Electrospray Mass Spectrometry of Iron Bleomycin II: Investigation of the Reaction of Fe(III)−Bleomycin with Iodosylbenzene

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Abstract: The reaction of ferric bleomycin (Fe^{III}BLM) with iodosylbenzene (PhIO) has been investigated using electrospray ionization mass spectrometry (ESI-MS) and EPR spectroscopy. A rapid reaction occurs that ultimately yields several cleavage products of the drug, the structures of which have been deduced from their masses. The intermediates observed by ESI-MS suggest a mechanism for the reaction of Fe^{III}BLM with PhIO that does not involve hypervalent iron or activated oxygen but, instead, hypervalent iodine I(III) as the oxidant. Indeed, we could not detect the production of an iron oxene complex of BLM (O=Fe^{V}BLM) or hydroperoxyferric BLM (HOO−Fe^{III}-BLM). Furthermore, we show that the products formed when Fe^{III}BLM is reacted with PhIO are identical to those formed when Fe^{III}BLM is activated in air or when Fe^{III}BLM is reacted with H_2O_2. Surprisingly, the same products are obtained when Zn^{II}BLM or metal-free BLM is reacted with PhIO, indicating that a metal ion is not required for the oxidation of BLM by PhIO.

Introduction

Hypervalent iodine compounds (for reviews, see refs 1−3) have been used in many systems to model oxygen activation by heme and non-heme iron. Iodosylbenzene (PhIO), perhaps the best understood member of this class of reagents, has been shown to support the hydroxylation, epoxidation, and N-dealkylation of various hydrocarbons mediated by transition metal complexes or by cytochrome P-450.[4] In these reactions, PhIO has been proposed[4a] to act as a "single oxygen atom donor" by directly forming an iron(V) oxene,[5] Fe^{V}=O, from the ferric species. Our interest in this chemistry is related to a report by Murugesan et al.[6a] in which ferric bleomycin A_2 (Fe^{III}-BLM, Figure 1b), when reacted with PhIO, was claimed to produce O=Fe^{V}BLM, a species which reportedly carries out epoxidation reactions[6a] as well as DNA cleavage.[6b]

Bleomycin A_2 (BLM, Figure 1, for reviews see refs 7−9) is a glycopeptide antibiotic currently used as a chemotherapeutic agent.[10] Although the drug's mechanism of cytotoxicity remains unproven, it has been shown that BLM is able to bind iron, activate oxygen, and cleave DNA and RNA.[11−14] The form of the drug kinetically competent in the cleavage of DNA is known as activated BLM,[15] which can be formed from ferrous BLM (Fe^{II}BLM) plus dioxygen and a single reducing equivalent,[16,17] or from Fe^{III}BLM with H_2O_2.[15] Due to the similarity of these drug activation reactions to those of cytochrome P-450 and due to the similar chemical transformations supported by the two systems, it was proposed that activated BLM contains an iron oxene analogous to that proposed for the activated form of cytochrome P-450.[18−20] Indeed, the observation by Murugesan et al.[6a,18] that Fe^{III}BLM, when reacted with PhIO, was capable of transferring oxygen to olefinic substrates in a manner similar to cytochrome P-450 led them to propose the formulation of activated BLM as O=Fe^{V}BLM.

It is now known that activated BLM is instead a ferric peroxide, HOO−Fe^{III}BLM. Burger et al. demonstrated by EPR[15] and Mössbauer[21] spectroscopy that activated BLM contains low-spin ferric iron with a least one atom of oxygen, derived from O_2 or H_2O_2, bound to the iron atom. Furthermore, the low-spin EPR signal of activated BLM (g = 2.27, 2.17,

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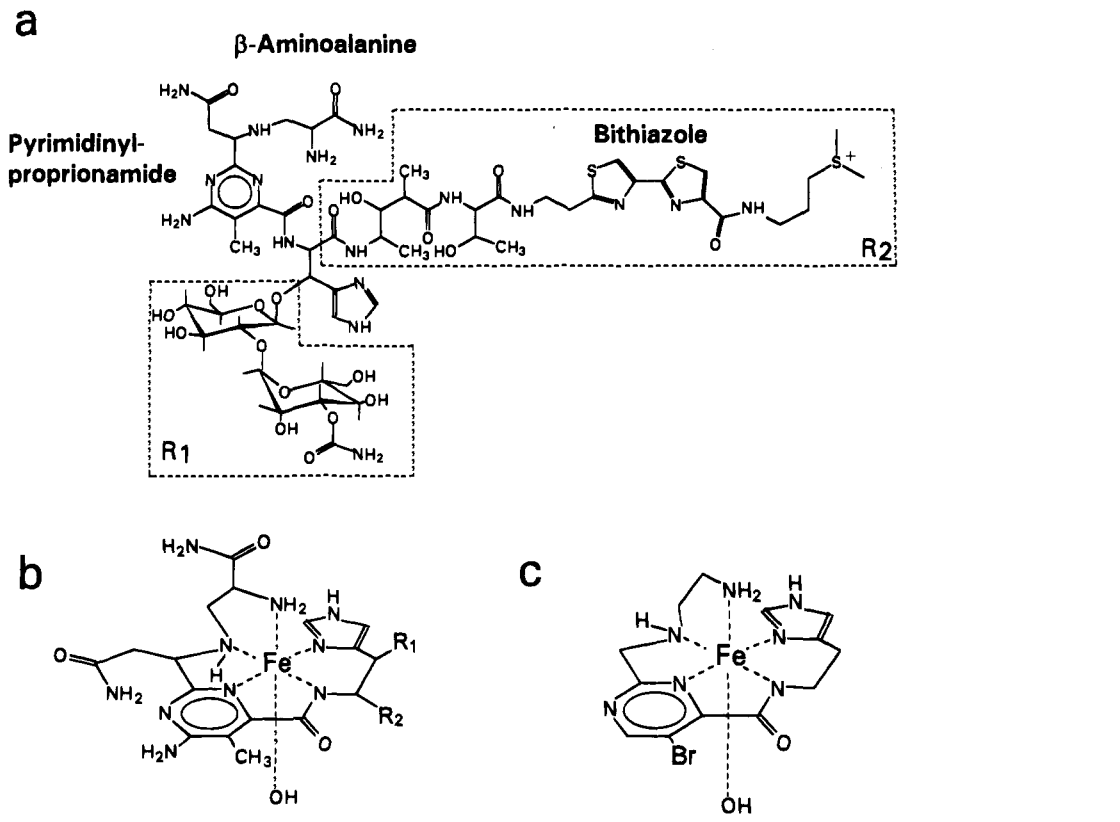

