Table V. Thermochemical Data Evaluated in This Study at 298 K[a]

		previous value[a]	ref
ΔH_f° (1)	70.7 ± 3.2,[b] $\geq 67.7 \pm 3$[c]	$\leq 58 \pm 5$	18
PA(1)	377 ± 2		
EA(c-C_5H_4)	$\leq 54.4 \pm 2$, $\geq 41.5 \pm 7$		
D°(c-C_5H_4-H^-)	103.9 ± 5.2,[b] $\geq 100.8 \pm 5$[c]		

[a] In kcal mol^{-1}. [b] From PA bracketing. [c] From H· affinity bracketing.

$C_5H_4^{-}\cdot$, it was considered essential to establish beyond reasonable doubt the skeletal structure and electronic configuration of this anionic species. The following reactions of this species deal with these structural questions.

a. The same ion–molecule chemistry (rate constants and products) results from the anion product from the following three reactions;[32] reactions ii[18] and iii are known to yield c-$C_5H_5^-$. These results confirm the skeletal structure of anion m/z 64 as c-$C_5H_4^{-}\cdot$.

(i) $C_5H_4^{-}\cdot + CH_3OH$ →
(ii) c-$C_5H_6 + O^-$ → c-$C_5H_5^-$
(iii) c-$C_5H_6 + CH_3O^-$ →

b. The radical behavior of anion m/z 64 is amply demonstrated by its H· abstraction reactions.

c. The reactivity of c-$C_5H_4^{-}\cdot$ as a base toward proton donors, especially ROH, is in keeping with the structure of a π-delocalized anion, but not that of a σ-localized carbanion. This conclusion is supported by the failure of c-$C_5H_4^{-}\cdot$ to react with N_2O.[21]

We, therefore, conclude that the anion radical m/z 64 produced from **2** in our FA experiments is c-$C_5H_4^{-}\cdot$ with the $\sigma^1\pi^2$ electronic configuration as represented in **1**.

Summary of Thermochemical Data from This Study. The important thermochemical data determined in the present study at 298 K are summarized in Table V.

The obvious disagreement in ΔH_f°(**1**) determined in this study and that previously reported[18] from the reaction

$$\text{c-}C_5H_6 + S^{-}\cdot \rightarrow C_5H_4^{-}\cdot + H_2S \qquad (23)$$

requires comment. Domenico et al.[18] assumed that this reaction proceeded by α-elimination of H^+ and H· from c-C_5H_6. We have been unable to produce anion m/z 64 by this reaction in the FA; the only observed product ion was c-$C_5H_5^-$ (m/z 65). We calculate for eq 23 that $\Delta H_{rx} = +15.7 \pm 3.4$ kcal mol^{-1}.[49] We conclude that the elementary processes for eq 23 are not α-elimination of the geminal hydrogens from c-C_5H_6 by $S^{-}\cdot$.[18] Further, using ΔH_f°(**1**) $\leq$ 58 kcal mol^{-1} yields an abnormally low value of D°(c-C_5H_4-H^-) $\leq 91.2 \pm 2$ kcal mol^{-1}.[33,38]

The present results portray the power of the flowing afterglow method for determination of the intrinsic reactivities of reactive intermediates. The results of this approach have added another dimension to our understanding of the physical organic chemistry of hypovalent anion radicals in the absence of solution and ion-pair phenomena. Studies with other carbene anion radicals, and with various carbene cation radicals, are in progress.

Acknowledgment. We wish to express our sincere appreciation to Dr. John Kolts for the original design of our flowing afterglow and for discussions during its construction, and to Mr. Al Nielson for his talents and advice in the FA construction. We gratefully acknowledge support of this research from the U.S. Army Research Office (DAAG29-77-G-0142) and the National Science Foundation (Equipment Grant CHE76-80382).

(49) $\Delta H_f^\circ(S^{-}\cdot) = 17.73$ kcal mol^{-1};[18] ΔH_f°(c-C_5H_6) = 32.4 kcal mol^{-1};[18,25] $\Delta H_f^\circ(H_2S) = -4.88 \pm 0.15$ kcal mol^{-1}.[31]

Oxidation of Amines by a 4a-Hydroperoxyflavin

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Abstract: Kinetic and product studies have been carried out for the reaction of 12 tertiary amines, secondary amines, and secondary hydroxylamines with the 4a-hydroperoxide of N^5-ethyl-3-methyllumiflavin (4a-FlEtOOH). All reactions were found to be first order in 4a-FlEtOOH and amine in *t*-BuOH solvent. Transfer from *t*-BuOH to the aprotic solvent dioxane decreases the second-order rate constant by ~threefold, but does not change the kinetic order in reactants (i.e., no external proton source is required). The reactions with the secondary and tertiary amines are quantitative, yielding secondary hydroxylamines and tertiary amine oxides along with the flavin pseudobase (4a-FlEtOH). Secondary hydroxylamines yield with 4a-FlEtOOH nitrones and 4a-FlEtOH. The free radical trap 2,6-di-*tert*-butyl-4-methylphenol does not influence the rate constants or product yields. This finding, along with the observation that rate constants are not related to the stability of cation radicals derived from amine, establishes that free radical processes are not involved in the N-oxidation reactions. The N-oxidation reactions are best explained as occurring through nucleophilic attack of amine nitrogen upon the terminal oxygen of the 4a-FlEtOOH molecule with back donation of the hydroperoxy hydrogen to the internal peroxy oxygen. Comparison of the second-order rate constants (on the basis of the amine pK_a's in H_2O) provides the nucleophilic order secondary hydroxylamines > tertiary amines > secondary amines. The disappearance of 4a-FlEtOOH from solution in the presence of primary amines is much slower than with secondary amines and the reaction does not follow a simple rate law nor is 4a-FlEtOH a major product. In *t*-BuOH the spontaneous first-order rate constant for decomposition of 4a-FlEtOOH exceeds that for the decomposition of H_2O_2 by more than 400-fold while the second-order rate constant for N-oxidation of *N,N*-dimethylbenzylamine by 4a-FlEtOOH exceeds that for N-oxidation by H_2O_2 by 36 000-fold (and N-oxidation by *t*-BuOOH by >400 000). These results are discussed in terms of the involvement of 4a-hydroperoxyflavin cofactor in the metabolism of amines by the hepatic flavoprotein microsomal oxidase.

