Activation of Fe(III)·Bleomycin by 10-Hydroperoxy-8,12-octadecadienoic Acid

Anand Natrajan,[†] Sidney M. Hecht,*[,†] Gijs A. van der Marel,[‡] and Jacques H. van Boom[‡]

Contribution from the Departments of Chemistry and Biology, University of Virginia, Charlottesville, Virginia 22901, and Department of Organic Chemistry, University of Leiden, Leiden, The Netherlands. *Received November 6, 1989*

Abstract: Treatment of 10-hydroperoxy-8,12 octadecadienoic acid with Fe(III)·bleomycin (BLM) resulted in reduction of the hydroperoxide to afford 10-oxo-8-decenoic acid, which must form by homolytic scission of the peroxide O–O bond. Although this reaction afforded a reactive species that could demethylate *N,N*-dimethylaniline and convert styrene to styrene oxide and phenylacetaldehyde, the extent of conversion was much less than that obtained following activation of Fe(III)·BLM with hydrogen peroxide. Further, unlike the activated species formed by admixture of Fe(III)·BLM + H_2O_2 or ethyl hydroperoxide, the activated Fe·BLM formed via the agency of 10-hydroperoxy-8,12-octadecadienoic acid would not mediate the destruction of the self-complementary oligonucleotide 5′-d($CGCT_3A_3GCG$), nor would it support naphthalene hydroxylation. These results suggest that the activated Fe·BLM formed from Fe(III)·BLM + H_2O_2 may differ from the species generated when Fe(III)·BLM reacts with alkyl peroxides that undergo homolytic O–O bond scission.

The bleomycins are glycopeptide-derived antitumor antibiotics that are used clinically both as single agents and in combination with other drugs such as *cis*-platinum.[1] The efficacy of these compounds as antitumor agents is believed to result from their ability to degrade DNA in cancer cells, a process that has been studied intensively by using isolated DNA in a variety of cell-free systems. Required cofactors for bleomycin-mediated DNA degradation include a metal ion such as Fe, Cu, or Mn and either O_2 or an oxidant such as H_2O_2.[2] Early studies by Sausville et al.[3] demonstrated that DNA degradation occurred following reductive activation of dioxygen in the presence of Fe(II)·BLM; subsequently, Burger et al.[4] showed that a chemically similar activated species could be formed by admixture of Fe(III)·BLM + H_2O_2. Although it is generally accepted that this activated species is an oxygenated metallobleomycin,[4,5] no detailed characterization of any activated metallobleomycin has been reported. The EPR and Mössbauer spectra of the last detectable iron–oxygen species were consistent with a ferric peroxide structure.[4,6] However, chemical studies that employed reducing agents such as I^- to titrate activated Fe·BLM[7] established that approximately two electrons were required to reduce this activated BLM to Fe(III)·BLM; accordingly, a perferryl (Fe^V=O) species may be the best chemical representation for what is now commonly referred to as activated Fe·BLM. Consistent with this view, it may be noted that in addition to mediating DNA degradation, activated bleomycin is also capable of effecting chemical transformations characteristic of heme proteins such as cytochrome P-450,[8] including olefin oxygenation, hydroxylation of aromatic substrates, and demethylation of *N,N*-dimethylaniline.[9]

The formation of a high-valent metal–oxo intermediate from Fe(II)·BLM + O_2 or Fe(III)·BLM + H_2O_2 must proceed via scission of the O–O bond; in principle, this process could occur either homolytically or heterolytically, affording metal–oxo complexes differing in oxidation state. Bruice and his co-workers have studied the interaction of metalloporphyrins with alkyl and acyl hydroperoxides. They found that the mode of O–O bond scission depended on the pK_a of the leaving group; peracids supported predominantly heterolytic O–O bond scission, while homolytic scission was obtained when alkyl hydroperoxides were employed.[10]

Recently, Padbury et al.[11] reported that activation of Fe(III)·BLM in the presence of 10-hydroperoxy-8,12-octadecadienoic acid (**1**) must occur via homolytic cleavage of the O–O bond, a conclusion based on the predominant formation of 10-oxo-8-decenoic acid (**3**) as a degradation product (Scheme I). In order to characterize this activated Fe·BLM, we have studied its reactive properties in comparison with activated Fe·BLMs obtained by other methods of activation. Presently, we demonstrate that Fe(III)·BLM does react with 10-hydroperoxy-8,12-octadecadienoic acid, and that the derived chemical species effects the oxygenation of styrene and the demethylation of *N,N*-dimethylaniline, albeit less efficiently than Fe(III)·BLM that had been activated with hydrogen peroxide. On the other hand, unlike Fe·BLMs activated with other oxidants, the activated species formed by admixture of Fe(III)·BLM + 10-hydroperoxy-8,12-octadecadienoic acid would neither hydroxylate naphthalene nor degrade the oligonucleotides d(CGCTTTAAAGCG) or d(CGCGCG) on a time scale shown to result in essentially complete consumption of the 10-hydroperoxy-8,12-octadecadienoic acid. Incubation of 10-hydroperoxy-8,12-octadecadienoic acid treated Fe(III)·BLM and d(CGCTTTAAAGCG) for much longer periods of time did result in some oligonucleotide degradation, but this was shown to result from the presence of reducing equivalents in the reaction mixture rather than from some persistent activated Fe·BLM. Unlike the activated Fe·BLM resulting initially from admixture of Fe-

* Address correspondence to this author at the Department of Chemistry.
† University of Virginia.
‡ University of Leiden.

