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PEROXIDASE-CATALYSED OXIDATION OF N^2,N^6-DIMETHYL-9-HYDROXYELLIPTICINIUM ACETATE

EVIDENCE FOR THE FORMATION OF AN ELECTROPHILIC QUINONE-IMINIUM DERIVATIVE

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Abstract—The activation of N^2,N^6-dimethyl-9-hydroxyellipticinium acetate (DMHE) by a peroxidase-H_2O_2 system leads to a reactive orthoquinone, or in the presence of a nucleophile like alanine, to adducts with a proposed benzoxazole structure. The stoichiometric and pH metric studies support the generation of a bicationic electrophilic intermediate, namely a quinone-iminium. Since no N^6-demethylation occurs during the oxidation process, DMHE is not a prodrug of Celiptium (N^2-methyl-9-hydroxy-ellipticinium acetate), but the high electrophilic properties of the species generated might explain its great cytotoxicity and antitumor properties. These results extend the possibility for N^6-methyl ellipticine derivatives of a biooxidative activation which can play a role in their cytotoxicity.

In the ellipticine series, only elliptinium acetate (N^2-methyl-9-hydroxyellipticinium acetate or NMHE)§ (Fig. 1) is currently used in clinical trials as an antitumor agent [1, 2]. Among all the various hypotheses on its mechanism of action, we have focused our attention on its biotransformation. This molecule is easily oxidized *in vitro* by the horseradish peroxidase (HRP) in the presence of hydrogen peroxide to an electrophilic quinone-imine derivative [3, 4] which can alkylate biological molecules [5, 6] and their models [7]. The identification of thiol-derivatives of NMHE in biological fluids of animals [8] or patients [9] treated with this antitumor agent confirms that such oxidation process occurs also *in vivo*. Furthermore, we have recently shown that nucleic acids of L1210 cells are alkylated by the drug after incubation with it [10].

Besides this drug, two other ellipticine compounds are highly cytotoxic and exhibit noticeable antitumor activities: 9-methoxyellipticine (9-OMe-E) [11] and N^2,N^6-dimethyl-9-hydroxyellipticinium acetate (DMHE) [12] (Fig. 1).

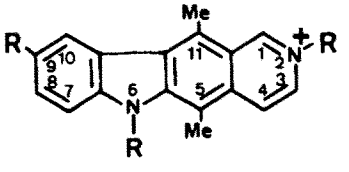
These two drugs are both masked *para* aminophenol structures, the first by methylation of the phenolic O–H and the second by methylation of the indolic N–H (Fig. 1). The consequence is an increase in their redox potential values (cyclic voltammetry shows anodic sweeps at +180, +500 and +420 mV for NMHE, 9-OMe-E and DMHE, respectively) which should decrease the ability of HRP to oxidise them.

In a recent report, we have shown that unexpectedly 9-OMe-E is demethoxylated by the HRP/H_2O_2 system leading to an electrophilic quinone-imine (as for NMHE) and methanol [13].

Here, we report that DMHE is easily oxidized by HRP but no demethylation occurs; the electrophilic intermediate (a quinone-iminium structure) can react with differents nucleophiles to give various adducts.

MATERIALS AND METHODS

Horseradish peroxidase (type VI, EC 1.11.1.7) was obtained from Sigma and used without further



Ring substituent			Compound
2	6	9	
Me	H	OH	NMHE
H	H	OMe	9-OMe-E
Me	Me	OH	DMHE

Fig. 1. Structure of ellipticine derivatives.

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§ Abbreviations used: NMHE, N^2-methyl-9-hydroxy-ellipticinium; 9-OMe-E, 9-methoxyellipticine; DMHE, N^6,N^6-dimethyl-9-hydroxyellipticinium; dioxo-NME, 9,10-dioxo-N^2-methylellipticinium; dioxo-DME, 9,10-dioxo-N^2,N^6-dimethylellipticinium; ala$_1$-DME, major alanine-DMHE adduct; ala$_2$-DME, minor alanine-DMHE adduct; HRP, horseradish peroxidase; H_2O_2, hydrogen peroxide; ala, alanine; M.S., mass spectrometry; DCI, desorption and chemical ionisation; ala-NME, alanine-NMHE adduct; RRT, relative retention time on HPLC compared to the retention time of DMHE under standard conditions (see experimental section).

purification. Enzyme solutions were calibrated spectrophotometrically [14]. DMHE was synthetised in the laboratory by Dr J Chenu according to a method previously described [15]. Hydrogen peroxide solution (30%) and ammonium hexafluorophosphate were obtained from Merck, L-alanine from Sigma, Sep-Pak C_{18} cartridges from Waters, LH20 gel from Pharmacia (Uppsala, Sweden) and XAD_2 resines from Serva (Heidelberg, W. Germany). Common laboratory reagents were purchased from Prolabo (Paris, France). The peroxide concentration was determined by iodometric titration [16].

