until the base consumption stopped, required between 92 and 105% of the calculated amount of NaOH.

Because of a diminutive leaking of base from the inlet, the half-lives of the very slowly hydrolyzed esters 7, 8, and 10 could not be measured by the titration technique but were estimated by TLC using butanol–HOAc–H_2O (4:1:1) as eluent. With intervals of 48 h, a sample of the hydrolysis mixture was withdrawn and applied on a silica gel plate (Merck) next to a freshly prepared solution, which was 5 mM with respect to both 1 and the ester. After development and drying, the plate was sprayed with a solution of ninhydrin in EtOH, and the intensities of the color obtained for the two spots from the hydrolysis mixture were compared with the spots from the standard solution.

Hydrolysis in Extract of Rat Brains. Six adult albino rats were decapitated and their brains were rapidly removed and homogenized in 20 mL of ice-cold 0.5 M KCl with a Warring blender for 5 min. The homogenate was dispersed with a 100-W MSE Ultrasonic disintegrator for 30 s twice with a 30-s interval. The suspension was centrifuged at 48000*g* for 20 min. The supernatant fluid was collected and made up to 30 mL by addition of 0.5 M KCl. The extract, which contained 15 mg of protein/mL,[19] was immediately divided into six portions and stored at −20 °C until needed. A mixture of 0.5 M KCl (45 mL), phoshate buffer (0.5 mL), and extract of rat brain (5.0 mL) was brought to pH 7.35, and 0.05 mmol of the ester was added. The pH was readjusted and maintained constant by the addition of 0.1 N NaOH. The hydrolysis was followed in the same manner as described above.

Pharmacology. Anticonvulsant Activity. The convulsions were induced by bicuculline, isoniazide, or by electroshock at times of 0.5, 1, 2, 3, 4, 5, 6, 17, and 24 h after injection (ip) of the ester. At each time interval, groups of at least five mice were used.

(a) Bicuculline. Bicuculline was injected in a dose of either 5 mg/kg subcutaneously or 0.63 mg/kg intravenously at each time.

(b) Isoniazide. The antagonistic effects of the esters against convulsions induced by isoniazide were determined by a previously described method.[20]

(c) Electroshock. Antagonism of maximal electroshock seizure (MES) was determined as described by Swinyard.[21] To induce the convulsions an electric high-voltage sinus wave stimulator (9.5 mA, 0.4 s) was used.

Acknowledgment. This work was supported by the Danish Medical Research Council. We thank Mrs. B. Hare for secretarial assistance and Mrs. U. Geneser and Mr. S. Stilling for technical assistance.

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Bioactivation of the Antitumor Drugs 9-Hydroxyellipticine and Derivatives by a Peroxidase–Hydrogen Peroxide System

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Hydroxylation in position 9 (see Table I) of various antitumor drugs derived from ellipticine results, in most cases, in the possible further oxidation of the hydroxylated drugs into free radicals and quinone products in the presence of a peroxidase–H_2O_2 system. Except for the N^6-methyl derivative, free radicals of hydroxyellipticines do not react with neighboring molecules. However, quinone products have been found to be strong electrophilic molecules. They can oxidize NADH into NAD^+ through a nonenzymatic process, and, moreover, quinone from N^2-methyl-9-hydroxyellipticine may undergo a nucleophilic attack, resulting in an irreversible binding of the drug to bovine serum albumin. Among the drugs tested, those which can be oxidized by peroxidase–H_2O_2 exhibit the most cytotoxic effect on L1210 cells in vitro.

Ellipticine [5,11-dimethyl-6*H*-pyrido[4,3-*b*]carbazole; see Table I] is a plant alkaloid which exhibits a high cytotoxic activity against murine leukemia L1210 and various other tumor cells.[1] Recent experiments have identified the phenolic derivative 9-hydroxyellipticine (9-OH-E; see Table I) as a main product of the oxidative metabolism in rats.[2,3] It has been further demonstrated that the hydroxylation of ellipticine depends on the microsomal drug-metabolizing system.[4] It has been recognized for several years[5,6] that the addition of an hydroxyl group on the ellipticine ring in position 9 results in a strong increase in both cytotoxic and antitumor activities. In the field of investigation dealing with the mechanism of action of these drugs, it is interesting to determine why the hydroxylation of ellipticine will increase its antitumor activity. Since biotransformation of chemical compounds to active metabolites appears to be a common prerequisite for the development of their biological effects, one possible hypothesis is that hydroxylation of ellipticine enables it to be biotransformed into an even more reactive compound(s). Along this line, preliminary investigations[7] indicate that 9-OH-E may act as a substrate for peroxidases, enzymes which catalyze the oxidation of various arylamines and phenols in the presence of hydrogen peroxide (H_2O_2).[8] Thus, using a peroxidase–H_2O_2 system as a model of biooxidation, we have studied the ability of various ellipticines (see Table I) to be oxidized and determined, in most cases, the nature and the reactivity of the products of oxidation.

(1) G. M. Svoboda, G. A. Poore, and M. L. Montfort, *J. Pharm. Sci.*, **57**, 1720 (1968).
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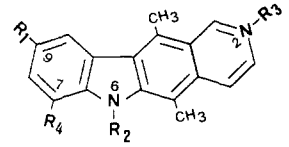
Table I. Structures of Ellipticine Derivatives



no.	R_1	R_2	R_3	R_4	name	abbreviation
1	H	H		H	ellipticine	E
2	H	H	CH_3	H	ellipticinium	N^2-Me-E
3	OH	H		H	9-hydroxyellipticine	9-OH-E
4	OH	H	CH_3	H	9-hydroxyellipticinium	N^2-Me-9-OH-E
5	OCH_3	H		H	9-methylellipticine	9-OMe-E
6	OH	CH_3	CH_3	H	N^6-methyl-9-hydroxyellipticinium	N^2,N^6-Me_2-9-OH-E
7	H	H		OH	7-hydroxyellipticine	7-OH-E

Results are discussed in terms of the relationship between the ability of the drugs to be activated and their cytotoxic activities.

