A STUDY OF SOME QUINONEIMINES AS POTENTIAL ANTI-TUMOR AGENTS. I. PREPARATION OF SOME DERIVATIVES OF LAPACHOL, A NATURALLY OCCURRING NAPHTHOQUINONE. II. A POLAROGRAPHIC STUDY OF SOME QUINONEIMINES

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CHAPTER I

INTRODUCTION

While investigating some quinoneimines as possible anti-tumor agents, it became evident that some knowledge of the relative redox potentials would be useful, particularly in studies correlating anti-tumor activity with various physical and chemical variables. Previous attempts to obtain electrochemical data on these compounds by potentiometric methods proved tedious and time consuming and were hampered by the extreme insolubility of these compounds in both water and 50% ethanolic solution.[1] Polarographic determination of the relative reduction potentials of these compounds would surmount any solubility problems as the optimum concentration for polarographic work is around 10^{-3}M. In addition, several runs could be made on a solution as the electroactive species is not destroyed by the determination. Therefore, a polarographic study was conducted on a series of quinone derivatives to obtain their half-wave potentials for use in correlations with biological activity.

The redox potential, E^{o}, determined potentiometrically and the half-wave potential, $E_{\frac{1}{2}}$, are not the same value but they are related. To obtain E^{o} from polarographic data, the pH dependence of the $E_{\frac{1}{2}}$ values is determined. The value of E^{o} is obtained by extrapolation to pH of 0 and correcting for the resistance of the system.

Lapachol, a naturally occurring napthoquinone, is active against

the Walker 256 carcinosarcoma. Increasing the water solubility of lapachol by synthesizing suitable derivatives might result in a derivative with a greater and more general anti-tumor activity. Two potentially useful reagents for the synthesis of such compounds might be the ketone-water-solubilizing Girard reagents P and T (see Figure 1). The activities of the resulting derivatives would then be determined to see if the chemical modification improved the biological activity.

CHAPTER II

HISTORICAL

Biological Activity of Quinones

Quinones are an important class of biological compounds which are widespread in nature and are involved in the important biochemical processes of respiration and photosynthesis.[2] The key to their activity in these reaction sequences is their reversible reduction to the corresponding hydroquinones, a reaction which occurs under very mild conditions. Quinones and hydroquinones function as a part of the chain of coupled redox reactions, the electron-transport chain, which allows potential energy (initially in the form of the redox potential) to be converted to biologically useful energy. The biological activity of a quinone, whether it is inhibition of a bacterial disease such as malaria or the acceleration of an enzymic reaction, is most probably due to the redox reactions in which it participates.

Although many different quinones exhibit biological activity, it can not be inferred that the mechanism of action is the same in all cases. In fact, it is known that the opposite is true. The inhibition of enzymes by quinones may be due to the oxidation of functional groups of the enzyme (especially the -SH group), reaction with substrates of the enzyme, competition with quinonoid or phenolic substrates, or the formation of metal complexes. Any of the above mechanisms may give rise to the biological activity exhibited by some quinones in accelerating

or inhibiting respiratory processes. In addition, a quinone may act as an electron donor or acceptor or it may form an alternative route for electron transfer. The 3-alkyl-1,4-naphthoquinones to which lapachol is related block specific sites in the electron-transport chain. The mechanism of this activity is unknown and is possibly related to some of those previously mentioned.[3] Some quinones also are known to act as uncouplers in oxidative phosphorylation by related mechanisms. The most obvious and important observation which can be made upon studying the biological activity of quinones is that the effects are diverse, in one case a quinone may inhibit a biological reaction while in another system the same quinone may accelerate a reaction. The biological activity of quinones is probably linked to the ease with which they undergo redox reactions.

Lapachol Derivatives

Lapachol (1) is a naturally occurring naphthoquinone which is found in the wood of several species of the family Bignoniaceae. Its chemistry was first studied in the second half of the 19th century by Paterno.[4] Samuel C. Hooker and co-workers studied the chemistry of lapachol extensively during the periods 1889-96 and 1905-35.[5] In the 1940's Fieser and co-workers, in conjunction with a study of antimalarials, synthesized and studied the properties of some 2-hydroxy-3-alkyl-1,4-naphthoquinones including lapachol.[6]

In recent years, there has been a resurgence of interest in lapachol and its derivatives since it exhibits a wide variety of biological activity, especially anti-tumor activity. In fact, it is currently used commercially in Brazil as an anti-tumor drug[7] and is

undergoing human clinical trials as an anti-tumor agent in this country.[8] Rao, McBride, and Oleson screened lapachol for antitumor activity against five tumor systems and observed highly significant activities only against the Walker 256 carcinosarcoma system, especially when the drug is taken orally twice daily.[8] It has also shown some significant activity against the Murphy-Sturm lymphosarcoma.[7] In another study the acetylglucosylation of lapachol yielded a derivative which was active against mouse lymphocytic leukemia P-388.[7] As its biological activity has been recognized, various toxicologic studies of lapachol have been conducted prior to its clinical trial as an anti-tumor agent.[9,10,11] Toxic and lethal doses as well as the physiological effects were determined in dogs and monkeys.

How lapachol acts to inhibit tumor systems is not known with certainty although many studies have been conducted in attempts to elucidate the mechanism of action. In 1947, Ball and co-workers studied the inhibitory action of naphthoquinones on respiratory processes.[12] Succinate oxidase isolated from beef heart was suspended in a buffer which contained enough cytochrome c to saturate the enzyme and then treated with a solution of the quinone being investigated. From their studies, Ball and co-workers concluded that lapachol is a very potent respiratory poison which blocks the electron transport chain at a position between cytochromes b and c. They suggested that the quinone might inhibit an unknown enzyme which controls the reaction between these two cytochromes.

In 1961, Tong and Chaikoff studied the effects of some quinones, including lapachol, upon the utilization of iodine by cell-free sheep thyroid tissue preparation. Lapachol was found to inhibit this process.[13]

Howland found that lapachol and other 2-hydroxy-3-alkyl-1,4-naphthoquinones inhibited the coupled oxidation of N,N,N',N'-tetramethyl-p-phenylene-diamine (TMPD) by acting as uncouplers, stimulating mitochondrial ATPase, an enzyme in the electron-transport chain.[14] The fact that succinate oxidation was also inhibited indicated interference in the electron transport chain in the vicinity of cytochromes b and c. A dual mechanism, involving stimulation of ATPase and inhibition of the redox couples of the electron transport system was postulated to explain these results.[14]

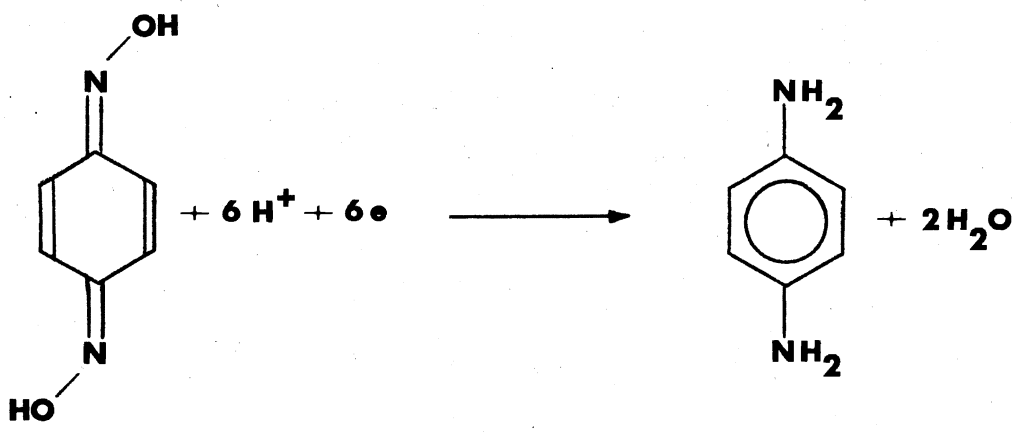
Polarography

Since its invention by Heyrovsky in 1922, polarography has proven to be a very useful electrochemical technique.[15] It is a branch of voltammetry which studies the effect of variation in potential upon the current that flows through an electrolysis cell. Polarography differs from voltammetry in that one electrode is the dropping mercury electrode. Both methods use working electrodes of small surface area so that the electrode is polarized by the large current density it experiences as the potential is applied across the cell, this results in reduction or oxidation, depending upon the potential, of the electroactive species present in a small layer surrounding the mercury drop. By studying the effects of many variables such as the pH and height of mercury, the mechanism of the electrode reaction can be studied. Since many organic compounds undergo irreversible reduction, potentiometric methods are useless. In polarography, a reversible redox couple is not necessary for the appearance of a polarographic wave and therefore the determination of the half-wave potential, the potential at the half height of the

wave is possible. This useful fact led to the rapid growth of organic polarography.

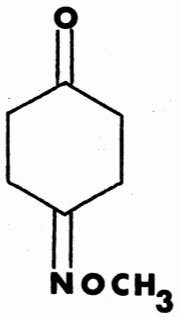
The polarography of quinonoid compounds makes up a large part of the polarographic literature.[16] While the polarographic behavior of the azomethine group and quinonoid compounds have both been studied, data on the quinoneimines is sketchy.[17] The study of the oxidation of aminophenols to the corresponding quinoneimines was not possible over the entire pH range because of the instability of the electrolysis product.[18]

In 1956, Elofson and Atkinson attempted to consolidate previous work on quinone monoximes and to study the electrochemistry of the corresponding dioximes.[19] They studied the mono- and dioximes of 1,4-benzoquinone, 1,2- and 1,4-naphthoquinone, and 9,10-anthraquinone. They observed a four-electron reduction for the monoximes studied. This agreed with the reports of previous workers.[20,21] For the dioximes, they observed waves corresponding to a six-electron reduction and postulated the following reduction mechanism:

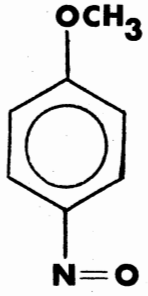


In neutral solution, this reaction occurred in two steps.[19] Because the lower wave was kinetic in nature, the authors postulated that isomerization occurred at the electrode before reduction. They invoked the classical oxime-nitrosophenol tautomerization. The products of electrolysis were not isolated nor were the results of ongoing spectral analyses reported.

In 1972, Castetbon and Bonastre reported on their extensive electrochemical study of some quinonemono- and dioximes.[22,23,24] They studied the reduction of these compounds using conventional polarography and cyclic voltammetry, a technique especially useful for studying electrode kinetics, and controlled potential electrolysis. By studying the electrode mechanism of the "blocked" forms of the nitrosophenol-oxime tautomer pair, (2 and 3) the authors were able to conclude that the initial step in the reaction involved the oxime tautomer. Results from



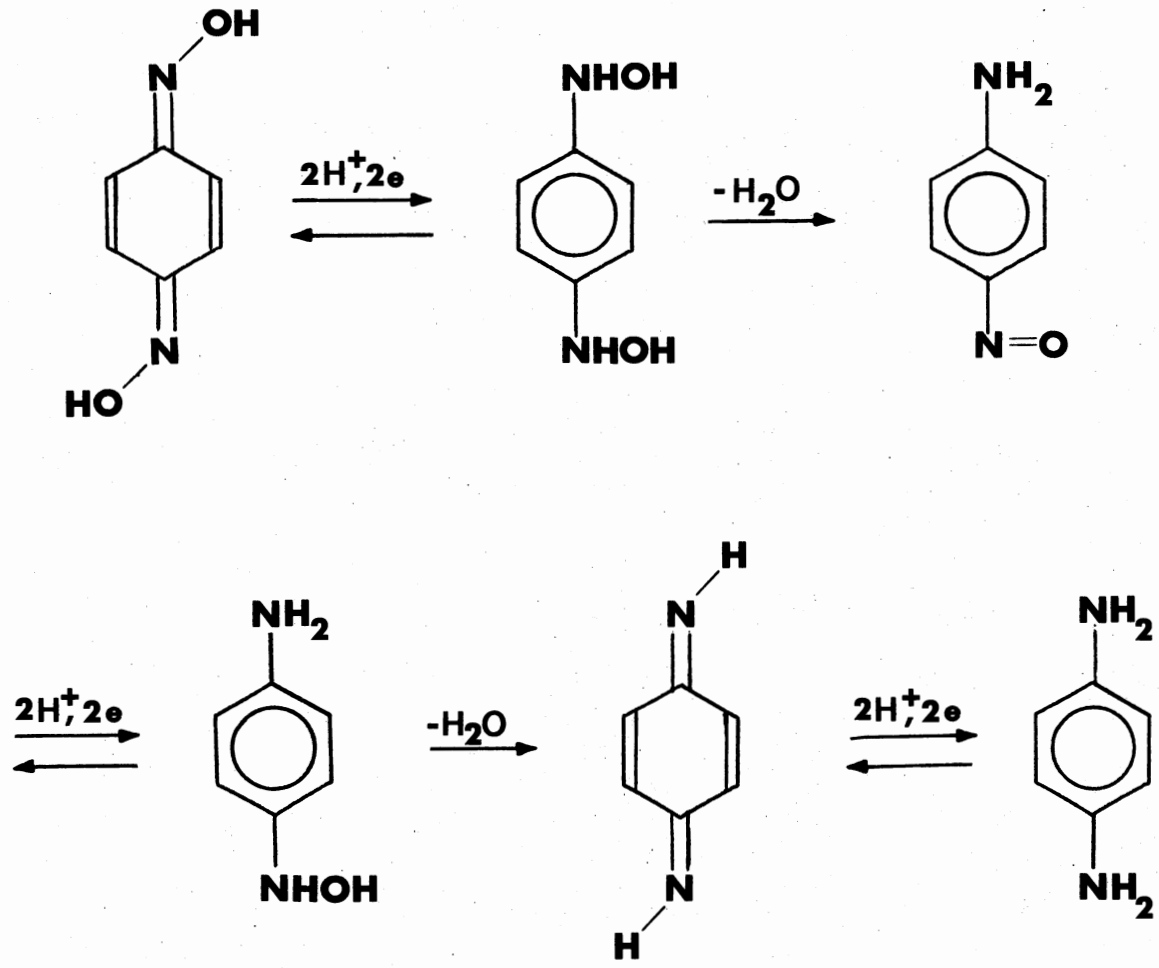
(2)



(3)

cyclic voltammetry identified the reversible steps in the mechanism. The mechanism of reduction of the dioxime of 1,4-benzoquinone is shown

as a representive example of the reduction of these compounds.