Spectrometric methods. NMR Spectra were recorded on a Bruker WM 250 (250 MHz) spectrometer operated in the Fourier transform mode. The chemical shifts are expressed in ppm (δ) with tetramethylsilane as internal standard. A Riber 10–10 apparatus was used for mass spectrometric data. The technique of the desorption and chemical ionisation (DCI) with NH_3 as vector gas was employed. U.v.-visible spectra were obtained with a Beckman Acta III spectrophotometer.

Chromatographic methods. All HPLC studies were carried out on a Waters chromatograph using a μ Bondapak C_{18} column and a mixture of methanol/10 mM ammonium acetate (7/3, v:v or 5/5, v:v) as eluent after acidification to pH 4.5 with acetic acid. The detection of ellipticine derivatives was monitored by a uv-visible spectrophotometer at 254, 280 or 313 nm. An RRT value was associated with each peak of the HPLC chromatograms. The different products were quantified from linear calibration curves (peak areas vs concentration) constructed with known quantities of each original sample.

Preparation of dioxo-DME (PF_6 salt). A solution of 12 mg (34 μmol) of DHME in 25 ml of phosphate buffer (0.1 M, pH 5), 100 μmol of H_2O_2, and 0.05 μmol HRP were incubated 10 min at 25°. The reaction was stopped by precipitation with 420 mg (2.5 mmol) of NH_4PF_6. The dark-green precipitate was washed twice with diluted aqueous solution of NH_4PF_6 and filtered through a Sep-Pak C_{18} cartridge (Waters) conditioned by acetonitrile and then water prior to use. The purified compound was collected with acetonitrile, evaporated and dried under vacuum: 6.4 mg of dioxo-DME were obtained (40% yield) with PF_6^- as counterion for the drug (RRT = 0.54).

Identification of dioxo-DME (PF_6 salt). U.v.-visible (H_2O): λ_{max}, 226 nm ($\varepsilon = 32{,}000\ M^{-1}\,cm^{-1}$), 285 (40,000), 418 (7,100). MS data (DCI): m/z at 307 ($M + 2H^+$) and 341 ($M + 2NH_4^+$). ^1H NMR data (DMSO-d_6); δ ppm 3.12 (s, 3H, Me_5); 3.39 (s, 3H, Me_{11}); 4.29 (s, 3H, N_6-Me); 4.48 (s, 3H, N_2-Me); 6.62 (d, 1H, $J_{H8-H7} = 9.9$ Hz, H_8); 8.09 (d, 1H, $J_{H7-H8} = 9.9$ Hz, H_7); 8.47 (d, 1H, $J_{H3-H4} = 6.9$ Hz, H_3); 8.65 (d, 1H, $J_{H4-H3} = 6.9$ Hz, H_4) and 10.01 (s, 1H, H_1).

Preparation of the alanine adducts of DMHE. To a solution of 18.5 mg (52 μmol) of DMHE and 3.34 mg (37.5 mmol) of L-alanine, 100 μmol of H_2O_2 and 0.02 μmol of HRP were added. The reaction was allowed to incubate 10 min at 25° and was stopped by precipitation with 50 mmol of NH_4PF_6. The yellow precipitate was washed twice with a diluted aqueous solution of NH_4PF_6 and then loaded on an XAD_2 type column. The two main compounds were eluted using a step gradient of 50 mM ammonium acetate/methanol. After elimination of methanol by evaporation, the remaining solutions were precipitated by NH_4PF_6. The precipitates were (as for dioxo-DME) filtered through Sep-Pak C_{18} cartridges condition by acetonitrile and water prior to use. The two purified compounds were collected with acetonitrile, evaporated and dried under vacuum. 4.5 mg of ala_1-DME (RRT = 2.9, 22% yield) and 0.7 mg of ala_2-DME (RRT = 1.7, 3% yield) were both obtained with PF_6^- as counterion.