Peroxidase–H_2O_2 System as a Model of Bioactivation. Peroxidases are enzymes known to catalyze the oxidation of various organic compounds, such as arylamines and phenols, in the presence of H_2O_2 to act as electron acceptors.[8] It has been further demonstrated[9] that free radicals of electron-donor molecules are formed in the peroxidase reaction according to the mechanism shown in Scheme I, where AH_2 is the electron donor.

Scheme I

$$\text{peroxidase} + H_2O_2 \xrightarrow{2H^+} \text{compound I} + 2H_2O \quad (1)$$

$$\text{compound I} + AH_2 \rightarrow AH\cdot + \text{compound II} \quad (2)$$

$$\text{compound II} + AH_2 \rightarrow AH\cdot + \text{peroxidase} \quad (3)$$

$$AH\cdot + AH\cdot \rightleftharpoons AH_2 + A \quad (4)$$

$$\text{or } AH\cdot + AH\cdot \rightarrow AH\text{–}AH \quad (5)$$

$$\text{or } (AH\cdot)_n \rightarrow (AH\text{–}AH\ldots)_n \quad (6)$$

Free radicals generate either two-electron-oxidized products of the donor through a dismutation process (eq 4) or dimers and/or polymers (eq 5 and 6). Both free radicals, as well as two-electron-oxidized products from phenols and arylamines, are usually electrophilic and reactive molecules. Thus, peroxidase-catalyzed oxidation of organic molecules may be considered as a biological mechanism of chemical activation.

Results

Oxidation of 9-OH-E by a Peroxidase–H_2O_2 System. As already pointed out, peroxidases are enzymes which catalyze the oxidation of various organic compounds which have suitable electron-releasing groups in their structures. The monooxygenation of ellipticines results in the formation of a phenolic derivative which exhibits the required structure for undergoing further oxidation through catalysis by peroxidases. Accordingly, in the presence of H_2O_2 various peroxidases, such as horse radish peroxidase (HRP) or human myeloperoxidase (MPO), catalyze the oxidation of 9-OH-E into a compound identified as the two-electron-oxidized product of 9-OH-E (see Experimental Section). This compound exhibits a quinone imine structure (Figure 1) and has been denominated 9-oxoellipticine (9-oxo-E). The visible absorption spectrum of 9-oxo-E is shown in Figure 2. This spectrum, as well as the extinction coefficient of the pure product, proves useful in

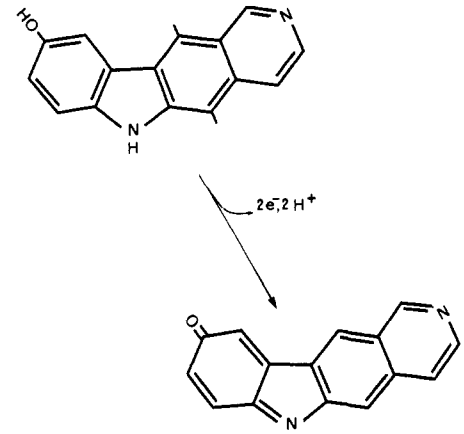


Figure 1. Oxidation of 9-OH-E into 9-oxo-E.

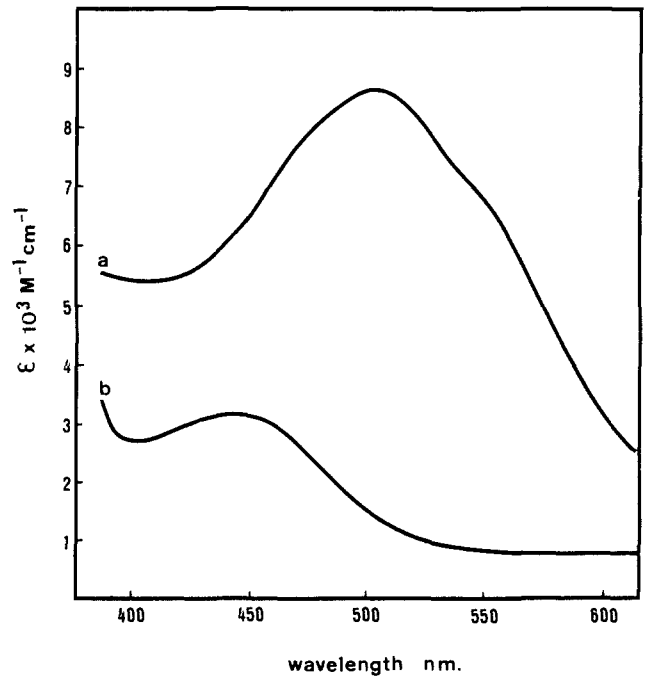


Figure 2. Visible absorption spectra of 9-oxo-E (a) and 9-OH-E (b) dissolved in Me_2SO.

the identification and the quantitative determination of the quinone products obtained from various ellipticines. Therefore, in further experiments the generation of quinones have been determined by the change in absorbance at 515 nm, which is the visible λ_{max} of the difference spectrum between 9-OH-E and 9-oxo-E.

Oxidation of Various Ellipticine Derivatives by the MPO–H_2O_2 System. In agreement with the mechanism

(9) I. Yamazaki and L. H. Piette, *Biochim. Biophys. Acta*, 77, 47 (1963).

