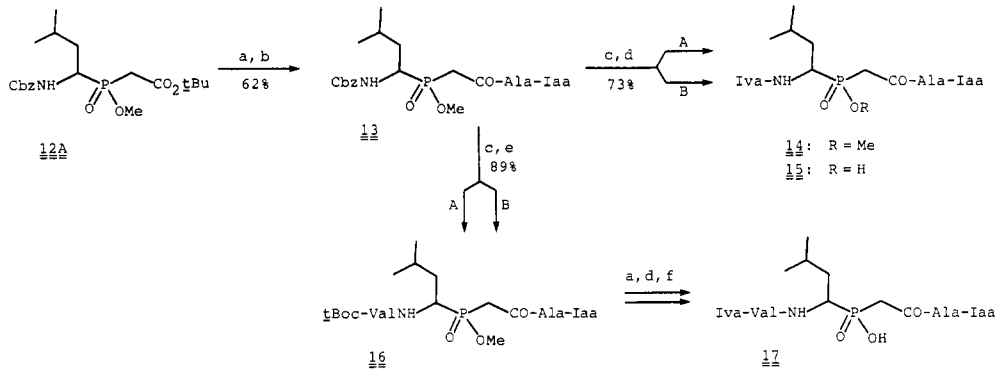
Scheme II[a]



[a] (a) TFA, 21 °C; (b) Ala-Iaa, HOBT, DCC, CH_2Cl_2, 21 °C; (c) TsOH, H_2/Pd-C, MeOH, 21 °C; (d) IvaOH, DCC, Et_3N, CH_2Cl_2/DMF, 21 °C; (e) *t*-Boc-ValOH, DCC, Et_3N, DMF, CH_2Cl_2/DMF, 21 °C; (f) LiS-*n*-Pr, HMPA (ref 9).

Table II. Binding of Tetrapeptide Analogues to Pepsin

inhibitor	K_D, nM	$t_{1/2}$ (→)	K_i, nM
Iva-Val-D-Sta^P-Ala-Iaa (**17A**)	*d*		200[a]
Iva-Val-L-Sta^P-Ala-Iaa (**17B**)	7	115 min	<0.07[b]
Iva-Val-L-Sta-Ala-Iaa (**5**)	60	<10 s	1.1[c]
Iva-Val-L-Sto-Ala-Iaa (**4**)	*d*		56[e]
Iva-Val-Val-L-Sta-Ala-Sta (Pepstatin, **3**)	13	~30 s	0.046[f]

[a] Determined at 37 °C at pH 3.5 (0.1 M NaOAc) with Z-His-*p*NO_2Phe-Phe-OMe as substrate. [b] As *a* with Lys-Pro-Ala-Glu-Phe-*p*NO_2Phe-Arg-Leu as substrate (ref 10). [c] Reference 11. [d] Slow binding not observed. [e] Reference 4. [f] Reference 12.

the L configuration; in contrast, the longer IvaValNH moiety appears to be restricted to one extended pocket.

The inhibition observed with **17B** is not of the simple competitive type, however, as a marked increase in the degree of inhibition occurs during the course of a 10- or 15-min assay. Such behavior is frequently observed with tightly-bound inhibitors of pepsin and has been well-characterized by Rich and his co-workers as involving the two-stage association sequence represented by eq 1.[11]

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E{\cdot}I \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E{\cdot}I^* \quad (1)$$

$$K_D = k_2/k_1 \quad (2)$$

$$K_i = \frac{k_2}{k_1}\frac{k_4}{(k_3 + k_4)} \quad (3)$$

From the chemical nature of the phosphinic acid moiety, we expect that **17B** is binding to the enzyme in a reversible and noncovalent fashion. Accurate determination of its binding affinity is not straightforward, however. The binding constant K_D for the "loose" complex E·I can be readily determined by steady-state methods in the case of inhibitor **17B**, since the rate of isomerization to the "tightened" complex is very slow ($t_{1/2} \approx 2$ h). By treatment of the isomerization of E·I ⇌ E·I* as an irreversible process, it is possible to determine a value for k_3 of 0.36 h^{-1} ($t_{1/2}$ = 115 min) at 37 °C and a ratio of $k_4/k_3 < 0.01$.[13] If the binding process is in fact reversible, the overall K_i must therefore be less than 70 pM.