Identification of ala_1-DME. U.v.-visible (CH_3CN), λ_{max} 247 nm ($\varepsilon = 19{,}200\ M^{-1}\,cm^{-1}$), 290 (23,000), 313 (32,400), 444 (3,400). MS data (DCI): m/z at 330 (M^+). ^1H NMR (CD_3CN); δ 2.74 (s, 3H, Me-ala); 3.02 (s, 3H, Me-5); 3.74 (s, 3H, Me-11); 4.18 (s, 3H, N_6-Me); 4.37 (s, 3H, N_2-Me); 7.51 (d, 1H, $J_{H8-H7} = 8.8$ Hz, H_8); 7.84 (d, 1H, $J_{H7-H8} = 8.8$ Hz, H_7); 8.09 (d, 1H, $J_{H3-H4} = 7.3$ Hz, H_3); 8.31 (d, 1H, $J_{H4-H3} = 7.3$ Hz, H_4) and 9.46 (s, 1H, H_1).

Chemical synthesis and identification of the reference compound ala_1-DME. Two milligrams of the adduct ala-NME were synthetized as previously described [17]. To this product was added 0.5 mg of NaH (50%) in 100 μl DMF. The purple mixture was shaken for 30 min and 200 μl of CH_3I was then added to give a yellow colour. After 10 min, the excess of CH_3I was evaporated and the product precipitated with diethyl ether. This was washed three times with water and then with a diluted solution of acetic acid in ether. The product was dried under vacuum to give 1.2 mg of a yellow ochre powder with an iodide (I^-) counterion (RRT = 2.9; Yield = 50%). U.v.-visible (CH_3CN/CH_3COOH; 9/1: λ_{max} 247 nm ($\varepsilon = 19{,}200\ M^{-1}\,cm^{-1}$); 290 (22,800); 313 (32,000); 444 (3,400). MS data (DCI): m/z at 330 (M^+). ^1H NMR data (CD_3COOD) δ ppm 2.83 (s, 3H, Me-ala); 3.24 (s, 3H, Me-5) 4.02 (s, 3H, Me-11); 4.40 (s, 3H, N_6-Me); 4.61 (s, 3H, N_2-Me); 7.68 (d, 1H, $J_{H8-H7} = 8.8$ Hz, H_8); 7.97 (d, 1H, $J_{H7-H8} = 8.8$ Hz, H_7); 8.42 (d, 1H, $J_{H3-H4} = 7.3$ Hz, H_3); 8.53 (d, 1H, $J_{H4-H3} = 7.3$ Hz, H_4) and 9.96 (s, 1H, H_1).

Identification of ala_2-DME. U.v.-visible (CH_3CN); λ_{max} 256 nm ($\varepsilon = 33{,}800\ M^{-1}\,cm^{-1}$); 279 (28,000); 311 (33,200); 442 (3,400). MS data (DCI): m/z at 330 (M^+). ^1H NMR data (CD_3CN) δ ppm 2.73 (s, 3H, Me-ala); 3.08 (s, 3H, Me-5); 3.51 (s, 3H, Me-11); 4.23 (s, 3H, N_6-Me); 4.41 (s, 3H, N_2-Me); 7.55 (d, 1H, $J_{H8-H7} = 8.6$ Hz, H_8); 7.90 (d, 1H, $J_{H7-H8} = 8.6$ Hz, H_7); 8.14 (d, 1H, $J_{H3-H4} = 7.1$ Hz, H_3); 8.39 (d, 1H, $J_{H4-H3} = 7.1$ Hz, H_4) and 9.56 (s, 1H, H_1).