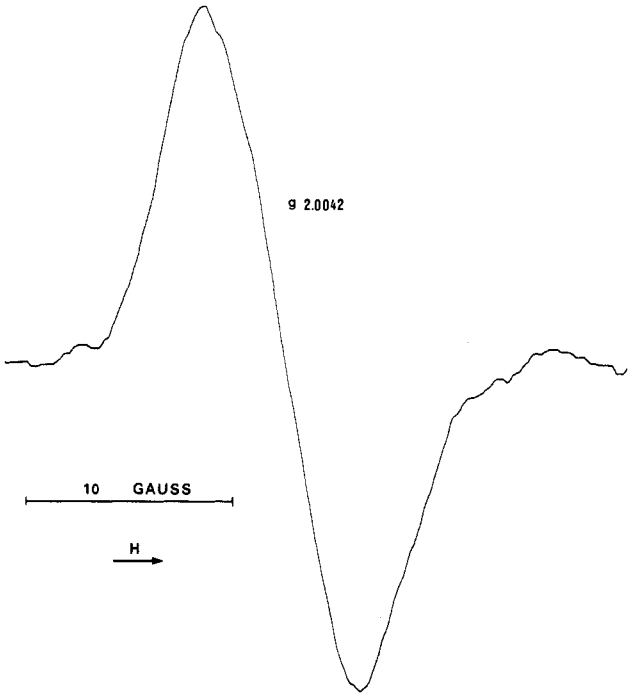


Figure 3. EPR sepctrum of 9-OH-E when mixed with I_2 in Me_2SO. Experiments were carried out as described under Experimental Section. Microwave frequency was 9753 GHz, modulation 1 G, microwave power 250 mW, and temperature 110 K.

Table II. Generation of Free Radicals during MPO-H_2O_2 Catalyzed Oxidation of Ellipticines[a]

compd	generation of free radical detectable at g = 2.0042
E	−
N^2-Me-E	−
9-OH-E	+
N^2-Me-9-OH-E	+
9-OMe-E	−
N^2,N^6-Me_2-9-OH-E	+
7-OH-E	−

[a] Free radicals were detected by EPR as described under Experimental Section. In all the experiments, spectra were performed at 110 K with a microwave frequency in the 9750-GHz range, 1 G modulation, and 250-mW microwave power.

of peroxidase-catalyzed reactions as indicated in eq 1–6, the one-electron oxidation of the initial compound occurs first (eq 2 and 3). This process generates free radicals which can be detected by EPR spectroscopy. Therefore, in an attempt to determine the ellipticine derivatives which will undergo oxidation in the presence of MPO–H_2O_2, we performed a series of EPR experiments as follows: first of all, in order to obtain the reference signal of the hydroxyellipticine radical, we attempted to generate the radical in iodine solution.[10] When mixing a solution of 9-OH-E and a solution of iodine dissolved in Me_2SO (see Experimental Section), a free radical at $g = 2.0042 \pm 0.0002$ can be effectively detected at low temperature (Figure 3). The signal is related to the presence of the radical cation of 9-OH-E. In the presence of the MPO–H_2O_2 system, free radicals of various hydroxyellipticines can also be detected at low temperature in phosphate buffer, as indicated under Experimental Section. All detected signals exhibit a g factor of 2.0042, which is identical with the one observed with the iodine solution. Results summarized in Table II

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Table III. Generation Rate of Quinone Products during the MPO-H_2O_2 Catalyzed Oxidation of Ellipticines[a]

compd	change in absorbance at 515 nm (ΔD_0/min)
E	0
N^2-Me-E	0
9-OH-E	0.080
N^2-Me-9-OH-E	0.085
9-OMe-E	0
N^2,N^6-Me_2-9-OH-E	0
7-OH-E	0

[a] Oxidation reactions were carried out at 25 °C in a 0.05 M phosphate buffer (pH 7.40) containing 10^{-4} M H_2O_2, 10^{-7} M MPO, and 10^{-4} M of each compound tested. Values of ΔD_0/min were the means of the ΔD_0 measured during the three first minutes after the addition of MPO.

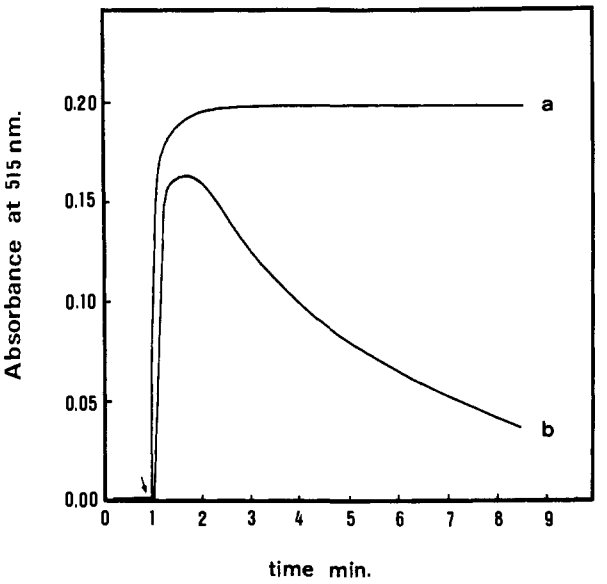


Figure 4. Kinetics of formation of 9-oxo-E (a) and N^2-Me-9-OH-E oxidized product (b) in the presence of the MPO–H_2O_2 system. Experiments were carried out at 25 °C in a medium containing 0.05 M phosphate buffer (pH 7.40), 10^{-4} M H_2O_2, 5×10^{-5} M 9-OH-E, or N^2-Me-9-OH-E and 5×10^{-6} M MPO.