Introduction

Mammalian liver contains two separate enzyme systems with different pathways for the oxidation of N-substituted amine drugs.[1] One system containing a flavoprotein (NADPH cytochrome P-450 reductase) and cytochrome P-450 catalyzes C-oxidation of amines resulting in N-dealkylation. The other system involves N-oxidation of secondary and tertiary amines catalyzed by a flavoprotein free of cytochromes, iron, and copper in an NADPH- and O_2-dependent process. Spectral studies establish that oxygen reacts with the reduced flavoprotein monooxygenase (Enz-FlH_2) to form an enzyme-bound 4a-hydroperoxyflavin (Enz-4a-FlHOOH).[2,17] In

(1) B. S. S. Masters and D. M. Ziegler, *Arch. Biochem. Biophys.*, **145**, 358 (1971).

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a subsequent reaction, the Enz-4a-FlHOOH reacts with amine to form the N-oxidized amine, enzyme-bound oxidized flavin (Enz-Fl_{ox}), and water. The sequence of reactions can be visualized as occurring via eq 1–3:

$$\text{NADPH} + \text{Enz-Fl}_{ox} + \text{H}^+ \rightarrow \text{Enz-FlH}_2 + \text{NADP}^+ \quad (1)$$

$$\text{Enz-FlH}_2 + \text{O}_2 \rightarrow \text{Enz-4a-FlHOOH} \quad (2)$$

$$\text{Enz-4a-FlHOOH} + \text{R}_3\text{N} \rightarrow \text{R}_3\text{NO} + \text{Enz-Fl}_{ox} + \text{H}_2\text{O} \quad (3)$$

The substrate specificities of the cytochrome P-450 and flavoenzyme hepatic monooxygenase systems are still not well defined. Important in the thinking of investigators has been the proposal of Gorrod[3a] that the more basic amines are preferred substrates for the flavoprotein monooxygenase whereas the less basic amines are handled by the P-450 monooxygenase. However, there are many exceptions to this generalization.[3b] A question of interest is whether the specificity of the flavoprotein monooxygenase is determined by the reactivity of the enzyme bound 4a-hydroperoxyflavin. In such a multistep process as that indicated by eq 1–3, the direct reaction of substrate with the Enz-4a-FlHOOH is obscured. Recently, the synthesis of 5-alkyl-4a-hydroperoxyflavins (4a-FlROOH) has been reported.[4] The 4a-FlROOH compounds have been found to possess monooxygenase activity in the oxidation of aldehydes[4] (a chemiluminescent model for bacterial luciferase), sulfides,[4b,5] and tertiary amines (for a preliminary account, see ref 5). We report herein the results of our studies on the N-oxidation of secondary, tertiary, and hydroxyl amines by 4a-FlEtOOH.

Experimental Section

Materials. *tert*-Butyl alcohol was refluxed over calcium hydride, distilled under an atmosphere of dry nitrogen, and subjected to several cycles of freeze–thaw to remove final traces of oxygen. Dioxane was refluxed under dry nitrogen over sodium and benzophenone until the intensely blue color of the benzophenone anion radical persisted. Distillation of dioxane from the blue solution was followed by several cycles of freeze–thaw. Hydrogen peroxide (90%) was diluted with *t*-BuOH and the solution subjected to several cycles of freeze–thaw. *tert*-Butyl hydroperoxide was distilled under reduced pressure followed by several cycles of freeze–thaw. Amines, with the exception of *N*-methyl-*N*-benzylhydroxylamine, were obtained from commercial sources, distilled under dry nitrogen, and purged with vanadous-scrubbed argon for at least 2 h. *N*-Methyl-*N*-benzylhydroxylamine was synthesized from benzaldehyde and *N*-methylhydroxylamine as described elsewhere.[6] The glacial acetic acid, methanol, and 95% ethanol used for the hydroperoxide and *N*-oxide analyses were obtained from commercial sources and deoxygenated without further purification. Acetic acid was deoxygenated by several cycles of freeze–thaw. Methanol and 95% ethanol were purged with nitrogen or vanadous-scrubbed argon for several hours to remove dissolved oxygen. All deoxygenated materials were stored within a glovebox under an atmosphere of dry nitrogen. The materials for hydroxylamine analysis (*n*-amyl acetate, acetic acid, sodium acetate, bathophenanthroline, ferric nitrate, and EDTA (disodium salt)) were obtained from commercial sources and used without further purification. The materials for formaldehyde determination (ammonium acetate, acetic acid, and 2,4-pentanedione) were also obtained from commercial sources and used without further purification. The N^5-ethyl-3-methyllumiflavinium perchlorate ($Fl_{ox}{}^+EtClO_4{}^-$) and its corresponding 4a-pseudobase were synthesized from 3-methyllumiflavin as described by Hemmerich.[7] 4a-Hydroperoxy-5-ethyl-3-methyllumiflavin (4a-FlEtOOH) was synthesized as described previously.[4a] The synthesis of 3-methyllumiflavin is described elsewhere.[8] The tertiary amine *N*-oxides, *N,N*-dimethylbenzylamine *N*-oxide,[9] *N,N*-dimethylaniline *N*-oxide,[10] and *N*-methylmorpholine *N*-oxide,[11] were prepared by the reaction of excess 30% H_2O_2 with the corresponding tertiary amine in methanol as described by Cope for the preparation of *N,N*-dimethylbenzylamine *N*-oxide.[9] The *N*-oxides were isolated and stored as their hydrochloride salts and generated for use in *t*-BuOH solutions by reaction with a stoichiometric quantity of potassium *tert*-butoxide. *N*-Methylbenzaldoxime was obtained as a synthetic precursor to *N*-methyl-*N*-benzylhydroxylamine (see above). The 2,6-di-*tert*-butyl-4-methylphenol (BHT) was obtained commercially and sublimed prior to use.

Apparatus. Kinetic measurements and spectra were made on a Cary 15 spectrophotometer, the sample compartment of which was enclosed in a glovebox under an atmosphere of nitrogen. pH measurements were made using a Radiometer Model 26 pH meter equipped with a standardized GK-2302 C Radiometer electrode at 30 °C. Rate constants were calculated using a Hewlett-Packard 9825 A calculator.

