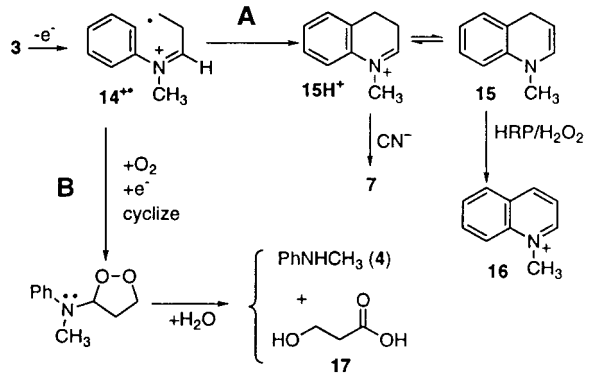
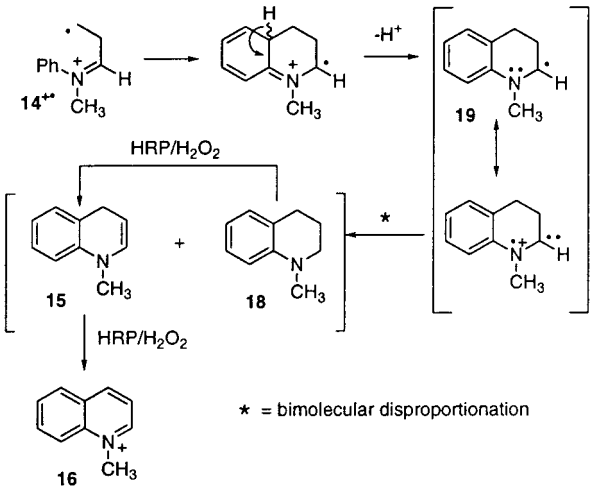
Scheme 5



Scheme 6



18. In separate experiments, HRP was shown to oxidize **18** to **16**, whereas in the presence of cyanide both **16** and **7** were formed from **18**. To account for this aspect of the reaction we propose the $\mathbf{14}^{+\bullet}$ undergoes cyclization and deprotonation to generate radical **19** as shown in Scheme 6; bimolecular disproportionation of **19** would then yield **18** and **15**, both of which are ultimately oxidized to quinolinium ion **16**.

Conclusions

Results presented above indicate that the cyclopropylaminium ion generated by HRP oxidation of **3** reacts exclusively by ring-opening fragmentation to the acyclic distonic radical $\mathbf{14}^{+\bullet}$. This is in sharp contrast to the reactivity of the related aminium ions $\mathbf{8}^{+\bullet}$ and $\mathbf{9}^{+\bullet}$, both of which undergo exclusive deprotonation on their methyl group, and no doubt reflects the kinetic[9−11] and thermodynamic[8] instability of $\mathbf{3}^{+\bullet}$ vs that of its isomer $\mathbf{14}^{+\bullet}$. Once formed, $\mathbf{14}^{+\bullet}$ reacts via two competing pathways, one unimolecular and one bimolecular and oxygen-dependent (Scheme 5). Intramolecular cyclization of $\mathbf{14}^{+\bullet}$ into its phenyl ring, followed by deprotonation and a second one-electron oxidation, generates the iminium ion $\mathbf{15H}^{+}$ and its conjugate base **15**. In incubations with cyanide present, $\mathbf{15H}^{+}$ is trapped as **7**; otherwise **15** is further oxidized by HRP to quinolinium ion **16**, which is the exclusive metabolite of **3** in the absence of both cyanide and oxygen. In competition with intramolecular cyclization, cation radical $\mathbf{14}^{+\bullet}$ can also react with dissolved oxygen via C−O bond formation leading ultimately to formation of the N-dealkylated metabolite **4** plus 3-hydroxypropionic acid (**17**) derived from the cyclopropyl moiety of **3**. The synthetic and analytical methods developed in the course of these studies should facilitate the application of cyclopropylamine-containing mechanistic probes to reactions catalyzed by cytochrome P450 enzymes.