Although an accurate determination of k_4 (and thus K_i) is not possible without radiolabeled material, it is clear that the phosphinate **17B** is an exceedingly potent inhibitor of pepsin, approaching the affinity of pepstatin itself (Table II). The use of phosphorus analogues to mimic tetrahedral intermediates therefore appears to be an effective strategy for inhibition of the aspartic peptidases as well as the zinc and serine peptidases. We hope to be able to extend these results to related enzymes of this class and to probe the nature of the exceedingly slow-binding transition.

Acknowledgment. We thank Professor B. M. Dunn and Dr. Robert M. Scarborough for generous gifts of the octapeptide substrate and Professor D. H. Rich for helpful discussions. Support for this research was provided by a grant from the National Institutes of Health (CA-22747).

Supplementary Material Available: Experimental procedures for the preparation of compounds **8–17** and description of inhibitor assay procedures (12 pages). Ordering information is given on any current masthead page.

(11) Rich, D. H.; Sun, E. T. O. *Biochem. Pharmacol.* **1980**, *29*, 2205–2212. Rich, D. H.; Sun, E. T. O.; Ulm, E. *J. Med. Chem.* **1980**, *23*, 27–33. Rich, D. H.; Bernatowicz, P. G. *Ibid.* **1982**, *25*, 791.

(12) Workman, R. J.; Burkitt, D. W. *Arch. Biochem. Biophys.* **1979**, *194*, 157–164.

(13) On incubation of the enzyme at 50 nM and inhibitor **17B** at 100 nm (=14K_D) concentrations, the enzyme is present initially as E·I, isomerizing with time to an equilibrium mixture of E·I and E·I*. The extent of isomerization can be determined by removing aliquots and diluting them 100-fold into excess octapeptide substrate at 250 μM (=5K_m) concentration. E·I dissociates relatively rapidly, whereas E·I* does not, hence the enzymatic activity observed in the diluted aliquot reflects how much of the enzyme has *not* been transformed into E·I*. After 30 h, when equilibrium has been reached between E·I and E·I*, less than 1% of the control activity recovers on dilution, suggesting that $k_4/(k_3 + k_4) \approx k_4/k_3 < 0.01$.

Iron(II)-Induced Activation of Hydrogen Peroxide to Ferryl Ion (FeO^{2+}) and Singlet Oxygen ($^1O_2$) in Acetonitrile: Monoxygenations, Dehydrogenations, and Dioxygenations of Organic Substrates

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Received April 27, 1984
Revised Manuscript Received May 30, 1984

Although activation of H_2O_2 by iron(II) (Fenton chemistry) has been thoroughly characterized in aqueous media[1] and shown

$$Fe(II) + H_2O_2 \xrightarrow{H_2O} Fe^{III}(OH^-) + {\cdot}OH \quad (1)$$

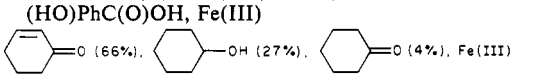
to have substrate reactions that are identical with those for hydroxyl radical (·OH),[1,2] the nature of this system in an anhydrous, noncomplexing solvent has not been evaluated. Here we report that the slow addition of dilute H_2O_2 (in dry acetonitrile (MeCN)) to a solution that contains iron(II) and an organic substrate (RH) in dry MeCN (<0.005% H_2O) results in the monooxygenation or dehydrogenation of RH. Table I compares the products that result from the Fe(II)–H_2O_2–RH/MeCN system with those from

(1) Walling, C. *Acc. Chem. Res.* **1976**, *9*, 175.

(2) Dorfman, L. M.; Adams, G. E. "Reactivity of the Hydroxyl Radical in Aqueous Solutions"; NSRDS-NBS 46, SD Catalog No. 13.48:46, U.S. Department Printing Office: Washington, DC; June, 1978.

0002-7863/84/1506-4283$01.50/0

Table I. Products from the Iron(II)-Induced Oxygenation/Dehydrogenation of Organic Substrates (RH) by H_2O_2 in Dry Acetonitrile and, for Comparison, the Products for the Fe(II)/H_2O_2/RH Systems under Aqueous Conditions (Fenton Chemistry)