Kinetic Measurements. All kinetic measurements were conducted at 30 ± 0.2 °C. The reactions of 4a-FlEtOOH with amines in either *t*-BuOH or dioxane were followed under the conditions of [amine] ≫ [4a-FlEtOOH] by observing the decrease in absorbance of the reaction mixtures at 370 nm (4a-FlEtOOH) with time. Solutions for kinetic studies were prepared under N_2 in the glovebox which also housed the sample compartment of the spectrophotometer. Solutions of *t*-BuOH were prevented from crystallizing by maintenance at ~25 and 30 °C. Reactions were initiated by transferring 1 mL of a *t*-BuOH or dioxane solution of 4a-FlEtOOH into a 1-cm absorbtion cell containing 2 mL of a *t*-BuOH or dioxane solution of the appropriate amine. The 4a-FlEtOOH solutions ($3–6 \times 10^{-4}$ mmol/mL) were prepared by dissolving a weighed quantity of 4a-FlEtOOH of determined purity (~90%) in a measured volume of *t*-BuOH or dioxane. By employing rapid and efficient stirring, solution of the 4a-FlEtOOH in *t*-BuOH was achieved in less than 5 min. The 4a-FlEtOOH dissolved readily in dioxane. Solutions of amines in *t*-BuOH were prepared by diluting the appropriate volume of amine with *t*-BuOH. Densities of the amines were obtained either from the literature or measured gravimetrically. Volumes of these amine solutions were transferred to a 1-cm absorbtion cell and diluted to 2 mL with *t*-BuOH. Amine solutions in dioxane were prepared similarly. Solutions of BHT or *N*-methyl-*N*-benzylhydroxylamine in *t*-BuOH were prepared by dissolving a weighed quantity of the solid in *t*-BuOH and diluting to the appropriate volume. For those runs in which BHT was included in the reaction medium, an appropriate volume of BHT solution was added to the 1-cm absorbtion cell containing a *t*-BuOH solution of amine. The resultant mixture was diluted to 2 mL with *t*-BuOH and the reaction initiated as described above.

The reactions of H_2O_2 and *t*-BuOOH with amines were conducted in oxygen-free *t*-BuOH by observing the decrease in hydroperoxide under the conditions of [amine] ≫ [hydroperoxide]. Unreacted hydroperoxide was determined by following the production of $I_3{}^-$ at 358 nm under N_2 when 0.1 mL of the reaction mixture was transferred to 3 mL of acetic acid–ethanol 0.1 M in NaI. For H_2O_2, the assay medium was composed of 3 volumes of acetic acid diluted to 25 volumes with ethanol. The pseudo-first-order rate constant for the formation of $I_3{}^-$ under these conditions was found to be 2×10^{-3} s^{-1}. For *t*-BuOOH, the assay medium was composed of 10 volumes of acetic acid diluted to 25 volumes with ethanol. The pseudo-first-order rate constant for the formation of $I_3{}^-$ under these conditions was found to be 1×10^{-3} s^{-1}.

Reaction Products. The fate of the 4a-FlEtOOH in the *N*-oxidation of amines was determined by comparison of the spectral characteristics of the final reaction mixtures with those of the 4a-pseudobase of the 3-methyllumiflavin cation [4a-FlEtOH] λ_{max} 352 nm, ϵ 8000 M^{-1} cm^{-1} (*t*-BuOH); λ_{max} 343, ϵ 8400 M^{-1} cm^{-1} (dioxane). In neither *t*-BuOH nor dioxane was the 4a-FlEtOH a major product of spontaneous 4a-FlEtOOH decomposition. Further confirmation of the identity of the flavin product from the reaction of 4a-FlEtOOH in *t*-BuOH with all of the amines investigated was obtained by acidification of 1 mL of the *t*-BuOH reaction mixtures with 2 mL of 1 M HCl. In all cases the characteristic spectrum of $Fl_{ox}{}^+Et$ appeared (λ_{max} at 545 nm and 430 nm, $\epsilon_{545} \simeq 8000$ M^{-1} cm^{-1}).

Analysis for the *N*-oxide of *N,N*-dimethylaniline from the reactions of 4a-FlEtOOH with *N,N*-dimethylaniline in *t*-BuOH was conducted by the method of Zeigler and Pettit[12] with some modification. In this procedure, 2 mL of the reaction mixture is transferred aerobically to 5

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mL of H_2O and the pH adjusted to ~11 by addition of 0.01 mL of 1 M NaOH. This aqueous solution is extracted four times with 10 mL of dichloromethane to remove unreacted dimethylaniline. It was determined that this procedure quantitatively removes up to 1 M dimethylaniline in *t*-BuOH. After extraction with dichloromethane, 0.01 mL of 3 M trichloroacetic acid is added to the aqueous phase which adjusts the pH to ~2.3; 0.1 mL of 4.5 M $NaNO_2$ is added, and the solution is heated for 5 min at 60 °C. This converts the *N*-oxide of dimethylaniline to the amine and subsequently to its *p*-nitroso derivative (λ_{max} 420 nm, ϵ_{420} 8200 M^{-1} cm^{-1}). The hot yellow solution was cooled, diluted to 10 mL, and the absorbance read at 420 nm. A small correction was applied for the blank determination.

The yield of the *N*-oxide of dimethylbenzylamine from the reactions of 4a-FlEtOOH with dimethylbenzylamine was determined by transferring under nitrogen 0.2 mL of the *t*-BuOH reaction mixtures to 3 mL of deoxygenated AcOH 0.1 M in NaI. A control run employing an authentic sample of the *N*-oxide of dimethylbenzylamine under virtually identical conditions was conducted simultaneously. The 4a-FlEtOH present in the reaction mixture is converted to $Fl_{ox}{}^{+}Et$ upon transfer to glacial acetic acid which rapidly liberates an equivalent amount of I_3^-. The *N*-oxide of dimethylbenzylamine reacts much more slowly with NaI under these conditions (30 °C) with an observed pseudo-first-order rate constant of 4×10^{-5} s^{-1}. The yield of *N*-oxide was determined according to eq 4 from the amount of I_3^- formed and the identity of the oxide

$$R_3NO + 3I^- + 2H^+ \rightarrow R_3N + I_3^- + H_2O \quad (4)$$

affirmed from the rate constant for I_3^- appearance. This same procedure was used to identify the product of the reaction of *N,N*-dimethylbenzylamine with H_2O_2 in *t*-BuOH. The *N*-oxidation product from the reaction of 4a-FlEtOOH with *N*-methylmorpholine in *t*-BuOH was determined as the *N*-oxide of *N*-methylmorpholine essentially as described above for the *N*-oxide of dimethylbenzylamine. In this case, however, 0.4 mL of the *t*-BuOH reaction mixtures was transferred to 3 mL of AcOH 0.1 M in NaI. The *N*-oxide of *N*-methylmorpholine was found to react with NaI under these conditions (30 °C) with an observed pseudo-first-order rate constant of 1.6×10^{-5} s^{-1}.