Depending upon the pH, the species initially reduced is either protonated, neutral, or anionic. The monooxime is reduced by a similar mechanism involving 4 electrons overall and the loss of only one molecular of water.

CHAPTER III

EXPERIMENTAL

Lapachol Derivatives

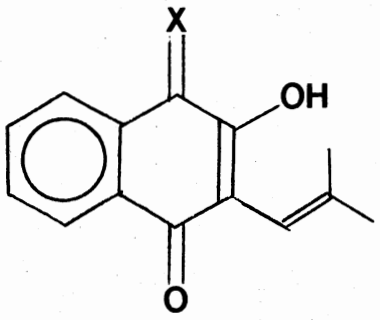
Materials

Two different samples of lapachol were used in this study. One was supplied by Dr. Harry Wood of the National Cancer Institute in Bethesda, Md. It was a Pfizer sample, lot #1052-176-1. The second sample was purchased from the Aldrich Chemical Co., lot #120427.

Preparation of Lapachol Derivatives

The structures of all new compounds were verified by NMR and IR spectroscopy and by chemical modifications. The results of these studies are presented later in this chapter.

Preparation of Lapachol-1-oxime (2-hydroxy-3-(3-methyl-2-butenyl)-1,4-naphthoquinone-1-oxime) (4, Figure 1).[25] Lapachol (3 g, 0.012 moles) was dissolved in 5% aqueous sodium hydroxide (80 ml.). Hydroxylamine hydrochloride (1.25 g, 0.018 moles) was added. The solution changed from deep red to orange brown in a few minutes. After 15 minutes, the solution was diluted with distilled water (300 ml) and acidified with dilute acetic acid. The pale yellow precipitate was collected and recrystallized from ethanol. Yield 2.1 g, (0.009 mole, 68% of theory) mp, 165^{o}-170^{0} (d) (lit., 160^{o}-180^{0}).[25]



1: X = O — Lapachol or 2-hydroxy-3(3-methyl-2-butenyl)-1,4-naphthoquinone

4: X = N-OH — Lapachol-1-oxime or 2-hydroxy-3(3-methyl-2-butenyl)-1,4-naphthoquinone 1-oxime

5: X = N-N-C-NH$_2$ (N, S) — Lapachol-1-thiosemicarbazone or 2-hydroxy-3-(3-methyl-2-butenyl)-1,4-naphthoquinone 1-thiosemicarbazone

6: X = N-N-C-CH$_2$ ⊖ ⊕N Cl, (CH$_3$)$_3$ (H, O) — Girard T derivative of Lapachol or 2-hydroxy-3-(3-methyl-2-butenyl)-1,4-naphthoquinone-1-((carboxymethyl)trimethylammonium chloride hydrazone)

7: X = N-N-C-CH$_2$ ⊖ ⊕N Cl (H, O) — Girard P derivative of Lapachol or 2-hydroxy-3(3-methyl-2-butenyl)-1,4-naphthoquinone-1-((carboxymethyl)pyridinium chloride hydrazone)

8: X = N-N-C-NH$_3$ ⊕ Cl ⊖ (H, NH) — Lapachol-1-amidinohydrazone, hydrochloride or 2-hydroxy-3-(3-methyl-2-butenyl)-1,4-naphthoquinone-1-(amidinohydrazone hydrochloride)

Figure 1. Lapachol Derivatives

Preparation of Lapachol-1-thiosemicarbazone (2-hydroxy-3-(3-methyl-2-butenyl)-1,4-naphthoquinone-1-thiosemicarbazone (5).[25,26] Thiosemicarbazide (0.45 g, 0.05 moles) was dissolved in distilled water (15 ml) and glacial acetic acid (1 ml) on a steam bath. Lapachol (1.21 g, 0.05 moles) was dissolved in alkaline ethanol (5% NaOH, 80 ml). The thiosemicarbazide solution was added and the solution was warmed on a steam bath for one hour, then allowed to stand at room temperature for three days. The solution was acidified with dilute hydrochloric acid. The yellow precipitate was collected and recrystallized from absolute ethanol. Yield, 0.75 g (0.0024 moles, 48% of theory). The product was fine yellow crystals with a melting point of 265°.

Preparation of Lapachol-1-thiosemicarbazone (2-hydroxy-3-(3-methyl-2-butenyl)-1,4-naphthoquinone-1-thiosemicarbazone) (5).[25,26] Thiosemicarbazide (6.86 g, 0.02 moles) was dissolved in distilled water (60 ml) and glacial acetic acid (4 ml) on a steam bath. Lapachol (5 g, 0.02 moles) was dissolved in alkaline ethanol (5% NaOH, 320 ml). The thiosemicarbazide solution was added and the solution was warmed on a hot plate for 2 hours. The reaction mixture was stirred for three days at room temperature. The solution was acidified with dilute hydrochloric acid. The yellow precipitate was collected and recrystallized from absolute ethanol. Fine yellow-orange needles were recovered. 4.8 g (0.015 moles, 75% of theory), mp, 188°-190°.

Preparation of the Girard T derivative of Lapachol (2-hydroxy-3-(3-methyl-2-butenyl)-1,4-naphthoquinone-1-(carboxymethyl)trimethylammonium chloride hydrazone) (6).[25,27] Lapachol (3 gm, 0.012 moles) was dissolved in aqueous 5% sodium hydroxide (80 ml). Girard Reagent T

((carboxymethyl)-trimethylammonium chloride hydrazide) (2.08 g, 0.012 moles) was added and the solution was stirred at room temperature for three days. The solution was diluted with distilled water (320 ml) and acidified with dilute hydrochloric acid. The red-brown precipitate was collected and recrystallized from 95% ethanol. Golden brown crystals, 2.67 g (0.0076 moles, 63.3% of theory) were collected, mp, 140^{0}-144^{o}.

Preparation of the Girard P derivative of Lapachol (2-hydroxy-3-(3-methyl-2-butenyl)-1,4-naphthoquinone-1-(carboxymethyl)pyridinium chloride hydrazone) (7).[25,27] Lapachol (5 g, 0.02 moles) was dissolved in 5% aqueous sodium hydroxide (135 ml). Girard Reagent P ((carboxymethyl) pyridinium chloride hydrazide) (4.7 g, 0.028 moles) was added to the solution. The reaction mixture was allowed to stand at room temperature with stirring for 3 days. The green solution was diluted with distilled water (350 ml). The resulting red solution was acidified with dilute hydrochloric acid. The red-brown precipitate was collected and recrystallized from 95% ethanol. Red-brown crystals (4.77 g, 0.012 moles, 58% of theory) were collected, mp, 120^{o}-126^{o}.

Preparation of 2-hydroxy-3-(3-methyl-2-butenyl)-1,4-naphthoquinone-1-amidinohydrazone hydrochloride (8).[28] Lapachol (1 g, 0.004 moles) was dissolved in concentrated ammonium hydroxide (20 ml) and distilled water (20 ml). Aminoguanidine bicarbonate (0.6 g, 0.004 moles) was added. The mixture was stirred at room temperature for three days. The orange precipitate was collected, washed with dilute hydrochloric acid (3 x 5 ml) and dried. An orange solid product was collected, 0.02 gm (0.0029 moles, 71% of theory) mp, 225^{o}-230^{o}(d).

Attempted preparation of N-(trihydroxymethyl)methyl-2-hydroxy-3-(3-methyl-2-butenyl)-1,4-naphthoquinone-1-imine.[25] Lapachol (2 g, 0.008 moles) was dissolved in 5% aqueous sodium hydroxide (40 ml). 2-Amino-2- hydroxymethyl -1,3-propanediol (1.8 g, 0.015 moles) was added. The resulting red paste was stirred and heated gently for 3 hours. A thick red precipitate formed which was acidified with dilute hydrochloric acid. The yellow precipitate was recrystallized from 95% ethanol. The yellow crystals had a melting point of 137° (mixed mp with lapachol, 137°). It was therefore concluded that the starting material was recovered.

Attempted preparation of N-(trihydroxymethyl)methyl-2-hydroxy-3-(3-methyl-2-butenyl)-1,4-naphthoquinone-1-imine.[28] Lapachol (1 g, 0.004 moles) and 2-amino-2-hydroxymethyl-1,3-propanediol (0.6 g, 0.005 moles) was dissolved in pyridine (10 ml). The reaction mixture was stirred at room temperature for three days in a nitrogen atmosphere. The solvent was removed by evaporation and dilute hydrochloric acid (5 ml) was added to the solid residue. The solution was diluted with distilled water (200 ml). The yellow precipitate was recrystallized from absolute methanol. Golden yellow crystals were recovered, mp 132°. The pmr spectrum indicated that no reaction took place at either C-1 or C-4 and that only the starting material was recovered.

Attempted preparation of N-(trihydroxymethyl)methyl-2-hydroxy-3-(3-methyl-2-butenyl)-1,4-naphthoquinone-1-imine.[28] Lapachol (1 g, 0.004 moles) and 2-amino-2-hydroxymethyl-1,3-propanediol (0.6 g, 0.005 moles) was suspended in 80% trifluoroacetic acid (15 ml). The solution was stirred under nitrogen at room temperature for two days. The solu-

tion was diluted with distilled water (200 ml). The orange precipatate was collected by vacuum filtration and recrystallized from 95% ethanol. Dark red-orange crystals were recovered, mp, 145°. The crystals were insoluble in sodium hydroxide solution, indicating formation of a β-lapachone. The sodium fusion test for nitrogen was negative.

Attempted preparation of N-(trihydroxymethyl)methyl-2-hydroxy-3-(3-methyl-2-butenyl)-1,4-naphthoquinone-1-imine. Lapachol (1 g, 0.004 moles), 2-amino-2-hydroxymethyl-1,3-propanediol (0.6 g, 0.005 moles) and $FeCl_3$ (1 g, 0.006 moles) were suspended in benzene (100 ml) and placed in a 250 ml round-bottomed flask equipped with a Dean-Stark trap and a reflux condenser. The mixture was heated for three days. The solution was placed in a separatory funnel, washed with distilled water (2 x 50 ml), 6N NaOH (2 x 50 ml). The aqueous layer was filtered and acidified with dilute hydrochloric acid. The yellow precipitate was collected and recrystallized from 95% ethanol. The yellow needles recovered had a mp of 137° (mixed with lapachol, 137°). Only starting material was recovered.

Attempted preparation of 2-hydroxy-3-(3-methyl-2-butenyl)-1,4-naphthoquinone-1-thiosemicarbazone.[28,29] Lapachol (1.21 g, 0.005 moles) and thisemicarbazide (0.45 g, 0.005 moles) were dissolved in a mixture of 95% ethanol (33 ml), glacial acetic acid (65 ml) and concentrated hydrochloric acid. The reaction mixture was heated under reflux for 1 day. The red-orange product was insoluble in aqueous sodium hydroxide, indicating the formation of a β-lapachone.

Structural Conformation

Both chemical and spectral methods were used to confirm the structure of the lapacholhydrazones and imines produced. Proton magnetic resonance spectra were obtained using a Varian model XL-100 analytical nuclear magnetic resonance spectrometer equipped with a NMR-FT unit. Proton magnetic resonance spectra were obtained by analysis with TMS as an internal standard. Carbon-13 magnetic resonance (CMR) spectra were obtained by Fourier Transform analysis with TMS as an internal standard. For the ^{13}C spectra, 10-15% of a deuterium-containing solvent was used to accommodate the deuterium lock of the FT unit. All NMR spectra were run in dimethylsulfoxide-d_6. Infrared spectra were run on a Beckman IR-5A spectrophotometer. All IR spectra were obtained from potassium bromide pellets.

Proton magnetic spectra were used to assign structures to the new compounds. In lapachol (1) there are two types of aromatic protons, those at C-5 and C-8 and those at C-6 and C-7 (see Figure 1).[30] However in lapacholoxime, one of the original carbonyls is now a C=N bond. The electronic environment of the aromatic protons is now different, none of these protons are equivalent. Those protons at C-6 and C-7 are in a similar environment. They show up as a multiplet at δ 7.66. The proton adjacent to the carbonyl at C-5 produces a multiplet at δ 8.13 while the one at C-8, adjacent to the C=N bond, has a multiplet at δ 8.95. The appearance of three signals in the aromatic region was used as conformation of the formation of a carbon-nitrogen double bond at either C-1 or C-4. The pmr spectra of lapachol and its various derivatives are shown in Figures 2-7.