	MeCN[a]		H_2O (pH 4)[a,b,c]	
substrate	reactn[d] efficiency, %	products[e]	reactn[d,f] efficiency, %	products
blank (H_2O_2)	100	O_2, H_2O, Fe(II)	100	Fe(III)
Ph_3P	100	Ph_3PO	NR (insol)	Fe(III)
Me_2SO	100	Me_2SO_2	100	CH_4, C_2H_6, MeOH, Me_2SO_2, others,[g] Fe(III)
Ph_2SO	100	Ph_2SO_2	NR (insol)	Fe(III)
EtOH	70	MeCH(O) (90%), MeC(O)OH (10%), O_2	100	MeCH(O) (4%), many others (96%), Fe(III)
$PhCH_2OH$	100	PhCH(O)	20 (hetero)	PhCH(O) (70%), 4 others (30%), Fe(III)
c-$C_6H_{11}OH$	47	$C_6H_{10}(O)$, O_2	44	$C_6H_{10}(O)$ (70%), several $C_6H_{10}(OH)_2$ (30%), Fe(III)
MeCH(O)	20	MeC(O)OH, O_2	100	many products,[h] MeC(O)OH (minor), Fe(III)
$Me_2C(O)$	NR	O_2	100	many products, Fe(III)
PhCH(O)	28	PhC(O)OH, O_2	17 (hetero)	black tar, PhC(O)OH, (HO)PhCH(O), (HO)PhC(O)OH, Fe(III)
cyclohexene	NR	O_2	20	
1,4-c-C_6H_8	59	PhH, O_2	50 (hetero)	PhH (60%), Ph(OH) (40%), Fe(III)
PhNHNHPh	100	PhN=NPh	50 (hetero)	polymers, PhN=NPh, (HO)PhN=NPh(OH), (HO)PhN=NPh, PhOH, Fe(III)
PhI	NR	O_2	10 (hetero)	(HO)PhI, Fe(III)
H_2S	100[i]	H_2SO_4	100	S_8(s), Fe(II)
H_2O (56 mM)	100	Fe(III)		

[a] Product solution [from the slow addition (~5 min to give a final 2 mM concentration) of 1 M H_2O_2 (98% H_2O_2 in MeCN) to a solution of 1 mM $Fe^{II}(ClO_4)_2$·4MeCN plus 2 mM substrate] analyzed by gas chromotography and assayed for residual Fe(II) by MnO_4^- titration and by colorimetry with 1,10-*o*-phenanthroline. [b] Radical species indicated as the primary product on the basis of identified stable secondary products and their consistency with aqueous Fenton and ·OH radical studies.[1,2] [c] The same products result with a 90:10 MeCN:H_2O solvent. [d] 100% represents one substrate oxygenation or dehydrogenation per H_2O_2 added to the system (oxidation in the case of the aqueous systems); NR, less than 2% substrate reaction within 5 min. [e] The Fe(II) remains unoxidized in the absence of H_2O or other complexing solvents and substrates. The Fe(II)–H_2O_2 system does not attack the solvent within the 5-min duration of the experiments, and control experiments of O_2 plus Fe(II) without H_2O_2 and of H_2O_2 without Fe(II) result in less than 2% reaction for each substrate. [f] (insol), substrate insoluble; (hetero), heterogeneous reaction matrix. [g] Reference 3. [h] Including $[CH_2CH(O)]_2$, $[CH_2C(O)OH]_2$, and (O)$CHCH_2CH_2C(O)OH$. [i] 100%, one H_2S converted to H_2SO_4 per four H_2O_2 added.

aqueous Fenton chemistry for several organic substrates (the Fe(II) is oxidized via eq 1 and by ·OH).[1-3]

In sharp contrast, the products for the Fe(II)–H_2O_2–substrate systems in dry MeCN are characteristic of monoxygenase or dehydrogenation reactions. The total absence of products from ·OH radical chemistry and of Fe(III) in the product solutions confirms that reaction 1 does not occur in dry MeCN (with H_2O >10%, products are the same as for aqueous Fenton chemistry (Table I)).

Reaction 1 does not occur in dry acetonitrile because the reduction potential for the Fe(III)/Fe(II) couple is +1.62 V vs. NHE, rather than +0.4 V (H_2O at pH 7). With H_2O (pH 7) reaction 1 is favored by 0.1 V ($H_2O_2 + e^- \rightarrow OH^- + \cdot OH$; $E^{\circ\prime}$ +0.51 V). In contrast to the facile Fe(II)–H_2O_2 reaction, the combination of Fe(II) and Me_3COOH in dry MeCN is essentially unreactive.