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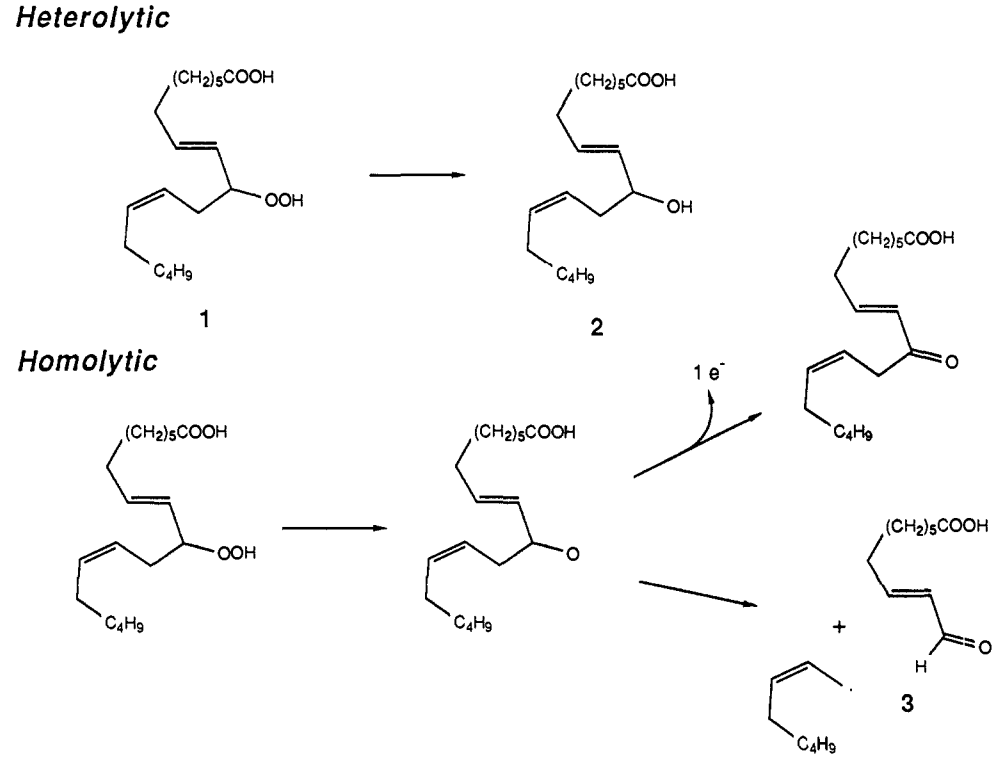
Scheme I. Decomposition of 10-Hydroperoxy-8,12-Octadecadienoic Acid (**1**) to 10-Hydroxy-8,12-Octadecadienoic Acid (**2**) and 10-Oxo-8-decenoic Acid (**3**) via Heterolytic and Homolytic Pathways, Respectively



(II)·BLM + O_2, the species formed upon Fe(III)·BLM + 10-hydroperoxy-8,12-octadecadienoic acid would not oxidize I^- after consumption of hydroperoxide **1**, suggesting that no high-valent metal–oxo species was sustained in solution following reaction between Fe(III)·BLM and the fatty acid hydroperoxide.

These results indicate that the putative "homolytically activated" Fe·BLM is unlikely to be the therapeutically relevant form of bleomycin if the drug functions at the level of DNA degradation. Further, they imply that the activated Fe·BLM actually responsible for DNA degradation must result from heterolytic O–O bond scission.

Results

DNA Oligonucleotide Degradation. Recently, Padbury et al.[11] reported that incubation of a plasmid DNA in the presence of 10.8 μM Fe(III)·blenoxane and 500 μM 10-hydroperoxy-8,12-octadecadienoic acid (**1**) resulted in degradation of the plasmid DNA. Limitations of the reported work included substantial DNA degradation obtained even in the absence of added hydroperoxide **1**, the inability to quantify DNA lesions in a system known to produce both heterolytic and homolytic degradation products from **1**, and the lack of any data concerning the nature of the DNA products formed.

As an alternative, we have employed the self-complementary dodecanucleotide d(CGCTTTAAAGCG), an efficient substrate for bleomycin that has been shown previously to undergo damage predominantly at cytidine$_3$ and cytidine$_{11}$ following treatment with Fe(II)·BLM + O_2 or Fe(III)·BLM + H_2O_2.[12] This substrate has been shown to afford products analogous to those obtained when Fe·BLM is used to degrade DNA (Scheme II);[12] further, the strand selectivity (i.e., C_3 vs C_{11}) of oligonucleotide modification has been shown to vary substantially among BLM congeners that differ within the metal-binding domain[12b] and may therefore be thought to be characteristic of the shape and reactivity of individual activated bleomycins. In fact, this dodecanucleotide has recently been used as an analytical tool to characterize the nature of Fe(III)·BLM activated electrochemically at a glassy carbon electrode.[13]

Table I. Degradation of d(CGCTTTAAAGCG) and d(CGCGCG) by Fe·Bleomycin in the Presence of Each of Several Oxidants[a]

oxidant (mM)	C + C propenal,[d] mM	recovered intact oligonucleotide, mM (%)
d(CGCT$_3$A$_3$GCG)		
10-hydroperoxy-8,12-octadecadienoic acid (**1**) (2)	<0.01	2.8 (100)
ethyl hydroperoxide (2)	0.07	1.9 (67)
hydrogen peroxide (2)	0.50	0 (0)
O_2 (atm tension)[b]	0.38	0.32 (11)
d(CGCGCG)		
10-hydroperoxy-8,12-octadecadienoic acid (**1**) (2)	0.02	*c*
hydrogen peroxide (2)	0.60	*c*

[a] Carried out in the presence of 1 mM Fe(III)·BLM A_2 and either 2.8 mM d(CGCT$_3$A$_3$GCG) or 2.5 mM (CGCGCG) (final nucleotide concentration) in 50 mM sodium cacodylate, pH 7.2, at 25 °C for 1 h. [b] 1 mM Fe(II)·BLM A_2. [c] Not measured. [d] Cytosine + cytosine propenal.

Table I summarizes the data obtained for the degradation of d(CGCT$_3$A$_3$GCG) by Fe·BLM in the presence of each of several oxidants; recorded in each case is the total amount of cytosine and cytosine propenal formed[14] and the amount of recovered d(CGCT$_3$A$_3$GCG). As shown in the table, treatment of the dodecanucleotide with Fe(III)·BLM A_2 + 10-hydroperoxy-8,12-octadecadienoic acid gave negligible amounts of products and essentially total recovery of unreacted d(CGCT$_3$A$_3$GCG). In contrast, ethyl hydroperoxide and hydrogen peroxide both gave significant amounts of the products usually obtained from d(CGCT$_3$A$_3$GCG) with activated Fe·BLM,[12] with proportional decreases in the amounts of unreacted dodecanucleotide that could

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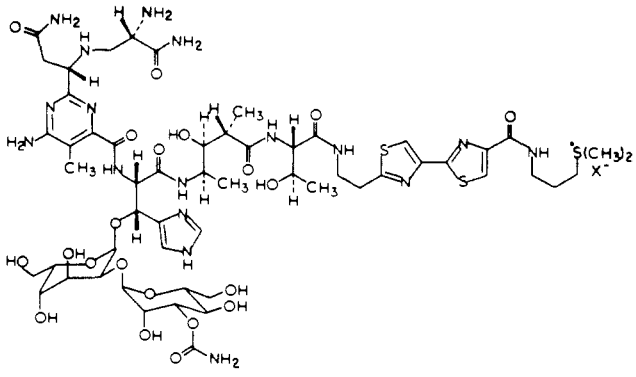
(14) Because modification of d(CGCT$_3$A$_3$GCG) occurred primarily at C_3 and C_{11}, the amount of cytosine + cytosine propenal provided an approximate measure of the amount of free bases and base propenals produced (the sum of which is equal to the number of DNA lesions (cf. Scheme II)).[12]



bleomycin A_2

be recovered from the reaction mixtures. Also assayed in parallel was the degradation of d($CGCT_3A_3GCG$) by Fe(II)·BLM A_2 + O_2. As indicated in the table, aerobically activated Fe(II)·BLM A_2 also effected degradation of the oligonucleotide, with concomitant production of cytosine and cytosine propenal.[15] Studied in parallel was the hexanucleotide d(CGCGCG), which has also been shown to undergo degradation following treatment with Fe(II)·BLM + O_2 or Fe(III)·BLM + H_2O_2.[16] As shown in Table I, incubation of d(CGCGCG) with Fe(III)·BLM + hydroperoxide **1** afforded little cytosine or cytosine propenal.