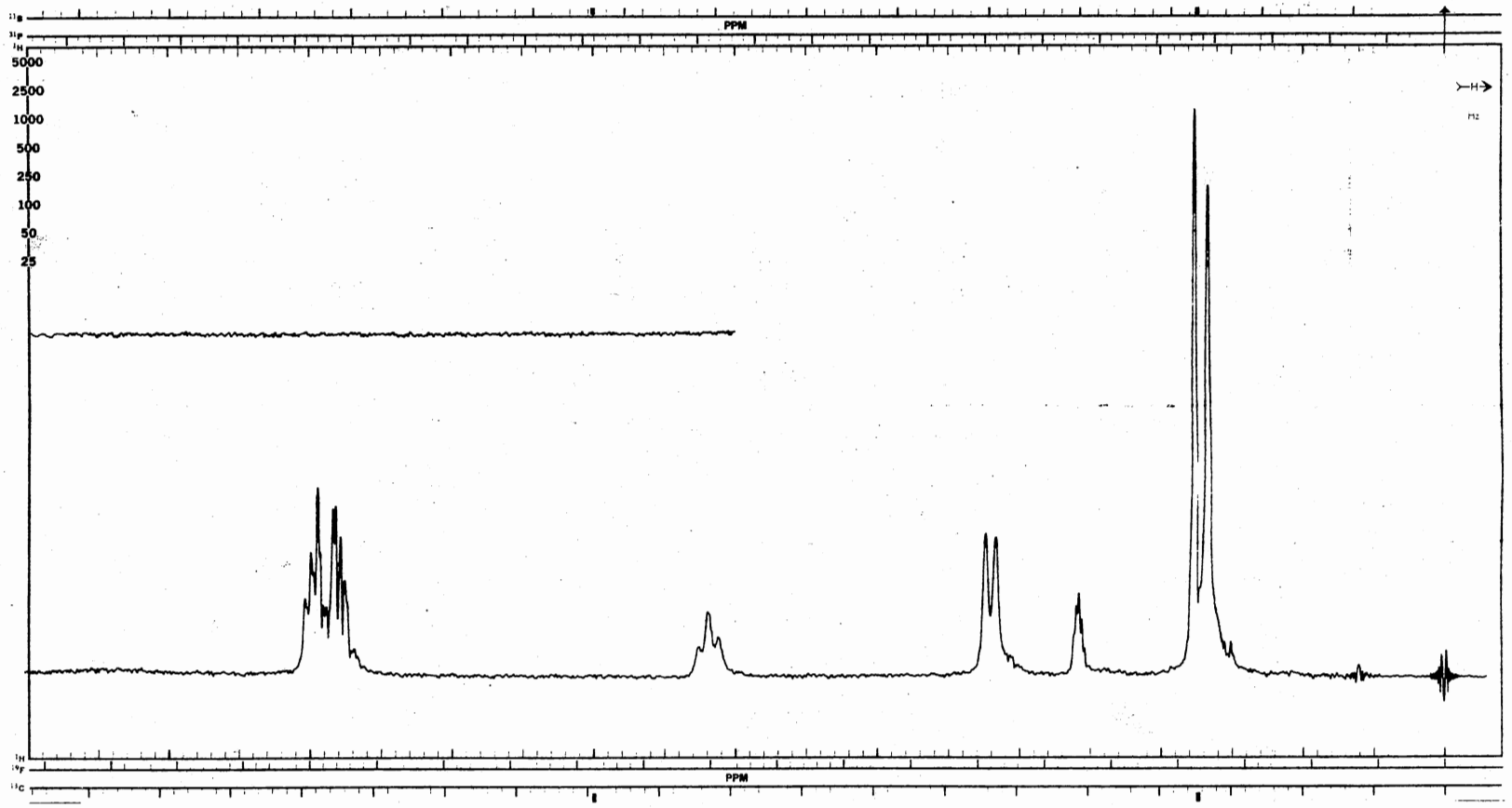


Figure 2. Proton Magnetic Resonance Spectrum of Lapachol (1) in DMSO-d_6

... 100 MHz R.F. . . . 57 dB S.W. . . . 1000 Hz S.A. . . . 1.25
F.B. . 2 Hz S.T. . . 250 sec S.O. . . 83701 Hz

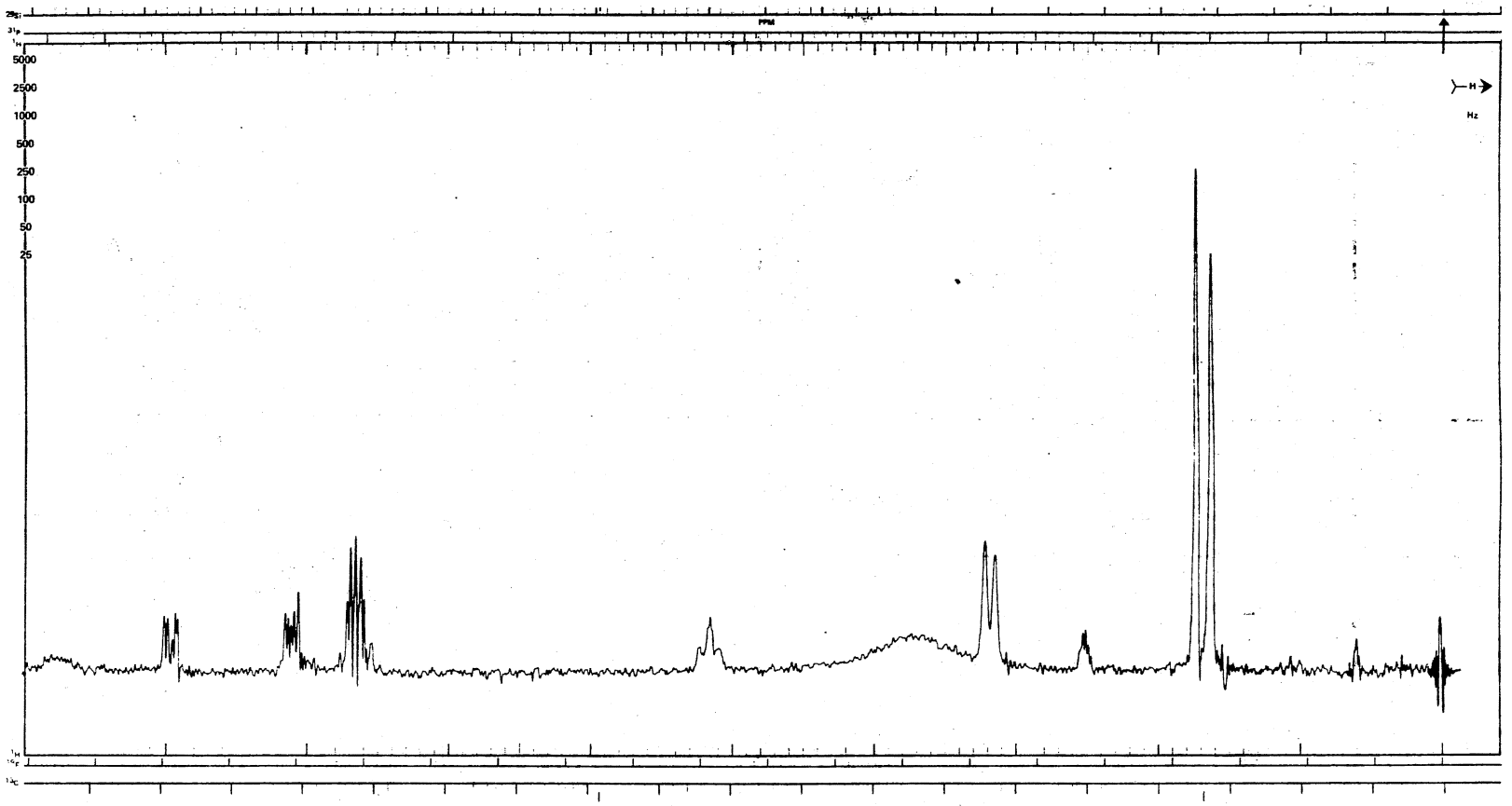


Figure 3. Proton Magnetic Resonance of Lapachol 1-Oxime (4) in DMSO-d_6

... 100 MHz	R.F. . . . 71 dB	S.W. . . . 1000 Hz	S.A. . . . 8.0
F.B. . 2 Hz	S.T. . . 250 sec	S.O. . . 83701 Hz	

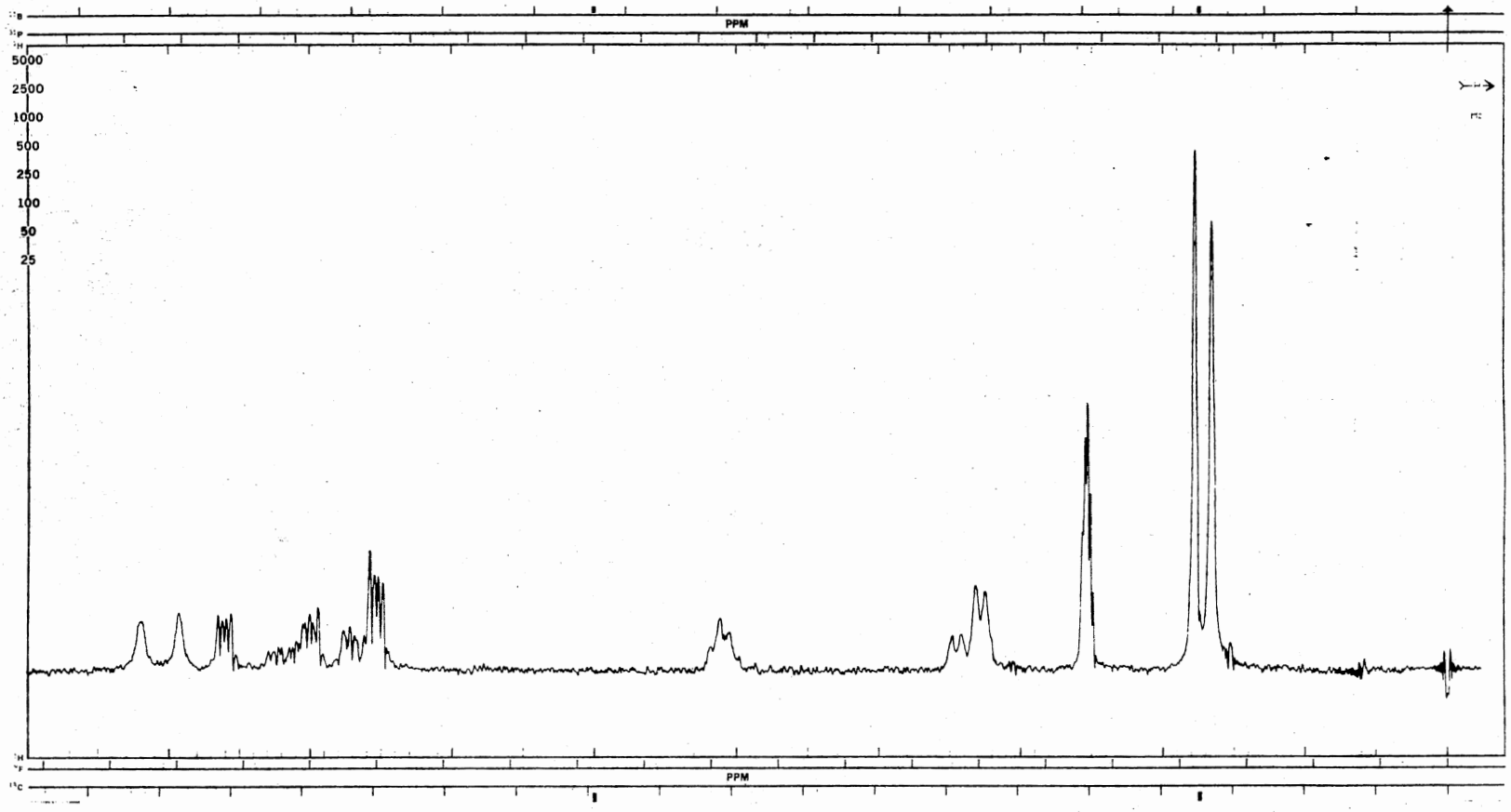


Figure 4. Proton Magnetic Resonance of Lapachol-1-thiosemicarbazone (5) in DMSO-d_6

... 100 MHz R.F. . . . 60 dB S.W. . . . 1000 Hz S.A. . . . 2.5
F.B. . 2 Hz S.T. . . 250 sec S.O. . . 83701 Hz

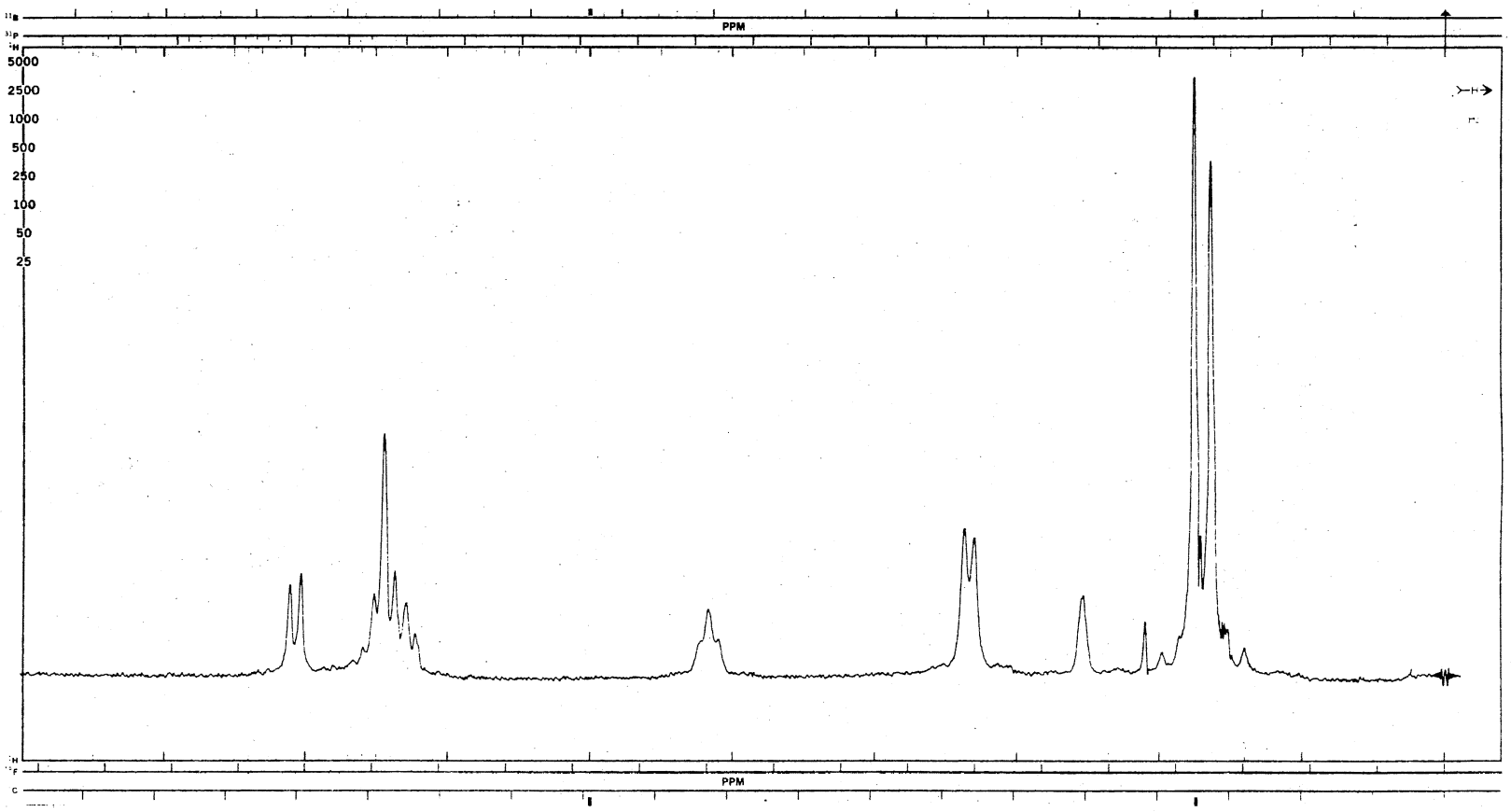


Figure 5. Proton Magnetic Resonance of Girard T Derivative of Lapachol (6) in DMSO-d_6