RESULTS

(a) *Dioxo-DME formation*

DMHE is easily and completely oxidized by HRP leading to an orthoquinone: the dioxo-DME (Fig. 2). This compound was characterised by mass spectrometry and ^1H NMR data (see Materials and Methods). Two main peaks are observed for this orthoquinone in the mass spectrum: the first at 307 corresponding to the theoretical molecular mass of dioxo-DME plus two mass units, and the second at 341 which can be attributed to the same product plus the mass of two NH_4^+ molecules. This analytical

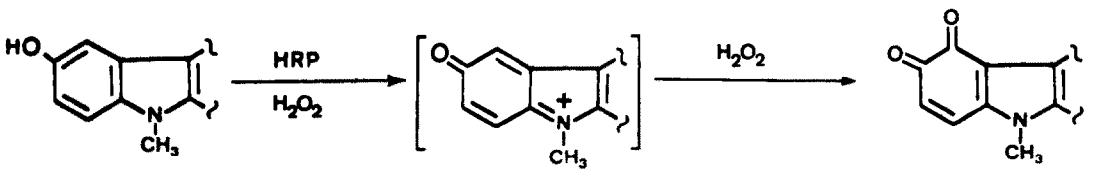

Fig. 2. Proposed mechanism for the formation of dioxo-DME during the peroxidase oxidation of DMHE.

method confirms that the N^6-methyl group is retained in the structure of dioxo-DME.

The ^1H NMR data confirm the orthoquinone structure for dioxo-DME in so far as they are in accordance with the data obtained for the parent orthoquinone dioxo-NME [4]. Furthermore, this compound, as dioxo-NME, displays in the u.v.-visible spectrum a maximum around 290 nm and a green colour [4].

Therefore, we can note that during the oxidation of DMHE there is no demethylating process. This unexpected result shows that this peroxidase oxidation differs completely from that of 9-OMe-E for which an O-demethylation occurs. In addition, during the enzymatic oxidation of DMHE, we cannot detect the apparition of a red colour significative of the quinone-imine observed when NMHE or 9-OMe-E are substrates [3, 4, 13]. Since there is formation of an orthoquinone for both NMHE and DMHE, and since dioxo-DME still possesses the methyl group on the indolic nitrogen after the oxidation step, we can suppose the formation of an intermediate, namely a quinone-iminium, in the oxidation of DMHE. This bicationic intermediate must be extremely reactive and therefore too unstable to be isolated (Fig. 2). In order to obtain evidence for the formation of this possible quinone-iminium structure, we have studied different parameters influencing the dioxo-DME generation.

The formation of dioxo-DME is pH dependent as illustrated in Fig. 3(a). The orthoquinone yield appears to be more quantitative at acidic pH (yield ~90% at pH 3). To know if this phenomenon correlates with the stability of the orthoquinone according to the pH, dioxo-DME was isolated, solubilised in acetonitrile and diluted with phosphate buffer at different pH values (Fig. 3b). The results show that dioxo-DME disappears at alkaline pH. This suggests that during the oxidation of DMHE to dioxo-DME (Fig. 4a) at alkaline pH, there is a degradation of the orthoquinone to products undetectable by HPLC. Furthermore, the rate of disappearance of DMHE is constant at different pH values (results not shown). These observations support the fact that dioxo-DME is more reactive than dioxo-NME. Effectively, dioxo-NME is formed preferentially at alkaline pH and is relatively stable after its formation [4]. The dioxo-DME is a molecule still endowed with electrophilic properties. For instance, with a nucleophile like the amino acid L-alanine, it forms an adduct

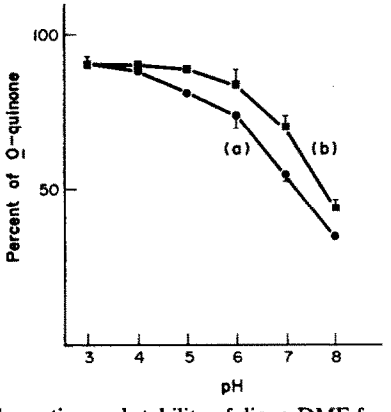

Fig. 3. Formation and stability of dioxo-DME formed during the pH dependent peroxidase oxidation of DMHE. (a) pH dependent peroxidase oxidation of DMHE to dioxo-DME. The reaction mixture (1.0 ml) contained 67 mM phosphate buffer (acidified with diluted HCl for the assays at pH 4 and at pH 3), 50 μM DMHE, 250 μM H_2O_2, 0.5 μM HRP. The reactions were initiated by addition of HRP at 20° and stopped 10 min later by direct injection in HPLC. The experiments were carried out in triplicate. The data presented are average values and are illustrated by black circles. (b) Stability of dioxo-DME at different pH. The mixture (1.0 ml) contained 500 μM DMHE, 2.5 mM H_2O_2, 67 mM phosphate buffer acidified with diluted HCl (pH 3) and 5 μM HRP. The reaction was initiated by addition of HRP at 20° and stopped 1 min later by precipitation with 500 mM NH_4PF_6 and centrifuged. The pellet was redissolved in 1 ml acetonitrile and then diluted 10-fold in 67 mM phosphate buffer at different pH. The reactions were terminated 10 min later by direct injection in HPLC. The experiments were carried out in triplicate. The data presented are average values and are illustrated by black squares.