The yield of hydroxylamine from the reactions of 4a-FlEtOOH with secondary amines was determined by a procedure modified from that reported by Kadlubar, McKee, and Ziegler.[13] In this procedure, 0.2 mL of the reaction mixture is transferred aerobically to 3 mL of *n*-amyl acetate and 3 mL of a 1 M acetate buffer pH 4.6; 1.2 mL of a solution composed of 3.33 mg/mL of bathophenanthroline in *n*-amyl acetate–ethanol (v/v, 15/1) was added; 0.3 mL of 0.01 M $Fe(NO_3)_3$ was added and the mixture was stirred vigorously for 4 min. Then 0.6 mL of 0.01 M EDTA was added and the mixture stirred briefly. The two phases were allowed to separate and the aqueous phase was discarded. The optical density of the red organic layer was read at the position of its visible absorbtion maximum (535 nm) and the yield of hydroxylamine was calculated by assuming 2 reducing equivalents per mole of hydroxylamine using an extinction coefficient of 19600 M^{-1} cm^{-1} for the ferrous bathophenanthroline complex. The hydroxylamines were found to decompose slowly in the final reaction mixtures. Thus, the analysis for hydroxylamine was conducted as soon as possible after 7 half-lives of reaction.

The identity of the N-oxidation products of *N*-methyl-*N*-benzylhydroxylamine was determined in part by examination of the spectral characteristics of the final reaction mixture. The reaction of 4a-FlEtOOH with *N*-methyl-*N*-benzylhydroxylamine in *t*-BuOH was initiated as described above such that the initial concentration of 4a-FlEtOOH was 2×10^{-4} M. Simultaneously, a 2×10^{-4} M solution of 4a-FlEtOH in *t*-BuOH containing the equivalent content of *N*-methyl-*N*-benzylhydroxylamine was prepared. After following the decomposition of the 4a-FlEtOOH to completion, the spectrum of the reaction mixture was scanned to affirm the yield of 4a-FlEtOH. The absorbance of the reaction mixture below 300 nm was too large for direct analysis, so the reaction mixture and the prepared 4a-FlEtOH solution were diluted with *t*-BuOH and the difference spectrum was recorded. The resulting difference spectrum was virtually superimposable on the spectrum of an authentic sample of *N*-methylbenzaldoxime in *t*-BuOH (λ_{max} 292 nm, ϵ_{292} 16000 M^{-1} cm^{-1}). The yield of product was calculated based on the absorbance of the difference spectrum at 292 nm. Additionally, formation of the benzyl nitrone was detected by its hydrolysis to formaldehyde[14] (eq 5) and determination of the formaldehyde produced by means of the

$$C_6H_5CH_2\overset{\overset{O}{\uparrow}}{N}=CH_2 + H_2O \longrightarrow C_6H_5CH_2NHOH + CH_2O \quad (5)$$

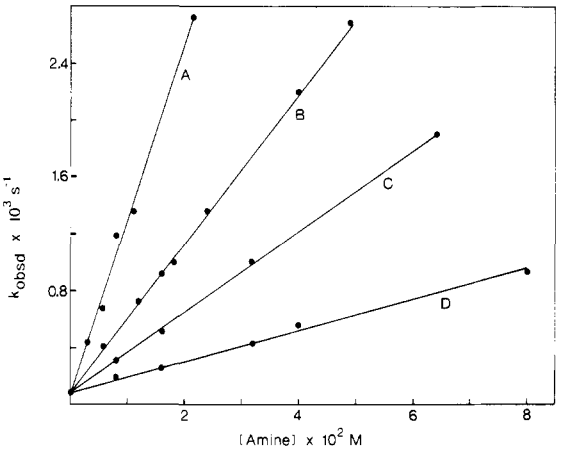


Figure 1. Plots of k_{obsd} for the reactions of 4a-FlEtOOH with amines in *t*-BuOH (30 °C) vs. amine concentration. (A) *N,N*-Dimethylbenzylamine. (B) *N*-Methylbenzylamine. (C) *N*-Methyl-*N*-benzylhydroxylamine. (D) Morpholine.

Hantzch reagent.[14] In this procedure, the entire 3-mL content of the reaction mixture was transferred to 5 mL of H_2O and the excess hydroxylamine removed by extraction with 3–10-mL portions of diethyl ether. (The presence of *N*-methylhydroxylamines interferes with formaldehyde estimation by use of the Hantzch reagent.[14]) The aqueous phase was diluted to 10 mL with Hantzch reagent and the spectrum of the yellow solution recorded. The yield of formaldehyde was calculated from the absorbance of this solution at λ_{max} 412 nm using an extinction coefficient for the 3,5-diacetyl-1,4-dihydrolutidine ϵ_{412} of 8000 M^{-1} cm^{-1}. A small correction was applied for the blank determination.

pK_a's of Amines. The pK_a's of *N,N*-diethylhydroxylamine and *N*-methyl-*N*-benzylhydroxylamine were determined in water by half-neutralization at 30 °C and $\mu \simeq 0.02$. The remainder of the pK_a's were obtained from the literature determinations also in water.

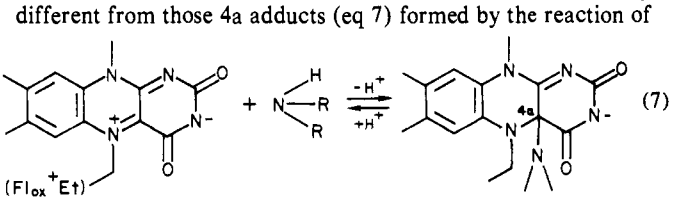
Results

The reactions of 4a-FlEtOOH with secondary, tertiary, and hydroxyl amines were conducted in absolute and oxygen free *t*-BuOH (30 °C) by following the disappearance of 4a-FlEtOOH at 370 nm. Under the experimental conditions of [amine] ≫ [4a-FlEtOOH], the decrease in [4a-FlEtOOH] was first order to at least 3 half-lives of reaction. Plots of the determined pseudo-first-order rate constants (k_{obsd}) vs. the concentration of amine for the reactions of 4a-FlEtOOH with all of the amines investigated were linear with the same positive intercept in k_{obsd} at [amine] = 0 (see Figure 1). Second-order rate constants (k_2) of eq 6 were determined from the slopes of these plots (see Table I). The value of k_1 for spontaneous decomposition of 4a-FlEtOOH was found to be 8×10^{-5} s^{-1}.