... 100 MHz R.F. . . . 52 dB S.W. . . . 1000 Hz S.A. . . . 1.0
F.B. . 2 Hz S.T. . . 250 sec S.O. . . 83701 Hz

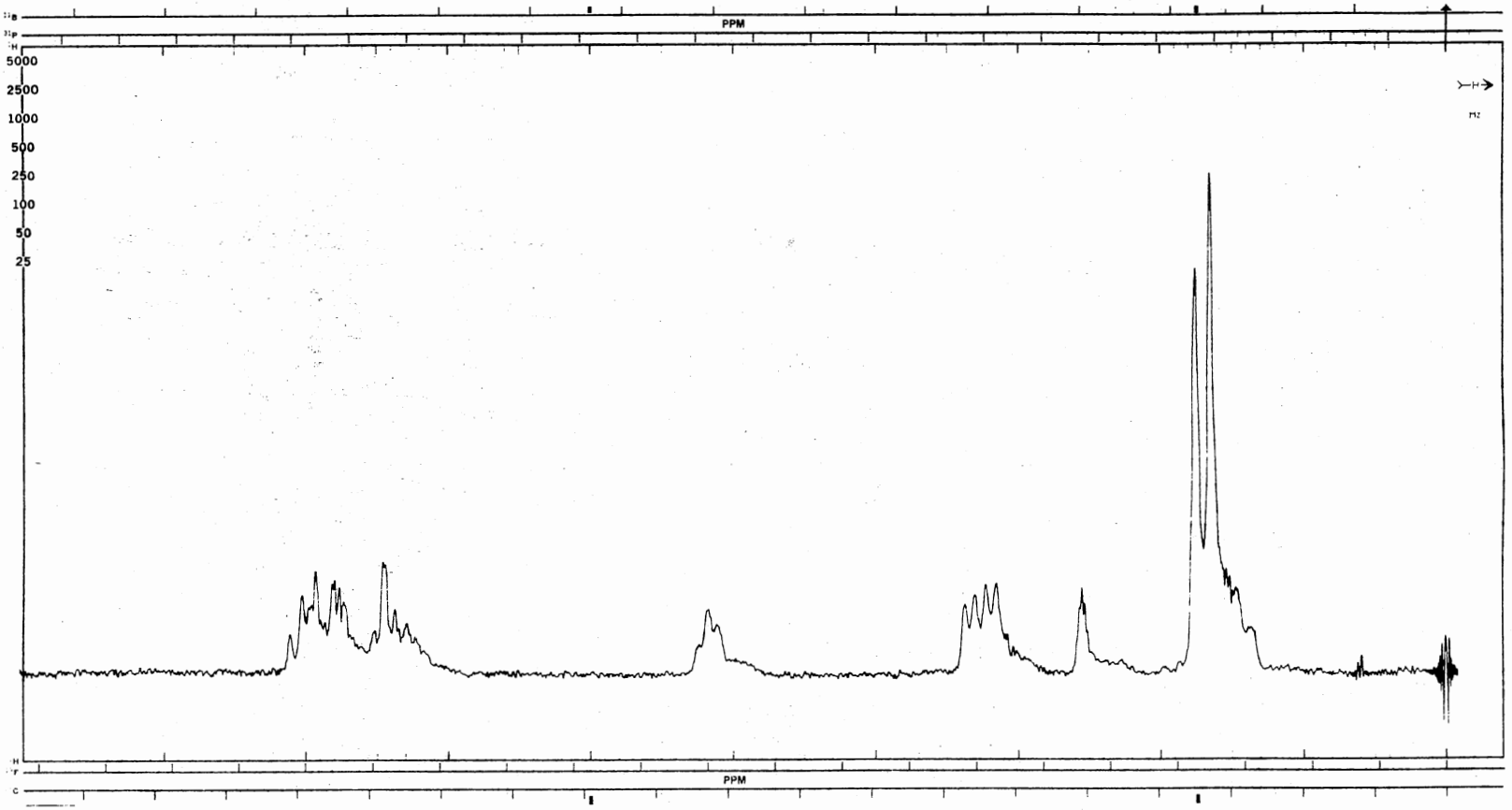


Figure 6. Proton Magnetic Resonance of Girard P Derivative of Lapachol (7) in DMSO-d_6

... 100 MHz	R.F. . . . 50 dB	S.W. . . . 1000 Hz	S.A. . . . 2.0
F.B. . 2 Hz	S.T. . . 250 sec	S.O. . . 83701 Hz	

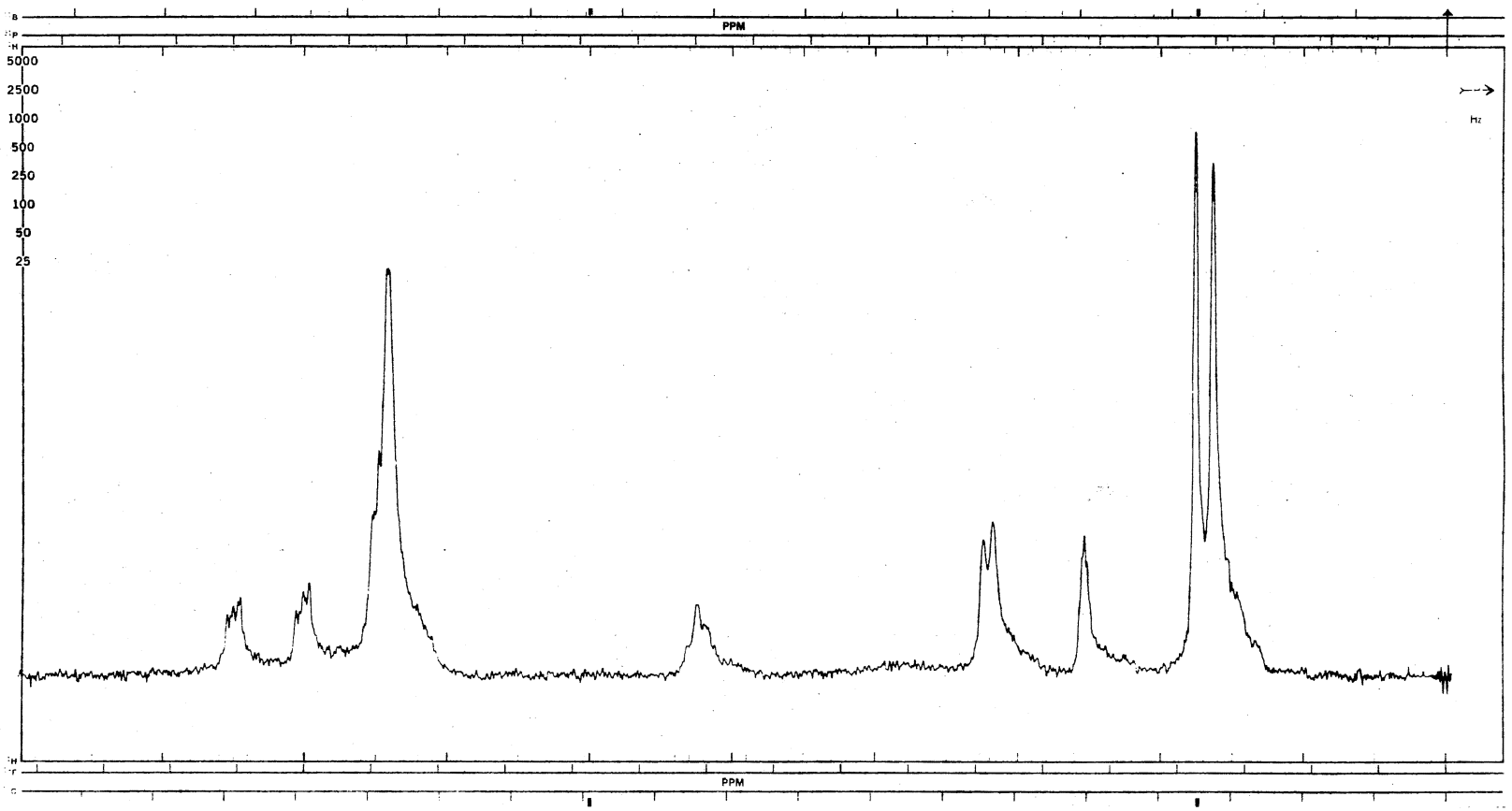


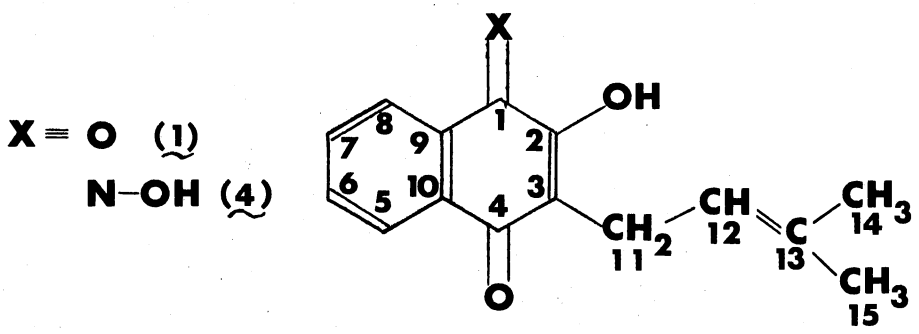
Figure 7. Proton Magnetic Resonance of Lapachol 1-Amidinohydrazone (8) in DMSO-d_6

... 100 MHz R.F. . . . 57 dB S.W. . . . 1000 Hz S.A. . . . 3.2
F.B. . 2 Hz S.T. . . 250 sec S.O. . . 83701 Hz

A method was needed to positively identify the position of the C=N bond in the new lapachol derivative. Therefore a ^{13}C magnetic resonance study of lapachol and lapachol 1-oxime was performed to see if the technique would prove useful in distinguishing between the C-1 and C-4 carbons. The CMR spectra of lapachol and lapachol 1-oxime appear in Figures 8 and 9.

The position of condensation of the oxime was confirmed by infrared spectra and chemical methods (see below). It was hoped that by the comparison of the two CMR spectra, the C-1 position could be identified with certainty. The position of all peaks are in ppm relative to TMS, whose signal is arbitrarily set at 0 ppm.

Some peak assignments can be made for lapachol based upon literature values. The signals at 183.6 and 180.6 ppm can be assigned to C-1 and C-4 based on the literature values of the carbonyls in 1,4-naphthoquinone.[31,32,33] However, it is not possible to determine which carbon corresponds to which signal. The three aliphatic carbons appear closest to TMS. The signal at 17.6 ppm is probably due to C-11 while the signals at 21.9 and 25.3 ppm are due to the methyl groups, cis (C-15) and trans (C-14) to C-11.