Experimental Section

Materials. Horseradish peroxidase (EC 1.11.1.7, RZ = 0.5) was obtained from Sigma Chemical Co. (St. Louis, MO) and dissolved in 0.4 M potassium phosphate buffer (pH 5.5) at a concentration of 102 μg/mL; this working stock solution was stable for months at 4 °C. Sodium [^{14}C]-formate (0.5 mCi, 55 mCi/mmol) was purchased from Moravek Biochemicals, Inc. (Brea, CA). Sodium [^{13}C]-formate (99% enrichment) and [1,2-$^{13}C_2$]-ethyl iodide (99% enrichment) were procured from Cambridge Isotope Labs (Cambridge, MA). Chemicals and solvents of reagent or HPLC grade were supplied by Aldrich Fine Chemical Co. (Milwaukee, WI), TCI America (Portland, OR) and Fisher Scientific (Pittsburgh, PA). Water for incubation reagents and chromatography was distilled, followed by passage through a Millipore Milli-Q Water System. Tetrahydrofuran (THF) was distilled under nitrogen from sodium and benzophenone, and MeCN and Et_2O were distilled under nitrogen from CaH_2 prior to use. Unless otherwise noted, all air and moisture sensitive reactions were performed under a nitrogen environment. Analytical thin-layer chromatography (TLC) was conducted on Analtech Uniplate 250 μm silica gel plates with detection by UV light and I_2. For flash chromatography, Selecto Scientific 63−200 mesh silica gel was employed. ^{1}H NMR spectra were obtained at either 400 or 500 MHz with Bruker DRX 400 and DRX 500 spectrometers, while ^{1}H-decoupled ^{13}C NMR spectra were obtained at 100.6 MHz on a Bruker 400 spectrometer. Proton chemical shifts are reported in ppm (δ) relative to tetramethylsilane as inferred from shifts of residual protons in the deuterated solvents (i.e., 7.26 ppm for $CDCl_3$, 4.80 ppm for D_2O, 2.50 ppm for d_6-DMSO, and 3.31 ppm for CD_3-OD); ^{13}C chemical shifts are in ppm relative to internal $CDCl_3$ (77.23 ppm).

Incubation Procedures. Incubations were generally conducted on a 1.0 mL scale in 1.5 dram (ca. 15 × 50 mm) screw-cap vials and contained, in order of addition, 962 μL of buffer (0.4 M potasium phosphate, pH 5.5), 8 μL of HRP stock solution (0.021 nmol HRP), 20 μL of substrate stock solution (25 mM in MeCN) and, to initiate reaction, 10 μL of 0.1 M aqueous hydrogen peroxide. Reactions were stirred magnetically at room temperature (22−23 °C) under air and stopped by addition of 670 μL of MeCN. Larger scale incubations used the same proportions and procedure. Anaerobic incubations were conducted similarly except that buffer, substrate, and HRP were combined in a tube closed by a septum penetrated by a long syringe needle (nitrogen inlet) and a short needle (nitrogen outlet). The solution was purged for 10−12 min by a moderate stream of nitrogen gas so as not to cause foaming or evaporative loss of substrate, and reaction was initated by addition of nitrogen-purged hydrogen peroxide solution. The nitrogen purge was maintained at a slow speed throughout the reaction. At the appropriate time nitrogen-purged MeCN was added to stop the reaction. Reactions under oxygen were conducted following the same protocol using oxygen instead of nitrogen.

Metabolite Analysis by HPLC and GC/EIMS. Procedures for direct analysis of incubations by HPLC, for derivatization of carbonyl metabolites with dinitrophenylhydrazine, and for analysis of incubation extracts by GC/EIMS have been described previously.[26] Standard solutions of potential carbonyl metabolites were prepared and analyzed similarly. Thus, formaldehyde (380 μL, 5.1 mmol, 37% (w/v) solution) was added dropwise with stirring to 0.15 M DNPH trapping solution (35 mL, 5.3 mmol); a yellow precipitate formed immediately. After 15 min the yellow crystals were isolated by suction filtration, washed with H_2O (100 mL), and recrystallized from absolute EtOH to afford *N*-(2,4-dinitrophenyl)-*N*′-methylene-hydrazine (978 mg, 92%) as yellow-orange crystals. Mp 166 °C, lit. mp 167 °C.[29] Similarly, acetone (370 μL, 5.0 mmol) was used to produce *N*-(2,4-dinitrophenyl)-*N*′-isopropylidene-hydrazine (869 mg, 73%) as yellow crystals. Mp 125 °C, lit. mp 128 °C.[29] Acrolein (340 μL, 5.1 mmol) afforded a cyclic DNP derivative 1-(2,4-dinitrophenyl)-4,5-dihydro-1*H*-pyrazole, 710 mg, 59%) as dark orange crystals. Mp 165 °C, lit. mp 165 °C.[29] 3,3-Diethoxy-1-propanol (500 μL, 3.2 mmol) was used as described above