indicate that only ellipticines with an OH group in position 9 will undergo a one-electron oxidation in the presence of MPO–H_2O_2. Where generated, free radicals may undergo dismutation and/or dimerization reactions (eq 4–6), thus yielding the terminal product of the oxidation. In the case of 9-OH-E the dismutation process of the radical generates a quinone product which can be detected by a change in absorbance at 515 nm. Results given in Table III show that of the ellipticines which undergo one-electron oxidation in the presence of MPO–H_2O_2, the N^6-methyl derivative (compound **6** in Table I) does not generate any quinone product. In addition and as expected, E and 7-OH-E (compounds **1** and **7** in Table I) do not yield quinone either. It should be pointed out that, whereas the quinone product obtained from 9-OH-E is stable in solution, the N^2-CH_3-9-OH-E (compound **4** in Table I) oxidized product appears to be unstable under these experimental conditions. Figure 4 shows the kinetics of formation of both 9-OH-E and N^2-CH_3-9-OH-E oxidized products at pH 7.40 in the presence of excess peroxidase. The instability of the N^2-CH_3-9-OH-E oxidized product is evident by the decay in absorbance at 515 nm. As indicated in Figure 5, the half decay time in absorbance at 515 nm strongly depends on both the pH and phosphate concentrations. Below pH 5.50, the N^2-CH_3-9-OH-E oxidized product becomes quite stable. The visible absorption spectrum of

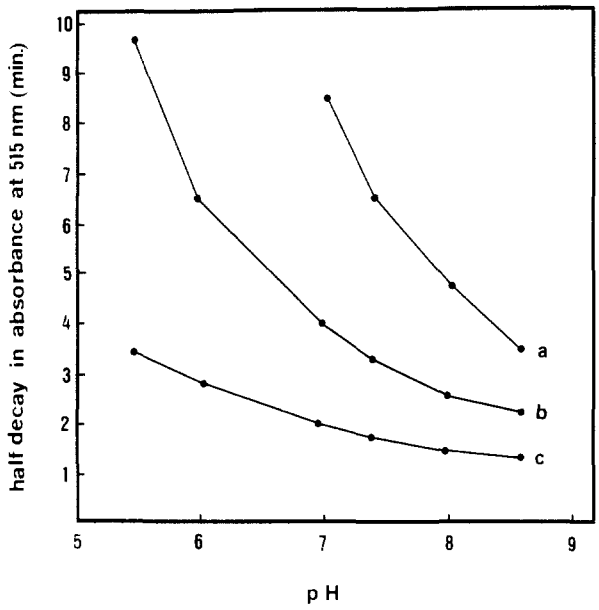


Figure 5. Effect of pH and phosphate concentration on the decay in absorbance at 515 nm of the N^2-Me-9-OH-E oxidized product. Experiments were performed at 25 °C in a medium containing in a, 0.025 M; in b, 0.050 M; in c, 0.100 M phosphate buffer at various pH. In all cases, mixtures contain 5×10^{-5} N^2-Me-9-OH-E, 10^{-4} M H_2O_2, and 5×10^{-6} M MPO. All kinetics were first order, and the observed rate constant varied from 0.069 to 0.53 min^{-1} for data indicated in the figure.

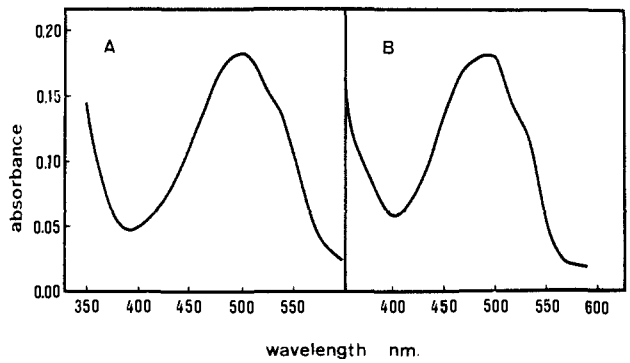


Figure 6. Visible absorption spectra of 9-oxo-E (A) and N^2-Me-9-OH-E oxidized product (B) in 0.005 M phosphate buffer (pH 5.50). Oxidation of both 9-OH-E and N^2-Me-9-OH-E were carried out at 25 °C in mixtures containing 5×10^{-5} M drugs, 10^{-4} M H_2O_2, and 10^{-7} M MPO. Spectra were recorded 3 min after the addition of the enzyme.

the red mixture containing this compound closely resembles the one performed under similar conditions using 9-OH-E (Figure 6), which suggests that the red compound obtained from N^2-CH_3-9-OH-E is the quinone imine N^2-CH_3-9-oxo-E as expected. Unfortunately, due to its reactivity, we were unable to purify this compound for further identification. Moreover, it should be pointed out that, according to the mechanism of peroxidase-catalyzed oxidation (see eq 1–6), the kinetics of N^2-CH_3-9-OH-E oxidation might be consistent with the primary production of a long-life free radical, which is followed by a dimerization reaction, as reported for the HRP-catalyzed oxidation of chlorpromazine.[11] The possibility of such an occurrence has been investigated by EPR spectroscopy. Unlike the results obtained with 9-OH-E, which yield a stable quinone, a free radical at $g = 2.0046 \pm 0.0002$ is