Figure 1. Structures of (a) metal-free BLM with relevant residues indicated; (b) Fe^{III}BLM (proposed in ref 38); and (c) Fe^{III}PMA, taken from ref 24.

1.94) is similar to that of many heme and non-heme ferric peroxides.[22] Recently, we used electrospray mass spectrometry (ESI-MS) and tandem mass spectroscopy (MS/MS) to demonstrate that activated BLM contains two oxygen atoms derived from hydrogen peroxide and is, therefore, a ferric peroxide and not an iron oxene.[23] The question we address in this report is whether Fe^{III}BLM can be activated to the same species with PhIO as it can with O_2 or H_2O_2. The chemistry of iron bleomycin plus iodosylbenzene is still at issue, though a recent report by Guajardo et al.[24] shows that Fe^{III}PMA (Figure 1c), a synthetic analog of Fe^{III}BLM, when reacted with PhIO plus 1 equiv of $^-$OH, generates a species with an EPR spectrum (g = 2.28, 2.17, 1.93) nearly identical to that of activated BLM. This result is remarkable in that (a) previous attempts at producing a species with the EPR signal of activated BLM from Fe^{III}-BLM and PhIO were unsuccessful[25] and (b) the apparent production of HOO–Fe^{III}PMA implies the initial production of an iron(V) oxene which is attacked by $^-$OH.[26] This could represent the reverse of the reaction reported for several other iron systems in which initial production of a ferric peroxide is followed by heterolysis of the O–O bond, generating an iron oxene.[27]

We have used EPR spectroscopy, ESI-MS, and MS/MS to investigate the reaction of Fe^{III}BLM with PhIO. We did not detect the production of activated BLM, despite our ability to observe its formation upon reacting Fe^{III}BLM with H_2O_2 under the same conditions. Furthermore, the intermediates produced upon reacting Fe^{III}BLM, Zn^{II}BLM, and even metal-free BLM with PhIO indicate that the reactions involve hypervalent iodine and not, as suggested by others,[18–20] hypervalent iron or activated oxygen.

Experimental Section

Sample Preparation. Bleomycin A_2 was purified from bleomycin sulfate (BLENOXANE), generously supplied by Bristol Myers Co. (Syracuse, NY), using a Source 15S FPLC column (Pharmacia, Piscataway, NJ) and eluting with a linear gradient of NH_4HCO_3. Fractions containing BLM were pooled and lyophilized repeatedly to remove buffer. Metal complexes of BLM were prepared by the addition of $Fe^{III}Cl_3$ (Aldrich) or $ZnCl_2$ (99.999% pure, Aldrich) to 1.1 equiv of BLM. The absence of iron in solutions of ZnBLM and metal-free BLM was confirmed by ESI-MS. Iodosylbenzene was prepared from iodobenzene diacetate by the addition of NaOH, as previously reported.[28]

EPR Spectroscopy. EPR spectra (77 K) were collected on an X-band Varian E112 spectrometer equipped with a Systron-Donner frequency counter, a Varian NMR gaussmeter, a liquid N_2 finger dewar, and a rectangular TE101 cavity. Spectroscopy was typically performed using 10–30 mW microwave power and 5 G modulation amplitude at 100 kHz.

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(26) While Guajardo et al.[24] explicitly proposed the production of an iron oxene preceding the formation of the ferric peroxide complex, Sauer-Masarwa et al. (Sauer-Masarwa, A.; Herron, N.; Fendrick, C. M.; Busch, D. H. *Inorg. Chem.* **1993**, *32*, 1086–1094), who first reported this chemistry, argued that other mechanistic interpretations of this reaction are possible.

(27) (a) Dawson, J. H. *Science* **1988**, *240*, 433–439. (b) Marnett, L. J.; Weller, P.; Battista, J. R. In *Cytochrome P-450, Structure, Mechanism, and Biochemistry*; Ortiz de Montellano, P. R., Ed.; Plenum Press: New York, 1986; pp 29–76.

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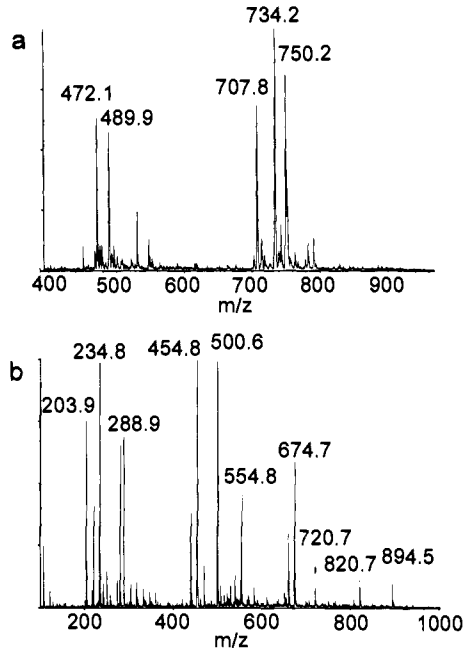

Figure 2. Mass spectra of (a) PhIO (1 mM) and (b) $Fe^{III}BLM$ (0.5 mM), both in MeOH. Relevant peaks are labeled, and their assignments are given in Table 1. The *y*-axis indicates relative peak intensity.