$$-d[FlEtOOH]/dt = (k_1 + k_2\,[amine])[4a\text{-}FlEtOOH] \quad (6)$$

$$k_{obsd} = (k_1 + k_2[amine])$$

The flavin product of all the N-oxidation reactions of this study is exclusively 4a-FlEtOH. The 4a-FlEtOH is not a major product of the spontaneous decomposition of 4a-FlEtOOH. In the case of the reactions of 4a-FlEtOOH with secondary amines, the spectra of the final reaction mixtures were virtually superimposable on those of 4a-FlEtOH under identical conditions, but distinctly different from those 4a adducts (eq 7) formed by the reaction of



(7)

the corresponding secondary amines with $Fl_{ox}{}^{+}Et$ ClO_4^- (see Table II).

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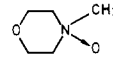
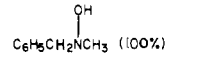
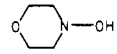
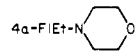
Table I. Second-Order Rate Constants and Product Yields for the Reactions of Amines with 4a-FlEtOOH in Oxygen Free *t*-BuOH (30 °C)

pK_a	amine	k_2 M^{-1} s^{-1}	products	
10.70	triethylamine	4.1×10^{-1}		4a-FlEtOH (100%)
9.97	*N*-methylpiperdine	1.2×10^{-1}		4a-FlEtOH (100%)
9.03	*N,N*-dimethylbenzylamine	1.2×10^{-1}	$C_6H_5CH_2N(O)(CH_3)_2$ (100%),	4a-FlEtOH (100%)
7.57	*N*-methylmorpholine	5.7×10^{-2}	 (100%),	4a-FlEtOH (100%)
5.15	*N,N*-dimethylaniline	9.6×10^{-4}	$C_6H_5N(O)(CH_3)_2$	4a adduct[a]
11.25	di-*n*-butylamine	7.7×10^{-2}	$(C_4H_9)_2NOH$ (100%)	4a-FlEtOH (100%)
10.50	diisobutylamine	1.9×10^{-2}	$(C_4H_9)_2NOH$ (100%)	4a-FlEtOH (100%)
9.42	*N*-methylbenzylamine	3.7×10^{-2}		4a-FlEtOH (100%)
8.60	morpholine	1.05×10^{-2}		4a-FlEtOH (100%)
4.85	*N*-methylaniline	8.6×10^{-4}	$C_6H_5N(OH)-CH_3$ (100%)	4a adduct[a]
5.60	*N,N*-diethylhydroxylamine	6.3×10^{-2}		4a-FlEtOH (100%)
4.60	$C_6H_5CH_2NOHCH_3$	2.8×10^{-2}	$C_6H_5CH{=}N(O)CH_3$ (53%) $C_6H_5CH_2N(O){=}CH_2$ (24%)	4a-FlEtOH (100%)
6.72	2,6-lutidine	$<10^{-4}$	-	-
5.25	pyridine	$<10^{-4}$	-	-

[a] In the cases of aromatic amines, the presence of the excess amine required to facilitate the *N*-oxidation reaction interfered with the direct observation of the spectrum of 4a-FlEtOH from the final reaction mixtures. Thus the flavin product of these reactions was identified as a 4a-adduct by acidification to generate $Fl_{ox}{}^+Et$.

Table II. Spectral Characteristics of 4a-FlEt-X Adducts in *t*-BuOH (30 °C)

4a-FlEt-X	λ_{max}, nm	$E_{\lambda_{max}}$, M^{-1} cm^{-1}
4a-FlEtOOH	365	9000
4a-FlEtOH	352	8000
4a-FlEt-N$(C_4H_9)_2$	349	6400
4a-FlEt-N(isobutyl)$_2$	347	4000[a]
4a-FlEt-N$(CH_3)CH_2C_6H_5$	347	6700
	346	5300

[a] When 6×10^{-4} mm of $Flox^+Et$ ClO_4^- was dissolved in 3 mL of *t*-BuOH 2×10^{-2} M in diisobutylamine, an increasing absorbance ($t_{1/2}$ of 630 s) was observed in the region of 350 nm. The λ_{max} value of 347 nm and the E_{347} nm are reported for the spectrum of the product of that reaction.

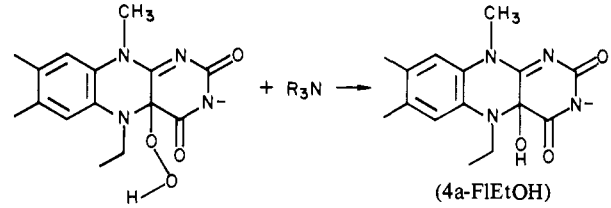
Table III. Product Yields from the Spontaneous (k_1) and Amine-Dependent (k_2[Amine]) Reactions of 4a-FlEtOOH in *t*-BuOH

	[amine] × 10^2 M	product yields, % 4a-FlEtOH[a]	N-oxidation products	k_2[amine]/$k_1 + k_2$[amine][c]
			N-oxide	
N,N-dimethyl-	0	21[b]	0	0
aniline	6.7	46	50	44
	16.7	-	68	67
	33.3	85	77	80
	66.7	100	80	88
			hydroxylamine	
diisobutyl-	0.4	60	53	49
amine	0.8	74	64	66
	1.6	89	79	79
	3.2	100	87	88

[a] The yield of 4a-FlEtOH includes that initially present in the ~90% pure sample of 4a-FlEtOOH. In the remainder of the flavin product, the isoalloxazine ring system has been destroyed. [b] In the case of the spontaneous 4a-FlEtOOH decomposition, the yield of 4a-FlEtOH includes 10% of FlEt. [c] Calculated yield of N-oxidation products, %.

