Enzymatic *N*-Dealkylation of an *N*-Cyclopropylamine: An Unusual Fate for the Cyclopropyl Group

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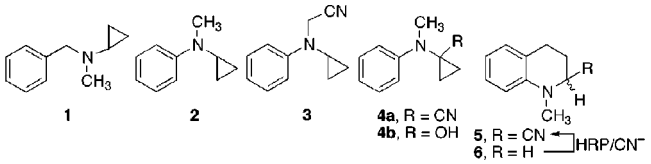
The cyclopropylamine substructure is found in numerous drugs and drug candidates, many of which undergo cytochrome P450-catalyzed *N*-dealkylation with loss of the cyclopropyl group. Some cyclopropylamines, such as *N*-cyclopropylbenzylamine (**1**), are also suicide substrates for P450 enzymes.[1−3] The latter property is general among but unique to *cyclo*propylamines; acylating the nitrogen, separating it from the cyclopropane moiety, or enlarging the ring to cyclobutyl greatly reduces or eliminates suicide substrate activity.[4,5] Despite numerous studies of the P450-catalyzed oxidation of cyclopropanes,[6−9] the fate of the cyclopropyl groups lost during *N*-dealkylation and the nature of the reactive intermediates involved in their inactivation of P450 enzymes remain obscure.



1 **2** **3** **4a**, R = CN; **4b**, R = OH **5**, R = CN; **6**, R = H

Monoamine oxidase, a flavoprotein, also catalyzes the *N*-dealkylation of amines and is covalently inactivated by cyclopropylamines including **1**.[10,11] In this case product analysis showed that the cyclopropyl group was converted to the *acyclic* metabolite acrolein.[12] Similarly, oxidation of cyclopropanone hydrate by horseradish peroxidase (HRP), a heme enzyme whose redox cycle involves a ferryl group similar to that in P450 enzymes, leads to inactivation of the enzyme due to covalent modification of a meso carbon of the porphyrin periphery by a 2-carboxyethyl substituent (i.e. a net two-electron oxidation).[13] While P450 can oxidize a wide range of substrates by several mechanisms,[14,15] extensive data support the hypothesis that horseradish peroxidase, cytochromes P450, and monoamine oxidase can each oxidize at least some of their substrates by an initial step involving single electron transfer (SET) to generate a radical-cation.[11,15−18] In agreement with their calculated instability,[19] cyclopropylaminium radical-cations ($C_3H_5NH_2^{+\bullet}$) undergo ring-opening extraordinarily rapidly.[20−22] Ring-opening generates an acyclic, distonic radical−cation that could, conceivably, covalently modify enzyme active sites or go on to form stable acyclic metabolites. On the other hand, *N*-dealkylation via the classical process of hydrogen atom abstraction followed by hydroxyl recombination at C-1 of the cyclopropyl moiety should produce the ring-intact metabolite cyclopropanone (which undergoes extensive hydration in solution[23]).

To validate the use of cyclopropylamines as reporters for differentiating between the SET vs classical pathways of *N*-dealkylation, we sought to identify the metabolites derived from the cyclopropyl group of a cyclopropylamine undergoing SET-initiated *N*-dealkylation. We began our studies using HRP because of its well-known action as an SET oxidant and we chose *N*-cyclopropyl-*N*-methylaniline (**2**) as a relatively low-$E_{1/2}$ substrate because HRP oxidizes many aniline derivatives but does not oxidize amines such as **1** due to their higher oxidation potentials and pK_a values.[16,24] Incubation of **2**[25] with HRP under standard conditions[26] resulted in its complete oxidation within 30 min. Product analysis by direct HPLC[27] revealed a single UV-absorbing peak identical with that of *N*-methylaniline (NMA), and the *absence* of *N*-cyclopropylaniline (NCA). An aliquot of reaction mixture was treated with 2,4-dinitrophenylhydrazine (DNP) reagent[28] and analyzed by HPLC and capillary GC/MS.[29] These analyses revealed the *absence* of formaldehyde, acrolein, and 3-hydroxypropionaldehyde (from *N*-demethylation vs *N*-dealkylation of **2**, respectively), although control experiments showed these compounds to be readily detectable at levels corresponding to as little as 5% conversion of substrate. Unfortunately, despite considerable effort, we found that cyclopropanone hydrate in dilute aqueous solution (or even in more-concentrated solution) does not form a DNP derivative. We could rule out its formation, however, on the basis that amounts corresponding to 50% conversion of **2** completely inactivated HRP