Mass Spectrometry. All mass spectra were acquired using an API III triple-quadrupole mass spectrometer equipped with an ionspray interface (PE-Sciex, Thornhill, ON, Canada), as previously reported.[23] Samples were introduced using a dual syringe pump (Harvard Apparatus, South Natick, MA) and Hamilton syringes (Hamilton Co., Reno, NE) feeding into a low dead volume mixing tee (Upchurch, Oak Harbor, WA) that interfaced with the ionspray source. For most of the experiments, the mass resolution of the spectrometer was tuned to give a constant peak width of 0.5 Da (at 10% peak height) across the mass range of interest, so that the charge state of the ions could be unambiguously identified. Typically, 10 scans of 10–20 s duration were acquired and added to yield a mass spectrum. Fragment ion (tandem) mass spectra (MS/MS) were obtained by collision-induced dissociation of precursor ions selected by their *m/z* value in the first quadrupole. Collisional activation was accomplished by introducing argon into the second (rf-only) quadrupole, and the resulting fragment ions were analyzed in the third quadrupole of the instrument. In these experiments, a target-gas thickness of 1.5×10^{14} atoms/cm^2 and laboratory-frame collisional energies of 40–60 eV for the doubly charged BLM complexes were typically employed.

Results

Intermediates in the Reaction of $Fe^{III}BLM$ with PhIO. Figure 2a shows the m/z = 400–960 region (m/z = mass to charge ratio) of the mass spectrum of $Fe^{III}BLM$ in MeOH; the assignments of the ions referred to in this paper are compiled in Table 1. The peaks corresponding to BLM, $Fe^{III}BLM$,[29] and $Fe^{III}BLM$ complexed with various anions can be seen and have been previously observed[23] in the mass spectra of $Fe^{III}BLM$ in H_2O, with the exception of an ion with m/z = 750.2. This peak is attributable to the methoxide complex of $Fe^{III}BLM$ ($Fe^{III}BLM^{4+} + MeO^- - H^+$; the m/z for this species is 1500.5/2, or 750.2). It should be noted that the charge (z) of each ion is easily determined from the spacing of the peaks of a given ion, which correspond to differences in mass by 1 Da due to the presence of naturally abundant heavy isotopes; i.e., a z = 2 ion has peaks spaced 0.5 m/z units apart, whereas a z = 1 ion has peaks separated by a single m/z unit; the molecular mass of each ion can then be calculated directly from the observed m/z. The mass spectrum of PhIO dissolved in MeOH is provided in Figure 2b. As reported by Schardt and Hill[30] and verified by our observation of ions with m/z = 234.8, 288.9, 500.6, etc., PhIO in MeOH undergoes extensive solvolysis, although a small peak with m/z = 220.9, attributable to the parent compound, is seen. Also, several polymeric forms, e.g., with m/z = 454.8, 500.6, etc. (Figure 2b), are observed.

We then acquired the mass spectra of the ions produced 2–600 s after mixing methanolic solutions of $Fe^{III}BLM$ and PhIO (not shown). Although several new peaks with intensities that rise and fall as a function of reaction time are observed (see below), we cannot detect the formation of activated BLM (m/z = 751.3) nor BLM–Fe^{V}=O (m/z = 742.3) in any of the spectra. Furthermore, the addition of neither base (NaOH) nor H_2O generates these species. However, since methanolic solutions were used (in an attempt to mimic the conditions used by Guajardo et al.[24]), it is difficult to rule out the production of a small amount of activated BLM that is masked by the large m/z = 750.2 peak due to the methoxide complex of $Fe^{III}BLM$.

Table 1. Proposed Assignments of the Ions Cited in This Paper[a]