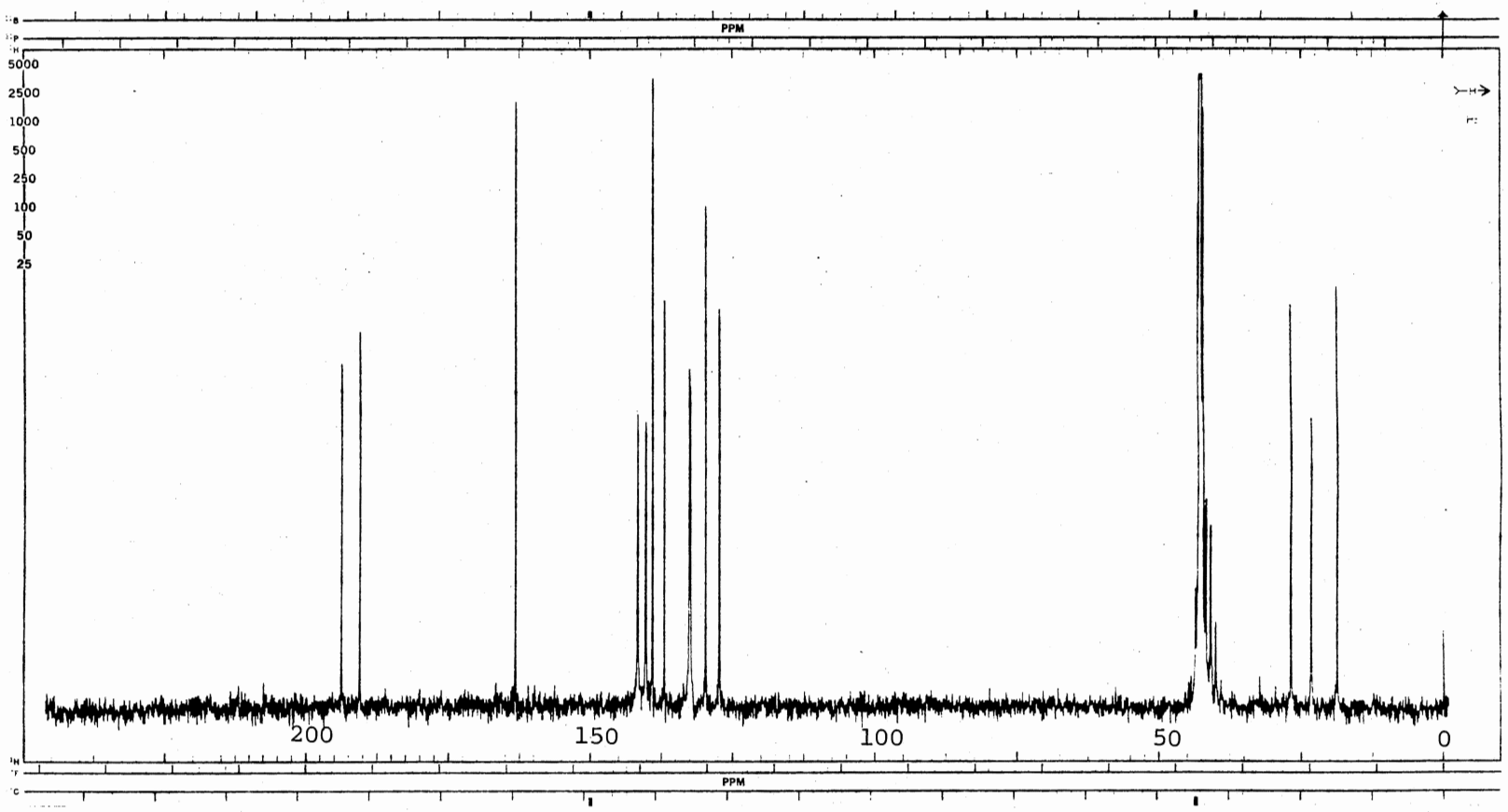


Figure 8. ^{13}C Magnetic Resonance Spectrum of Lapachol (1) in DMSO-d_6

... 25.2 MHz S.W. . . . 6000 Hz S.O. . . . 35351 Hz

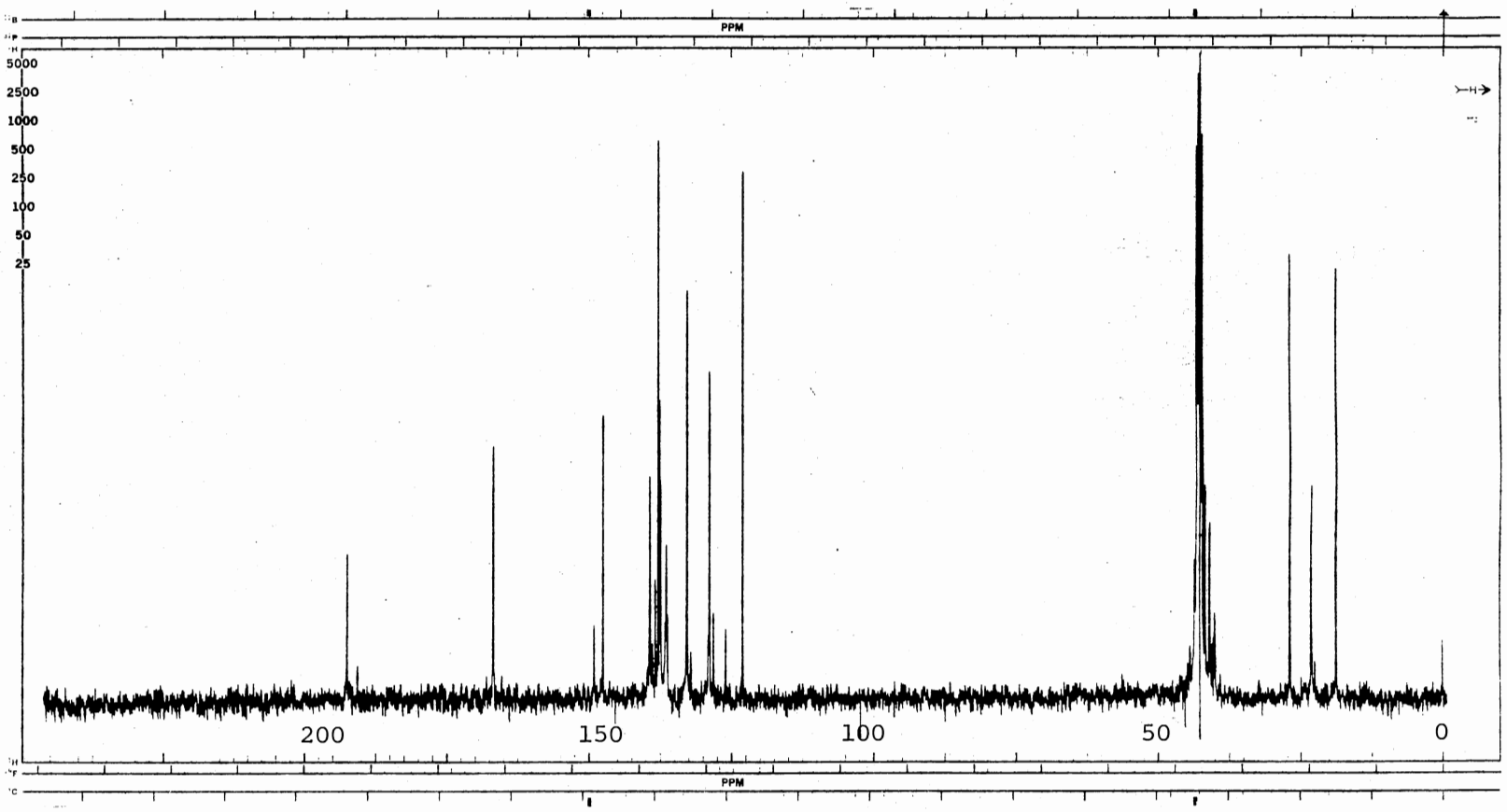


Figure 9. ^{13}C Magnetic Resonance Spectrum of Lapachol 1-Oxime (4) in DMSO-d_6

... 25.2 MHz S.W. . . . 6000 Hz S.O. . . . 35351 Hz

C-2 is assigned the signal of 154.6 ppm based on the CMR spectra of 1,4-naphthoquinone and other naphthoquinones.[31,32,33] This carbon in 1,4-naphthoquinone has a signal of 138.5 ppm, but the hydroxy substituent would be expected to shift the signal downfield. Assignment of the other carbons is more difficult, each carbon has a different electronic environment and gives a different signal. The peaks of the two vinylic and the remaining seven carbons appear between 120.4 and 134.0 ppm.

In the CMR spectrum of lapachol 1-oxime, the signal at 182.1 ppm corresponds to C-4, one of the original carbonyls. Whether this peak shifts downfield from 180.6 ppm or upfield from 183.6 ppm can not be determined, therefore nothing can be said about which carbon is responsible for the original signal in lapachol. By studying the literature on ketones and their oximes, the conclusion that C-1 gives rise to the signal at 139.3 ppm is made. The carbon shift observed in changing from a carbonyl to a C=N bond is 43.1 ppm; this is the only major change seen. In a paper by Hawkes, Herwig, and Roberts the average shift observed between ketones and their oximes is 48 ppm.[34] There is also an increase in the number of signals in the aromatic region which can be explained by postulating the formation of both geometric isomers of the oxime, the syn and anti forms. The small peak observed at 140.8 ppm in the CMR spectrum of lapachol 1-oxime is possibly due to the other isomer with the OH of the oxime anti to the 2-OH group. The difference of 1.5 ppm observed compares favorably with the differences observed for the different isomers of other ketoximes.[34,35] Since no definite statement could be made concerning which signal was due to the C-1 atom in lapachol, CMR could not be used to positively identify the position of

densation.

Infrared spectra of lapachol and the derivatives prepared in this study are shown in Figures 10-15. Lapachol oxime shows a broad peak at 3330 cm^{-1} which is due to hydrogen bonding between the oxime nitrogen and the 2-hydroxy group whereas lapachol exhibits no such band. This broad band is present in the IR spectra of all the new derivatives, indicating hydrogen bonding between the group introduced at C-1 and the hydroxy group at C-2. If the condensation had occurred at C-4, no hydrogen bonding would be observed.

Chemical methods were also used to confirm the position of the new C=N bond. The imine or hydrazone was first reduced to the corresponding aminophenol using $SnCl_2$ and HCl. The aminophenol was then subjected to air oxidation at pH 8 to yield the phenoxazone.[36]

Conversion of 2-hydroxy-3-(3-methyl-2-butenyl)-1,4-naphthoquinone-1-imine or 1-hydrazone (9) to 9-hydroxy-6,8-(3-methyl-2-butenyl)-dibenzo(aj)phenoxazin-5-one (11).[26,28,36] The imine or hydrazone (9) (4.05 mmoles), stannous chloride dihydrate (3.0 g., 0.013 moles), water (20 ml), concentrated hydrochloric acid (10 ml), and 95% ethanol (5 ml) were heated under reflux until complete solution had occurred. The solution was filtered and concentrated hydrochloric acid (10 ml) was added to the filtrate. The white precipitate of the aminophenol (10) was collected after cooling. The aminophenol (10) was dissolved in distilled water (30 ml). Sodium acetate (1.7 g, 0.012 moles) was dissolved in distilled water (100 ml). The two solutions were combined and were stirred overnight open to the atmosphere. The dark green precipitate was collected and triturated with ether and ether-insoluble solid collected. The color reactions and TLC properties of this compound were

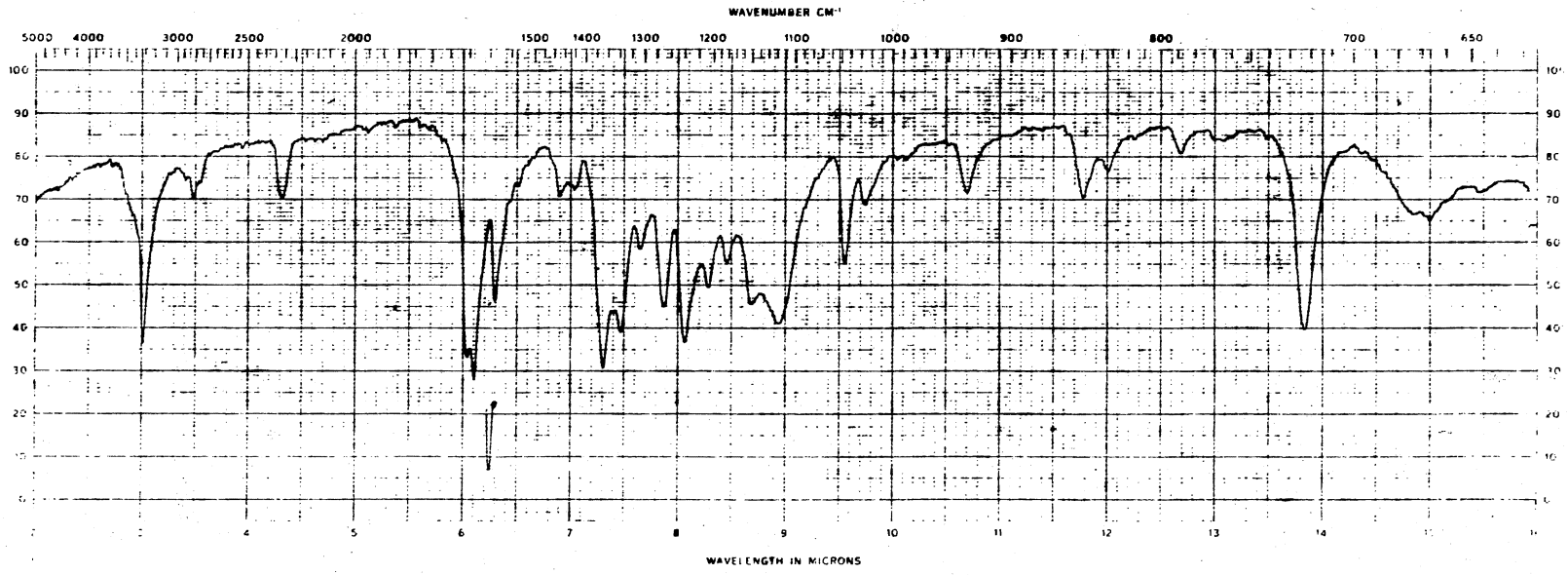


Figure 10. Infrared Spectrum of Lapachol (1)-KBr Pellet

C-H stretching, 3310 cm^{-1}; C=O stretching, 1635 cm^{-1}; C=C stretching, 1580 cm^{-1}; O-H bending, 1372 cm^{-1}

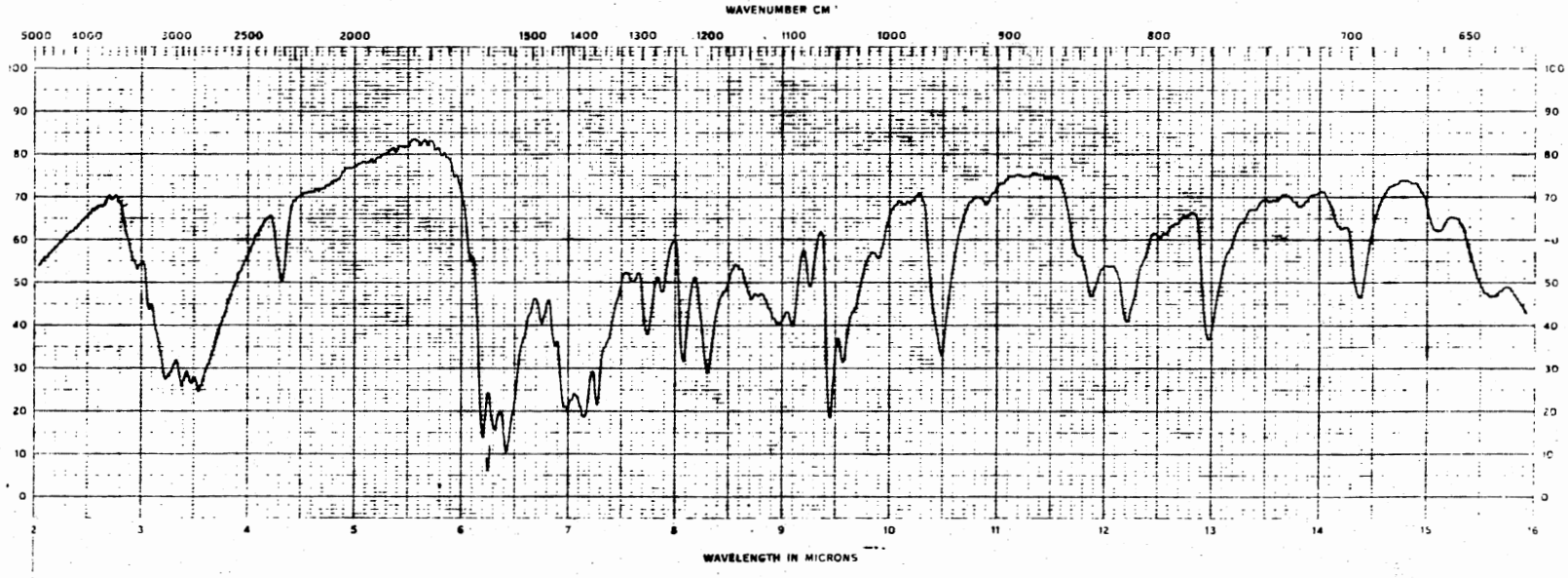


Figure 11. Infrared Spectrum of Lapachol 1-Oxime (4)-KBr Pellet O-H stretching, 2800-3400 cm^{-1}; C=O stretching, 1620 cm^{-1}; C=N stretching, 1553 cm^{-1}; O-H bending, 1399 cm^{-1}; N-O stretching, 952 cm^{-1}