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(22) We could find no reported rate constant for ring-opening of a cyclopropylaminium ion, but protonated dialkylaminium cation radicals are (10^2−10^5)-fold more reactive than their neutral dialkylaminyl radical counterparts, and the *N*-methyl-*N*-(*trans*-2-phenylcyclopropyl)aminyl radical ring-opens with a rate constant of 7.2×10^{11} s^{-1}. Musa, O. M.; Horner, J. H.; Shahin, H.; Newcomb, M. *J. Am. Chem. Soc.* **1996**, *118*, 3862−3868.
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(25) Compound **2**, a colorless oil, was synthesized in 31% yield by treating *N*-methylformanilide with Ti(O-^{i}Pr)$_4$ and EtMgBr as described by Chaplinski and de Meijere (Chaplinski, V.; de Meijere, A. *Angew. Chem., Int. Ed. Engl.* **1996**, *35*, 413−414). Purity (>99% after preparative HPLC on silica gel) and identity were confirmed by capillary GC/MS, ^{1}H NMR, and ^{13}C NMR.
(26) Incubations were conducted at room temperature under air and contained 0.82 μg (0.021 nmol) of HRP (Sigma, RZ = 0.5), 500 nmol of substrate, and 1000 nmol of H_2O_2 in a final volume of 1.0 mL of potassium phosphate buffer (0.4 M, pH 5.5). For HPLC analysis incubations were quenched by addition of 0.67 mL of MeCN, which controls showed to stop the oxidations completely.
(27) Aliquots of quenched incubations (20 μL) were injected onto a Vydac C-18 column (5 μm, 4.6 × 250 mm) eluted at 1 mL/min with the following two-step gradient: 0−12 min, 0−45% solvent B (MeCN) in solvent A (40% MeCN in 50 mM NH_4OAc); 12−17 min, hold at 45% B in A; 17−25 min, 45−100% B. Column effluent was passed through a UV detector (254 nm) in series with a Ramona radioactivity flow detector with a solid scintillant cell. Data were collected by a SRI chromatography data system and analyzed using Peak Simple software.

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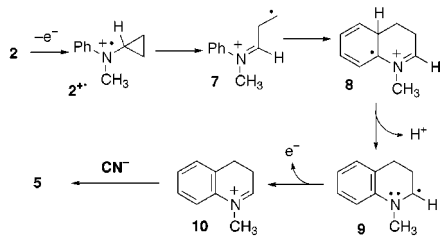
almost immediately, whereas no loss of HRP activity was observed after complete oxidation of **2**.

To focus more specifically on the fate of the 3-carbon cyclopropyl moiety we resorted to radiolabeling. Incubation of [1′-^{14}C]-**2**[30] with HRP[26] followed by HPLC analysis[27] revealed a single peak of radioactivity in the solvent front with little associated UV absorbance (254 nm). Upon reducing or eliminating the organic modifier from the HPLC mobile phase, the ^{14}C remained at the solvent front and was not retained on the C_{18} column, nor was it affected by (a) lyophilization and reconstitution, (b) refluxing in 1 M HCl for 24 h, (c) extraction with organic solvents, and (d) treatment with DNP reagent.

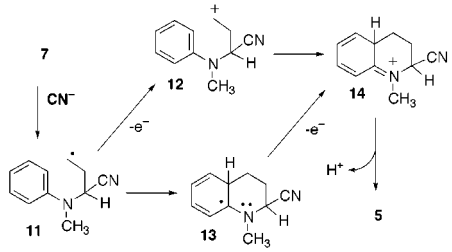
Oxidative *N*-dealkylation reactions often involve iminium ion intermediates which can be trapped with cyanide ion.[31−33] Thus, we examined the oxidation of [1′-^{14}C]-**2** under standard conditions[26] with 1 mM KCN added. Since cyanide inhibits HRP, these reactions took (3−4)-fold longer to reach completion. Examination of product mixtures by direct HPLC (A_{254}) revealed a sharp new peak at $t_R = 9.0$ min in addition to NMA and traces of aniline, but no NCA. The radiochromatogram showed a significantly diminished solvent front peak and a sharp new peak at $t_R = 9.0$ min (metabolite **M1**). Throughout the entire incubation period (0−100% consumption of **2**) the ^{14}C in the solvent front peak plus that in the **M1** peak equalled the amount of substrate consumed, and the metabolite ratio (**M1**/solvent front) was constant at ca. 60:40. Extraction of product mixtures with ether efficiently removed **M1**, which capillary GC/MS showed to be a single compound having *m*/*z* 172 (which corresponds to **2** − H + CN).