m/z	z	assignment
203.9	1	$I(C_6H_4)_2I + H^+$
220.9	1	$PhIO + H^+$
234.8	1	PhI^+OMe
288.9	1	$PhI(OMe)_2 + Na^+$
454.8	1	$PhIO + PhI^+OMe$
472.2	3	$Blm^+ + 2H^+$
489.9	3	$Blm^+ + Fe^{3+} - H^+$
500.8	1	$PhI(OMe)_2 + PhI^+OMe$
554.8	1	$(PhI(OMe)_2)_2 + Na^+$
654.2	2	$[Blm^+ + {}^{2+}IPh - H^+] - {}^{+}IPh - CONH_2 - S(CH_3)_2 + H^+$
664.3	2	$Blm^+ - {}^{-}NHCH_2CH(NH_2)CONH_2 + O$
664.8	2	$Blm^+ - {}^{+}CH_2CH(NH_2)CONH_2 + 2H^+$
665.8	2	$[Blm^+ + Fe^{3+} + PhI(OMe)_2 - 2H^+] - PhI - 2MeOH - S(CH_3)_2 - CH(NH_2)CONH_2$
670.8	2	$Blm^+ - {}^{-}CH(NH_2)CONH_2$
674.8	1	$(PhIO)_2 + PhI^+OMe$
675.8	2	$[Blm^+ + {}^{2+}IPh - H^+] - IPh - S(CH_3)_2$
680.8	2	$[Blm^+ + Fe^{3+} + PhI(OMe)_2 - 2H^+] - PhIOMe - MeOH - S(CH_3)_2 - CONH_2$
690.8	2	$Blm^+ + Fe^{3+} - CH_2CH(NH_2)CONH_2 - 2H$
702.3	2	$[Blm^+ + Fe^{3+} + PhI(OMe)_2 - 2H^+] - PhI - 2MeOH - S(CH_3)_2$
706.8	2	$Blm^+ - 2H + H^+$
707.8	2	$Blm^+ + H^+$
718.8	2	$Blm^+ + Na^+$
720.4	1	$(PhI^+OMe)_3 + O^{2-}$
734.3	2	$Blm^+ + Fe^{3+} - 2H^+$
742.3[b]	2	$Blm^+ + (Fe^{V}O^{2-}, Fe^{IV}O^-, Fe^{III}O^0)^{3+} - 2H^+$
743.3	2	$Blm^+ + Fe^{3+} + {}^{-}OH - H^+$
750.3	2	$Blm^+ + Fe^{3+} + {}^{-}OMe - H^+$
751.3[c]	2	$Blm^+ + Fe^{3+} + {}^{-}OOH - H^+$
808.7	2	$Blm^+ + {}^{2+}IPh - H^+$
820.7	1	$(PhI(OMe)_2)_3 + Na^+$
835.7	2	$Blm^+ + Fe^{3+} + {}^{+}IPh - 3H^+$
851.3	2	$Blm^+ + Fe^{3+} + PhI^+OMe - 3H^+$
858.3	2	$Blm^+ + Fe^{3+} + PhI^+OEt - 3H^+$
867.2	2	$Blm^+ + Fe^{3+} + PhI(OMe)_2 - 2H^+$
874.2	2	$Blm^+ + Fe^{3+} + PhI(OMe)(OEt) - 2H^+$
881.2	2	$Blm^+ + Fe^{3+} + PhI(OEt)_2 - 2H^+$
894.5	1	$(PhIO)_3 + PhI^+OMe$

[a] The m/z values indicated are calculated from the assignment proposed; the slight deviations from the m/z values cited in the text are attributable to experimental error. As an aid to the reader, brackets have been included to indicate the composition of the precursor ion that yielded the assigned fragment ion upon collisional activation. [b] This ion was not observed. [c] Activated BLM.

(29) The binding of Fe^{3+} to BLM^+ displaces three protons from the drug, forming $[Fe^{III}BLM]^+$ (see ref 9). Upon ionization, this species gains another proton, producing $[Fe^{III}BLM]^{2+}$ which has m/z = 734.3 (Fe(III) + BLM − 2H; cf. Table 1).

(30) Schardt, B.; Hill, C. *Inorg. Chem.* **1983**, *22*, 1563–1565.

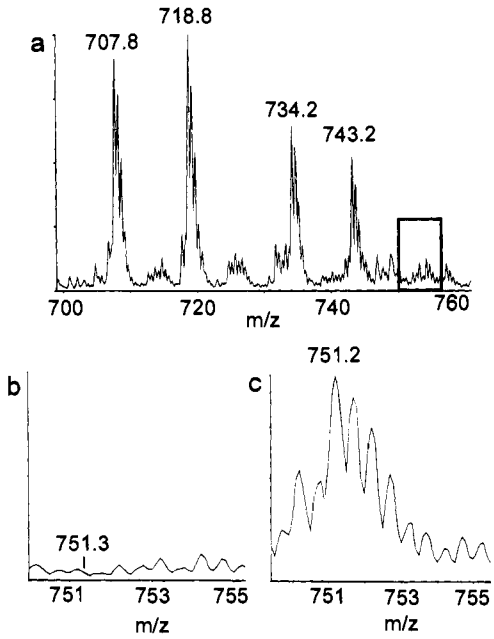

Figure 3. Mass spectra of Fe^{III}BLM (1 mM) in EtOH reacted with PhIO (2 mM) (a,b) or H_2O_2 (10 mM)[39] (c). Spectra b and c are expanded views of the spectral region indicated by the box in a. Relevant peaks are labeled, and their assignments are given in Table 1. The *y*-axis indicates relative peak intensity.

Thus, solutions of Fe^{III}BLM and PhIO in 9:1 EtOH/H_2O were rapidly mixed and analyzed by ESI-MS. The spectrum obtained 15 s after mixing is shown in Figure 3a; Figure 3b contains an expanded region of the spectrum in Figure 3a, illustrating the absence of an m/z = 751.3 species. These results are in sharp contrast to the mass spectrum of Fe^{III}BLM reacted with H_2O_2 in 9:1 EtOH/H_2O in which a large peak at m/z = 751.2 is seen (Figure 3c). Thus, the absence of activated BLM upon reacting Fe^{III}BLM with PhIO is not due to an inherent instability of activated BLM under the present conditions nor due to an inadequacy of the mass spectrometric parameters used in these experiments.

We also examined the reaction of Fe^{III}BLM with PhIO by EPR spectroscopy. Figure 4a contains the spectrum obtained approximately 30 s after reacting (MeOH, −78 °C) Fe^{III}BLM with PhIO followed by the addition of NaOH. The EPR signal in Figure 4a is typical of low-spin HO–Fe^{III}BLM (g = 2.43, 2.18, 1.89). Similar results are obtained (1) at longer reaction times, i.e., after thawing and refreezing the solution, (2) when MeOH/CH_3CN is employed as the solvent,[31] and (3) in the absence of base (data not shown). Activated BLM, produced by reacting Fe^{III}BLM with H_2O_2 (EtOH, 24 °C), exhibits an EPR signal with g = 2.26, 2.17, 1.93, as shown in Figure 4b. Thus, using both ESI-MS and EPR spectroscopy, we could not detect production of activated BLM upon reacting PhIO with Fe^{III}BLM with or without the addition of base.