to produce *N*-(2,4-dinitrophenyl)-*N*′-(3-hydroxypropylidene)-hydrazine, the DNP derivative of 3-hydroxypropanal, (138 mg, 17%) obtained as gold crystals after flash chromatography (5% MeOH in CH_2Cl_2). Mp 123−124 °C; TLC (CH_2Cl_2:MeOH, 9.5:0.5) $R_f = 0.34$; ^{1}H NMR (400 MHz, CD_3OD) δ 2.65 (q, $J = 6.0$ Hz, 2H), 3.87 (t, $J = 6.3$ Hz, 2H), 7.79 (t, $J = 5.3$ Hz, 1H), 8.01 (d, $J = 9.6$ Hz, 1H), 8.36 (dd, $J = 9.6$, 2.6 Hz, 1H), 9.06 (d, $J = 2.6$ Hz, 1H); GC $t_R = 23.7$ min; EIMS: $[M - 18]^+ = 236$; Elemental analysis calcd for $C_9H_{10}N_4O_5$ C: 42.53, H: 3.97, N: 22.04; found, C: 42.75, H: 3.68, N: 21.60.

NMR Analysis of Metabolites. Incubations were conducted as described above and contained 33 μg (0.87 nmol) of HRP, 10 μmol of ^{13}C-enriched **3** and 20 μmol of H_2O_2 in a final volume of 10 mL of potassium phosphate buffer (0.4 M, pH 5.5). After complete oxidation of **3** (as determined by HPLC), the incubation was quenched with MeCN (6.7 mL) and concentrated in vacuo to ca. 2 mL. The solvent front metabolite peak ($t_R = 2.7-3.9$ min) was isolated by preparative HPLC (as described for aniline HPLC analysis) by collecting the column effluent of seven injections. The isolated effluent was concentrated in vacuo to ca. 500 μL and added to a 5 mm NMR tube containing 50 μL D_2O, and the ^{1}H-decoupled ^{13}C NMR spectrum was obtained by a Bruker 500 MHz NMR at a spectral frequency of 125.8 MHz (NS = 57730). Spectra of authentic standards of **16** and **17** were obtained similarly: **16** ^{1}H NMR (400 MHz, D_2O) δ 4.58 (s, 3H), 7.94 (m, 2H), 8.17 (t, $J = 7.4$ Hz, 1H), 8.27 (m, 2H), 9.02 (d, $J = 8.4$ Hz, 1H), 9.14 (d, $J = 5.7$ Hz, 1H); ^{13}C NMR (100.6 MHz, D_2O) δ 45.18, 118.01, 121.35, 129.39, 129.91, 130.24, 135.69, 138.47, 147.36, 149.00; **17** ^{1}H NMR (400 MHz, D_2O) δ 2.43 (t, $J = 6.0$ Hz, 2H), 3.72 (t, $J = 5.9$ Hz, 2H); ^{13}C NMR (100.6 MHz, D_2O) δ 38.64, 58.46, 178.87.

***N*-Cyclopropyl-*N*-methylaniline (3)[41] and Its Isotopically Labeled Forms.** Ethylmagnesium bromide (3.0 M in Et_2O, 4 mL, 12 mmol) was added dropwise to a vigorously stirred solution of *N*-methylformanilide (500 μL, 4.1 mmol) and titanium isopropoxide (1.6 mL, 5.4 mmol) in THF (20 mL). The solution was heated at 65 °C for 30 min and then stirred at room temperature for 24 h. After quenching the reaction with saturated NH_4Cl (15 mL) and removing the THF under reduced pressure, the crude solution was diluted with 1 N HCl (15 mL), extracted with Et_2O (5 × 5 mL), dried ($MgSO_4$), and concentrated in vacuo. The crude oil was purified by flash chromatography (2.4% Et_2O in pentane) to afford **3** (187 mg, 31%) as a colorless oil. TLC (pentane:Et_2O, 40:1) $R_f = 0.70$; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.62 (m, 2H), 0.81 (m, 2H), 2.37 (m, $J = 3.4$ Hz, 1H), 2.97 (s, 3H), 6.76 (t, $J = 7.2$ Hz, 1H), 6.99 (d, $J = 7.9$ Hz, 2H), 7.25 (t, $J = 8.0$ Hz, 2H); ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 9.26, 33.48, 39.30, 113.97, 117.57, 129.01, 151.05; GC $t_R = 12.1$ min; EIMS: $[M]^+ = 147$.