It was further observed that in the reaction of 4a-FlEtOOH with secondary, tertiary, or hydroxyl amine, the absorbance of the reaction mixtures at 370 nm after 7 half-lives of reaction drifted slowly downward. The 4a-FlEtOH shows a similar decrease in absorbance at 370 nm in *t*-BuOH containing a secondary, tertiary, or hydroxyl amine. In contrast, the absorbances at 370 nm of the secondary amine adducts of $Fl_{ox}{}^+Et$ in *t*-BuOH are far more stable. Thus, the flavin product of the *N*-oxidation reactions of 4a-FlEtOOH with secondary amines exhibits behavioral characteristics of 4a-FlEtOH and not secondary amine adducts of $Fl_{ox}{}^+Et$. When an attempt was made to determine the spectrum of 4a-FlEt-$ON(C_2H_5)_2$ in *t*-BuOH by mixing $Fl_{ox}{}^+Et$ ClO_4^- with excess $(C_2H_5)_2NOH$, the formation of the radical of N^5-ethyl-3-methyllumiflavin (FlEt·) occurred rapidly and in ~74% yield. The final spectrum of the reaction mixture of 4a-FlEtOOH with $(C_2H_5)_2NOH$ was found to have an absorption maximum at 352 nm indicative of 4a-FlEtOH and virtually no absorbance at 640 nm where the FlEt· absorbs (ϵ 5000 M^{-1} cm^{-1} in *t*-BuOH). Similar results were obtained for *N*-methyl-*N*-benzylhydroxylamine.

In the case of the reactions of 4a-FlEtOOH with tertiary amines, the *N*-oxidation products found were tertiary amine *N*-oxides (see eq 8). The 4a-FlEtOOH reacted with secondary

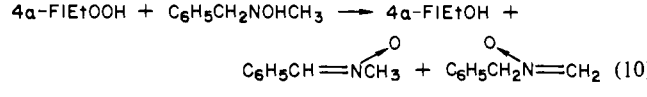


(8)

amines to form the corresponding hydroxylamines (eq 9). With

$$4a\text{-FlEtOOH} + R_2NH \rightarrow 4a\text{-FlEtOH} + R_2NOH \quad (9)$$

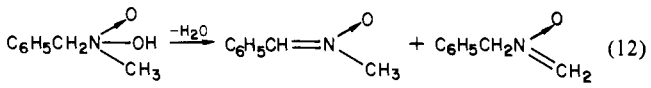
N-methyl-*N*-benzylhydroxylamine, the 4a-FlEtOOH reacts to form a mixture of nitrones (eq 10). The alternate nitrones can



(10)

occur via dehydration of a common intermediate (eq 11 and 12).[14]

(11)



(12)

The primary amines *n*-butylamine and benzylamine were found to react with 4a-FlEtOOH much more slowly than secondary amines of comparable pK_a's. The disappearance of 4a-FlEtOOH in the reactions was not first order under pseudo-first-order conditions, nor was 4a-FlEtOH a major product. No further investigation of these reactions was made. The spontaneous decomposition of 4a-FlEtOOH was enhanced by either pyridine or 2,6-lutidine with k_{obsd} for the reaction 1.4×10^{-4} s^{-1} independent of amine concentration over the range 4×10^{-3} M–3.2×10^{-1} M (compare with 8×10^{-5} s^{-1}). The 4a-FlEtOH was not a product of the reaction.

It was found that the reactions of 4a-FlEtOOH with *N,N*-dimethylbenzylamine and with *N*-methylbenzylamine did not exhibit any trend toward saturation kinetics up to k_{obsd} values corresponding to $t_{1/2}$ of $\simeq$ 30 s. Inclusion of 4×10^{-3} M–3.2×10^{-1} M 2,6-di-*tert*-butyl-4-methylphenol (BHT) into the *t*-BuOH reaction mixtures with *N,N*-dimethylbenzylamine, *N*-methylbenzylamine, or *N,N*-diethylhydroxylamine did not affect the rate of 4a-FlEtOOH decomposition, nor did it alter the yield of 4a-FlEtOH. It was also found that the inclusion of 4×10^{-2} M BHT into the reaction of 1.6×10^{-2} M *N*-methylbenzylamine with 1×10^{-4} M 4a-FlEtOOH did not alter the yield of hydroxylamine (100%). When 4a-FlEtOO$^-$ was generated in *t*-BuOH containing *N,N*-dimethylaniline by addition of *t*-BuOK ([*t*-BuOK] = [4a-FlEtOOH]), formation of the *N*-oxide of *N,N*-dimethylaniline was not observed. The dissolution of solid NaO_2 in *t*-BuOH 1 M in *N,N*-dimethylaniline under anaerobic conditions also did not lead to the formation of *N*-oxide.

Analyses for the N-oxidation products of *N,N*-dimethylaniline and of diisobutylamine and for 4a-FlEtOH from the N-oxidation reactions with 4a-FlEtOOH were conducted as a function of amine concentration (see Experimental Section). Under conditions such that k_2[amine] of eq 6 approaches k_1 for spontaneous 4a-FlEtOOH decomposition, the yield of N-oxidation products and of 4a-FlEtOH was found to decrease accordingly (see Table III). Inspection of Table III indicates that the percent yield of the N-oxidation products may be calculated on the assumption that the N-oxidation products arise from the bimolecular reaction of 4a-FlEtOOH with amine.

The reactions of the tertiary amine *N,N*-dimethylbenzylamine and the secondary amine *N*-methylbenzylamine with 4a-FlEtOOH were also conducted in the aprotic solvent dioxane. Under the experimental conditions of [amine] $\gg$ [4a-FlEtOOH], the disappearance of 4a-FlEtOOH was pseudo first order to at least 3 half-lives of reaction. Plots of the determined pseudo-first-order rate constants (k_{obsd}) vs. the concentration of amine were linear with the same positive intercept in k_{obsd} at [amine] = 0. Second-order rate constants k_2 of eq 6 determined from the slopes of these plots were found to be 4.7×10^{-2} and 9.5×10^{-3} M^{-1} s^{-1} for the reactions of 4a-FlEtOOH with *N,N*-dimethylbenzylamine and *N*-methylbenzylamine, respectively. The k_1 value of eq 6 for spontaneous decomposition of 4a-FlEtOOH in dioxane was found to be 2.5×10^{-5} s^{-1}.