Although no activated BLM is detected, we do observe several new species, e.g., with m/z = 808.8, 835.7, 851.3, and 867.2, in the mass spectrum acquired after reacting PhIO with Fe^{III}BLM in MeOH, as shown in Figure 5a. Recently, we reported the use of tandem mass spectrometry as an aid in determining the assignments of peaks in the mass spectra of BLM complexes;[23] we have now applied the same methods to the investigation of the intermediates of the reaction of Fe^{III}-

(31) The solvent used by Guajardo et al.[24] was an unspecified mixture of CH_3CN and MeOH. We reacted Fe^{III}BLM and PhIO in 1:4:5 CH_3CN/MeOH/ethylene glycol, 3:2 CH_3CN/MeOH, and 10:1 DMF/H_2O, and in each case, we could not detect the production of activated BLM.

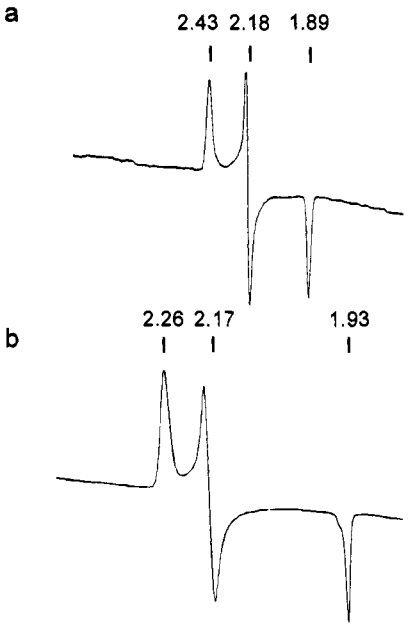

Figure 4. EPR spectrum of Fe^{III}BLM (1 mM) reacted with (a) PhIO (1.5 mM) and NaOH (3 mM) in MeOH and (b) H_2O_2 (20 mM)[39] in EtOH. Solutions were diluted 1:1 with ethylene glycol immediately prior to freezing in liquid N_2. The g-values of the peaks are indicated. Spectroscopic parameters are provided in the Experimental Section.

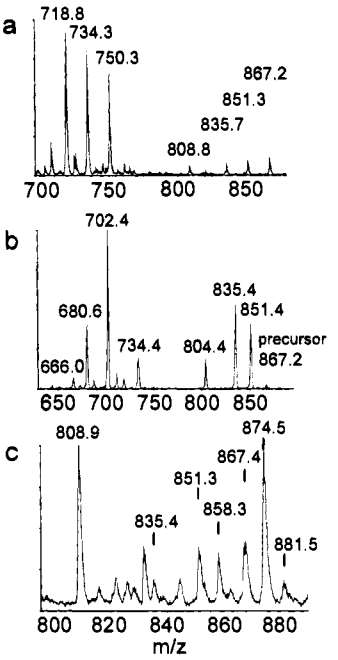

Figure 5. (a) Mass spectrum of Fe^{III}BLM (1 mM) reacted with PhIO (1 mM) in MeOH. (b) Tandem mass spectrum of the m/z = 867.2 ion in spectrum a. (c) Mass spectrum of Fe^{III}BLM (0.5 mM) reacted with PhIO (0.5 mM) in 4:1 EtOH/MeOH. Relevant peaks are labeled, and their assignments are given in Table 1. The *y*-axis indicates relative peak intensity.

BLM and PhIO. The m/z = 867.2 and 851.3 species have masses corresponding to Fe^{III}BLM complexed with $PhI(OMe)_2$ and $PhI^+(OMe)$, respectively, and were the first ions studied by MS/MS. As shown in Figure 5b, the observed fragmentation pattern of the m/z = 867.2 ion agrees well with its assignment as the iodobenzene dimethoxide ($PhI(OMe)_2$) complex of Fe^{III}-BLM, which can shed one or both of its methoxide groups as methanol, producing the m/z = 851.4 and 835.4 fragments. Furthermore, ions with m/z = 734.4, 702.4, 680.6, and 666.0

can also be seen in Figure 5b. These fragment ions have been observed previously in the MS/MS spectra of Fe^{III}BLM complexed with various anions.[23] The presence of these ions in Figure 5b demonstrates that the $PhI(OMe)_2$ moiety can dissociate, upon collisional activation, from the Fe^{III}BLM molecule, which then gives the same fragments as Fe^{III}BLM complexed with various anions. Thus, the m/z = 867.2 ion is assigned as ferric BLM with $PhI(OMe)_2$ bound to iron ($PhI(OMe)_2$–Fe^{III}-BLM). Additionally, the MS/MS spectrum of the m/z = 851.3 ion (data not shown) contains the same fragment ions, e.g., with m/z = 835.7, 734.4, 702.4, 680.7, and 666.0, as in Figure 5b and is also in agreement with the assignment of this species as PhI^+OMe–Fe^{III}BLM.

Also observed as an intermediate in the reaction of Fe^{III}BLM with PhIO is an ion with m/z = 835.7 (Figure 5a); this mass to charge ratio corresponds to the PhI^+ complex of Fe^{III}BLM, which might be formed by loss of $CH_3O^\bullet$ from PhI^+OMe–Fe^{III}BLM. The tandem mass spectrum of this ion (not shown) contains fragment ions from the unmodified Fe^{III}BLM moiety with m/z = 734.4, 680.7, and 665.8, consistent with the assignment as $Fe^{III}PhI^+$–BLM. MS/MS was also performed on the m/z = 808.7 intermediate (data not shown). The mass of this ion corresponds to the PhI^{2+} complex of metal-free BLM. Accordingly, the fragment ions produced exhibit m/z values of 675.8 and 654.2 and correspond to the metal-free equivalents of the m/z = 702.3 and 680.8 ions observed in the MS/MS spectra of the iron-containing intermediates in the reaction.