In the absence of substrate in dry MeCN, iron(II) catalyzes the rapid disproportionation of H_2O_2 to O_2 and H_2O but remains unoxidized (Table I). This is further confirmation that H_2O_2 cannot oxidize Fe(II) in MeCN via a one-electron process (eq 1). Hence, the facile redox reactivity between Fe(II) and H_2O_2 must involve a two-electron (oxygen atom transfer) mechanism to produce ferryl ion (FeO^{2+}) in the primary step

$$Fe(II) + H_2O_2 \rightarrow FeO^{2+} + H_2O \quad (2)$$

$$FeO^{2+} \equiv [Fe^{II}(O) \leftrightarrow Fe^{IV}{=}O \leftrightarrow Fe^{III}(O^{-}\cdot)]$$

with ferryl ion depicted as a resonance hybrid of three iron oxidation states (the structure of ferryl is not known, FeO^{2+} is a convenient symbol to represent $Fe(H_2O_2)^{2+}$).

The rapid decomposition of H_2O_2 in MeCN that is induced by trace levels of Fe(II) is consistent with a rapid two-electron oxidation of H_2O_2 by FeO^{2+} (Table I).

$$FeO^{2+} + H_2O_2 \rightarrow Fe(II) + O_2 + H_2O \quad (3)$$

The controlled introduction of dilute H_2O_2 into a Fe(II) (1 mM)–substrate (2 mM) solution limits the concentration of H_2O_2 and ensures that a ferryl–substrate reaction can be competitive with reaction 3.

The results in Table I strongly support the conclusion that the FeO^{2+} species in dry MeCN has the characteristics of $Fe^{II}(O)$ and acts as a monoxygenase or dehydrogenase.[4]

$$FeO^{2+} + RH \rightarrow ROH + Fe(II) \quad (4a)$$

$$FeO^{2+} + 1,4\text{-cyclohexadiene} \rightarrow PhH + H_2O + Fe(II) \quad (4b)$$

The presence of 2% H_2O in MeCN shifts the redox potential for the Fe(III)/Fe(II) couple to a much less positive value and makes reaction 1 feasible. Such water levels also may promote the hydrolysis of ferryl ion

$$FeO^{2+} + H_2O \rightarrow Fe^{III}(OH)^{2+} + \cdot OH \quad (5)$$

Although only cyclohexanone is produced from cyclohexanol under anhydrous conditions, the presence of 1–5% H_2O (or a large excess of substrate) yields a spectrum of hydroxylated products (analogous to a previous study).[5]

Singlet Oxygen. Table II summarizes the reaction efficiencies and dioxygenated products that result from the addition of 1 mM $Fe^{II}(ClO_4)$·4MeCN to a solution of 2 mM H_2O_2 and 2 mM substrate (RH) in dry MeCN. The results indicate that the dioxygen product of reaction 3 [or the $Fe(H_2O_2)_2^{2+}$ species] has the reactivity of the singlet ($^1\Delta_g$) state; in the absence of substrate the overall stoichiometry is consistent with the summation of reactions 2 and 3.

$$2H_2O_2 \xrightarrow{Fe(II)} 2H_2O + {}^1O_2 \quad (6)$$

The extensive reactivity of diphenylisobenzofuran (DPBF), 9,10-diphenylanthracene, and rubrene to form exclusively dioxygenated products is consistent with the conclusion that the Fe(II)–H_2O_2 system produces singlet oxygen.[6]

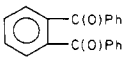
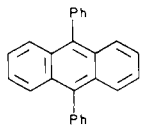
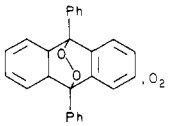
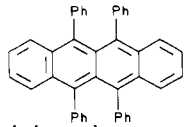
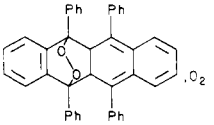
(3) Gilbert, B. C.; Norman, R. O. C.; Sealy, R. C. *J. Chem. Soc., Perkins Trans. 2* **1975**, 303.

(4) For those substrates that react stoichiometrically with the Fe(II)/H_2O_2 system, reaction 4 is dominant and O_2 is not produced. The absence of O_2 precludes the possibility of a transient Fe(III) species that is reduced by H_2O_2.

(5) Groves, J. T.; VanDerPuy, M. *J. Am. Chem. Soc.* **1976**, *98*, 5290.

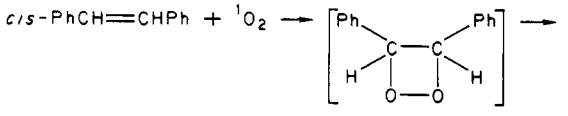
(6) Foote, C. S. In "Biochemical and Clinical Aspects of Oxygen"; Caughey, W. S., Ed.; Academic Press: New York, 1979; pp 603–626.