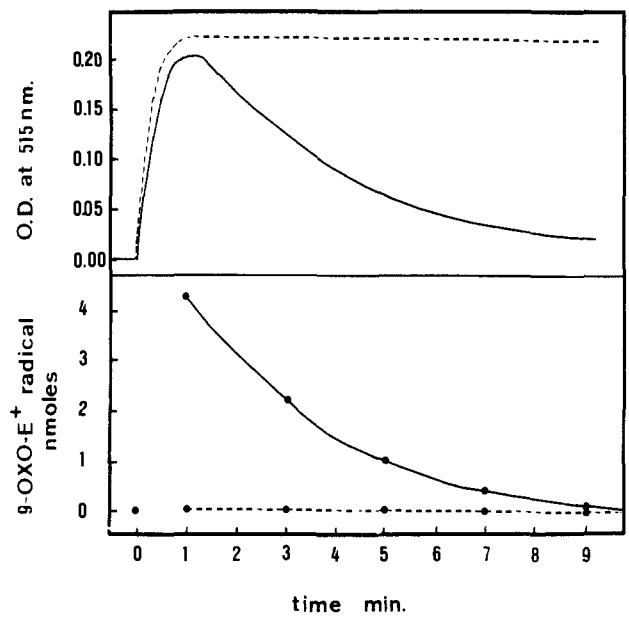


Figure 7. Comparison between the change in absorbance at 515 nm and the kinetics of N^2-Me-9-oxo-E degradation during the peroxidase-catalyzed oxidation of N^2-Me-9-OH-E. Experiments were performed at 25 °C in 0.05 M phosphate buffer (pH 7.40, —; pH 5.50, ----) containing 10^{-4} M H_2O_2 and 25×10^{-6} M N^2-Me-9-OH-E. At time 0 min, 2×10^{-6} M MPO was added and the change in absorbance was recorded. EPR experiments were carried out as described under Experimental Section. In these assays, microwave frequency was in the 9750-GHz range, modulation 1 G, microwave power 250 mW. At pH 7.40, the decay of the radical concentration showed a first-order kinetics with a rate constant of 0.36 min^{-1}.

detectable at room temperature during the MPO–H_2O_2 catalyzed N^2-CH_3-9-OH-E oxidation. The radical concentration decreases in relationship to the decay in absorbance at 515 nm and disappears after the termination of the reaction (Figure 7). However, it should be noted that the signal is detected only at pH 7.40. At pH 5.50, under which condition N^2-CH_3-9-oxo-E is stable, no signal is detected. The presence of the radical at nearly neutral or alkaline pH, in relationship to the instability of the quinone, suggests that the radical is not a long-life radical but a steady-state radical involved as an intermediate of the degradation of the quinone.

Chemical Reactivity of the Oxidized Product of 9-Hydroxyellipticines. 9-Hydroxyellipticine derivatives in their reduced state are likely to be oxidizing compounds, since their standard redox potential of the one-electron oxidation as determined polarographically is estimated to be $E_o' = +0.50$ V (unpublished data). Therefore, the oxidized products obtained from 9-OH-E derivatives should be strong oxidizing species. In order to obtain information about the oxidizing power of the various products obtained from hydroxyellipticines in the presence of MPO–H_2O_2, we utilized measurements of the NADH oxidation rate as a probe, in a system containing MPO, H_2O_2, NADH, and the ellipticine tested. Table IV summarizes the result obtained with 9-OH-E at various pH. As expected, the MPO-H_2O_2 system catalyzes the aerobic oxidation of NADH at acidic pH.[12] At neutral pH and above, no oxidation occurs. The addition of 9-OH-E to the assay medium is followed by the rapid oxidation of NADH over

(11) L. H. Piette, G. Bulon, and I. Yamazaki, *Biochim. Biophys. Acta*, 88, 120 (1964).

(12) K. Yokota and I. Yamazaki, *Biochim. Biophys. Acta*, **105**, 301 (1965).

(13) I. Odajima and I. Yamazaki, *Biochim. Biophys. Acta*, **206**, 71 (1970).

Table IV. Effect of 9-OH-E on NADH Oxidation in the Presence of the MPO-H_2O_2 System at Various pH[a]

	minus 9-OH-E		plus 9-OH-E	
pH	NADH oxidation	oxygen uptake	NADH oxidation	oxygen uptake
6.0	1.2	0.52	5.5	0.00
6.8	0.5	0.28	7.8	0.00
7.4	0.0	0.00	10.2	0.00
7.8	0.0	0.00	7.2	0.00
8.3	0.0	0.00	6.5	0.00

[a] All reactions were carried out in 0.05 M phosphate buffer at 25 °C. The assay medium was composed in each experiment of 10^{-4} M H_2O_2, 10^{-4} M NADH, and 2×10^{-8} M MPO. Where indicated, 9-OH-E was added to a concentration of 5×10^{-5} M. NADH oxidation was estimated by the decrease in absorbance at 340 nm, using a molar extinction of 6200 M^{-1} cm^{-1}. Oxygen consumptions were measured under identical conditions as indicated under Experimental Section. All values were expressed nanomoles/minute.

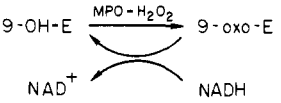
Table V. Effect of Various Ellipticine Derivatives on NADH Oxidation in the Presence of MPO-H_2O_2[a]

compd	NADH oxidized, nmol/min	oxygen consumed, nmol/min
E	0	0
N^2-Me-E	0	0
9-OH-E	10.50	0
N^2-Me-9-OH-E	12.20	0
9-OMe-E	0.22	0
N^2,N^6-Me_2-9-OH-E	16.15	7.70
7-OH-E	0.17	0

[a] All the experiments were performed at 25 °C in 0.05 M phosphate buffer (pH 7.40) essentially as described in Table IV, except that 10^{-5} M ellipticines were present in the assay media.

the whole pH range. In the presence of 9-OH-E, no oxygen consumption occurs and superoxide dismutase (O_2^- oxidoreductase, EC 1.15.1.1.), an enzyme known to inhibit the myeloperoxidase compound III [(perox)Fe^{3+}–O_2^-], does not affect the NADH oxidation rate (data not indicated). Moreover, in this system, 9-OH-E is continually regenerated, suggesting that the reaction occurs in a cyclic process as follows:

Scheme II



Results obtained in this system in the presence of various hydroxyellipticines are indicated in Table V and show that all the compounds yielding a quinone product in the presence of MPO–H_2O_2 will promote NADH oxidation at neutral pH. We noticed that N^6-Me-9-OH-E induces an aerobic NADH oxidation. This reaction is thought to proceed according to the sequence shown in Scheme III,

Scheme III

$$2R\text{–}OH \xrightarrow{MPO\text{–}H_2O_2} 2RO\cdot + 2H_2O \quad (7)$$

$$R\text{–}O\cdot + NADH \rightarrow R\text{–}OH + NAD\cdot \quad (8)$$

$$NAD\cdot + O_2 \rightarrow NAD^+ + O_2^{-}\cdot \quad (9)$$

$$2O_2^{-}\cdot + 2H^+ \rightarrow H_2O_2 + O_2 \quad (10)$$

where ROH is the N^6-Me-9-OH-E derivative. These results indicate that both one- and two-electron oxidized products of hydroxyellipticines are powerful oxidizing compounds and can be considered potent electrophilic molecules. Such

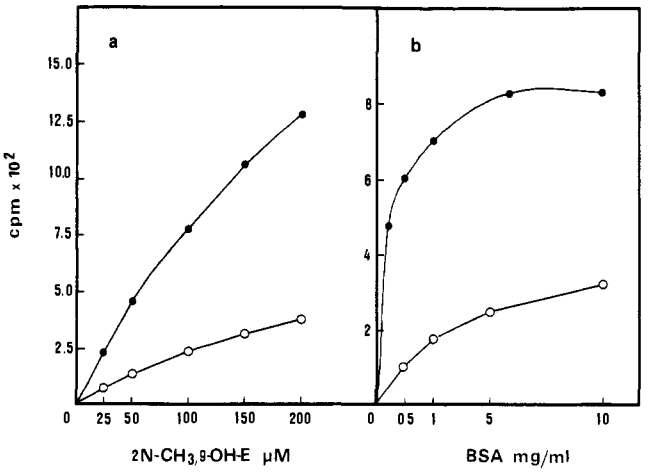


Figure 8. Effect of N^2-Me-9-OH-E concentration (a) and BSA concentration (b) on the amount of drug bound to BSA at pH 5.50 (●–●) and pH 7.40 (○–○). Experiments resulting in binding of N^2-Me-9-OH-E on BSA were performed as described under Experimental Section. Calibration curves indicated that 0.625 nmol of N^2-Me-9-OH-E exhibited a radioactivity corresponding to 100 cpm.

molecules may undergo a nucleophilic attack, resulting in covalent binding with the nucleophilic molecule. In order to test this possibility, we have studied the formation of irreversible binding of the reactive N^2-Me-9-oxo-E with the biological nucleophile bovine serum albumin (BSA). After purification of BSA from a mixture of (pH 7.40) containing N^2-Me-9-oxo-[10-^{3}H]E (see Experimental Section) generated by the MPO–H_2O_2 system, the radioactivity was found to be irreversibly associated with BSA, suggesting that N^2-Me-9-oxo-E is covalently bound to the protein. Figure 8a indicates that the binding efficiency strongly depends on the pH of the medium, and Figure 8a indicates that the amount of N^2-Me-9-oxo-E bound on BSA is quite linear with respect to the initial concentration of the drug in the assay mixtures. The low binding efficiency at pH 7.40 is likely due to an increase in the polymerization rate of N^2-Me-9-oxo-E which competes with the binding.

Discussion

Experimental data presented in this work clearly indicate that the addition of an OH group in position 9 of the ellipticine ring may result in further oxidation of the drug. This oxidation can be catalyzed by a biological system such as peroxidase–H_2O_2. As expected for the peroxidase-catalyzed reaction, the primary products of the oxidation process are free radicals. These chemical species are usually reactive compounds and their reactivities may be related to the one-electron redox potential of the parent bivalent redox molecules. In the case of 9-OH-E, the redox potential E_0' of one-electron transfer is estimated to be +0.50 V, which indicates, as expected for the radical of stable phenols, that radicals of hydroxyellipticines are potent oxidizing compounds. However, except for N^6,N^2-Me_2-9-OH-E, ellipticine radicals preferentially undergo a dismutation reaction and do not react with neighboring molecules even in the presence of a potent electron donor such as NADH. This is obvious from the lack of oxygen consumption in the MPO–H_2O_2–NADH system.

The quinone derivatives of 9-hydroxyellipticine are strong oxidizing agents which is obvious from the spontaneous reaction that occurs between these compounds and NADH. Moreover, it appears that the chemical reactivity of the quinone products depends on the charge of the nitrogen in pyridine. In the absence of a suitable electron donor, 9-oxo-E remains stable in solution, whereas N^2-

Table VI. Cytotoxic and Antitumor Activities of Ellipticine Derivatives

drug	ID_{50},[a] μM	LD_0,[b] mg/kg	ILS,[c] %	reactive compds generated in the presence of MPO/H_2O_2
E	0.99	50	68	none
N^2-Me-E	1.68	12.5	18	none
9-OH-E	0.015	50	53	quinone
N^2-Me-9-OH-E	0.050	5	62	quinone
9-OMe-E	0.60	70	70	none
N^2,N^6-Me_2-9-OH-E	0.041	15	78	free radical
7-OH-E	5.44	nontoxic	inact	none

[a] Dose which reduces by 50%, after 48 h, the cell growth as compared to controls. [b] Highest nonlethal dose (ip treatment). [c] Increase in life span over controls (10^5 cells): mouse by ip route; single ip injection 2 h after cell grafting. Dose of drug injected = 1 LD_0. Significant antitumor effect for ILS ≥ 25%.