We obtained further support for the assignments of the m/z = 808.7, 835.7, 851.3, and 867.2 ions by using 4:1 EtOH/MeOH as the solvent in the reaction. Under these conditions, ions with m/z = 858.3, 874.5, and 881.5 are observed (Figure 5c), indicating the replacement of methoxide in the m/z = 851.3 and 867.2 ions by ethoxide with the concomitant increase in mass by 14 Da and m/z by 7. In contrast, the m/z = 808.7 or 835.7 species remain unchanged. These data, in conjunction with the MS/MS results, provide compelling evidence for the assignments of these ions given in Table 1. Furthermore, the identification of these intermediates is intriguing in that a mechanism for the reaction of Fe^{III}BLM with $PhI(OMe)_2$ (Figure 6) can be proposed that accounts for all of the observed intermediates and yet does not involve species containing activated oxygen or hypervalent iron. The absence of activated oxygen and hypervalent iron will be addressed in more detail below and in the Discussion section.

Products of the Reaction of Fe^{III}BLM with PhIO. It has been shown that upon reaction of a large excess of PhIO with Fe^{III}BLM the fluorescence properties and metal-binding capacity of the drug are rapidly altered, implying modification at the bithiazole moiety and N-terminal (metal-binding) region of the Fe^{III}BLM molecule.[25a] Furthermore, these alterations of the BLM molecule are similar to those observed in the autoxidation of activated BLM.[23,32,33] Hence, we used ESI-MS to identify the terminal products formed by reacting Fe^{III}BLM with PhIO. Since it has been demonstrated here that no iron oxene- or ferric peroxide-containing intermediates are observed in the reaction of Fe^{III}BLM with PhIO, and in addition, since it has previously been shown that Zn^{II}BLM is capable of supporting the oxidation of stilbene by PhIO (a reaction presumed to require hypervalent iron),[34] the question of how or whether iron and/or hypervalent oxidation states facilitate the oxidation of BLM by PhIO is raised. We therefore compared the products of the reaction of

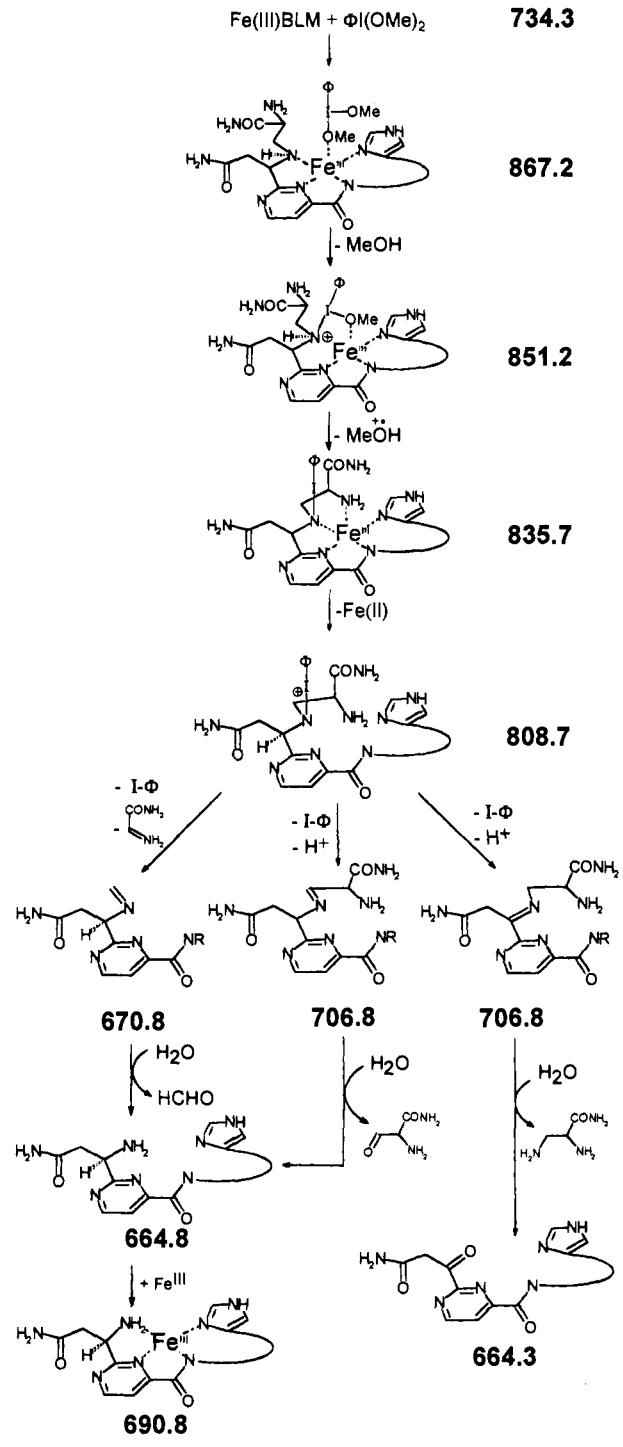

Figure 6. Proposed mechanism for the reaction of Fe^{III}BLM with PhIO in H_2O. The m/z values of relevant species are labeled; see Table 1 for the assignments of these ions.