To ensure that the reaction conditions employed actually resulted in decomposition of the added 10-hydroperoxy-8,12-octadecadienoic acid, we also measured the amount of recoverable (i.e., unreacted) hydroperoxide **1**, as well as the β-scission product 10-oxo-8-decenoic acid (**3**) (Scheme I).[11,17] As shown in Table II, under conditions very similar to those employed for the experiments outlined in Table I, hydroperoxide **1** had largely decomposed. In agreement with the results of Padbury et al.,[11] we found that the decomposition was dependent on the presence of Fe(III)·BLM and afforded 10-oxo-8-decenoic acid (**3**) as the predominant product.

Although hydroperoxide **1** had largely disappeared within 1 h in the presence of Fe(III)·BLM (Table II) without concomitant oligonucleotide degradation or the production of significant amounts of cytosine or cytosine propenal (Table I), the degradation of d($CGCT_3A_3GCG$) was also assayed after longer periods of incubation (Table III). While the amounts of cytosine and cytosine propenal formed following activation of Fe(III)·BLM with hydroperoxide **1** were not large, the amounts of these products did increase steadily as a function of time; after 24 h hydroperoxide **1** mediated products were ~20% as abundant as those formed by Fe(III)·BLM + H_2O_2 after a 1-h reaction. To investigate the source of the formed products, the activation of Fe(III)·BLM with hydroperoxide **1** was repeated. After 1 h, when hydroperoxide **1** would largely have been consumed (Table II), an aliquot of the reaction was treated with 1 equiv of Cu(II) to displace Fe from BLM with concomitant formation of Cu(II)·BLM.[18] After 1 min, an aliquot of the Cu(II)-treated sample was treated with an additional equivalent of (preformed) Fe(III)·BLM. The three reaction mixtures were each maintained in the presence of d($CGCT_3A_3GCG$) for a total of 24 h and then analyzed for formation of cytosine + cytosine propenal. As shown in Table IV, product formation was suppressed by the addition of Cu(II), but largely restored by subsequent addition of Fe(III)·BLM. Because

Table II. Decomposition of 10-Hydroperoxy-8,12-octadecadienoic Acid (**1**) by Fe(III)·BLM[a]

catalyst	reaction time, min	**1** dec, mM (%)	**3** formed, mM (%)
Fe(III)·BLM A_2	15	1.70 (85)	1.44 (72)
Fe(III)·BLM A_2	60	1.80 (90)	1.58 (78)
	60	<0.1 (5)	
Fe(III)	60	<0.1 (5)	

[a] Carried out with 1 mM Fe(III) or Fe(III)·BLM, 2 mM 10-hydroperoxy-8,12-octadecadienoic acid and 2.5 mM calf thymus DNA in 50 mM cacodylate, pH 7.2 at 25 °C.

Table III. Degradation of d($CGCT_3A_3GCG$) by Fe·Bleomycin at Long Reaction Times[a]

time, h	C + C propenal,[b] mM	time, h	C + C propenal,[b] mM
	10-Hydroperoxy-8,12-octadecadienoic acid (2 mM)		
1	0.03	6	0.06
3	0.05	24	0.12
	Hydrogen Peroxide (2 mM)		
1	0.58	6	0.62
3	0.62	24	0.54

[a] Carried out in the presence of 1 mM Fe(III)·BLM A_2 and 2.8 mM d($CGCT_3A_3GCG$) (final nucleotide concentration) in 50 mM sodium cacodylate, pH 7.2, at 25 °C. [b] Cytosine + cytosine propenal.

Table IV. Degradation of d($CGCT_3A_3GCG$) by Fe·Bleomycin at Long Reaction Times after Treatment with Cu(II)[a]

time, h	species added at 1 h (mM)	C + C propenal,[b] mM
1		0.04
24		0.09
24	$CuCl_2$ (1.0)	0.03
24	$CuCl_2$ (1.0); then Fe(III)·BLM (1.0)[b]	0.07

[a] Carried out in the presence of 1 mM Fe(III)·BLM A_2, 2.0 mM d($CGCT_3A_3GCG$) (final nucleotide concentration), and 4 mM 10-hydroperoxy-8,12-octadecadienoic acid in 50 mM sodium cacodylate, pH 7.2, at 25 °C. [b] Addition of Fe(II)·BLM was carried out after a delay (~1 min) sufficient to allow Cu(II) to displace Fe from the Fe·BLM present in the reaction mixture initially. [c] Cytosine + cytosine propenal.

Cu(II) is often used to quench Fe·BLM-mediated reactions, we infer the involvement of Fe-BLM in the formation of cytosine and cytosine propenal at long reaction times. However, because product formation could be restored by addition of fresh Fe(III)·BLM subsequent to consumption of hydroperoxide **1** and (irreversible) conversion of initially added Fe·BLM to Cu(II)·BLM, product formation could not have been due to some persistent metal–oxo derivative of Fe·BLM that effected slow oxidative degradation of the oligonucleotide. We suggest that the product formation observed after extended reaction times is due to the presence of small amounts of reducing equivalents present in the reaction mixture that originally contained Fe(III)·BLM + hydroperoxide **1**. This would result in the formation of limited amounts of Fe(II)·BLM, which could undergo aerobic activation and subsequently effect oligonucleotide degradation. Two additional experiments support this thesis. 10-Hydroperoxy-8,12-octadecadienoic acid (**1**) and Fe(III)·BLM A_2 were combined and maintained under ambient conditions for 1 h, i.e., until hydroperoxide **1** had been consumed (cf. Table II). The reaction mixture was treated with I^- in an effort to effect reduction of any activated Fe·BLM that might be present. As shown in Figure 1, no I_2 was formed. In contrast, an equimolar amount of Fe(II)·BLM maintained under aerobic conditions produced I_2 from I^- in amounts comparable to those reported by Burger et al.[7] A second reaction mixture, constituted similarly with Fe(III)·BLM + hydroperoxide **1**, was admixed with a 149-nucleotide 5′-^{32}P-end-labeled DNA fragment and maintained at 25 °C for 6 h. Analysis of this BLM-treated DNA sample by polyacrylamide

(15) For each of the oxidants that supported the formation of significant amounts of products, the products formed included all of those expected from d($CGCT_3A_3GCG$) based on earlier reports (Scheme II);[12] further, the observed ratios of bases to base propenals and strand selectivities were the same within experimental error for each oxidant (data not shown).

