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ELECTROCHEMICAL AND ELECTRON SPIN RESONANCE STUDIES OF ACTINOMYCIN D AND OTHER PHENOXAZONES

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Electrochemical studies on actinomycin D (1) and two analogs, 2-amino-3-phenoxazone (2) and 1,2,4-trichloro-7-nitrophenoxazone (3) were analyzed by polarography and ESR spectroscopy. The polarograms of the three compounds in acetonitrile all show two reduction waves. ESR experiments confirm that the first reduction wave corresponds to a one-electron transfer process which produces a phenoxazone free radical anion and the second wave corresponds to a subsequent one-electron transfer producing a diamagnetic dianion. Substitution with electron-withdrawing groups such as NO_2 (at C-7) and chloro (at C-1, C-2 and C-4)3 facilitated the reduction of the phenoxazone ring system to a free radical (i.e., half-wave potentials; 1, −0.815 V; 2, −0.920 V; 3, −0.135 V). It was found, by computer simulation of the ESR spectra, that the spin density in the electrochemically generated free radicals from 1, 2 and 3 was preferentially located in the benzenoid ring and at the N-10 nitrogen. For radicals obtained from 1 and 2, only a small residual spin density could be detected in the quinoid ring. Since 1 can be metabolized to a free radical in cells, these free radical forms of 1 and its analogs may represent reactive forms of the phenoxazone nucleus.

1. Introduction

The actinomycins are a family of antibiotics produced by soil actinomycetes [1]. In general, these compounds consist of a substituted phenoxazone ring system, with two cyclic pentapeptide lactones attached at the C-1 and C-9 positions. One of these compounds, 1 (fig. 1), was found to have limited but effective action in the treatment of Wilm's tumor [2] and gestational choriocarcinoma [3]. Recently, some analogs of 1 have been synthesized which seem to have a broader range of antitumor activity and/or lower toxicity in animals [4,5]. Our studies on the metabolism and mechanism of action of quinone-containing anticancer drugs [6–8] have shown that drug free radicals are generated as biochemical metabolites. Similarly, we observed that the quinone imine structure of 1 was a site for the bioreduction of this antibiotic to a free radical [9]. Since both quinone- and quinone imine-containing antibiotics have the capacity to cause macromolecular intracellular damage, it is possible that a mechanism of cytotoxicity occurs through a biochemically mediated free radical process.

This paper reports the free radical formation from actinomycin D (1), 2-amino-3-phenoxazone (2) and 1,2,4-trichloro-7-nitrophenoxazone (3) (fig. 1) by controlled potential electrolysis. Chemical reduction of the same compounds was reported earlier [10] but did not lead to the formation of free radicals with well resolved ESR spectra. With

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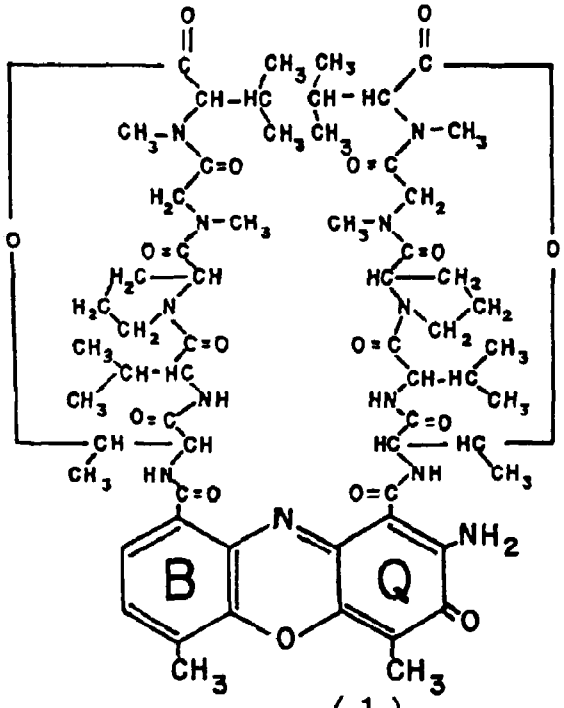
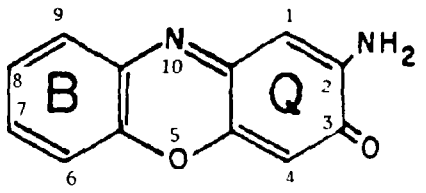
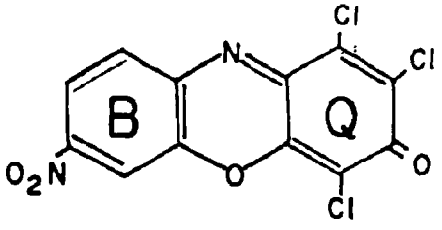