Zn^{II}BLM or metal-free BLM with PhIO to those formed with Fe^{III}BLM. For these experiments, the reagents were mixed and incubated for approximately 30 min, and the mass spectra were acquired. Panels a–c of Figure 7 display the m/z = 660–710 region of the mass spectra of the products obtained from PhIO reacted in MeOH with Fe^{III}BLM, Zn^{II}BLM, and BLM, respectively (the other regions of these spectra contain only ions from unreacted starting materials). The most striking feature of these spectra is that the same products are produced, albeit in different distributions, despite the differences in the metal employed, or lack thereof. Furthermore, the ions observed in the spectra, e.g., m/z = 706.8, 670.8, and 664.8 (Figures 7a–c and 6), suggest

(32) Nakamura, M.; Peisach, J. *J. Antibiot.* **1988**, *41*, 638–647.
(33) Takita, T.; Muraoka, Y.; Nakatani, T.; Fujii, A.; Iitaka, Y.; Umezawa, H. *J. Antibiot.* **1978**, *31*, 1073–1077.
(34) Moriarty, R.; Penmasta, R.; Prakash, I. *Tetrahedron Lett.* **1985**, *26*, 4699–4702.

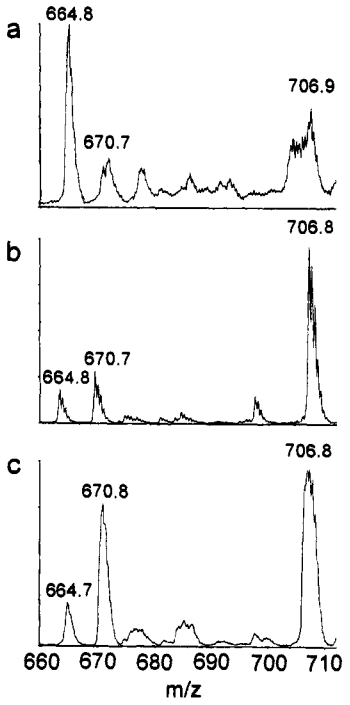

Figure 7. Mass spectra of PhIO (2 mM) reacted with (a) Fe^{III}BLM (1 mM), (b) Zn^{II}BLM (1 mM), and (c) metal-free BLM (1.1 mM) in MeOH. Relevant peaks are labeled, and their assignments are given in Table 1. The *y*-axis indicates relative peak intensity.

that the major reaction, in all cases, is N-dealkylation of the β-aminoalanine secondary amine (Figure 1).

N-Dealkylation of the drug also occurs in the autoxidation of Fe^{III}BLM activated with H_2O_2 and Fe^{II}BLM activated with O_2.[33] However, the latter reactions were performed using aqueous solutions, and the major product obtained was the 2-(2-carbamoyl-1-oxoethyl)pyrimidinyl derivative (Figure 6; m/z = 664.3) while the 2-(2-carbamoyl-1-aminoethyl)pyrimidinyl derivative (Figure 6; m/z = 690.8) was a secondary product; interestingly, the m/z = 664.8 and 706.8 ions were not observed. We therefore reacted Fe^{III}BLM with PhIO using 1:1 MeOH/H_2O as the solvent. Under these conditions, the m/z = 664.3 derivative is, as predicted, an additional product (data not shown). Furthermore, we observe an increase in the yields of the products with m/z = 664.3, 664.8, and 690.8 relative to those with m/z = 670.8 and 706.8 (data not shown). This indicates an increase in the solvolysis of the imine derivatives, i.e., the m/z = 670.8 and 706.8 ions.

Lastly, we compared the rates of the reactions of BLM, Zn^{II}-BLM, and Fe^{III}BLM with PhIO in MeOH by ESI-MS. Figure 8 displays the rate of formation of the m/z = 664.8 product versus time. The data indicate that the rate of oxidation of BLM by PhIO is enhanced approximately 2-fold by the presence of Zn(II) and nearly 30-fold by the addition of Fe(III). These results, and their interpretation, will be discussed below.

Discussion

For nearly two decades, researchers have used iodosylbenzene as a "single oxygen atom donor" to directly generate hypervalent metal oxenes from their normal-valent counterparts. In particular, Murugesan et al.[6a] demonstrated that Fe^{III}BLM reacted with PhIO is capable of epoxidizing olefinic substrates. They further postulated that Fe^{III}BLM reacted with PhIO produces activated BLM, formulated in their scheme as $O{=}Fe^{V}$BLM, and that this species is responsible for the transfer of oxygen to the substrate.[18] However, it was subsequently shown (1) that under

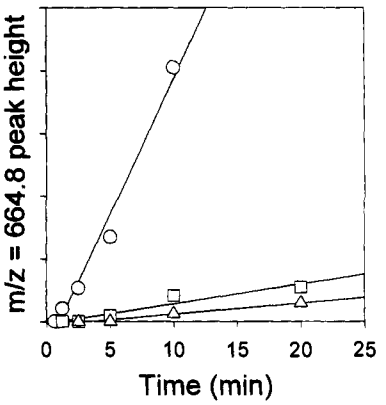

Figure 8. Rate of oxidation of BLM when Fe^{III}BLM (○), Zn^{II}BLM (□), or metal-free BLM (△) is reacted with PhIO in MeOH. The lines represent least-squares fitting; the error of measurement was estimated at 5%. The *y*-axis indicates the relative peak height of the m/z = 664.8 ion; the assignment of this ion is provided in Table 1 and Figure 6.

the conditions employed by Murugesan et al.[6a,18] (involving, for example, a 20-fold excess of PhIO), Fe^{III}BLM is rapidly degraded and no activated BLM is detectable by EPR spectroscopy,[25] (2) that activated bleomycin is, in fact, a low-spin ferric peroxide complex,[15,21,23] and (3) that Zn^{II}BLM, a species presumably incapable of forming a hypervalent metal oxo intermediate, supports the epoxidation of stilbene by PhIO.[34] Although it is difficult to reconcile these results with the scheme proposed by Murugesan et al.,[6a,18] it has recently been demonstrated that Fe^{III}PMA, a synthetic Fe^{III}BLM analog, reacted with PhIO and then $^{-}$OH or H_2O, generates a species with an EPR signal almost identical to that of activated BLM.[15,24] These findings were interpreted as initial formation of $O{=}Fe^{V}$PMA followed by addition of $^{-}$OH to form the ferric peroxide complex, $HOO{-}Fe^{III}$PMA or "activated PMA".[24]