The reaction of *N,N*-dimethylbenzylamine with H_2O_2 was conducted in oxygen-free *t*-BuOH by observing the decrease in H_2O_2 iodometrically (see Experimental Section) under the conditions of [amine] $\gg$ [H_2O_2]. A plot of the determined pseudo-first-order rate constants (k_{obsd}) vs. the concentration of amine was linear with positive intercept with k_{obsd} at [amine] = 0 (Figure 2). The second-order rate constant k_2 of eq 13 determined from

$$-d[H_2O_2]/dt = (k_1 + k_2[\text{amine}])[H_2O_2] \quad (13)$$

$$k_{obsd} = (k_1 + k_2[\text{amine}])$$

the slope of this plot was found to be 3.3×10^{-6} M^{-1} s^{-1}. The k_1 value of eq 13 for spontaneous H_2O_2 decomposition in *t*-BuOH was found to be less than 2×10^{-7} s^{-1}. The *N*-oxide of *N,N*-dimethylbenzylamine was identified as the product of the reaction in 90% yield. The reaction of *t*-BuOOH with *N,N*-dimethylbenzylamine in oxygen-free *t*-BuOH was also followed iodometrically under the conditions of [amine] $\gg$ [hydroperoxide]. However, the reaction is at least an order of magnitude slower than the N-oxidation of *N,N*-dimethylbenzylamine in *t*-BuOH by H_2O_2 and thus too slow for further investigation.

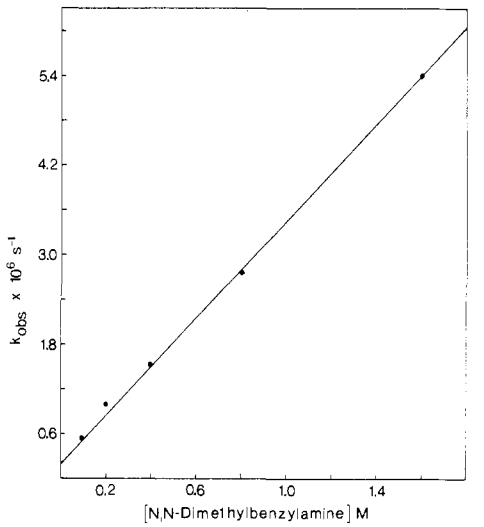


Figure 2. A plot of k_{obsd} for the reactions of H_2O_2 with *N,N*-dimethylbenzylamine in *t*-BuOH (30 °C) vs. concentration of *N,N*-dimethylbenzylamine.

Discussion

The 4a-FlEtOOH reacts with secondary, tertiary, and hydroxyl amines in *t*-BuOH to produce the same N-oxidation products found in the enzymic oxidation of these amines by the hepatic flavoprotein microsomal oxidase.[14,16] In the case of tertiary amines, the N-oxidation products are the tertiary amine N-oxides. With secondary amines, the corresponding hydroxylamines are formed. The similarity of the model and enzymc reactions is also seen in the ratios of nitrone products obtained in the N-oxidation of *N*-methyl-*N*-benzylhydroxylamine (eq 10). The ratio of the nitrones obtained in the enzymatic reaction was 3:1 in favor of the more stable nitrone isomer. The less stable benzyl nitrone hydrolyzes to formaldehyde and was identified as such.[14] The ratio of nitrones obtained by 4a-FlEtOOH oxidation in *t*-BuOH is 2.2:1 in favor of the more stable nitrone isomer (see Table I). Aliphatic primary amines are not oxidized by the hepatic flavoprotein microsomal oxidase.[16] Primary amines in *t*-BuOH destroy the isoalloxazine ring system of the 4a-FlEtOOH in a reaction slower than that seen with secondary and tertiary amines and that is not first order under the conditions of [amine] $\gg$ [4a-FlEtOOH].

To assess the change in reactivity of the 4a-FlEtOOH toward N-oxidation to changes in structure of the amine, a plot of the logarithm of the second-order rate constants (k_2) for the reactions of 4a-FlEtOOH with amines in *t*-BuOH vs. pK_a of the amines in H_2O was constructed (see Figure 3). Inspection of Figure 3 indicates that the relative reactivity of the 4a-FlEtOOH toward N-oxidation of amines of comparable pK_a's is hydroxylamines > tertiary amines > secondary amines. A small α effect[18] is observed for the reactions of 4a-FlEtOOH with hydroxylamines. This same order of reactivity has been found for other nucleophilic dis-

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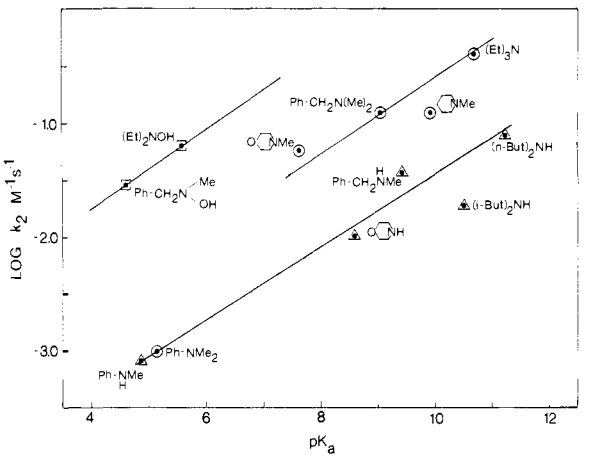


Figure 3. Plots of log k_2 for the reactions of 4a-FlEtOOH with amines in *t*-BuOH (30 °C) vs. pK_a of the amine in H_2O.

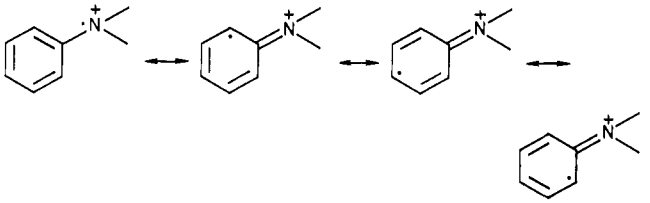
placement reactions.[19] The change in reactivity of the amine with 4a-FlEtOOH with change in pK_a indicated by a β_{nuc} value of ~0.3 for the reactions is also consistent with a nucleophilic displacement reaction.[19]

Dioxygen transfer from 4a-FlEtO$_2^-$ to the anions of 2,6-di-*tert*-butyl-4-methylphenol, 3,5-di-*tert*-butylcatechol, 10-ethoxy-9-phenanthrol, and 10-methyl-9-phenanthrol has been reported.[20,21] However, the decomposition of 4a-FlEtO$_2^-$ in *t*-BuOH containing *N,N*-dimethylaniline does not result in the formation of *N*-oxide. The equilibrium of eq 14 is unimportant in the present investi-

$$\text{4a-FlEtOOH} + R_3N \rightleftarrows \text{4a-FlEtO}_2^- + R_3\overset{+}{N}H \quad (14)$$