[1′-^{14}C]-3 was synthesized as described above from [*formyl*-^{14}C]-*N*-methylformanilide, which was obtained in two steps as follows. Dried, finely ground sodium formate (67.6 mg, 0.994 mmol) and sodium [^{14}C]-formate (9.09×10^{-3} mmol in 5 mL of aqueous EtOH, 500 μCi) were transferred to a 16 × 100 mm glass culture tube and concentrated to dryness in vacuo. Methanol (6 mL) and 18-crown-6 (568 mg, 2.2 mmol) were added to the culture tube, and the homogeneous solution was concentrated slowly under reduced pressure to afford an amorphous solid. THF (5 mL) was added, and the tube was sealed with a Teflon-lined screw-cap and heated to 70 °C. After being heated for 24 h, the tube was cooled to room temperature, benzyl bromide (150 μL, 1.3 mmol) was added, and the solution was again heated at 70 °C for another 60 h. Upon cooling, the THF was removed by careful Vigreux distillation, and the residue was diluted with H_2O (8 mL), extracted with Et_2O (3 × 4 mL), dried ($MgSO_4$), and concentrated by Vigreux distillation. The crude oil was purified by flash chromatography (9% Et_2O in pentane) to afford benzyl [^{14}C]-formate (282 μCi, 56%) as a colorless oil. TLC (pentane:Et_2O, 10:1) $R_f = 0.47$. The latter was combined with Kugelrohr-distilled *N*-methylaniline (500 μL, 4.6 mmol) and heated at 95 °C for 48 h in a screw-cap culture tube sealed under N_2. After being cooled to room temperature, the reaction solution was diluted with 1 N HCl (5 mL) and extracted with Et_2O (5 × 2 mL). The combined extracts were dried ($MgSO_4$) and concentrated by Vigreux distillation, and the crude oil was purified by flash chromatography (33% Et_2O in pentane) to yield *N*-methyl-[^{14}C]-formanilide (195 μCi, 69%) as a pale yellow oil which was stored in 6 mL of solvent (pentane:Et_2O:EtOAc, 4:1:1) at 0 °C. TLC (pentane:Et_2O, 2:1) $R_f = 0.22$. Using the same procedure as described for the synthesis of **3**, *N*-methyl-[^{14}C]-formanilide (195 μCi, 0.39 mmol), titanium isopropoxide (250 μL, 0.85 mmol), ethylmagnesium bromide (3.0 M in Et_2O, 600 μL, 1.8 mmol), and THF (5 mL) afforded [1′-^{14}C]-**3** (56 μCi, 29%) as a colorless oil, which was stored in 5 mL of pentane at −4 °C. TLC (pentane:Et_2O, 40:1) $R_f = 0.70$. Prior to its use in incubations, [1′-^{14}C]-**3** was purified (>99%) by preparative HPLC using an Alltech Silica column (10 $\mu\mu$, 4.6 × 250 mm), eluting with 0.1% EtOAc in hexanes at a flow rate of 1 mL/min. This step was necessary to remove small amounts of *N*-methyl-*N*-propylaniline and *N*-methyl-*N*-(3-pentyl)aniline formed as minor byproducts in the cyclopropanation reaction.