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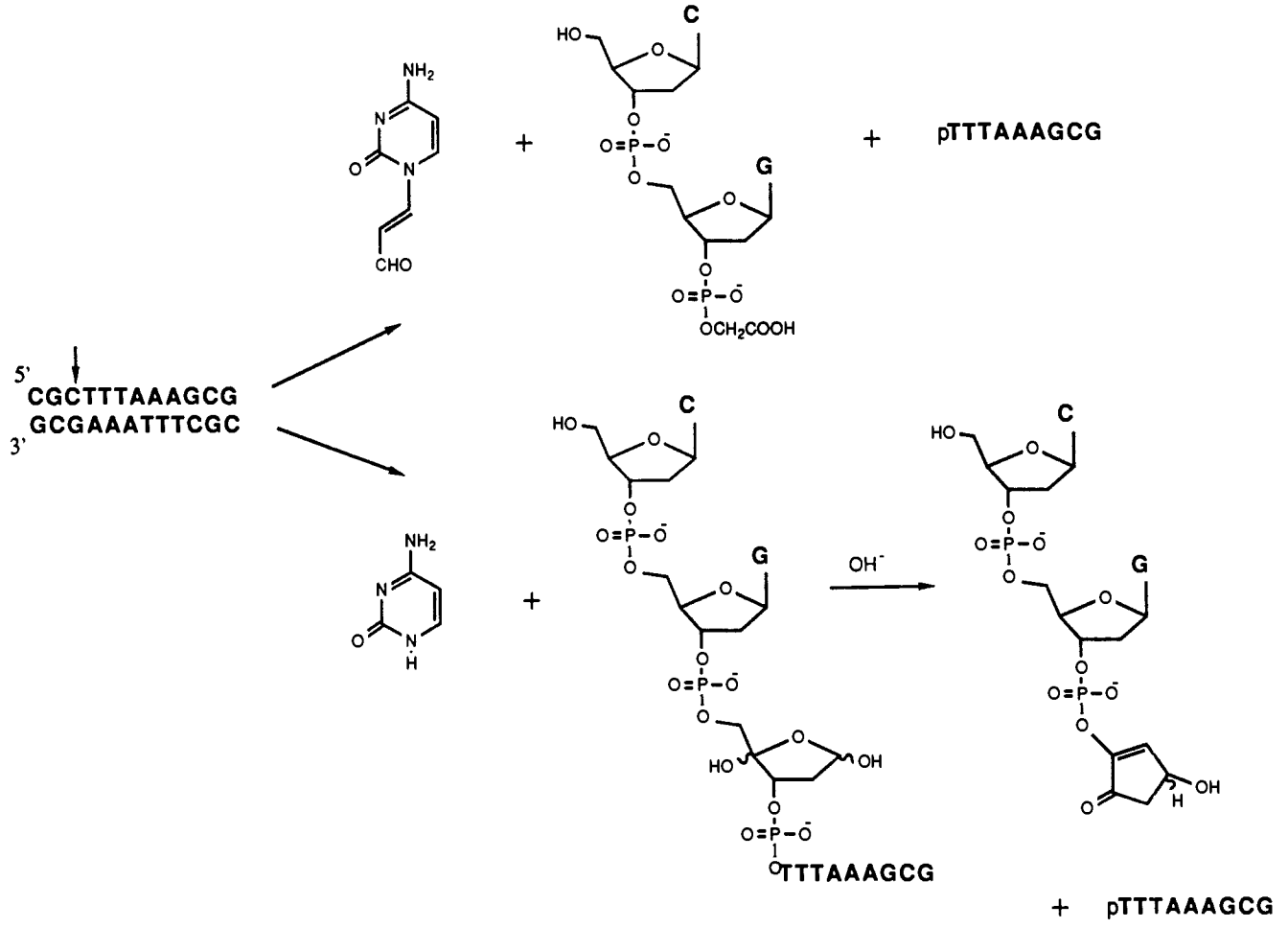
Scheme II. Degradation Products Resulting from Treatment of d(CGCT$_3$A$_3$GCG) with Activated Fe·BLM



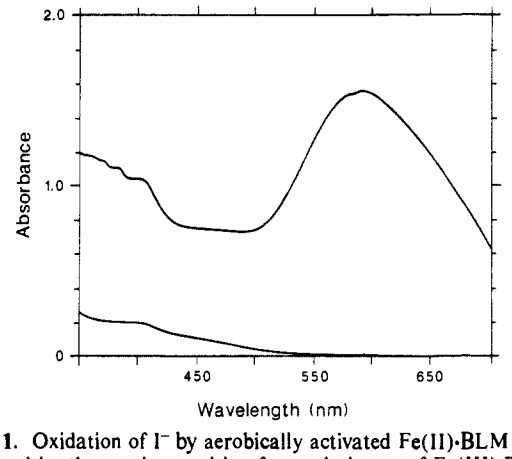


Figure 1. Oxidation of I^- by aerobically activated Fe(II)·BLM (upper trace) and by the species resulting from admixture of Fe(III)·BLM + 10-hydroperoxy-8,12-octadecadienoic acid (lower trace).

gel electrophoresis after the extended incubation period indicated a pattern of DNA cleavage identical with, albeit much weaker than, the pattern of DNA cleavage afforded by Fe(III)·BLM activated with H_2O_2; the latter has also been shown to afford DNA cleavage patterns identical with those produced by aerobically activated Fe(II)·BLM (not shown). That the pattern of DNA cleavage was identical with that obtained from aerobically activated Fe·BLM, in spite of the absence of a titratable activated species at the outset of the DNA cleavage reaction, suggests that activation of Fe·BLM (i) occurred subsequent to the consumption of hydroperoxide **1** and (ii) did not involve the formation of any diffusible radical species as suggested by Padbury et al.[11] since the latter would produce a characteristic pattern of DNA cleavage very different from that of BLM.[19]

Table V. Hydroxylation of Naphthalene by Fe(III)·BLM[a]

oxidant (mM)	1-naphthol, mM	2-naphthol, mM	total product, mM
10-hydroperoxy-8,12-octadecadenoic acid (**1**) (15)	0	0	0
hydroperoxide mixture from linoleic acid (50)[b]	0	0	0
ethyl hydroperoxide (15)	trace (<0.1)	0	
hydrogen peroxide (15)	9.9	4.6	14.5

[a] Carried out in 9:1 CH_3OH–H_2O containing 1.38 mM Fe(III)·BLM and 50 mM naphthalene at 25 °C for 1.5–2.0 h. [b] ^{1}H NMR analysis indicated >45 mM (90%) decomposition of the hydroperoxides at the conclusion of the experiment.