(1)
ACTINOMYCIN D



(2)
2-AMINO-3-PHENOXAZONE



(3)
1,2,4-TRICHLORO-7-NITRO-PHENOXAZONE

Fig. 1. Structure of actinomycin D, 2-amino-3-phenoxazone and 1,2,4-trichloro-7-nitrophenoxazone. B and Q denote the benzenoid and quinoid rings, respectively, of the phenoxazone system.

electrolysis it was possible to control the number of electrons involved (one or two) in a reduction process as well as to prepare sufficient yields of the reduced products to allow their identification and characterization. The electron-withdrawing NO_2 group has been reported to be responsible for an increase in the biological activity of actinomycin [11]. Compounds 2 and 3 are therefore useful for studying the effects of substituents (e.g., NO_2) on free radical formation and on spin density delocalization in the phenoxazone system.

2. Experimental section

Actinomycin D was obtained from the Division of Cancer Treatment, National Cancer Institute (Bethesda, MD), and used without further purification. Compounds 2 and 3 were synthesized as previously described [10]. Tetraethylammonium perchlorate (TEAP) was purchased from Eastman Kodak Co. (Rochester, NY), recrystalized three times from distilled water and dried at 65°C in vacuo [11]. Spectrograde acetonitrile from Matheson, Coleman and Bell Manufacturing Chemists (Norwood, OH) was used without further purification. Triple-distilled mercury from Bethlehem Apparatus Co. (Hellerton, PA) was used in all experiments.

2.1. Polarographic measurements

Polarographic measurements were made at room temperature (approx. 22°C) on a model 174A

polarographic analyzer and model 303 mercury drop electrode detector (Princeton Applied Research Inc., Princeton, NJ). Polarograms were recorded on a Varian model F-100 X-Y recorder. An Ag/AgCl electrode with a Teflon membrane (Yellow Springs Instrument Co., Yellow Springs, OH) was used as a reference electrode, and a platinum wire was used as a counter electrode. The mercury drop electrode had a drop time of 1.0 s in 0.1 M TEAP/acetonitrile solution. Before taking a polarogram, the solution was purged of oxygen by bubbling nitrogen gas for 2 min. A Teflon membrane instead of Vycor fitted glass tip was used which enhanced the stability of the Ag/AgCl reference electrode.

2.2. *ESR and electrolytic measurements*

ESR spectra were obtained on a Varian E-109 Century Series spectrometer with a rectangular TE-104 dual cavity and a strong pitch standard in the reference cavity. The strong pitch ($g = 2.0028$) was used to evaluate g values. The magnetic field was calibrated relative to an alkaline solution of potassium peroxylamine disulfonate (Fremy's salt): $g = 2.0055$ and $a(N) = 13.0$ G. Millimolar quantities of sample were dissolved in an acetonitrile solution containing 0.1 M TEAP and transferred to an ESR electrolytic flat cell (J.F. Scanlon Co., Solvang, CA) and placed in the spectrometer cavity for measurement. In situ electrolysis was performed as follows: The cathode was provided by placing mercury in contact with a platinum wire at the bottom of the ESR cell. Another platinum wire was inserted through a side port of the cell and used as a counter electrode. The reference electrode was Ag/AgCl. The potential across the solution was measured between the cathode and the reference electrode with a Fluke 800 A digital multimeter. A Sorensen QPR 40-2 power supply was used in all experiments.

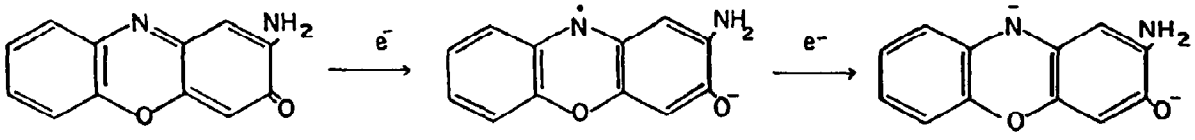
Computer simulations of ESR spectra were performed using the program RESIM written by Dr. Ray C. Perkins, Jr (Varian Associates).