[1′-^{13}C]-3 was prepared from sodium [^{13}C]-formate using the methods described above. Sodium [^{13}C]-formate (415 mg, 6.0 mmol), 18-crown-6 (3.41 g, 12.9 mmol), THF (15 mL), and benzyl bromide (900 μL, 7.6 mmol) afforded benzyl [^{13}C]-formate (636.7 mg, 77%) as a colorless oil. TLC (pentane:Et_2O, 10:1) $R_f = 0.48$; ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.21 (d, $^3J_{C-H} = 3$ Hz, 2H), 7.37 (m, 5H), 8.14 (d, $^1J_{C-H} = 226$ Hz, 1H); EIMS: $[M]^+ = 137$. This intermediate was converted to *N*-methyl-[^{13}C]-formanilide in 84% isolated yield (485 mg). TLC (pentane:Et_2O, 2:1) $R_f = 0.21$; ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.33 (d, $^3J_{C-H} = 3$ Hz, 3H), 7.18 (d, $J = 7.6$ Hz, 2H), 7.28 (t, $J = 7.2$ Hz, 1H), 7.42 (t, $J = 7.9$ Hz, 2H), 8.48 (d, $^1J_{C-H} = 197$ Hz, 1H); EIMS: $[M]^+ = 136$. Conversion to [1′-^{13}C]-**3** was effected using the same procedure as described for **3**, i.e., *N*-methyl-[^{13}C]-formanilide (415 mg, 3.1 mmol), titanium isopropoxide (1.3 mL, 4.4 mmol), ethylmagnesium bromide (3.0 M in Et_2O, 3.9 mL, 11.7 mmol) and THF (15 mL) afforded [1′-^{13}C]-**3** (165.9 mg, 37%) as a colorless oil. TLC (pentane:Et_2O, 40:1) $R_f = 0.71$; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.62 (m, 2H), 0.80 (m, 2H), 2.37 (m, $^1J_{C-H} = 173$ Hz, 1H), 2.98 (d, $^3J_{C-H} = 3$ Hz, 3H), 6.76 (t, $J = 7.3$ Hz, 1H), 6.99 (d, $J = 8.7$ Hz, 2H), 7.24 (t, $J = 5.4$ Hz, 2H); EIMS: $[M]^+ = 148$.

[2′,3′-$^{13}C_2$]-3 was prepared using [1,2-$^{13}C_2$]-ethyl iodide as the source of the Grignard reagent. Thus, [1,2-$^{13}C_2$]-iodoethane (500 mg, 3.2 mmol) and Et_2O (1 mL) were drawn into a syringe and added dropwise to a flask containing freshly crushed magnesium turnings (84.3 mg, 3.5 mmol), a tiny crystal of elemental iodine, and Et_2O (3 drops). The solution was stirred until refluxing ceased and nearly all the Mg was consumed. The [1,2-$^{13}C_2$]-EtMgI ethereal solution (~3.2 M) was immediately added via syringe to a mixture of *N*-methylformanilide (190 μL, 1.5 mmol), titanium isopropoxide (600 μL, 2.0 mmol), and THF (7.5 mL). Standard workup afforded [2′,3′-$^{13}C_2$]-**3** (31.5 mg, 7%) as a colorless oil. TLC (pentane:Et_2O, 40:1) $R_f = 0.67$; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.62 (dm, $^1J_{C-H} = 162$ Hz, 2H), 0.82 (dm, $^1J_{C-H} = 158$ Hz, 2H), 2.37 (m, $J = 3.2$ Hz, 1H), 2.98 (s, 3H), 6.76 (t, $J = 6.9$ Hz, 1H), 6.99 (d, $J = 7.8$ Hz, 2H), 7.25 (t, $J = 8.0$ Hz, 2H); ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 9.24, 33.13 ($^1J_{C-C} = 37.7$ Hz), 39.31, 114.01, 117.61, 129.02, 151.06; EIMS: $[M]^+ = 149$.

General Procedure for the Synthesis of 9, 10, and 18. Sodium cyanoborohydride (15 mmol) was added to a stirred solution of the respective Kugelrohr-distilled aniline analogue (5 mmol) and the requisite carbonyl compound (15 mmol) in MeCN (10 mL).[42] Glacial acetic acid (500 μL, 9.3 mmol) was added to the solution over a 10 min period, and the reaction was stirred at room temperature. After 2 h, glacial acetic acid (500 μL, 9.3 mmol) was again added to the solution, which was stirred at room temperature for an additional 15 h. The reaction was quenched with 2 N KOH (20 mL) and extracted with Et_2O (5 × 5 mL), and the extracts were washed with brine (10 mL), dried (Na_2SO_4), and concentrated in vacuo. The residue was purified by flash chromatography (9% Et_2O in pentane) to afford the desired compound. ***N*-Isopropyl-*N*-methylaniline** (**9**, from **4** and acetone) was obtained as a colorless oil (29% yield). TLC (pentane:Et_2O, 10:1) $R_f = 0.65$; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.16 (d, $J = 6.6$ Hz, 6H), 2.72 (s, 3H), 4.09 (sp, $J = 6.6$ Hz, 1H), 6.69 (d, $J = 7.3$ Hz, 1H), 6.79 (d, $J = 8.0$ Hz, 2H), 7.23 (t, $J = 8.0$ Hz, 2H); ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 19.52, 29.97, 49.09, 113.50, 116.58, 129.31, 150.41; GC $t_R = 11.3$ min; EIMS: $[M]^+ = 149$. ***N*-Isopropylaniline**