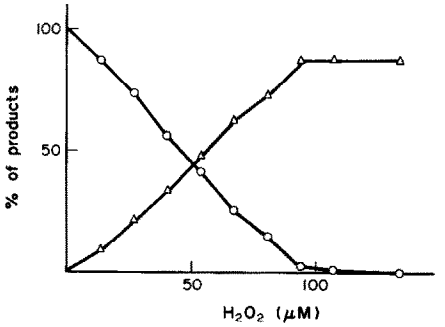

Fig. 4. Stoichiometry of DMHE consumption and of dioxo-DME formation during the HRP–H_2O_2 oxidation of DMHE with H_2O_2 as the variable substrate. The reaction mixture (1.0 ml) contained 67 mM phosphate buffer acidified with diluted HCl (pH 3), 50 μM DMHE, 1 μM HRP and H_2O_2 as indicated. The reactions were initiated by addition of HRP at 20° and stopped 10 min later by direct injection in HPLC. (○) DMHE and (△) dioxo-DME were dosed. The experiments were carried out in triplicate. The data presented are average values.

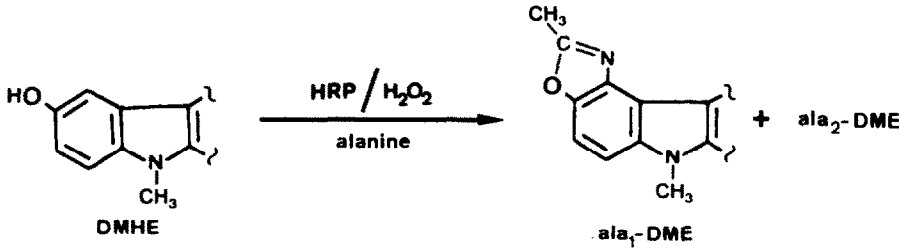

Fig. 5. Peroxidasic oxidation of DMHE in the presence of L-alanine to alanine-DMHE adducts.

whose structure was not determined. The study of the stoichiometry of the DMHE consumption and of the dioxo-DME generation was monitored at pH 3 (Fig. 4). In these experiments, where the amount of H_2O_2 is variable, it appears that two equivalents of H_2O_2 are required to induce the transformation of one equivalent DMHE to one equivalent dioxo-DME. These results are in accordance with the formation of a reactive electrophilic intermediate (a quinone-iminium) that appears after the first oxidation step of DMHE and then is attacked by a second H_2O_2 molecule to give the orthoquinone (Fig. 2).

(b) *Alanine-DMHE adducts formation*

To demonstrate the presence of a quinone-iminium intermediate in the peroxidase oxidation of DMHE, the reaction was carried out in the presence of a nucleophilic model: L-alanine. Two adducts were detected (ala_1-DME and ala_2-DME) (Fig. 5), and subsequently isolated, purified and characterized (see materials and methods).

The yield of these adducts increases with respect to the amount of nucleophile added (Fig. 6) and reaches a maximum at 10^4 fold excess of the nucleophile (adduct yield ~65%). For the oxidation of NMHE in the presence of alanine, there was just one adduct formed and 10 equivalents of nucleophile were sufficient to reach the maximal yield of the adduct [17]. The decrease in yield of the adducts with DMHE could be attributed to a great reactivity of the quinone-iminium which polymerises or decomposes before reacting with alanine. The formation of the alanine adducts according to the pH (Fig. 7) shows that the major adduct (ala_1-DME) appears preferentially at pH 7. This fact supports nucleophilic attack of the α-amino group as observed for the generation of the alanine-NMHE adduct [17]. On the other hand, ala_2-DME displays its maximum formation at pH 5.