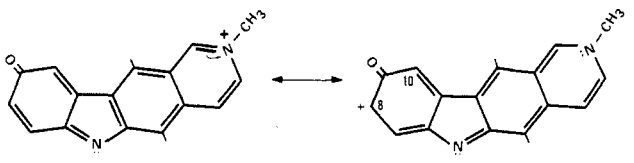


Figure 9. Possible nucleophilic addition process on N^2-Me-9-oxo-E.

Me-9-oxo-E appears to be a very unstable molecule. This instability is related to the high susceptibility of the quinone imine to nucleophilic addition, which is strongly facilated by the electrophilic property of the nitrogen in the pyridinium ion (Figure 9). Moreover, the nitrogen in the amino group is a weak base (pK_a = 5) and may act in its unprotonated form as an efficient nucleophile. When this happens, the quinone exhibits both nucleophilic and electrophilic reactivities which may result in cationic polymerization. We should keep in mind that the degradation rate of N^2-Me-9-oxo-E is strongly dependent on the phosphate concentration, suggesting that phosphate may act as a nucleophile and react with the quinone.

In the presence of a nucleophilic macromolecule such as BSA, in suitable conditions, N^2-Me-9-oxo-E undergoes irreversible binding with the protein. The amount of quinone bound to BSA, as determined by measurement of the radioactivity associated with the protein, appears to be strongly dependent on the pH. The low binding efficiency at neutral pH and above indicates that polymerization or reaction with phosphate preferentially occurs rather than binding with the protein.

A striking feature of the bioactivation of 9-hydroxyellipticines is that one of the reactive forms of these molecules is an electrophilic quinone. This chemical process can be related to the bioreductive alkylation reaction as described by Moore,[14] except that in the case of ellipticines the initial reduction step is not required since, unlike the anthraquinone derivatives, their stable form is a phenolic compound. Moreover, free radicals of hydroxyellipticines, which are intermediates in their oxidation, are oxidizing compounds, whereas free radicals of anthraquinone derivatives, which are intermediates of their reduction, are potent reducing radicals. The occurrence of 9-hydroxyellipticine activation into either oxidizing radicals or quinones in cells and the relationship between this possible activation and the cytotoxic properties of the drugs remain to be demonstrated. However, along this line it is interesting to compare the cytotoxic activity and the antitumor efficiency of the drugs to their ability to be activated through an oxidation process. Such a comparison is drawn in Table VI. Results obtained in previous work about the cytotoxic activity and antitumor efficiency of various ellipticines indicate, first of all, that drugs with an OH group in position 9 exhibit more cytotoxic activity against L1210 cells in vitro. These drugs are those which can be oxidized into reactive compounds. However, it should be noted that this correlation does not hold true neither for the toxicity of the drugs in mice, as expressed by LD_0, nor for their antitumor efficiency in vivo, as expressed by ILS (see Experimental Section). It appears that the presence of an OH group in position 9 and the presence of a quaternary ammonium on pyridine are both required for the most efficient antitumor activity. It is of interest to note that N^2-Me-9-OH-E which exhibits both structures, generates the most reactive oxidized product.

Experimental Section

Absorption spectra, as well as others spectrophotometric studies, were performed on a Beckman Acta III spectrophotometer. Mass spectra were performed on a Varian CH7 spectrometer, NMR spectra on a Bruker WH90 apparatus (90 MHz), and EPR studies on a Bruker B-ER 420 apparatus. Oxygen tension was measured using an oxygen electrode from a Yellow Spring instrument recorded to a Gilson oxygraph. High-performance LC was monitored on a Waters apparatus using a microbondapak C18 column.

Chemicals. Superoxide dismutase and horse radish peroxidase were obtained from Sigma Chemical Co. (St Louis). NADH was obtained from Boehringer (Mannheim, Germany). Ellipticines derivatives, E, N^2-Me-E, 9-OH-E, N^2-Me-9-OH-E, 9-OMe-E and N^2,N^6-Me_2-9-OH-E were synthesized according to Dalton et al.[15] and were a gift of Dr. Dat-Xuong.[16] 7-OH-E was synthesized according to Lallemand et al.[18] and was a gift of Dr. Mansuy.[17] N^2-Me-9-OH-E (5 Ci/mmol) was obtained from the C.E.A. (France).

Preparation of Myeloperoxidase. Human myeloperoxidase/donor (H_2O_2 oxidoreductase, EC 1.2.1.7) was extracted and purified from polymorphonuclear leucocytes of patients with untreated chronic myeloid leukemia, according to a procedure previously described.[13] The concentration of purified MPO was calculated on the basis of a value of 95 mM^{-1} cm^{-1} for the extinction coefficient at 450 nm. The A_{430}/A_{280} ratio for the enzyme used in the experiment was 0.77.

Oxidation of 9-OH-E by MPO–H_2O_2 and Purification of the Oxidized Product. Oxidation of 9-OH-E was carried out in 0.1 M phosphate buffer (pH 7.80) containing 10^{-5} M 9-OH-E, 10^{-4} M H_2O_2, and about 10^{-6} M MPO. The solution was allowed to stand in the dark for 30 min. The reddish material was extracted by methylene chloride. The extract was dried with calcium sulfate, filtered, and evaporated. The remaining material was dissolved in a mixture containing chloroform and ethyl acetate (v/v), then applied to a silica gel column, and eluted with a chloroform–ethyl acetate mixture. The red product was collected and accounted for 95% of the theorical yield. The purity was checked by high-performance LC using a reverse-phase column

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and a mixture of 56% methanol and 44% water containing 1 g of heptane sulfonate and 2 mL of acetic acid.