Oxidative Transformations of Low Molecular Weight Substrates. In addition to its ability to mediate DNA degradation, activated Fe·BLM has also been shown to effect the oxidation and oxygenation of low molecular weight substrates.[9] To characterize further the reactive properties of the activated species resulting from admixture of Fe·BLM and each of several oxidants, we next employed representative low molecular weight substrates.

Activated Fe·BLM has been reported to hydroxylate naphthalene,[9c] a reaction that bears at least superficial similarity to the initial events leading to DNA degradation by Fe·BLM. Accordingly, we used naphthalene as a substrate for Fe(III)·BLM following admixture of the latter with a suitable peroxide. As shown in Table V, neither 10-hydroperoxy-8,12-octadecadienoic acid (**1**) nor the mixture of hydroperoxides resulting from photooxygenation of linoleic acid[17] was able to support the hydroxylation of naphthalene following admixture with Fe(III)·BLM. The same was also true of ethyl hydroperoxide, although Fe-

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Table VI. Oxygenation of Styrene by Fe(III)·BLM[a]

oxidant (mM)	styrene oxide, mM	phenylacetaldehyde, mM	total product, mM
10-hydroperoxy-8,12-octadecadienoic acid (1) (15)	0.58	0.17	0.75
hydroperoxide mixture from linoleic acid (50)[b]	0.51	0.27	0.78
ethyl hydroperoxide (15)	0.13	0.12	0.25
hydrogen peroxide (15)	4.00	3.10	7.10

[a] Carried out in 5:1 CH_3OH–H_2O containing 2.3 mM Fe(III)·BLM and 229 mM styrene at −13 °C for 2 h. [b] ^1H NMR analysis indicated >45 mM (90%) decomposition of the hydroperoxides at the conclusion of the experiment.

Table VII. N-demethylation of *N,N*-Dimethylaniline by Fe(III)·BLM[a]

oxidant (mM)	*N*-methylaniline, mM
10-hydroperoxy-8,12-octadecadienoic acid (1) (15)	5.3
hydroperoxide mixture from linoleic acid (50)	4.3
ethyl hydroperoxide (15)	3.5
hydrogen peroxide (15)	13.5

[a] Carried out in 9:1 CH_3OH–H_2O containing 1.38 mM Fe(III)·BLM and 50 mM *N,N*-dimethylaniline at 25 °C for 1 h.

(III)·BLM A_2 + C_2H_5OOH did produce a species capable of DNA oligonucleotide degradation (cf. Table I). On the other hand, following treatment with H_2O_2, Fe(III)·BLM produced 1-naphthol and 2-naphthol in amounts essentially identical with the amount of hydrogen peroxide employed for Fe(III)·BLM activation.

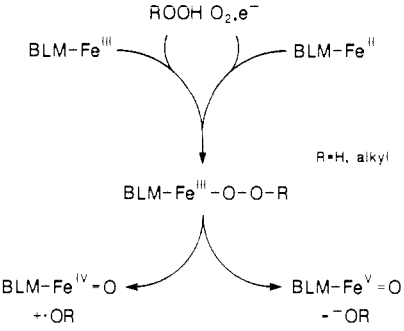
We have demonstrated previously that in the presence of hydrogen peroxide or iodosobenzene, Fe(III)·BLM acts as a catalyst for the oxidation and oxygenation of olefins such as *cis*-stilbene and styrene.[9] The oxygenation of *cis*-stilbene resulted in the formation of *cis*-stilbene oxide and deoxybenzoin;[9] ^{18}O-labeling studies were consistent with the derivation of both products from a common intermediate.[9d,e] Similarly, styrene was shown to afford both styrene oxide and smaller amounts of rearranged products.[9c] Since the process that resulted in these oxygenation reactions appeared to differ mechanistically from the hydroxylation of naphthalene, we tested the ability of several peroxides to support the oxygenation of styrene in the presence of Fe(III)·BLM. As shown in Table VI, all of the oxidants studied were able to promote the oxygenation of styrene to produce both styrene oxide and phenylacetaldehyde. While 10-hydroperoxy-8,12-octadecadienoic acid (1) supported the Fe(III)·BLM-mediated oxygenation reaction, the amount of product was only ~10% of that obtained with hydrogen peroxide. Ethyl hydroperoxide also supported the formation of styrene oxide and phenylacetaldehyde, albeit only in low yields.

In contrast to the foregoing reactions, each of the oxidants investigated proved to be effective in supporting the oxidative demethylation of *N,N*-dimethylaniline by Fe(III)·BLM (Table VII). These results were consistent with the results of Padbury and Sligar,[20] who reported the facile N-demethylation of *N,N*-dimethylaniline by Fe(III)·BLM supported by primary, secondary, and tertiary alkyl peroxides. It may be noted that the same transformation can also be effected with Fe(III)·BLM + C_6H_5IO[9c,20] or Fe(II)·BLM + O_2 + ascorbate.[9c]

Discussion

Previously, Burger et al. characterized an activated Fe·BLM accessible either by admixture of Fe(II) salts and BLM under aerobic conditions or by treatment of Fe(III)·BLM with H_2O_2.[4] The activated species contained Fe, BLM, and at least one oxygen atom and was found to have $t_{1/2}$ ~2 min; in the absence of substrate this activated Fe·BLM underwent rapid self-inactivation,[4,13,21] consistent with its representation as a highly reactive intermediate. That this activated Fe·BLM is best represented as a perferryl (BLM·Fe^V=O) species is suggested by the findings that a two-electron titration with either iodide or thio-NADH is sufficient to regenerate Fe(III)·BLM, that the chemistry of this activated BLM is remarkably similar to that of cytochrome P-450,[2b] and that DNA strand scission by activated Fe·BLM can also be achieved following admixture of Fe(III)·BLM and (mono)oxygen surrogates.[22]