The properties of **M1**, together with the context of its formation, suggested it might be either **3** or **4a**, but after synthesizing **3**[34] it was found to be separable from **M1** by both HPLC and capillary GC/MS. We isolated **M1** by preparative HPLC from larger scale incubations and examined it by ^{1}H and ^{13}C NMR as well as COSY, HMQC, and HMBC. These data clearly showed that **M1** was not **4a**,[35] but was very likely **5**,[36] which we synthesized by means of HRP oxidation of *N*-methyl-1,2,3,4-tetrahydroquinoline (**6**) in the presence of cyanide. This generated a single product indistinguishable from **M1** by HPLC or capillary GC/MS. Thus, SET oxidation of **2** gives rise to a reactive intermediate which can be trapped efficiently by dilute cyanide ion to yield **5** as the stable end product. In the absence of cyanide this intermediate is converted to highly polar material which has thus far resisted identification.

Scheme 1



Scheme 2



The formation of **5** from **2** requires a net two-electron oxidation. A mechanism for this transformation is suggested in Scheme 1. As reviewed by Chen et al.,[37] the initial step is a one-electron oxidation of **2** to **2**$^{+\bullet}$ by the oxidized (ferryl) form of HRP. The next (and *key*) step is the *unimolecular* ring-opening of **2**$^{+\bullet}$ to give the distonic cation radical **7**. If ring-opening of the cyclopropane moiety were nucleophile-assisted, like the ring-opening of arylcyclopropane cation radicals,[38,39] products other than **5** would have been formed. This point is crucial to the application of cyclopropylamines to probe for the involvement of SET mechanisms in enzyme-catalyzed *N*-dealkylations because active site nucleophiles (including solvent water) might not be readily available, leading potentially to "false-negative" results.

Formation of **5** from **7** ultimately requires several additional steps, the order of which is uncertain (and unimportant to the intended application of cyclopropylamines for detecting SET mechanisms). One plausible sequence is shown in Scheme 1; an alternate, if perhaps less plausible, mechanism is shown in Scheme 2. Because cyanide failed to trap iminium ion precursors to **3**, **4a**, or **4b**, we conclude that ring-opening of **2**$^{+\bullet}$ is substantially faster than its deprotonation on either the methyl group or the cyclopropyl C-1′ position. This again reinforces the suitability of cyclopropylamines as reporters for detecting aminium ion intermediates in oxidation reactions through the formation of ring-opened products. The identity of the solvent front metabolite(s) whose yield is reduced in the presence of cyanide is under investigation and will be reported separately.

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(28) DNP reagent (0.15 M 2,4-dinitrophenylhydrazine dissolved in a mixture of concentrated H_2SO_4/water/absolute ethanol, 3:4:14, v/v/v) was extracted thoroughly with hexanes (to remove numerous interfering contaminants) immediately before use. After quenching a 1.0 mL incubation with 0.67 mL of MeCN, hexanes−extracted DNP reagent (15 μL) was added. The mixture was stirred at room temperature for 30 min and shaken vigorously with EtOAc (300 μL). After phase separation a 1 μL aliquot of the EtOAc layer was analyzed by GC/MS and/or a 10 μL aliquot was analyzed by HPLC (Alltech cyano bonded phase column, 10 μm, 4.6 × 250 mm) eluting with 10% EtOAc in hexanes (solvent A) for 12 min followed by gradient elution with solvent B (1% i-PrOH in CH_2Cl_2; 0−100% over 23 min) at a flow rate of 1.5 mL/min. Standard solutions of expected carbonyl metabolites were prepared and analyzed similarly.

(29) GC/MS analyses were performed on a HP 5890 Series II GC fitted with a DB-5 capillary column coupled to a HP 5971A Mass Selective Detector. Data were collected in the full spectrum mode (40−350 amu, 0.77 scans/s) and analyzed using an HP 5970 ChemStation. Quenched incubations were extracted with 300 μL of ether and a 1 μL aliquot of the organic phase was analyzed by the temperature program: 60 °C for 3 min; 10 °C/min to 250 °C; hold at 250 °C for 5 min.

(30) For the synthesis of [1′-^{14}C]-**2**, sodium [^{14}C]-formate (1.0 mmol, 500 μCi, Moravek Biochemicals, Brea, CA) was combined with 2.2 mmol of 18-crown-6 and 1.3 mmol of benzyl bromide in THF (5 mL) and heated in a sealed screw-cap culture tube (70 °C, 60 h) maintained behind an adequate safety shield. After cooling, concentration by Vigreux distillation and flash chromatography (silica gel; 9% ether in pentane), the resulting benzyl [^{14}C]-formate (0.56 mmol, 282 μCi) was heated with *N*-methylaniline (500 μL, 4.6 mmol) in a screw-cap culture tube sealed under N_2 (95 °C, 48 h). After cooling, dilution with excess HCl, extraction with ether, concentration by Vigreux distillation, and flash chromatography (silica gel; 33% ether in pentane), the resulting *N*-methyl-[^{14}C]-formanilide (0.39 mmol, 195 μCi) was converted to [1′-^{14}C]-**2** (yield 0.11 mmol, 56 μCi) as described above.

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