3. Results and discussion

Berg [12] showed that actinomycin C has two half-wave potentials in 80% propanol or in 80% acetone solutions containing 0.1 M LiCl as a supporting electolyte. In our experiment, half-wave potentials (vs. Ag/AgCl electrode) were obtained for 1, 2, and 3 in acetonitrile containing 0.1 M TEAP. In all compounds, the first half-wave in table 1 corresponds to the reduction potential of the parent compound producing an anion radical. The second wave corresponds to a second electron transfer to the anion radical producing a diamagnetic dianion (reaction 1):

The reduction potentials are affected by the nature and number of substituents [13] *. The fact that the half-wave potential of 3 is significantly more positive than those of 1 and 2 implies that electron-withdrawing groups such as NO_2 and chloro contribute to the ease of free radical formation.

The radical anions of 1, 2 and 3 (fig. 2a, b and c, respectively) were generated by holding the potential between the first and second wave at -1.05 V for 1 and 2 and at -0.45 V for 3. In all cases, ESR signals were first observed approx. 30



(1)

* A cyclic voltammogram of 3 was obtained in dimethyl sulfoxide containing 0.1 M TEAP at a scan rate of 400 mV/s on a glassy carbon electrode. Scanning from +1.0 to −1.9 V, four peaks were observed at −0.03, −0.38, −1.23 and −1.38 V (vs. Ag/AgCl), respectively. The peaks at −1.23 and −1.38 V may account for the reduction of the NO_2 group at the B ring.

Table 1

Half-wave potentials observed for 10^{-3} M actinomycin D and its analogs in acetonitrile [a]

Compound	Half-wave potential [b] (in V vs. Ag/AgCl)	
	E_1	E_2
Actinomycin D	-0.815 ± 0.002	-1.192 ± 0.006
2-Amino-3-phenoxazone	-0.920 ± 0.002	-1.284 ± 0.013
1,2,4-Trichloro-7-nitrophenoxazone	-0.135 ± 0.003	-0.505 ± 0.003

[a] 0.1 M tetraethylammonium perchlorate (TEAP) was used as supporting electrolyte.

[b] Represents the average of at least three independent determinations.

min after the beginning of electrolysis. The intensities of the ESR signals were stable for about 1 h. When the potential was made more negative than the second half-wave potential the ESR spectra of all compounds decreased sharply with time and eventually disappeared within 30–45 min after the potential change.

Kinetic measurements of the conversion of the anion radical to the dianion species were difficult to make because of the long diffusion time of the free radicals in the ESR cell. The reduction process occurs at the mercury cathode located at the bottom of the cell so that our reported 30 min delay for the appearance of radical signals may

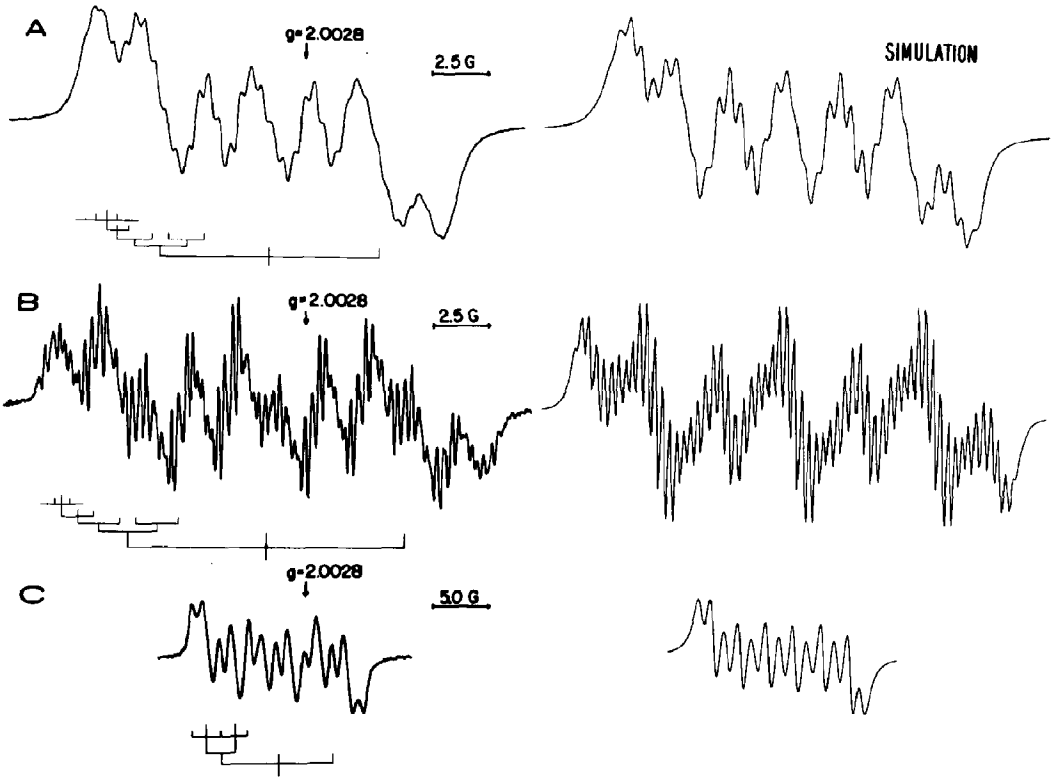
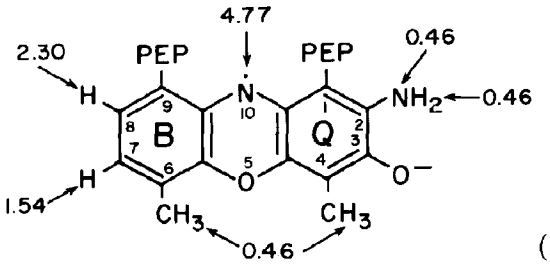