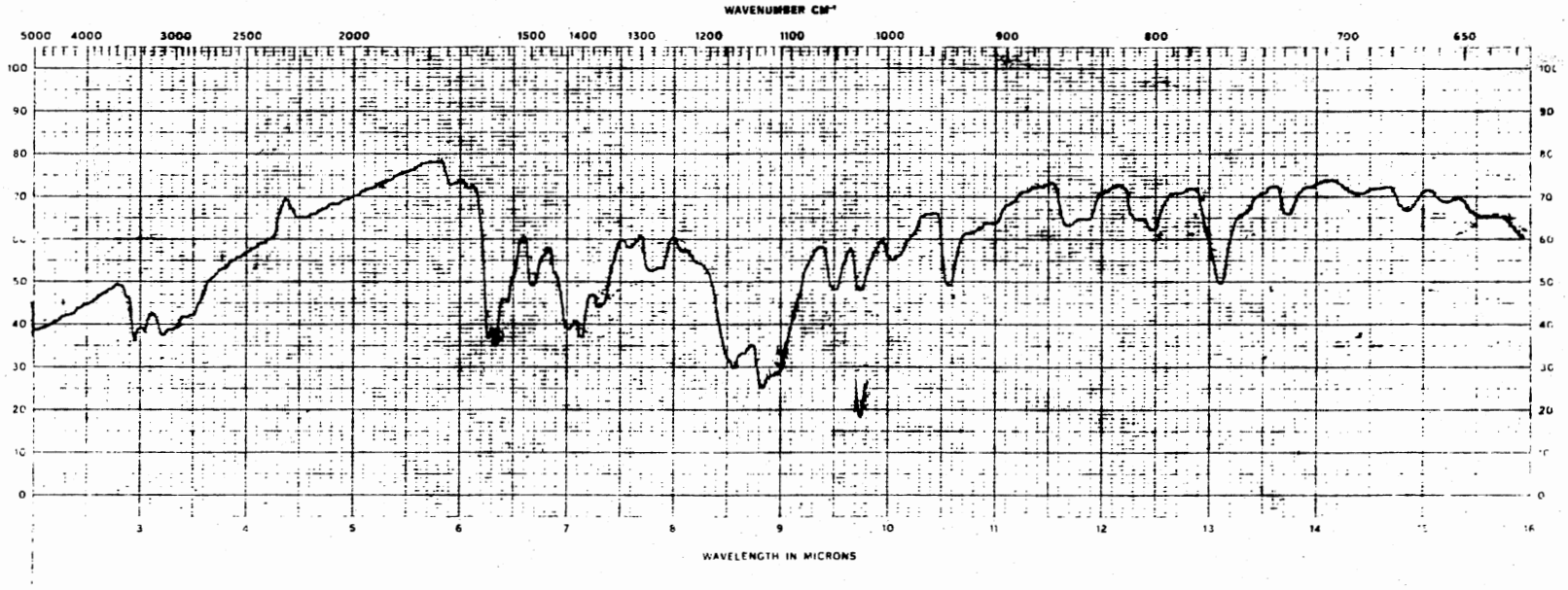


Figure 12. Infrared Spectrum of Lapachol-1-thiosemicarbazone (5)-KBr Pellet

O-H stretching, 2850-3100 cm^{-1}; C=O stretching, 1599 cm^{-1}; C=N stretching, 1570 cm^{-1}

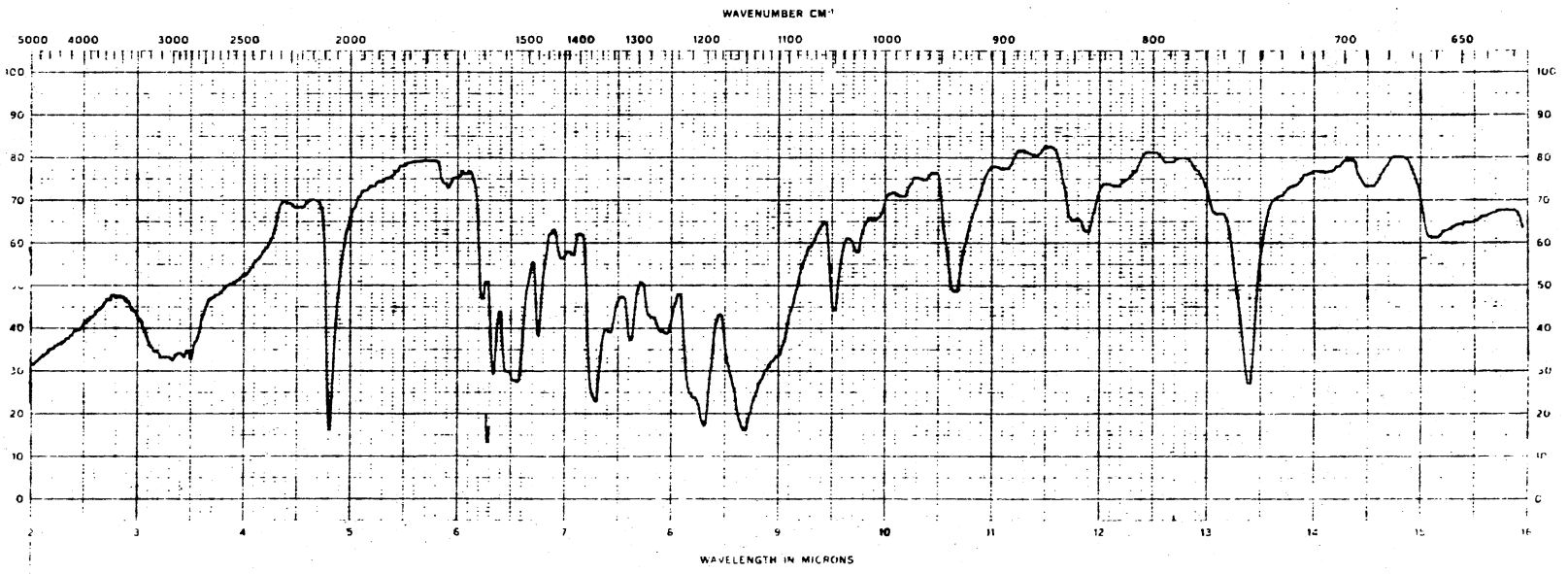


Figure 13. Infrared Spectrum of Girard T Derivative of Lapachol (6)-KBr Pellet O-H stretching, 2855-3215 cm^{-1}; +N-H stretching, 2070 cm^{-1}; C=O stretching, 1580 cm^{-1}; C=N stretching, 1525 cm^{-1}; O-H bending, 1372 cm^{-1}

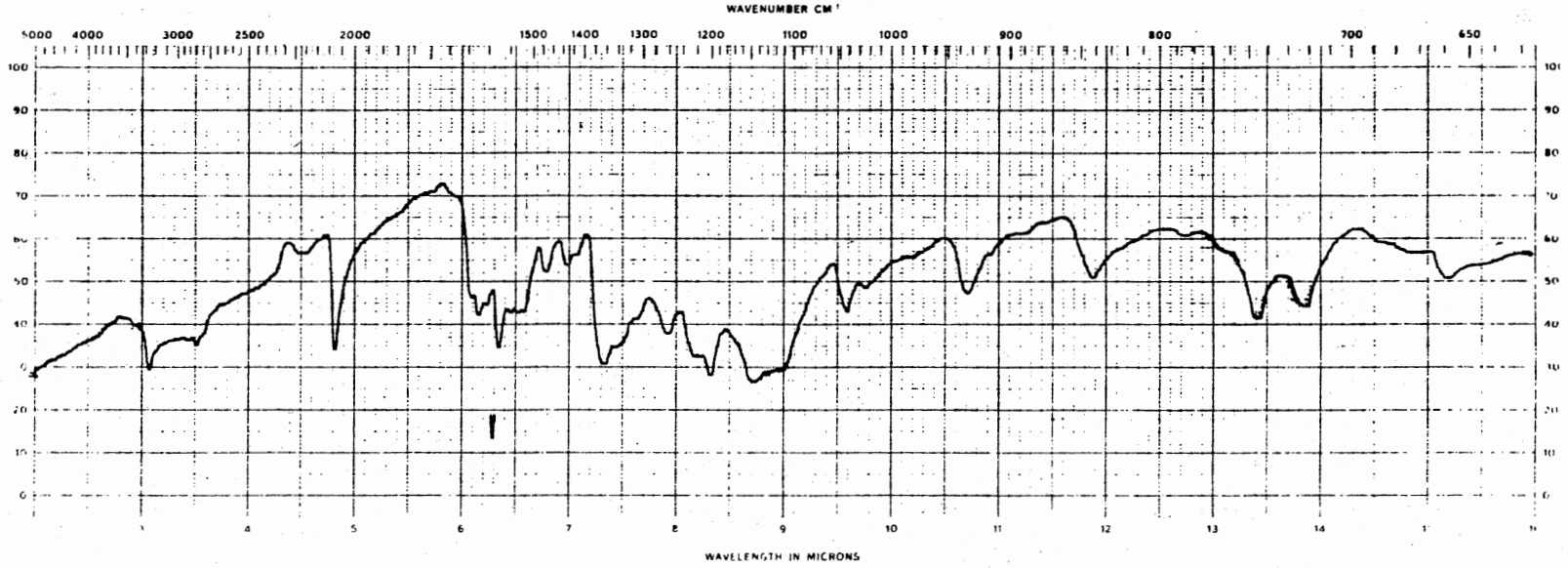


Figure 14. Infrared Spectrum of Girard P Derivative of Lapachol (7)-KBr Pellet O-H stretching, 2850-3280 cm^{-1}; +N-H stretching, 2080 cm^{-1}; C=O stretching, 1630 cm^{-1}; C=N stretching, 1580 cm^{-1}; O-H bending, 1369 cm^{-1}

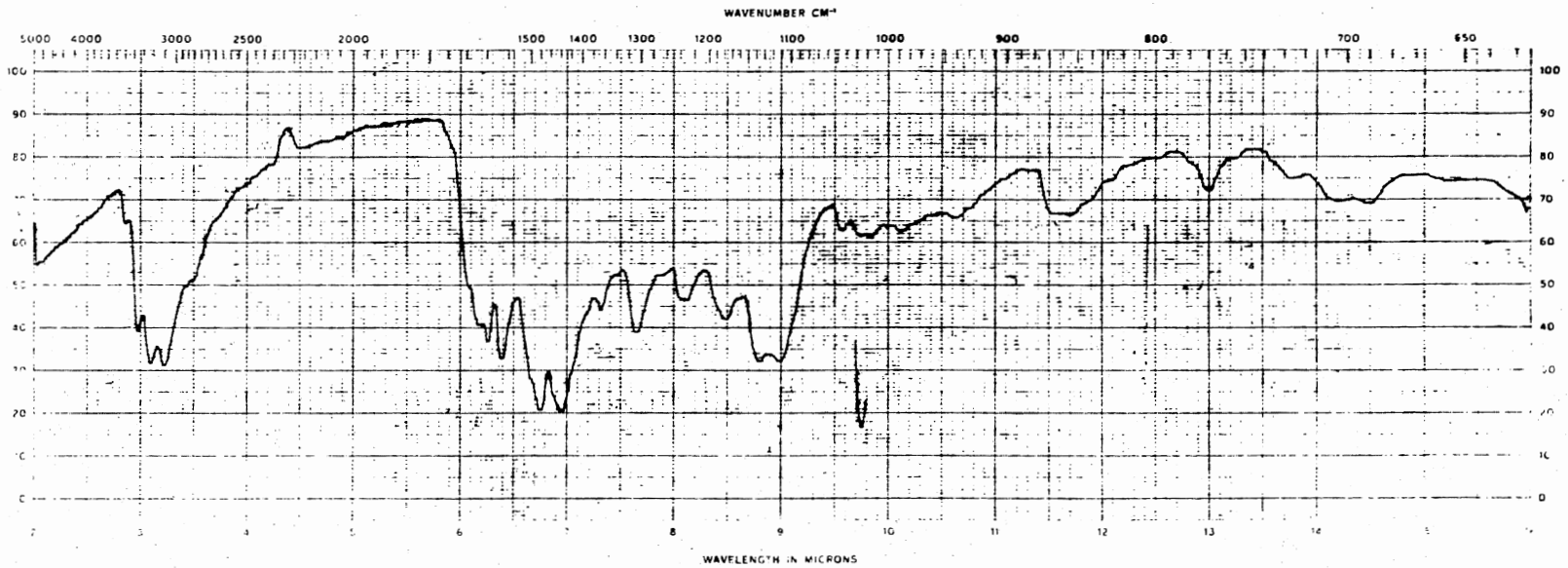


Figure 15. Infrared Spectrum of Lapachol-1-amidinohydrazone Hydrochloride (8)-KBr Pellet

O-H bending, 3090-3340 cm^{-1}; C=O stretching, 1590 cm^{-1}; O=H stretching, 1565 cm^{-1}

determined.[26,28,36] TLC solvents used were: (1) the organic layer of a mixture of benzene, acetic acid and distilled water (2:2:1), and (2) a mixture of benzene, ethyl acetate, and acetic acid (9:1:1). All TLC plates had two zones in common, a purple one and a pink one. The results of both sets of experiments are summarized in Tables I and II.