Scheme III. Activation of Fe·BLM by Heterolytic and Homolytic Routes



An alternative representation of activated Fe·BLM posits the homolytic cleavage of the O–O bond,[11] which should produce a metal–oxo intermediate containing an additional electron (nominally BLM·Fe^{IV}=O) (Scheme III). Recently, Padbury et al. attempted to obtain an activated Fe·BLM formed unambiguously via homolytic cleavage of an O–O bond. This was done by admixture of Fe(III)·BLM and 10-hydroperoxy-8,12-octadecadienoic acid, the latter of which can form a stabilized allylic radical via homolytic O–O bond scission, and subsequent fragmentation of the resulting alkoxyl radical. That Fe·BLM activation actually did occur predominantly via homolytic cleavage of the O–O bond was indicated by the production of 10-oxo-8-decenoic acid (3) in amounts nearly stoichiometric with the amount of 10-hydroperoxy-8,12-octadecadienoic acid (1) decomposed (Scheme I).[11]

Padbury et al. demonstrated that the activated Fe·BLM produced by admixture of Fe(III)·BLM + 10-hydroperoxy-8,12-octadecadienoic acid was capable of degrading plasmid DNA,[11] but did not provide any estimate of the extent of DNA damage. Because the formation of "homolytically activated" oxygenated Fe·BLM was accompanied by the formation of several percent 10-hydroxy-8,12-octadecadienoic acid (2) (Scheme I), it seems logical to anticipate that some "heterolytically activated" oxygenated Fe·BLM could also have been present to this extent and could have been responsible for the plasmid DNA degradation observed.

In order to obtain a quantitative estimate of the ability of the putative homolytically activated Fe·BLM to degrade DNA, we employed this preparation for the degradation of the dodecanucleotide d($CGCT_3A_3GCG$). Surprisingly, when utilized for 1 h under conditions that resulted in extensive oligonucleotide degradation by activated Fe·BLMs prepared by admixture of Fe(II)·BLM + O_2 or Fe(III)·BLM + H_2O_2, the homolytically activated Fe·BLM gave no, or very low, levels of detectable products (Table I). In a parallel set of experiments, it was shown that ~85% of the 10-hydroperoxy-8,12-octadecadienoic acid added for Fe·BLM activation had been decomposed within 15 min, and that the majority had already been converted to 10-oxo-8-decenoic acid. Thus, the formation of the putative homolytically activated Fe·BLM should have been complete within 15 min, even though no degradation of DNA could be detected within 1 h.

To test the possibility that we had formed some novel activated Fe·BLM having reactive properties different from the species accessible by admixture of Fe(II)·BLM + O_2 or Fe(III)·BLM + H_2O_2, we extended the period of incubation of the putative

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(21) Nakamura, M.; Peisach, J. *J. Antibiot.* **1988**, *41*, 638.
(22) (a) Ehrenfeld, G. M.; Rodriguez, L. O.; Hecht, S. M.; Chang, C.; Basus, V. J.; Oppenheimer, N. J. *Biochemistry* **1985**, *24*, 81. (b) Pratviel, G.; Bernadou, J.; Meunier, B. *Biochem. Biophys. Res. Commun.* **1986**, *136*, 1013.

homolytically activated Fe·BLM with d($CGCT_3A_3GCG$). As indicated in Table III, after 24 h of incubation, product formation from the DNA oligonucleotide substrate was readily apparent. Subsequently, we measured the actual time course of product formation for aerobically activated Fe(II)·BLM, Fe(III)·BLM activated with H_2O_2, and Fe(III)·BLM activated with 10-hydroperoxy-8,12-octadecadienoic acid. As is clear from Table III, the putative homolytically activated Fe·BLM did react with DNA, affording products chemically identical with those produced by the other activated Fe·BLMs, but on a time scale that was dramatically different.

To characterize the nature of the activated Fe·BLMs produced by the two methods of activation outlined above, we titrated the various species with I^-. As shown in Figure 1, the activated Fe·BLMs prepared by admixture of Fe(II)·BLM + O_2 readily oxidized I^- to I_2 as evidenced by the presence of a strong absorption at ~575 nm. Starch iodide titration indicated that approximately two electrons were required for reduction of the activated species.[23] This observation was entirely consistent with the earlier report by Burger et al.[7] and supports the representation of this activated Fe·BLM as BLM·Fe^V=O. On the other hand, titration of the activated Fe·BLM produced by admixture of Fe(III)·BLM + 10-hydroperoxy-8,12-octadecadienoic acid for 1 h indicated the absence of any oxidizing ability, and the obvious absence of any activated Fe·BLM that could mediate oxidative DNA degradation. The small amounts of DNA oligonucleotide degradation products observed at long reaction times (Table III) could presumably result from slow activation of Fe(III)·BLM with $CH_3(CH_2)_4CH$=C-HCH_2OOH, the alkyl hydroperoxide itself being produced from 2-octenyl radicals, a fragmentation product of 10-hydroperoxy-8,12-octadecadienoic acid (Scheme I). Alternatively, the observed products could derive from aerobic activation of Fe(III)·BLM following its reduction to Fe(II)·BLM by small amounts of reducing agents present in the reaction mixture after 1 h of incubation. The foregoing explanations are both supported by the finding that product formation was not observed if Cu(II), which is known to displace Fe from Fe·BLM, was added to the reaction mixture after 1 h, but that product formation was restored by subsequent addition of a further equivalent of (preformed) Fe(III)·BLM.[24]

It may be worth noting that if bleomycin activation obtained by admixture of Fe(III)·BLM + H_2O_2 or Fe(II)·BLM + O_2 proceeded via a homolytic route, then a byproduct of activation would be ·OH (cf. Scheme III). It would be anticipated that this species would afford DNA lesions at several positions in proximity to the site of binding,[19] in contrast to the site-selective cleavage actually observed after long reaction times (vida supra).[12] Further, efforts to trap diffusible oxygen radicals during Fe·BLM-mediated DNA degradation have not been successful.[25] Nonetheless, we cannot exclude the possibility that ·OH radicals are produced specifically at the site in DNA that is observed to undergo reaction.

In contrast to the DNA degradation results, Fe(III)·BLM + 10-hydroperoxy-8,12-octadecadienoic acid did support the oxidative transformation of certain low molecular weight substrates. As outlined in Tables V–VII, while solutions of Fe(III)·BLM + 10-hydroperoxy-8,12-octadecadienoic acid would not hydroxylate naphthalene, the epoxidation of styrene and N-demethylation of *N,N*-dimethylaniline could be observed. These observations established unambiguously the presence of some reactive species derived from Fe(III)·BLM + 10-hydroperoxy-8,12-octadecadienoic acid, but not whether the transformations were mediated by an activated BLM species per se or, e.g., by alkylperoxy radicals produced by combination of 2-octenyl radicals (Scheme I) with O_2 or excess **1**.