Since we have recently had success using ESI-MS and MS/MS to analyze activated BLM produced from Fe^{II}BLM and O_2 or Fe^{III}BLM and H_2O_2, we applied the same techniques to the investigation of the reaction of Fe^{III}BLM with PhIO. Using reaction conditions similar to those of Guajardo et al.,[24] we did not detect production of activated BLM ($HOO{-}Fe^{III}$BLM) or $O{=}Fe^{V}$BLM by mass spectrometry, nor was the formation of activated BLM observed by EPR spectroscopy. Furthermore, we obtain identical results when Fe^{III}BLM is reacted with PhIO followed by the addition of either $^{-}$OH or H_2O. Since we are able to observe activated BLM produced by reacting Fe^{III}BLM with H_2O_2, we conclude that, under the conditions reported here, Fe^{III}BLM reacted with PhIO does not produce activated BLM or $O{=}Fe^{V}$BLM in amounts greater than the detection limit, about 5% of the total Fe^{III}BLM.

An unresolved issue in investigations of PhIO-mediated oxidations is the role of the metal ion. Some[18-20] have proposed that hypervalent metal oxo species are generated and that these are responsible for oxidation of substrate; the present experiments seem to contradict this mechanism for the oxidation of Fe^{III}BLM by PhIO. Others[34] contend that the metal serves as a Lewis acid by increasing the electrophilicity of I(III). The demonstration that Zn^{II}BLM with PhIO is able to oxidize stilbene[34] and, in the absence of substrate, is oxidized itself, at a rate greater than that of metal-free BLM (Figure 8), supports this contention. However, the increased rate of drug oxidation attributable to Fe(III) is an order of magnitude greater than that of Zn(II).[35] This may be due, for example, to differences in the Lewis acidity of the trivalent vs divalent metal complexes or in the three-dimensional conformations of the metal–BLM complexes, i.e., Fe^{III}BLM may have a geometry that facilitates,

relative to Zn^{II}BLM, attack of the iodine atom on the β-aminoalanine secondary amine nitrogen atom. Although the present experiments do not allow one to distinguish between these and other interpretations of the data, they do demonstrate that the metal ion is not *required* for the oxidation of BLM by PhIO.

Furthermore, we have identified, using ESI-MS and MS/MS, several intermediates (Figure 6) which indicate that the reaction of Fe^{III}BLM and PhIO does not involve activated oxygen or hypervalent iron but instead hypervalent iodine, I(III), as the reactive species. Although similar intermediates have been proposed in previous mechanistic studies,[34,36] the present results represent a rare direct observation of these species.[37]

It should be stressed that the reactions proposed in this paper (above; and Figure 6), while contrary to the commonly held view that PhIO acts as a biomimetic "single oxygen atom donor", are in accord with the large body of literature regarding the use of PhIO and related compounds in organic synthesis.[1-3] In the majority of the systems studied, the hypervalent iodine compound undergoes ligand exchange at the electrophilic I(III) atom followed by reductive elimination, releasing iodobenzene. Similarly, in the case of Fe^{III}BLM, attack of I(III) on the β-aminoalanine secondary amine nitrogen atom affords the iodobenzene derivative of BLM (Figure 6, m/z = 808.7) and 2 equiv of MeOH. Subsequent dehydrohalogenation yields iodobenzene and BLM oxidation products (Figure 6, m/z = 670.8 and 706.8). The imines thus formed are unstable and fragment upon solvent addition (Figure 6). Lastly, it is a remarkable testament of the utility of ESI-MS that essentially all of the intermediates in the mechanism proposed in Figure 6 are observable by this technique, and that, even without prior knowledge of the reactivity of PhIO, one could formulate this mechanism from the identities of the observed intermediates alone.

Acknowledgment. This investigation was supported by Grants RR09113 from the NIH, RR02583 and GM40168 from the United States Public Health Service (J.P.), a Biomedical Research Support grant from the AECOM (X.J.T.), and an MSTP training grant (T32 GM07288) from the NIGMS (J.W.S.).

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(35) Interestingly, Valentine and co-workers (VanAtta, R. B.; Franklin, C. C.; Valentine, J. S. *Inorg. Chem.* **1984**, *23*, 4121–4123) observed much higher rates of epoxidation using PhIO with $Fe^{III}(OTf)_3$ as compared to Mn(II), Co(II), or Cu(II) triflate complexes.

(36) Yang, Y.; Diederich, F.; Valentine, J. *J. Am. Chem. Soc.* **1990**, *112*, 7826–7828.

(37) The isolation and characterization of $[XMn^{IV}TPP(OIPh)_2O$ (X = Cl^- or Br^-, TPP = tetraphenylporphyrin) has been reported: Smegal, J. A.; Schardt, B. C.; Hill, C. L. *J. Am. Chem. Soc.* **1983**, *105*, 3510–3515.

(38) Sugiura, Y. *J. Am. Chem. Soc.* **1980**, *102*, 5208–5215.

(39) A greater number of equivalents of hydrogen peroxide, relative to iodosylbenzene, were used in these reactions since (1) it has previously been demonstrated that reacting a large excess of PhIO with Fe^{III}BLM causes rapid destruction of the drug without the production of activated BLM[25] and (2) the apparent K_m for H_2O_2 has been shown to be ~30 mM.[15]