Fig. 2. (a) ESR spectrum of one-electron reduced actinomycin D (10^{-3} M). Reduction potential -1.0 V vs. Ag/AgCl in CH_3CN containing 0.1 M TEAP. ESR conditions at room temperature: 10 mW incident microwave power, 0.005 G modulation amplitude and 3.1 G/min scan rate. (b) ESR spectrum of one-electron reduced 2-amino-3-phenoxazone (10^{-2} M). Reduction potential -1.1 V vs. Ag/AgCl in CH_3CN containing 0.1 M TEAP. ESR conditions at room temperature: 15 mW incident microwave power, 0.16 G modulation amplitude and 3.1 G/min scan rate. (c) ESR spectrum of one-electron reduced 1,2,4-trichloro-7-nitrophenoxazone (10^{-2} M). Reduction potential -0.45 V vs. Ag/AgCl in CH_3CN containing 0.1 M TEAP. ESR conditions at room temperature: 10 mW incident microwave power, 0.063 G modulation amplitude and 12.5 G/min scan rate. The arrows point to strong pitch ($g = 2.0028$) which was used as a reference.

very well represent the diffusion time of the free radical to the sensitive region of the ESR cell. It is reasonable to believe that free radical formation starts at the onset of electrolysis and that it takes about 30 min to accumulate a detectable concentration.

The ESR spectrum of the free radical anion of 1 consists of seven main lines modulated by additional hyperfine lines (fig. 2a). Simulation has shown that the seven lines of various widths and slopes can be accounted for by many overlapping lines. These lines arise from the interaction of the unpaired electron with the ^{14}N nucleus of the N-10 nitrogen atom and with the only two available ring protons at C-7 and C-8. In addition, there is a relatively weak coupling to the protons of the methyl groups at C-4 and C-6 and to the NH_2 group at C-2 (one nitrogen and two protons) (see structure I).



(I)

The observed data as well as those obtained by computer simulation are listed in table 2. A possible explanation for the line broadening in the observed spectrum could be that the phenoxazone ring in 1 is undergoing hindered rotational motion which can be responsible for changes in lineshapes

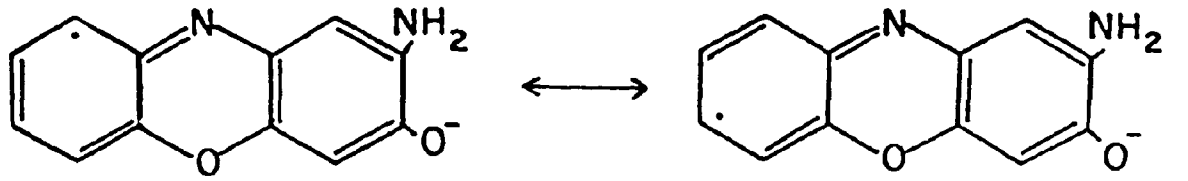
Table 2

ESR data of actinomycin D anion radical and its analogues

One-electron reduced compound	g value	Simulation parameters	
		$10^7 T_2$ (s)	a (G)
1	2.0037	1.3	4.77 (N-10) 2.30 (H at C-8) 1.54 (H at C-7) 0.46 (N at C-2) 0.46 ($2CH_3$ at C-4 and C-6 and 2H at NH_2)
2	2.0038	2.5	6.00 (N-10) 2.50 (H at C-9) 1.86 (H at C-7) 0.67 (N at C-2) 0.30 (2H at NH_2) 0.30 (4H at C-6, C-8, C-1, C-4)
3	2.0043	0.8	4.81 (N-10) 2.37 (H at C-9) 1.00 (2H at C-6 and C-8)

radical anion from 2 is shown in fig. 2b. It was found by computer simulation that the observed lines are attributed to the coupling of the unpaired electron to N-10, and probably to the two protons at C-7 and C-9 (reaction 2) as the spin density is again mostly delocalized into the benzenoid ring of the phenoxazone system.