Table II. Products and Conversion Efficiencies for the Iron(II)-Induced Dioxygenation of Organic Substrates (RH) by H_2O_2 in Dry Acetonitrile[a]

substrate (RH)	reactn[b] efficiency, %	products
blank (H_2O_2)	100	O_2, Fe(II)
	100	
	69	
	83	
cholesterol	NR	insoluble, O_2
$Ph_2C{=}CPh_2$	22	$Ph_2C(O)$, O_2
PhC≡CPh	42	PhC(O)C(O)Ph, O_2
PhC≡CMe	26	PhC(O)C(O)Me, O_2
PhC≡CH	12	PhC(O)CH(O), O_2
c-PhCH=CHPh	52	PhCH(O) (98%), PhC≡CPh (2%)[c], O_2
t-PhCH=CHPh	28	PhCH(O), O_2
PhCH=CHMe	32	PhCH(O) + MeCH(O) (85%), $\overline{PhCHCHOMe}$ (15%),[d] O_2

[a] Product solution [from the slow combination of 0.5 mM $Fe^{II}(ClO_4)_2 \cdot 4MeCN$ with 1 mM H_2O_2 (98%) and 1 mM substrate] analyzed by gas chromatography and assayed for residual Fe(II) by colorimetry with 1,10-*o*-phenanthroline. [b] 100%, one substrate dioxygenation per two H_2O_2 molecules added; NR, less than 2% substrate reaction within 5 min; for each reaction system the Fe(II) remains in its reduced state. Controls with Fe(II)/O_2/RH and H_2O_2/O_2/RH systems react less than 2% within 5 min. [c] Produced by the dehydrogenation of PhCH=CHPh by the FeO^{2+} intermediate of the Fe(II)–H_2O_2 process. With 100 mM Fe(II) and 20 mM PhCH=CHPh to which 20 mM H_2O_2 is added slowly, 43% of the product is PhC≡CPh [60% (FeO^{2+} + RH] process]. [d] Produced by the monoxygenation of PhCH=CHMe by the FeO^{2+} intermediate of the Fe(II)–H_2O_2 process. With 100 mM Fe(II) and 20 mM PhCH=CHMe to which 1 mM H_2O_2 is added slowly, 23% of the product is the epoxide [37% (FeO^{2+} + RH) process].

The other substrates (Table II) also undergo an initial dioxygenation that is characteristic of $^1O_2$. With *cis*-PhCH=CHPh, 0.26 mM of it is dioxygenated to give 0.52 mM PhCH(O); the stoichiometry of eq 6 indicates that 1.0 mM H_2O_2 yields, at most, 0.50 mM $^1O_2$. Hence, the reaction efficiency is 52% for a one-to-one $^1O_2$–substrate dioxygenation via a dioxetane intermediate (characteristic of singlet oxygen reactions with π-electron-rich unsaturated carbon–carbon bonds).[1,2]



2PhCH(O) (7)

trans-Stilbene is less reactive with the Fe(II)/H_2O_2 system, which is consistent with singlet oxygen chemistry.[6] For conditions where $^1O_2$ production is limiting,[7] the use of deuterated acetonitrile (MeCN-d_3) enhances the reaction efficiencies for the Fe(II)/H_2O_2–*cis*-PhCH=CHPh and –PhC≡CPh oxygenations by factors of 1.4 and 1.5, respectively.[8]

Formation of $^1O_2$ from H_2O_2 requires a two-electron oxidant that either transfers a hydride ion in a single step

$$FeO^{2+} + HOOH \rightarrow [Fe(OH)^+ \overline{O}\text{–}\overline{O}^+H] \rightarrow Fe(II) + H_2O + {}^1O_2 \quad (8a)$$

or a H atom followed by a radical electron within the reaction complex

$$FeO^{2+} + HOOH \rightarrow [Fe(OH)^{2+} \cdot \overline{O}\text{–}\overline{O}H] \rightarrow Fe(OH)^+ \overline{O}\text{–}\overline{O}^+H \rightarrow Fe(II) + H_2O + {}^1O_2 \quad (8b)$$

The second step is similar to the previously demonstrated oxidation by ferrocenium of superoxide to singlet oxygen.[3] Because any leakage of $HO_2\cdot$ from the solvent cage of the two-step process (eq 8b) and subsequent disproportionation to H_2O_2 and $^3O_2$ would decrease the yield of $^1O_2$, the hydride-transfer mechanism of eq 8a is favored in order to account for the 100% yields that are observed with DPBF as the trapping agent (rate constant for $^1O_2$–DPBF reaction, 8×10^8 M^{-1} s^{-1}).[9] [The reactive intermediate from reactions 2 and 8 may be $Fe(O)(H_2O_2)^{2+}$ or $Fe(H_2O_2)_2^+$ rather than free $^1O_2$.]