The description of an unambiguous route to a homolytically activated Fe·BLM[11] represents an important conceptual advance in our understanding of the chemistry of bleomycin activation. Although the homolytically activated Fe·BLM is probably not the species responsible for mediating the therapeutic effects of bleomycin, the characterization of this species helps to define the nature of the reactive species accessible by aerobic activation of Fe(II)·BLM.

Experimental Section

Ferrous ammonium sulfate was purchased from Alfa Products; ferric ammonium sulfate and ferric perchlorate were obtained from Fisher Scientific, as were methanol, tetrahydrofuran, 2-propanol, cyclohexane, ethyl acetate, and acetic acid. Naphthalene, styrene, linoleic acid, methylene blue, ethyl hydroperoxide, and hydrogen peroxide were obtained from Aldrich Chemicals. *N,N*-Dimethylaniline (Aldrich) was distilled under vacuum prior to use. Blenoxane was obtained as a gift from Bristol Laboratories through the courtesy of Dr. William Bradner; it was fractionated chromatographically to provide bleomycin A_2 as described.[26]

^1H NMR spectra were recorded in $CDCl_3$ on a General Electric QE-300 NMR spectrometer and are referenced to $CHCl_3$ (7.25 ppm). Gas chromatography was carried out on a Varian Model 3400 gas chromatograph equipped with a flame ionization detector; helium was employed as a carrier gas using a J&W Scientific 1701, 15-m capillary column. Mass spectra were recorded on a Finnigan MAT 4600 automated gas chromatograph–mass spectrometer. Reverse-phase HPLC analysis was performed on a Varian HPLC using a Rainin C_{18} column (4.6 mm × 10 cm; 3-μm particle size); quantitation of products was carried out with a Hewlett-Packard Model HP3394A integrator. Normal-phase HPLC analysis was carried out on a Waters HPLC equipped with a differential refractometer.

10-Hydroperoxy-8,12-octadecadienoic Acid (1).[17] A solution containing 2.0 g (7.2 mmol) of linoleic acid in 40 mL of methanol was treated with 12 mg (0.032 mmol) of methylene blue. Oxygen was bubbled through the dark blue solution, which was irradiated with a 250-W sun lamp. After 24 h, the methanol was concentrated under diminished pressure and the resulting oil was purified by flash chromatography[27] (Baker silica gel, 230–400 mesh; elution was with 85:15 cyclohexane–tetrahydrofuran containing 0.5% acetic acid). A mixture of monohydroperoxides was recovered as a viscous oil, yield 250 mg (11%). Separation of the individual hydroperoxides was accomplished by HPLC on a Whatman M-9 (10 mm × 500 mm; 10-μm particle size) silica gel column; elution was effected with 98:2:0.1 hexane-2-propanol–acetic acid at a flow rate of 5.0 mL/min. The retention times of the four hydroperoxides were 27, 29, 32, and 34 min.

The identification of 10-hydroperoxy-8,12-octadecadienoic acid was established by reducing each of the four hydroperoxides with 1 equiv of $Fe(II)SO_4$ to effect β-scission, as described by Labeque and Marnett,[17] and subjecting the crude product mixture to analysis by chemical ionization mass spectrometry. Thus, each hydroperoxide (2.6 mg, 8.3 μmol) was dissolved separately in 1.0 mL of 7:3 CH_3OH–H_2O and treated with 3.3 mg (8.3 μmol) of $Fe^{II}(NH_4)_2(SO_4)_2$. The solution turned yellow immediately and was stirred at 25 °C for 1 h. Each reaction mixture was diluted with water and extracted with 2 mL of $CHCl_3$. The $CHCl_3$ extract was dried ($MgSO_4$), concentrated, and subjected to mass spectral analysis. By this procedure, one of the hydroperoxides isolated initially (retention time, 32 min) gave an intense fragment ion corresponding to the expected β-scission product (M_r 184). 10-Hydroperoxy-8,12-octadecadienoic acid chemical ionization mass spectrometry (positive ion, isobutane) m/z (relative intensity) 313 (5, $(M + H)^+$), 295 (20), 279 (20), 185 (100); ^1H NMR ($CDCl_3$) 0.88 (t, 3, J = 6.6 Hz), 1.32 (br s, 12), 1.64 (m, 2), 2.05 (m, 4), 2.35 (t, 2, J = 7.5 Hz), 4.31 (m, 1), 5.54 (m, 3), 5.77 (m, 1) ppm; ^{13}C NMR ($CDCl_3$) 14.48, 22.99, 24.99, 27.80, 29.06 (2 C), 29.17, 29.63, 31.07, 31.94, 32.59, 34.23, 86.92, 124.29, 128.44, 133.03, 137.29, 179.75 ppm.

General Procedure for the Fe·BLM-Mediated Degradation of d-($CGCT_3A_3GCG$). A solution (50 μL total volume) of 50 mM sodium cacodylate, pH 7.2, containing 1.0 mM Fe(III)·BLM A_2, 2.8 mM d-

(23) The experimentally measured value of 1.92 equiv of I^- oxidized/mol of activated Fe·BLM compared favorably with the reported[7] value of 1.5 ± 0.25 equiv/mol of activated Fe·BLM.

(24) A third possibility, namely, that RO· generated during homolytic activation of Fe·BLM could be responsible for the chemistry observed at long reaction times (cf Mansuy, D.; Bartoli, J.-F.; Momenteau, M. *Tetrahedron Lett.* **1982**, *23*, 2781) would seem to be excluded by the short lifetimes of such species (Table II).

(25) Rodriguez, L. O.; Hecht, S. M. *Biochem. Biophys. Res. Commun.* **1982**, *104*, 1470. Incubation of 0.2 mM $Fe(NH_4)_2(SO_4)$ + 0.2 mM BLM in the presence of 2.5 mM d(CGCTTTAAAGCG) in 50 mM sodium cacodylate buffer, pH 7.2, containing 20% dimethyl sulfoxide resulted in the formation of ~29 μM cytosine propenal as opposed to 49 μM in H_2O. Thus, ~60% of the activity of Fe·BLM was retained even in reactions where dimethyl sulfoxide constituted 20% of the solvent.

(26) Chien, M.; Grollman, A. P.; Horwitz, S. B. *Biochemistry* **1977**, *16*, 3641.

(27) Still, W. C.; Kahn, J.; Mitra, A. *J. Org. Chem.* **1978**, *43*, 2923.