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(**10**, from aniline and acetone) was obtained as a colorless oil (66% yield). TLC (hexanes:EtOAc, 4:1) $R_f = 0.77$; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.25 (d, $J = 6.3$ Hz, 6H), 3.67 (sp, $J = 6.2$ Hz, 1H), 6.62 (d, $J = 8.6$ Hz, 2H), 6.71 (t, $J = 7.3$ Hz, 1H), 7.20 (t, $J = 7.5$ Hz, 2H); ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 23.18, 44.33, 113.38, 117.09, 129.44, 147.68; GC $t_R = 10.0$ min; EIMS: $[M]^+ = 135$. ***N*-Methyl-1,2,3,4-tetrahydroquinoline** (**18**, from 1,2,3,4-tetrahydroquinoline and formaldehyde) was obtained as a colorless oil (64% yield). TLC (pentane: Et_2O, 10:1) $R_f = 0.56$; ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.04 (m, $J = 6.3$ Hz, 2H), 2.82 (t, $J = 6.4$ Hz, 2H), 2.94 (s, 3H), 3.27 (t, $J = 5.7$ Hz, 2H), 6.67 (t, $J = 7.5$ Hz, 2H), 7.00 (d, $J = 7.1$ Hz, 1H), 7.13 (t, $J = 7.5$ Hz, 1H); ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 22.90, 28.23, 39.56, 51.72, 111.40, 116.64, 123.30, 127.48, 129.25, 147.19; GC $t_R = 13.6$ min; EIMS: $[M]^+ = 147$.

1-Ethoxycyclopropanol.[43] [(1-Ethoxycyclopropyl)oxy]trimethylsilane (3 mL, 14.9 mmol) was added all at once to MeOH (10 mL), and the solution was stirred at room temperature for 21 h. The title compound (965.2 mg, 64%) was isolated from the reaction solution by rotary evaporation as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.84 (d, $J = 10.2$ Hz, 4H), 1.13 (t, $J = 7.1$ Hz, 3H), 3.68 (q, $J = 7.1$ Hz, 2H), 4.04 (br s, 1H); ^{1}H NMR (400 MHz, D_2O) δ ppm 0.96 (dd, $J = 5.3$, 5.2 Hz, 4H), 1.19 (t, $J = 7.2$ Hz, 3H), 3.77 (q, $J = 7.2$ Hz, 2H); ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 14.08, 15.34, 61.90, 85.23.

Cyclopropanone Hydrate (12).[44] A solution of 1-ethoxycyclopropanol (7.8 mg, 76 μmol) in 500 μL of KH_2PO_4 buffer (0.4 M, pH 5.5) and 50 μL of D_2O was heated at 100 °C for 7 min in a 5 mm NMR tube to afford **12** in quantitative yield. The conversion of the hemiketal to **12** was observable by using ^{1}H NMR to monitor the disappearance of the two doublets from the methylene protons of 1-ethoxycyclopropanol and the concomitant appearance of a singlet for the methylene protons of **12**. ^{1}H NMR (400 MHz, D_2O) δ 0.86 (s, 4H); ^{13}C NMR (100.6 MHz, D_2O) δ 14.20, 79.87.

3-Hydroxypropanal (6).[45] Acrolein (**5**, 14 μL, 210 μmol), H_2O (493 μL), and D_2O (50 μL) were combined in a 5 mm NMR tube and left at room temperature for 14 days. Direct NMR analysis of the solution detected **6** in 50% yield. ^{1}H NMR (400 MHz, D_2O) δ 2.74 (td, $J = 5.8$, 1.7 Hz, 2H), 3.92 (t, $J = 5.8$ Hz, 2H), 9.69 (t, $J = 1.7$ Hz, 1H); ^{13}C NMR (100.6 MHz, D_2O) δ 39.78, 58.32, 207.12.

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Supporting Information Available: ^{1}H, ^{1}H−^{1}H COSY, heteronuclear multiple quantum correlation (HMQC), and heteronuclear multiple bond correlation (HMBC) spectra for incubation solutions containing **16** and **17** upon the aerobic oxidation of **3** by HRP/H_2O_2; complete ^{1}H and ^{13}C NMR chemical shift assignments for **16** and **7**. This material is available free of charge via the Internet at http://pubs.acs.org.

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