In Fig. 8, the peroxidase oxidation of DMHE is monitored in the presence of alanine and different amounts of H_2O_2. DMHE disappears almost completely with one equivalent of H_2O_2 (this corroborates the hypothesis of an intermediate quinone-iminium generated by one equivalent of H_2O_2). The yield of adducts plus residual DMHE is 30% for one equivalent of H_2O_2. The explanation of this phenomenon could be that the adducts that have to be oxidized by extra H_2O_2 to lead to their stable forms are not detected by HPLC. These adducts effectively appear more quantitatively (up to 65%) by increasing the H_2O_2/DMHE ratio above 1. Furthermore, for a H_2O_2/DMHE ratio ≤ 1, one can

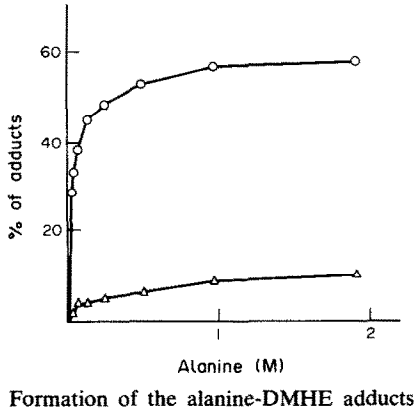

Fig. 6. Formation of the alanine-DMHE adducts in the peroxidase oxidation of DMHE with alanine as variable substrate. The reaction mixture (1.0 ml) contained water, 100 μM DMHE, 5 mM H_2O_2, 1 μM HRP and alanine as indicated. The reactions were initiated by addition of HRP at 20° (pH 7) and stopped 10 min later by direct injection in HPLC. (○) ala_1-DME and (△) ala_2-DME were dosed. The experiments were carried out in triplicate. The data presented are average values.

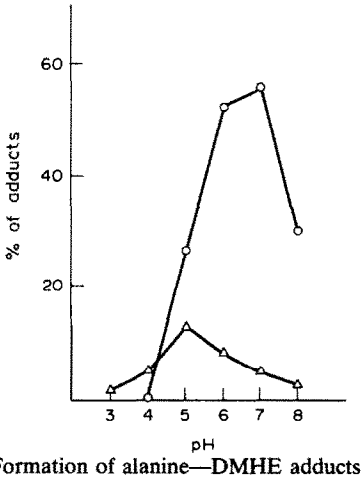

Fig. 7. Formation of alanine—DMHE adducts during the peroxidasic oxidation of DMHE in the presence of alanine at different pH. The reaction mixture (1.0 ml) contained 100 μM DMHE, 500 μM H_2O_2, 1.5 M L-alanine adjusted at different pH with diluted HCl or NaOH. 1 μM HRP was added to initiate the reaction at 20° which was stopped 10 min later by direct injection in HPLC. The adducts (○) ala_1-DME and (△) ala_2-DME were dosed. The experiments were carried out in triplicate. The data presented are average values.

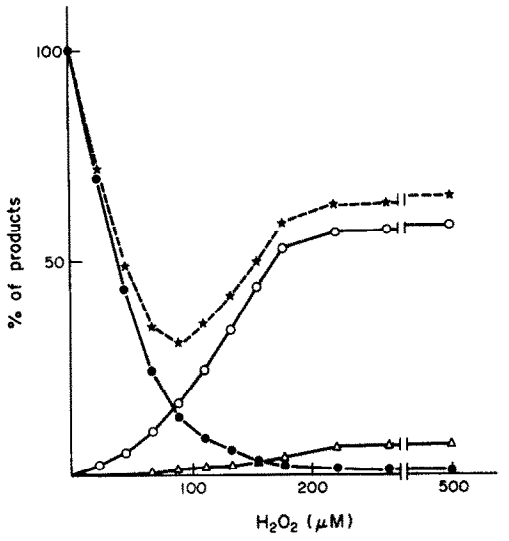

Fig. 8. H_2O_2-dependent formation of alanine—DMHE adducts during the HRP—H_2O_2 oxidation of DMHE in the presence of L-alanine. The reaction mixture (1.0 ml) contained water, 100 μM DMHE, 1.5 M L-alanine and H_2O_2 as indicated. 1 μM HRP was added to initiate the reaction at 20° (pH 7) which was stopped 10 min later by direct injection in HPLC. The products (●) DMHE, (○) ala_1-DME and (△) ala_2-DME were dosed and the calculated sum of their concentration was illustrated by symbol (★). The experiments were carried out in triplicate. The data are average values.

imagine that the low yield of adducts can be due to the dimerization and/or the polymerization of the oxidized DMHE, which is favoured by stacking interactions.