Additional but small couplings were also detected due to interactions with the amino group nitrogen at C-2 as well as with the remaining six protons at C-6, C-8, C-1, C-4, and on NH_2 at C-2 (see table



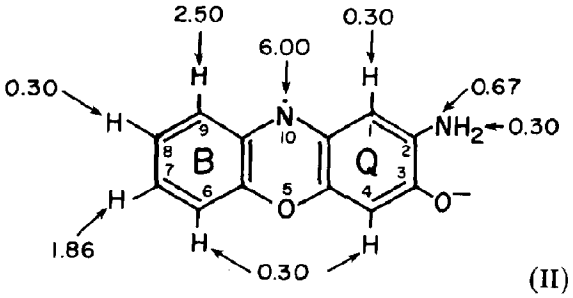
(2)

[14]. This is not unexpected since the molecular weight of the peptide moiety outweighs that of the phenoxazone ring by approx. 4-times.

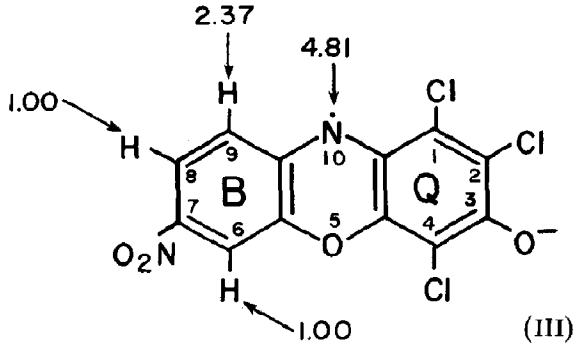
The ESR spectrum of the one-electron reduced

2). The simulation of the spectrum in fig. 2b was carried out assuming that these six protons (which have a relatively small hyperfine coupling constant, 0.30 G (6H)) are magnetically equivalent.

This assumption leads only to an approximate simulation, and a perfect fit between the observed and simulated spectra would require the introduction of some inequivalence between these protons. Nevertheless, the value of 0.30 G probably falls within the narrow range of the true values of hyperfine coupling constants for the different protons (structure II).



The ESR spectrum of the radical anion from 3 exhibited 13 overlapping lines (fig. 2c and table 2) and indicates the presence of coupling to N-10 and to the three protons at C-6, C-8 and C-9, two of which (probably at C-6 and C-8) are equivalent. No coupling was detected to the nitrogen of NO_2, since it is a strong electron-withdrawing group and hence little spin density is expected at C-7 (structure III).



The model compound 3 helps substantiate the assignment of the nitrogen coupling as that of N-10 in 1, 2 and 3. The nitrogen couplings are about the same (4.8 G) for radicals from 1 and 3 and must arise from the only common nitrogen position in both compounds, namely, N-10.

Our results show that most of the spin density of the free radicals from 1, 2 and 3 reside preferentially on the benzenoid (B), rather that on the quinoid (Q) portion of the phenoxazone system. This implies that, if actinomycin D free radicals are involved in the drug's binding to DNA, the benzenoid portion of the phenoxazone system will be most important in the binding mechanism. Other structure-related differences between the Q and B sites such as thermodynamic properties have been reported to be important in the binding of actinomycin D to deoxynucleotides [15].

The structural features of the phenoxazone ring are critical to the biological activity of 1 as it relates to the C-2 and C-7 substituents. For instance, all activity is lost when the quinoid function is blocked by reduction and alkylation [16]. Substitution at C-7 with electron-withdrawing groups modulates antibacterial activity. Bromination at C-7 increases activity by 50% whereas chlorination, nitration or amination reduces it [16]. Reduction of the quinoid function prior to drug use will alter the chemical structure (e.g., spin density distribution) of the actinomycin D free radical discussed here. These changes in spin density distribution could be related to the loss of drug activity. Electron-withdrawing groups at C-7 will tend to increase the spin density of the benzenoid ring, pointing to the importance of this region in the free radical theory of drug activity for these compounds. On the other hand, increased activity by bromination and decreased activity by chlorination and nitration at C-7 are difficult to correlate with our findings.

In general, our results point to an obligatory free radical intermediate that may be involved in the overall mechanism of action of actinomycin D.

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