With *cis*-PhCH=CHPh and PhCH=CHMe as substrates, the Fe(II)–H_2O_2 system yields some PhC≡CPh and $\overline{PhCHCHOMe}$, respectively, in addition to the aldehydes that result from the dominant $^1O_2$/dioxetane process. By appropriate adjustment of the experiments to favor reaction 2 and disfavor reaction 3, the FeO^{2+}/substrate process becomes competitive (~50% of the product mixture, Table II) with reaction 8.

$$FeO^{2+} + \textit{cis}\text{-PhCH=CHPh} \rightarrow PhC{\equiv}CPh + H_2O + Fe(II) \quad (9)$$

$$FeO^{2+} + PhCH{=}CHMe \rightarrow \overline{PhCHCHOMe} + Fe(II) \quad (10)$$

Such competition is in accord with a FeO^{2+}–H_2O_2 mechanistic pathway to formation of $^1O_2$.

The chemistry of reactions 2 and 3 is equivalent to that for *catalase* with FeO^{2+} acting as compound I.[10] Likewise, the chemistry of reactions 2 and 4a (with the products of Table I) has some similarity to that for *peroxidase*, with FeO^{2+} as compound II.[11] Although some of the ferryl–substrate reactions (eq 4, Table I) parallel the "oxene" monoxygenase chemistry that is catalyzed by *cytochrome P-450*,[12,13] the lack of significant reactivity with cyclohexene and norbornene indicates that FeO^{2+} is an inadequate model for the iron–oxygen center of the enzyme.

Additional studies are in progress to determine the effects of various ligands on the thermodynamics and kinetics of reactions 1–10 and the consequences for these reactions when organic peroxides are used in place of H_2O_2 as the source of "oxene" oxygen atoms. These should provide insight as to the possibility that Fe(II)-induced activation of H_2O_2 to FeO^{2+} and $^1O_2$ occurs in biological systems.

Acknowledgment. This work was supported by the National Science Foundation under Grant CHE82-12299. We are grateful to Professor M. A. J. Rogers, University of Texas, Austin, for helpful discussions and suggestions.

Registry No. DPBF, 5471-63-6; MeCN, 75-05-8; H_2O_2, 7722-84-1; Ph_3P, 603-35-0; Me_2SO, 67-68-5; EtOH, 64-17-5; $PhCH_2OH$, 100-51-6; MeCH(O), 75-07-0; PhCH(O), 100-52-7; PhNHNHPh, 122-66-7; 1,4-c-C_6H_8, 628-41-1; H_2S, 7783-06-4; $Ph_2C{=}CPh_2$, 632-51-9; PhC≡CPh, 501-65-5; *cis*-PhCH=CHPh, 645-49-8; PhC≡CMe, 673-32-5; PhC≡CH, 536-74-3; *trans*-PhCH=CHPh, 103-30-0; PhCH=CHMe, 637-50-3; Ph_2SO, 945-51-7; c-$C_6H_{11}OH$, 108-93-0; 9,10-diphenylanthracene, 1499-10-1; rubrene, 517-51-1.

(7) To 1 mmol of *cis*-PhCH=CHPh or 1 mmol of PhC≡CPh and 0.5 mmol of $Fe^{II}(ClO_4)_2 \cdot 4MeCN$ in 10 mL of MeCN or MeCN-d_3 was added 0.5 mmol of H_2O_2 (98%) in 0.3 mL of MeCN over a 5-min period.

(8) Lifetimes for $^1O_2$; 56 μs in MeCN and 610 μs in MeCN-d_3. (a) Ogilly, P. R.; Foote, C. S. *J. Am. Chem. Soc.* **1983**, *105*, 3423. (b) Rodgers, M. A. J., *J. Am. Chem. Soc.* **1983**, *105*, 6201.

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(12) Debrunner, P. G.; Gunzalus, I. C.; Sligar, S. G.; Wagner, G. C. In "Metal Ions in Biological Systems"; Sigel, H., Ed.; Marcel Dekker: New York, 1978; Vol. 7; pp 241–275.

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