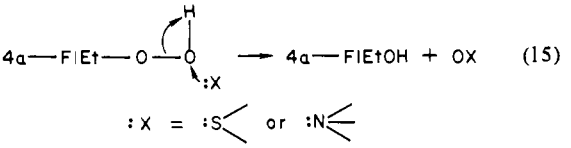
gations. This conclusion is based on the fact that the first-order rate constant for decomposition of 4a-FlEtO$_2^-$ in *t*-BuOH (4.6×10^{-2} s^{-1} at 30 °C)[21] greatly exceeds the k_{obsd} values of this study for the reactions of amines with flavin peroxide. If appreciable concentrations of peroxide anion were generated via eq 14, then the amines would have acted as catalysts for the 4a-FlEtOOH decomposition. This is not the case since disappearance of 4a-FlEtOOH due to its bimolecular reaction with amine is accompanied by *N*-oxidation (Table III). That the equilibrium of eq 14 must lie far to the left in *t*-BuOH (dielectric constant = 11) is not surprising since the separation of charge becomes less favorable as the polarity of the medium decreases.

The involvement of free radicals in the N-oxidation reactions of 4a-FlEtOOH was assessed by conducting the reactions in *t*-BuOH containing the free radical inhibitor BHT. No effect on the N-oxidation reactions was found. The observation that NaO_2 does not N-oxidize *N,N*-dimethylaniline suggests that $O_2^-\cdot$ is not involved in the N-oxidation reactions. Furthermore, the relative reactivities of amines toward N-oxidation by 4a-FlEtOOH parallels nucleophilicities of the amines rather than cation radical stability. Thus, N-oxidation of *N,N*-dimethylbenzylamine is more than two orders of magnitude more reactive than *N,N*-dimethylaniline toward N-oxidation by 4a-FlEtOOH. Yet, because of resonance stabilization of the cation radical of *N,N*-dimethylaniline (see below), the order of stabilities of the cation



radicals in *t*-BuOH should be reversed. That is, the cation radical of *N,N*-dimethylaniline is expected to be more stable in *t*-BuOH than the cation radical of *N,N*-dimethylbenzylamine. This is clearly not consistent with a radical mechanism for the N-oxidation reactions. Thus it appears that caged radicals, as well as free radicals, are not involved in the N-oxidation of amines by 4a-FlEtOOH.

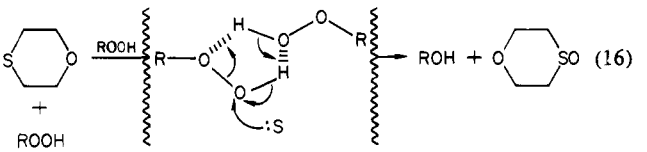
The N-oxidation of amines by 4a-FlEtOOH (eq 15) resembles



$$\text{4a—FlEt—O—O(H):X} \longrightarrow \text{4a—FlEtOH} + \text{OX} \quad (15)$$

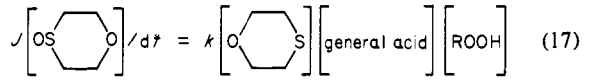
$$:X = :S< \text{ or } :N<$$

the *S*-oxidation of thioxane by hydroperoxides. Both represent, overall, nucleophilic displacement upon the terminal oxygen of the hydroperoxide. Edwards observed that the oxidations of thioxane by *t*-BuOOH and by hydrogen peroxide in the aprotic solvent dioxane are second order in these hydroperoxide species.[22] The second molecule of hydroperoxide was thought to serve as an essential proton source (eq 16). Edwards also found that an



$$\text{ROOH} + \text{ROOH} \longrightarrow \text{ROH} + \text{SO} \quad (16)$$

alcohol or carboxylic acid could replace the second molecule of hydroperoxide in eq 16. Thus the rate of S-oxidation of thioxane by hydroperoxides is given by eq 17. The N-oxidation reactions



$$-d[\text{OSO}]/dt = k[\text{OS}][\text{general acid}][\text{ROOH}] \quad (17)$$

of 4a-FlEtOOH with *N,N*-dimethylbenzylamine and *N*-methylbenzylamine in absolute dioxane are first order in 4a-FlEtOOH and plots of k_{obsd} vs. [amine] are linear. The S-oxidation of thioxane by 4a-FlEtOOH in absolute dioxane is also first order in 4a-FlEtOOH.[5] The lack of a requirement for general acid catalysis in the N-oxidation and S-oxidation reactions of 4a-FlEtOOH is attributed to the much greater oxygen transfer potential of the 4a-FlEtOOH compared with *t*-BuOOH and H_2O_2. Thus, the ratio of second-order rate constants for the N-oxidation of *N,N*-dimethylbenzylamine in *t*-BuOH is 4a-FlEtOOH: H_2O_2 = 3.6×10^4 : 1. The N-oxidation of *N,N*-dimethylbenzylamine by *t*-BuOOH is at least an order of magnitude slower than the N-oxidation by H_2O_2. The ratio of second-order rate constants for the S-oxidation of thioxane in CH_3OH is 4a-FlEtOOH:H_2O_2:*t*-BuOOH = 2×10^5:20:1.[4b]

In summary, we have shown that 4a-FlEtOOH reacts with secondary, tertiary, and hydroxyl amines in *t*-BuOH to produce the same N-oxidation products found in the enzymic oxidation of these amines by the hepatic flavoprotein microsomal oxidase. The flavin product of the N-oxidation reactions is 4a-FlEtOH. In the case of tertiary amines, the N-oxidation products are the tertiary amine N-oxides. With secondary amines, the corresponding hydroxylamines are formed. *N*-Methyl-*N*-benzylhydroxylamine is N-oxidized to a mixture of nitrones which can be attributed to dehydration of a common intermediate formed by N-oxidation of the parent hydroxylamine. It is suggested that the N-oxidation of amines by 4a-FlEtOOH occurs by a mechanism which involves nucleophilic displacement by sp^3-hybridized nitrogen on the terminal oxygen of the hydroperoxide. The efficiency of the 4a-FlEtOOH in the N-oxidation of *N,N*-dimethylbenzylamine is more than four orders of magnitude greater than the N-oxidizing ability of H_2O_2 and *t*-BuOOH.

Acknowledgment. This work was supported by a grant from the National Institutes of Health. We gratefully acknowledge the technical assistance of Eileen Wynne.

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