Identification of the 9-OH-E Oxidized Product. The purified oxidized product is a deep red powder: mp 345 °C; mass spectrum, m/e 260 (M^+). The UV spectrum displays a maximal absorption at 299 nm and in the visible region at 505 nm. The NMR spectrum is in agreement with the quinone imine structure: ^1H NMR (Me_4Si in $CDCl_3$) δ 9.393 (H_1, s), 8.679 and 8.617 (H_3, d), 7.785 and 7.723 (H_4, d, J_{3-4} = 5.58 Hz), 7.508 and 7.397 (H_7, d), 6.576 and 6.465 (H_8, q), 6.807 (H_{10}, s, J_{7-8} = 9.99 Hz and J_{8-10} = 1.98 Hz).

Electron Paramagnetic Resonance Studies of Free Radicals. (a) Formation of 9-OH-E Radical in Iodine Solution. 9-OH-E solution was prepared by dissolution of the solid compound in dry Me_2SO to a concentration of 10^{-3} M. The iodine solution was prepared by dissolving solid iodine in dry Me_2SO to a concentration of 10^{-2} M. Equal volumes of 9-OH-E and iodine were mixed. The solution was flushed with argon for 15 min, frozen in liquid nitrogen, and examined in an EPR spectrometer at 110 K.

(b) Formation of Free Radicals during MPO-H_2O_2 Mediated Oxidation of Ellipticine Derivatives. Experiments were performed in 0.05 M phosphate buffer containing 10^{-4} M H_2O_2 and 25×10^{-6} M of various ellipticines. The reaction was started by the addition of 2×10^{-6} M MPO. The mixture was frozen for 10 s after the addition of the enzyme and the EPR spectrum was recorded at 110 K. In these experiments, all components of the mixtures were prepared in argon, saturated solution. All signals detected under these conditions exhibited a g factor of 2.0042 ± 0.0002, determined according the following equation: $g = h\nu/\beta Hr$, where β = Bohr magneton, Hr = applied magnetic field at resonance, ν = frequency of the radiation field oscillation, h = Planck's constant.

(c) Formation of Steady State N^2-Me-9-OH-E Radical during the Degradation of the Quinone. Experiments were performed in 0.05 M phosphate Me-buffer (pH 7.40 or 5.50) containing 10^{-4} M H_2O_2 and 2.5×10^{-6} M N^2-Me-9-OH-E. Just after the addition of 2×10^{-6} M MPO, the mixtures were transferred to the EPR cell and the spectra were recorded at room temperature. The signal observed under these conditions exhibited a g factor of 2.0046 ± 0.0002, and the extent of the signal decreased as a function of time. In order to study the kinetics of the radical decay, a series of experiments were performed in which the mixtures were frozen in liquid nitrogen for 1, 3, 5, 7, and 9 min after MPO was added to them. Spectra were recorded at 110 K, and the radical concentrations were determined by comparing them to a standard solution of spin-label material [3-(2-iodoacetamido)-2,2,5,5-tetramethyl-1-pyrolidinyloxy].

Preparation of N^2-Me-9-oxo-E Irreversibly Bound to Bovine Serum Albumin (BSA). Standard Procedure. In phosphate buffer, 0.05 M (various pH), was dissolved 10^{-4} M H_2O_2 and 10^{-4} M N^2-Me-9-OH-E containing 0.5 mCi of N^2-Me-9-OH-[10-*Me*-^{3}H]E per mmol. MPO, 10^{-7} M, was added; 30 s after the addition of MPO, 15×10^{-6} M BSA was added and the mixture was allowed to stand at 37 °C for 30 min. After the mixture cooled, the BSA was precipitated by 0.6 N perchloric acid (3:1, v/v). The tubes were centrifuged at 5000g for 20 min. The supernatant was discarded, and the precipitate was washed with 0.6 N perchloric acid. This step was repeated twice. The final precipitate was dissolved in water and dialyzed overnight against distilled water. Gel filtration on Sephadex G-75 in 0.01 M phosphate buffer (pH 7.40) showed that the radioactivity remaining after these procedures was confined on the albumin peak. Moreover, incubation of BSA containing bound N^2-Me-9-oxo-E with cold excess 9-oxo-E did not remove any radioactivity from the protein. In these experiments, radioactivity was determined by liquid scintillation counting (ABAC SL40) in a vial containing 0.5 mL of BSA and 4.5 mL of scintillation counting mixture (Packard instrument).

Cytotoxic and Antitumor Tests. Inhibition of cell growth was determined with L1210 lymphocytic leukemia cells as previously described.[19] The inhibitory efficiency against cell multiplication is expressed in terms of ID_{50}, which represents the drug concentration which reduces the rate of cell multiplication by 50% as compared to the control. The highest nonlethal dose (LD_0) was determined for each drug after a single injection into DBA/2 or Swiss mice by an intraperitoneal route. The antitumoral tests were performed on DBA/2 mice that had been inoculated with 10^5 L1210 cells and treated 24 h latter by the same route. Antitumor efficiency is expressed in termof ILS (increase in life span) over controls, $[(T - C)/C] \times 100$. The values presented in the present work have already been published.[17]

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In Vitro Activity of 2-Alkyl-3-hydroxy-1,4-naphthoquinones against *Theileria parva*[1]

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A variety of naphthoquinones have been prepared and evaluated in vitro against the causative agent of the cattle disease East Coast Fever—*Theileria parva* infection. It is concluded from structure–activity studies that a 2-hydroxyl moiety is essential for high activity. The most active compounds tested were 2-hydroxy-3-alkyl-1,4-naphthoquinones in which the alkyl moiety was cyclohexyl, cyclohexylcyclohexyl, tridecyl, or tetradecyl.

The cattle disease East Coast Fever (ECF) is endemic in Eastern and Central Africa and causes serious losses.[2] Approximately half a million cattle each year die from the disease in East Africa alone.[3] Until recently no treatment existed for ECF, which is caused by the tick-borne protozoan *Theileria parva*. However, a major development

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