Biological Testing

Toxicities for each compound were determined in Swiss mice. Six male mice weighing at least seventeen grams were injected with a single intraperitoneal injection of a suspension of the compound in isotonic saline. The mice were weighed and given a predetermined dose in mg/kg. On the fourth day, mortalities were determined and the mice were reweighed. Runs were continued with variations in the dosage until that dosage causing a 50% mortality was determined. This corresponds to the LD_{50} value which was bracketed by determining the deaths and the weight loss, the latter is also an indication of toxicity. Table III lists the LD_{50} values for each compound tested.

The anti-tumor activity of these compounds were determined by measuring the inhibition of the ascitic SA 180 tumor system in Swiss mice. This activity was expressed as the T/C ratio which was calculated by determining the average life span of two sets of six Swiss white mice. On day one both sets were inoculated with the ascitic SA 180 tumor system obtained from a mouse with a week-old tumor. The mouse was sacrificed, the abdominal skin was removed and 1 ml of the peritoneal fluid was removed and diluted with 10 ml of Hepes buffer.[37] Whenever possible the fluid was removed without cutting the peritoneal membrane to avoid contamination of the fluid. After thorough mixing, 1 ml of diluted

TABLE I

COLOR REACTIONS OF PHENOXAZONES

Phenoxazone of	Acetone	Benzene	Ethyl Ether	Ethanol	Conc. H_2SO_4
II	Deep blue, purple-red fluores.	Light blue, purple-red fluores.	Light blue, purple-red fluores.	Deep blue, purple-red fluores.	Blue
XIII	Deep blue, purple-red fluores.	Light blue, purple-red fluores.	Light blue, purple-red fluores.	Deep blue, purple-red fluores.	Blue
XIV	Deep blue, purple-red fluores.	Light blue, purple-red fluores.	Light blue, purple-red fluores.	Deep blue, purple-red fluores.	Blue
XV	Deep blue, purple-red fluores.	Light blue, purple-red fluores.	Light blue, purple-red fluores.	Deep blue, purple-red fluores.	Blue
XVI	Deep blue, purple-red fluores.	Light blue, purple-red fluores.	Light blue, purple-red fluroes.	Deep blue, purple-red fluores.	Blue

TABLE II

THIN-LAYER CHROMATOGRAPHY RESULTS

Phenoxazone of	Solvent A		Solvent B	
	R_f (Purple Zone)	R_f (Pink Zone)	R_f (Purple Zone)	R_f (Pink Zone)
II	0.0084	0.592	0.079	0.465
XIII	0.038	0.574	0.037	0.452
XIV	0.070	0.588	0.093	0.486
XV	0.107	0.570	0.103	0.458
XVI	0.099	0.592	0.090	0.500

Solvent A = organic layer of benzene, acetic acid, and water, (2:2:1) (12).

Solvent B = benzene, ethyl acetate and acetic acid, (9:1:1) (26).

TABLE III

RESULTS OF BIOLOGICAL SCREENING

Compound	LD_{50}, mg/kg	Daily Dose mg/kg	5-Day Survivors	Average Wt. Gain, %		Survival Times, Days		T/C, %
				Treated	Controls	Treated	Controls	
1	400	100	6/6	10.5	10.1	11.0	9.5	116
4	300	100	6/6	10.1	8.0	13.8	13.0	106
5	165	32	6/6	15.1	15.9	16.0	11.5	139
6	350	40	6/6	14.1	15.9	14.3	11.5	124
7	250	50	6/6	12.8	5.3	14.7	12.7	116
8	800	40	6/6	16.7	15.9	15.3	11.5	133

fluid was removed and two drops of a 0.4% aqueous solution of tryphan blue is added, a stain which only stains the walls of those cells which are no longer viable. A hemocytometer was used to count the large, unstained cells and the suspension was diluted with the buffer so that each mouse received 1 x 10^6 cell in a 0.2-ml injection. On days 2, 3, and 4 the control was injected with isotonic saline and the mice, (the treated), were injected with a suspension in isotonic saline of the compound to be tested. The volume injected was adjusted to the weight of each mouse so that each mouse received a fixed mg/kg dose. The day of death was recorded, counting from the day of injection, the experiment was continued for sixty days. At the end of this time any surviving mice were sacrificed and examined for any signs of tumor cells. If no cells were present, the mouse was considered cured. The T/C ratio is the ratio of the average life span of the treated to the average life span of the control. Dosages were changed to obtain the optimum value of T/C, where the deaths due to toxicities were balanced by deaths due to the tumor system.

Currently, at least three experiments have been run for each compound. The optimum T/C value obtained so far are listed in Table III. The biological screening of these compounds is still in progress and will be published at a later date.

Polarographic Study

The polarograms were obtained with a Sargent Polarograph Model XV. pH measurements were made using a Beckman Research pH meter with a glass electrode and a saturated calomel electrode as reference.

The polarographic cell used was similar to that described by Lingane

and Laitinen.[38] A saturated potassium chloride agar plug held in place by a sintered glass disc made the connection with the reference electrode. The procedure for construction of the saturated calomel electrode is described by Meites.[39]

A water thermostat was used to maintain the temperature of all solutions at $25^{o} \pm 0.1^{o}$. Air was removed from the solution by bubbling nitrogen through the solution for 20 minutes.

The capillary used had the following characteristics: at a height, h, of 34.77 cm Hg, the drop time in 0.1 N potassium chloride was 5.88 seconds, m, the mass flow rate of mercury in mg sec^{-1} was 0.49, hence $m^{2/3} t^{1/6} = 0.84$. Both m and t were measured in an open circuit. Since the observed current is dependent upon the capillary characteristics, they are included here.

Polarograms were run on solutions of an approximate concentration of 10^{-4} M. The exact concentration was not known because of the extreme insolubility of the compounds studied.

The apparent pH values of all solutions were recorded after mixing with equal volumes of methyl alcohol and any deviation from the desired value was corrected before running the polarogram. All pH measurements were made at 25^{o}.

The composition of the buffers and electrolytes used are listed below:

Solution I, pH 5.40, a buffer 0.10 M in acetic acid and 0.1 M in sodium acetate in 50% methanol by volume.[40]

Solution II, pH 6.06, a buffer 0.0452 M in sodium acetate and 0.0095 M in acetic acid in 50% methanol by volume.[40]

Solution III, pH 7.00, an electrolyte 0.049 M in sodium acetate

in 50% methanol by volume, adjusted to pH of 7.00 with dilute acetic acid.

Solution IV, pH 7.97, an electrolyte 0.098 M in sodium acetate in 50% methanol by volume, adjusted to pH 7.97 with dilute acetic acid.

Solution V, pH 9.10, an electrolyte 0.195 M in sodium acetate in 50% methanol adjusted to pH 9.10 with dilute sodium hydroxide.

The lapacholoxime was prepared following the procedure of Hooker and Wilson.[25] The N-bromo- and N-chloroimines were synthesized by workers in this laboratory.[1]

To test the polarographic system, a 0.5 mM solution of cadmium ions was prepared by dissolving 0.128 g of $CdSO_4 \cdot 8/3H_2O$ in 1 liter of 1.0 N hydrochloric acid. The solution was deaerated and a polarogram was run from 0 to -2.0 volts with a current sensitivity of 0.020 μA/mm. The half-wave obtained was -0.654 volts vs. S.C.E., (lit., -0.642 volts vs. S.C.E.).[39] The half-wave potential obtained by oxidizing hydroquinone in the pH 5.40 buffer was +0.139 volts vs. S.C.E. (lit., +0.146 volts vs. S.C.E.)[40] and the value obtained by reducing 2,6-dimethyl-1,4-benzoquinone was +0.042 volts vs. S.C.E. (lit., +0.032 volts vs. S.C.E.).[40] The average deviation observed is +0.003 volts, this is probably due to differences in the resistance of the system. Since the difference will be a constant, it is not necessary to correct the half-wave potentials for use in correlations with biological activity.

Half-wave potentials were obtained by calculating the second derivative for each curve. The point of inflection was the point at which the sign of the second derivative changed; it was determined by interpolation. Figure 16 shows a typical polarogram with the second derivative superimposed. In the case of irregular polarograms, the in-

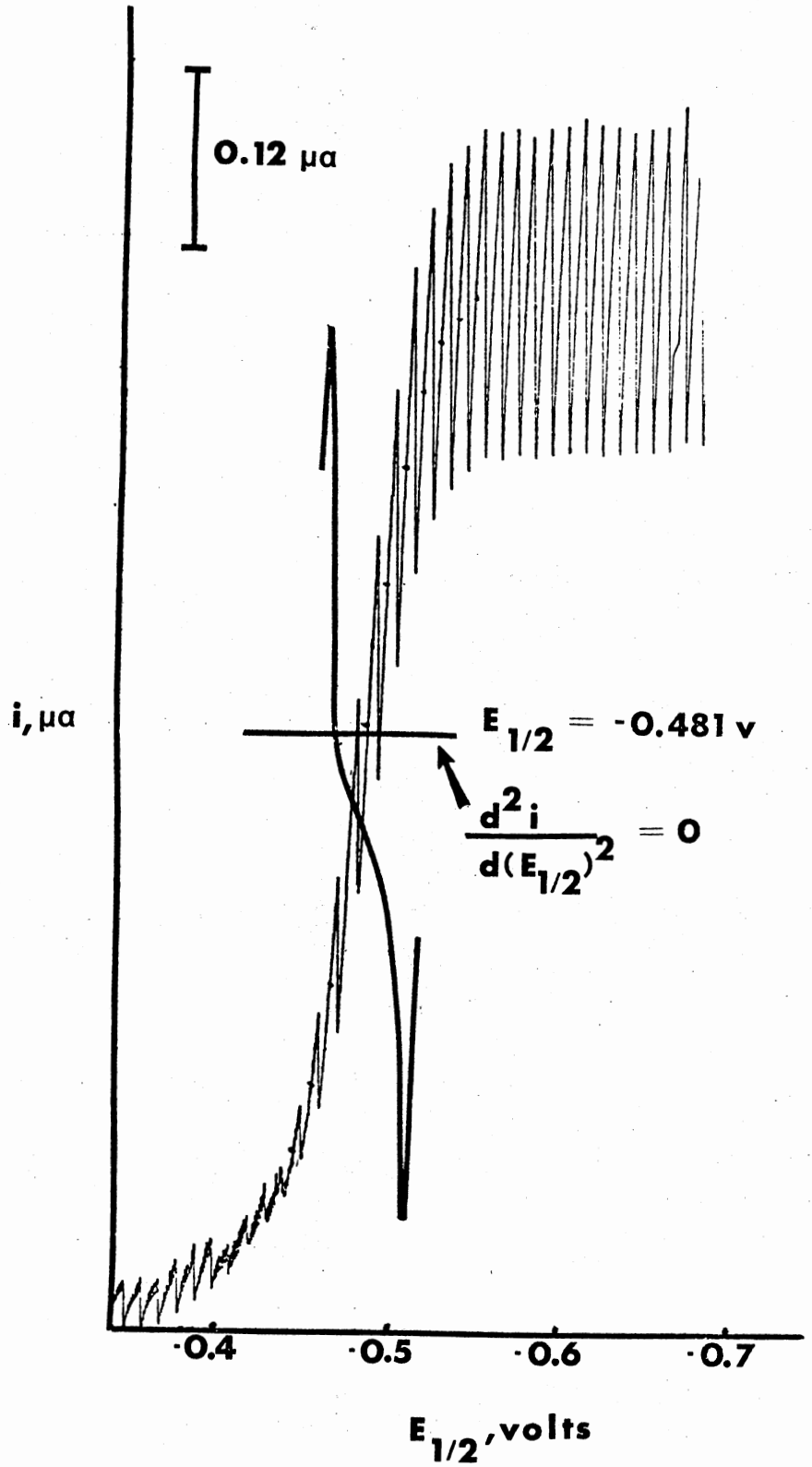


Figure 16. Polarogram of Lapachol-1-oxime in Solution IV With Second Derivative Plot Superimposed

flection point closest to the zero point of a log plot was taken to be the half-wave potential. Log plots were constructed by taking the logarithm of the ratio $\underline{i}/(\underline{i}_d-\underline{i})$ where $\underline{i}_d$ = the diffusion current and $\underline{i}$ = the current at the midpoint of the oscillation. The current is measured on the rising portion of the trace corresponding to the life of the mercury drop whenever possible since this is less steep. On those polarograms where there is excessive noise, $\underline{i}$ was measured on the falling portion of each oscillation. A plot of potential vs. log $(\underline{i}/(\underline{i}_d-\underline{i}))$ was constructed, the potential at the intercept was taken as the half-wave potential.

The biological activity was expressed as the T/C ratio. This was obtained by determining the average life span of two sets of white mice. One set, the control, was inoculated with SA 180 ascitic tumor system on day 1. On days 2-4 these mice are injected with isotonic saline. The other set (the treated) was treated in the same manner except on days 2, 3 and 4 the mice were injected with a suspension in isotonic saline of the compound being tested. The T/C ratio is the ratio of the average life span of the treated set to the average life span of the control. The % T/C is simply the T/C value multiplied by 100.

CHAPTER III

RESULTS AND DISCUSSION

Lapachol Derivatives

Five derivatives of lapachol were synthesized and characterized (4-8, Figure 1); the first four have not previously appeared in the literature.[41] The five new compounds and lapachol were all screened against the ascitic SA 180 tumor system in white mice.