(CGCTTTAAAGCG) (DNA nucleotide concentration), and 48 μM 7-methylguanine (as an internal standard) was treated with 2 mM oxidant (cf. Table I). Each reaction mixture was maintained at 25 °C for 1 h and then immediately analyzed by reverse-phase HPLC. The analysis was carried out with a Rainin Microsorb C_{18} (Short One) column (4.6 mm × 10 cm); elution with 0.1 M NH_4OAc at a flow rate of 1.4 mL/min afforded peaks for cytosine (1.4 min), 7-methylguanine (11.5 min), and cytosine propenal (17.5 min). For analysis of unreacted d($CGCT_3A_3GCG$), the same HPLC column was eluted with a linear gradient of aqueous NH_4OAc in CH_3CN (100% 0.1 M NH_4OAc → 70% 0.1 M NH_4OAc–30% CH_3CN) over a period of 30 min at a flow rate of 1.2 mL/min. The dodecanucleotide had a retention time of 12.5 min. The degradation of d(CGCGCG) was performed similarly.

Attempted Reduction of Activated Fe·BLM with I^-. The method employed was that of Burger et al.[7] A solution containing 500 μL (total volume) of 50 mM sodium cacodylate, pH 7.2, 200 μM Fe(III)·BLM A_2, and 200 μM 10-hydroperoxy-8,12-octadecadienoic acid was incubated at 25 °C for 2 h; aliquots were removed after 60 and 120 min and treated separately with 450 μL of 50 mM KI and 50 μL of a 0.5% starch solution. The ultraviolet spectrum was recorded (220–700 nm), but lacked any absorption at 575 nm characteristic of I_2. The assay system was validated by demonstrating that 100 μM Fe(II)·BLM + O_2 produced 1.56 A_{575} units of I_2 (48 mM; 96% of theoretical).

General Procedure for Quantitating the Decomposition of 10-Hydroperoxy-8,12-octadecadienoic Acid (1). A solution of 2.6 mL (total volume) of 50 mM sodium cacodylate, pH 7.2, containing 3.5 mg (2.5 μmol) of blenoxane, 1.2 mg (2.5 μmol) of ferric perchlorate, 2.4 mM calf thymus DNA (final nucleotide concentration), and 1.0 mg (3.6 μmol) of linoleic acid (as an internal standard) was treated with 1.6 mg (5.1 μmol) of 10-hydroperoxy-8,12-octadecadienoic acid. The combined solution was stirred at 25 °C for 1 h, then adjusted to pH 4.0 with 3 M HCl, and extracted twice with 10-mL portions of $CHCl_3$. The combined $CHCl_3$ extract was dried ($MgSO_4$) and concentrated under diminished pressure. Integration of key resonances in the ^1H NMR spectrum of the product mixture relative to the doubly allylic protons in linoleic acid (2.76 ppm) permitted determination of the extent of decomposition of 10-hydroperoxy-8,12-octadecadienoic acid (C*H*OOH; 4.3 ppm) and formation of 10-oxo-8-decenoic acid (C*H*O; 9.5 ppm).

General Procedure for the Hydroxylation of Naphthalene by Fe(III)·BLM. A reaction mixture containing 4.0 mg (2.75 μmol) of blenoxane and 1.4 mg (2.71 μmol) of ferric perchlorate in 200 μL of water was treated successively with 1.8 mL of CH_3OH, 12.8 mg (100 μmol) of naphthalene, and 2.0 μL (10.5 μmol) of 1-decanol (as an internal standard). The mixture was stirred vigorously for 2 min to dissolve the added naphthalene, and the resulting solution was then treated with the appropriate oxidant in the indicated amount (cf. Table V). After 1 h at 25 °C, the reaction mixture was diluted with 10 mL of water and extracted with 10 mL of $CHCl_3$. The $CHCl_3$ extract was dried ($MgSO_4$) and then analyzed by gas chromatography. The following temperature program was employed at a He gas flow rate of 5.0 mL/min: 100 °C for 2 min; 100 → 200 °C at 40 °C/min; then 200 °C for 15 min. Under these conditions, the observed retention times were as follows: naphthalene 3.4 min; 1-decanol, 5.4 min; 1-naphthol, 12.8 min; 2-naphthol, 12.9 min.

General Procedure for the Epoxidation of Styrene by Fe(III)·BLM. A reaction mixture containing 4.0 mg (2.75 μmol) of blenoxane and 1.4 mg (2.75 μmol) of ferric perchlorate in 200 μL of water was treated successively with 1.0 mL of methanol, 29 mg (32 μL, 275 μmol) of styrene, and 2.0 μL (14 μmol) of ethyl benzoate (as an internal standard). The resulting solution was cooled to −13 °C in a dry ice–benzonitrile bath and the appropriate oxidant was added to the final concentration indicated in Table VI. After 2 h at −13 °C under N_2, the reaction mixture was diluted with 10 mL of water and extracted with 10 mL of $CHCl_3$. The $CHCl_3$ extract was dried ($MgSO_4$) and analyzed by gas chromatography. The following temperature program was employed at a He gas flow rate of 1.0 mL/min: 45 °C for 2 min; 45 → 60 °C at 10 °C/min; 60 °C for 18 min; 60 → 85 °C at 10 °C/min; then 85 °C for 5 min. Under these conditions, the observed retention times were as follows: styrene, 5.4 min; styrene oxide, 18.2 min; phenylacetaldehyde, 18.5 min; ethyl benzoate, 28.3 min.

General Procedure for N-Demethylation of *N*,*N*-Dimethylaniline by Fe(III)·BLM. A reaction mixture containing 4.0 mg (2.75 μmol) of blenoxane and 1.4 mg (2.75 μmol) of ferric perchlorate in 200 μL of water was treated successively with 1.8 mL of methanol, 12.0 mg (12.6 μL, 100 μmol) of *N*,*N*-dimethylaniline, and 2.0 μL (14 μmol) of ethyl benzoate (as an internal standard). The reaction mixture was treated with the appropriate oxidant in the indicated amount (cf. Table VII). After 1 h at 25 °C, the reaction mixture was diluted with 10 mL of water and extracted with 10 mL of $CHCl_3$. The $CHCl_3$ extract was dried ($MgSO_4$) and then analyzed by gas chromatography. The following temperature program was employed at a He gas flow rate of 5.0 mL/min: 40 °C for 2 min; 40 → 70 °C at 10 °C/min; 70 °C for 10 min; 70 → 85 °C at 10 °C/min; then 85 °C for 10 min. Under these conditions, the observed retention times were as follows: *N*,*N*-dimethylaniline, 9.3 min; *N*-methylaniline, 10.3 min; ethyl benzoate, 14.7 min.

Acknowledgment. We thank Mr. James Quada for assistance in obtaining mass spectra and Dr. Erik de Vroom for performing the [^{32}P]DNA cleavage experiment. We thank Professor Lawrence Marnett for providing us with spectral data for 10-hydroperoxy-8,12-octadecadienoic acid. This work was supported by PHS Research Grant CA38544, awarded by the National Cancer Institute, DHHS.