(c) *Structural identification of alanine-DMHE adducts*

Two adducts have been detected during the peroxidase oxidation of DMHE in the presence of an excess of alanine. Both compounds have been isolated and purified. From spectroscopic data (MS and ^1H NMR) and chemical correlation, it is possible to attribute a benzoxazole structure to the major adduct, ala_1-DME (see Fig. 5). The corresponding benzoxazole adduct obtained after oxidation of NMHE in the presence of alanine [18] can be N^6-methylated, and gives an adduct that presents the same chromatographic and spectroscopic data as ala_1-DME. Concerning the minor adduct, which has been isolated despite its formation in low yield, the structure is not completely established. Surprisingly, the ala_2-DME adduct has the same mass spectrum as the major adduct, and furthermore, its ^1H NMR spectrum differs from that of ala_1-DME only by a shift of 0.2 ppm of the methyl group at position 11 of the ellipticine skeleton. Due to the small amount of available material, no further spectroscopic investigations were undertaken.

DISCUSSION

Different hypotheses have been recently proposed to explain the cytotoxicity and/or antitumoral activities of ellipticines, and can be divided in two classes.

(1) The first is where no biotransformation of the drug is involved. As examples, we can mention intercalation between DNA base-pairs [19], interaction with membranes [20] and DNA strand breaks via topoisomerase II action [21].

(2) The second class concerns cases where a biotransformation can be evoked: one-electron [22] or two-electron [3, 4] oxidation, generation of $HO^{\cdot}$ radicals [23], or covalent binding to nucleic acids *in vitro* [6] or *in vivo* [10]. The second approach is supported by the findings that ellipticine derivatives that have a free or a masked amino-phenol structure (Fig. 1) are among the most active antitumoral compounds in the series. All the recent studies on NMHE (Fig. 1) (a free amino-phenol structure) document the "biooxidative alkylation" hypothesis (see Refs 8 and 10 as leading references). Hence, this drug can be considered as a "pro-alkylant" molecule. The same hypothesis has been also applied to 9-methoxy-ellipticine (Fig. 1) (a masked amino-phenol structure on the oxygen atom), for which we have established that a peroxidase oxidation gives directly an electrophilic quinone-imine [13]. Consequently, it is of great interest concerning the DMHE derivative (a masked amino-phenol structure on the indolic nitrogen) to know: (i) if it can be oxidized by a peroxidase system used as a model of an extra-hepatic oxidation, (ii) if the indolic nitrogen is demethylated during the oxidation and (iii) if any electrophilic entities are generated. The present results answer the last three questions, at least *in vitro*. DMHE is rapidly oxidized by the HRP/H_2O_2 system and the ultimate oxidation product, an orthoquinone derivative, has been identified. The reactive quinone-iminium intermediate has not been isolated, due to its high reactivity. However, the addition compounds of this strong electrophile with a nucleophile model, alanine, have been detected and the major adduct, a benzoxazole compound, fully characterized. It must be noted that both orthoquinone and alanine-adducts still have the methyl group attached at position 6, which suggests that no N^6-demethylation occurred during the biochemical oxidation of the drug.

CONCLUSION

We have shown in the present article that a third amino-phenol (masked) ellipticine derivative, the one bearing a methyl group at the indolic nitrogen, DMHE, is readily oxidized by a peroxidase catalyzed reaction. The identification of adducts with nucleophilic models evidences the electrophilic properties of the intermediate quinone-iminium compound. No products resulting from a *N*-demethylation have been detected. So, in the hypothesis of a biooxidative alkylation as the mechanism of action for ellipticine derivatives, DMHE does not appear to be a pro-drug of NMHE: its high cytotoxic and antitumor properties (similar to that of NMHE) may have to be related to original electrophilic species generated during the oxidation step.

We are currently working on detailed metabolism studies with DMHE in order to confirm or not if such biooxidation might occur *in vivo*. These studies can be considered as the preliminary steps before answering the question: is the alkylation of cellular

macromolecules by ellipticine derivatives correlated or not to the cytotoxicity and/or antitumoral activities of these drugs?

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