The structures of the new compounds were confirmed by a combination of chemical and physical methods. Reduction of any lapachol-1-imine or -1-hydrazone (9) in Figure 17 will yield the same aminophenol (10) regardless of the original lapachol nitrogen derivative. This aminophenol (10) when oxidatively coupled at pH 8 yields the phenoxazone (11). Kehrman first synthesized compounds with the same basic ring structure from 1,4-naphthoquinone 1-oximes.[36] This phenoxazone has very characteristic and identifiable color reactions: in most organic solvents it exhibits a purple-blue color with dark red fluorescence. In sulfuric acid, it is deep blue. In 1969, Dudley and coworkers[26] and in 1970 Carroll and coworkers[27] used this reaction as a means of proving the structure of some hydrazone derivatives of 2-hydroxy-3-alkyl-1,4-naphthoquinones. The phenoxazones they obtained had the same color reactions as the Kehrman compound. All of the derivatives in this study yielded the same compound when subjected to reduction followed by oxidative

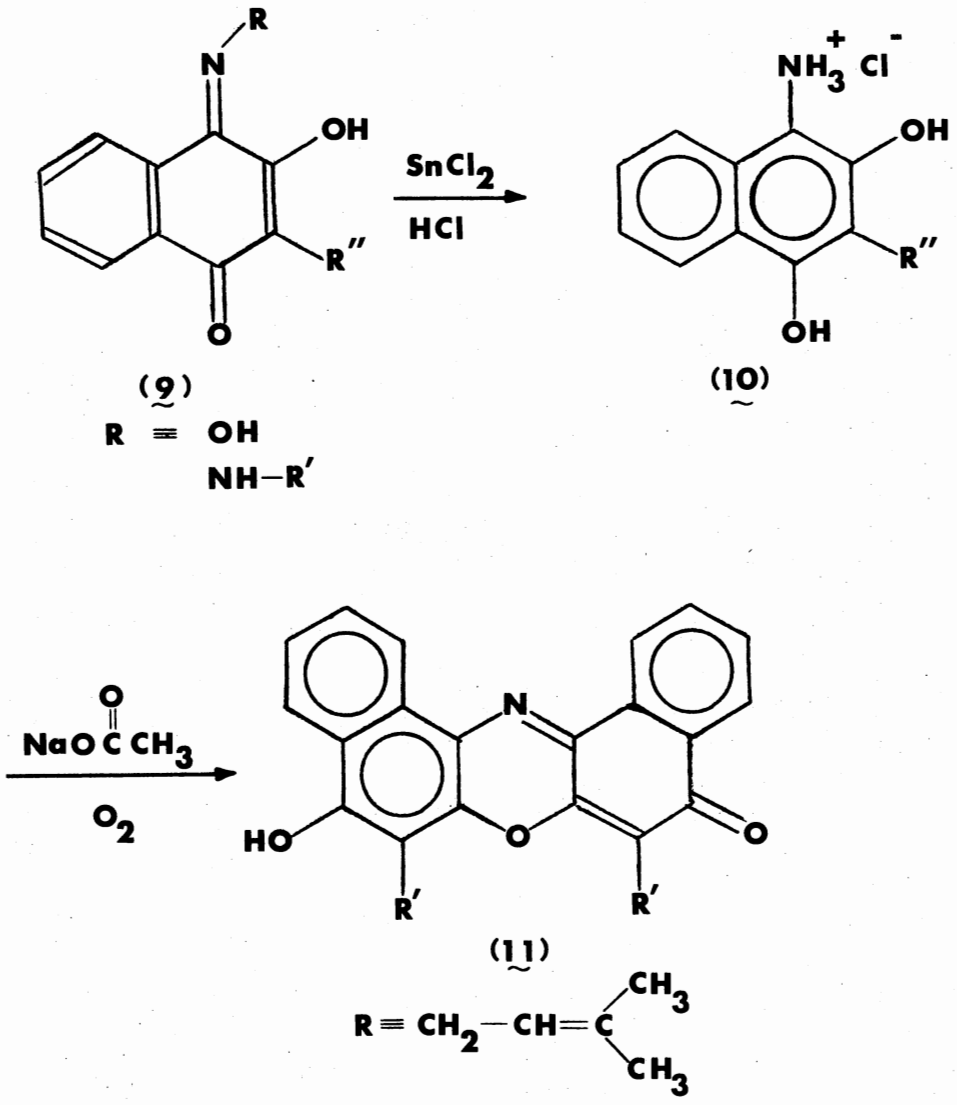


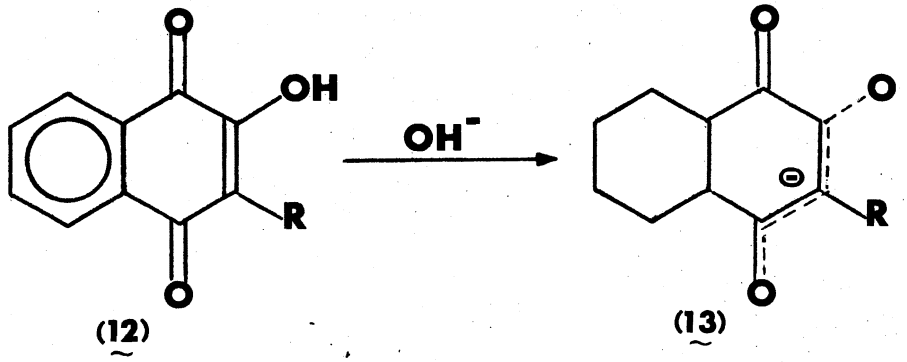
Figure 17. Reaction Scheme for the Formation of the Phenoxazone of Lapachol Hydrazones or Imines

coupling. The thin-layer chromatography data suggest that the compound was the same regardless of the starting material and correspond to the data of previous workers.[26,28] All of the TLC plates had two zones in common, the R_f values of these zones are listed in Table II. These values are close to one another indicating the same compound has been synthesized from the different lapachol derivatives. IR spectra and melting points could not be used to verify the identity of this compound as purification was difficult.

Spectral evidence for the formation of the C=N bond comes from comparison of the infrared spectra of lapachol oxime with that of lapachol. A broad band in the spectrum of lapachol oxime indicates hydrogen bonding. This same band is absent in spectrum of lapachol because there is no spatial opportunity for hydrogen bonding to occur. Since lapachol-1-oxime when reduced and subsequently oxidized at a pH of 8 yields the same highly colored product as the other lapachol derivatives do when treated similarly it appears that the other derivatives have also formed the hydrazone at the C-1 position. In addition, all of the other derivatives exhibit the broad band at about 3330 cm^{-1} indicative of hydrogen bonding between the nitrogen of the hydrazone and the 2-hydroxy group. If condensation had occurred at the carbonyl of C-4 no hydrogen bonding would be observed.

All attempts to make the Schiff base of lapachol and 2-amino-2-hydroxymethyl-1,3-propanediol failed. Ketones form Schiff bases sluggishly at best and acid catalysis is usually favored. However, with lapachol, basic conditions are preferred. Dudley and Carroll and coworkers offer a possible explanation.[26,27] 2-Hydroxy-1,4-naphthoquinones are weak acids (lapachol, pKa = 5.02)[42] and react with bases to yield the

conjugate base (13). The electronic structure of (13) with the negative charge delocalized through four atoms in the molecular offers a possible explanation why, under alkaline conditions, condensation occurs at C-1.



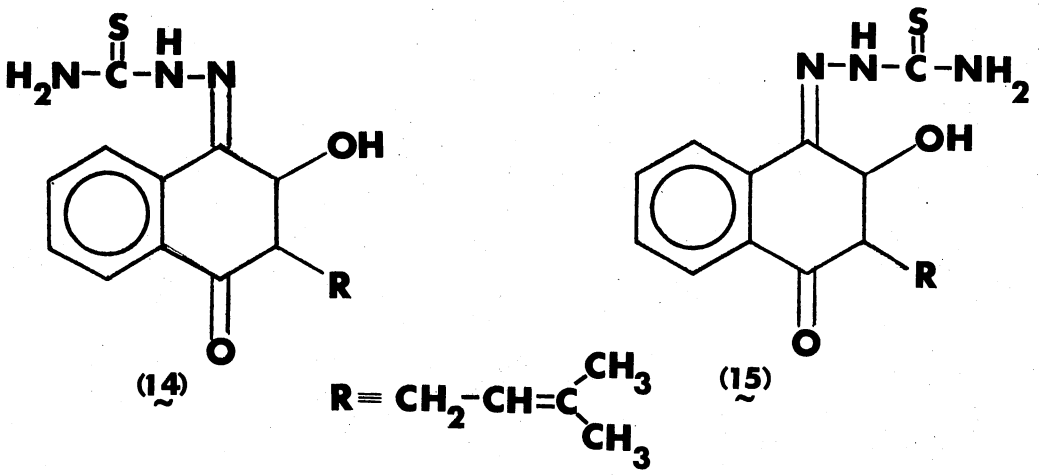
(12) (13)

The original intent of this study was to make derivatives of lapachol which have greater solubility in water and perhaps better antitumor activity. Difficulties were encountered synthesizing the Schiff base of lapachol and 2-amino-2-hydroxymethyl-1,3-propanediol, a compound which should be more water soluble than lapachol. Lapachol undergoes cyclization between the side-chain double bond and the hydroxy group with ease under strongly acidic conditions.[5,43] Reaction conditions must be either weakly acidic or alkaline. Therefore the method available for formation of Schiff bases of lapachol is limited.

The synthesis of hydrazones of lapachol under alkaline conditions was successful. Girard T and P reagents, quaternary ammonium hydrazides, were first prepared by Andre Girard and George Sandulesco in 1936.[27] These workers did not use these hydrazides themselves. However, as a result of their work, Reichstein was able to use the reagents to isolate many adrenocortical hormones from beef adrenal glands via the corresponding water-soluble hydrazones. These hydrazones were hydrolyzed to

regenerate the ketones.[45] These reagents were used to make hydrazone derivatives of lapachol.[44]

Although they are not noted for their water solubility amidinohydrazones and thiosemicarbazones have exhibited some anti-tumor activity.[46,47,48] The thiosemicarbazone of lapachol has been biologically screened by the National Institutes of Health in Maryland, but it has not been reported in the literature.[41] Two forms of the thiosemicarbazone were obtained, depending upon the reaction time and the amount of heat applied. One was a fine, yellow, crystalline solid with a melting point of 265° (14). The second was yellow-orange needles with a melting point of 188-90° (15). The 265° compound (14) is probably the kinetic product. The reaction conditions producing 15 favor the formation of the thermodynamic product as they include more heating and longer stirring. Both forms gave the same phenoxazone upon reduction and subsequent oxidation. Thin-layer chromatographic analysis of both solids in a mixture of benzene, ethyl ether, and acetone (2:1:1) showed two zones whose R_f values were the same for both compounds; one was yellow, the other orange. The yellow zone on the TL chromatogram for the 265° thiosemicarbazone was larger than for the orange thisemicarbazone, mp, 188-90°. The reverse was true with the orange zone. The NMR spectrum of each was similar in appearance. The infrared spectra indicated the orange form possessed more hydrogen bonding than the yellow form. From the facts obtained, the following structures were proposed for the two geometric isomers. Both the yellow and the orange solids were mixture of both isomers, with one form predominating in each product.



The thiosemicarbazone tested in the mice was the orange solid. Its NMR and infrared spectra appear in Figures 4 and 12.

Further work on nitrogen analogs of lapachol should be limited to hydrazone derivatives. The preparation of hydrazides of various water-soluble amines such as aminoglucose and 2-amino-2-hydroxymethyl-1,3-propanediol would permit synthesis of hydrazones of lapachol which might be more water soluble and possibly more biologically active.

Toxicities, in the form of LD_{50} values, and optimum T/C values are listed in Table III.

Polarographic Results

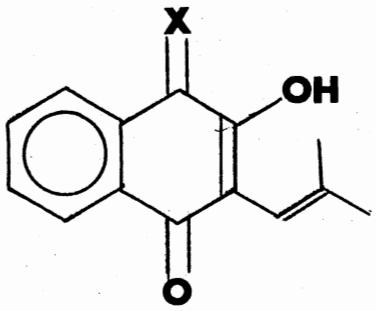
The variation of half-wave potential with pH was determined for seven compounds (1, 4, 16-21, Figure 18). Figures 17 - 22 and Table IV show the relationship between the half-wave potential and pH for the compounds tested. All compounds show a decrease in half-wave potential with increasing pH, a trend consistent with the reduction mechanisms postulated for quinones and quinone mono- and dioximes mentioned in Chapter II.[19,22,23,24,49] All the postulated mechanisms involve protons; as

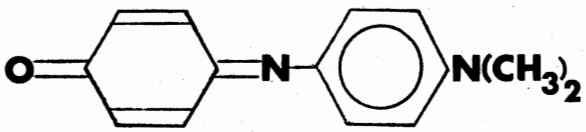
TABLE IV

EFFECT OF pH ON $E_{\frac{1}{2}}$ OF A SERIES OF QUINONOID COMPOUNDS

Compound	Solution I (pH = 5.40)	II (pH = 6.06)	III (pH = 7.00)	IV (pH = 7.97)	V (pH = 9.10)
Lapachol (1)	-0.292	-0.327	-0.396	-0.517	-0.695
Lapachol-1-oxime (4)	-0.257	-0.353	-0.366	-0.512	-0.618
N-Chloro-1,4-benzoquinone-4-imine (16)	+0.174	+0.132	+0.074	+0.014	-0.147
N,N'-Dichloro-1,4-benzoquinone-1,4-diimine (17)	+0.161	+0.044	+0.079	+0.048	-0.140
N-Bromo-1,4-benzoquinone-4-imine (18)	-0.019	-0.054	-0.102	-0.171	-0.202
N,N'-Dibromo-1,4-benzoquinone-1,4-diimine (19)	--	--	--	-0.168	-0.175
N,N-Dimethylindoaniline (20)	+0.082	_0.045	-0.034	-0.066	-0.147

All $E_{\frac{1}{2}}$ values vs. S.C.E.
Temperature = 25°.

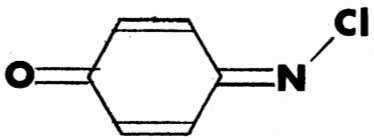


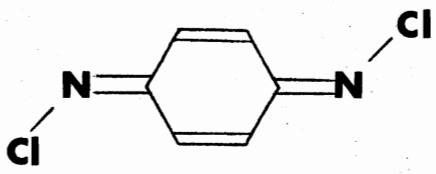


1: X = O, Lapachol

4: X = N-OH, Lapachol oxime

20: N,N-Dimethylindoaniline





16: N-Chloro-1,4-benzoquinone-4-imine

17: R=H, N,N'-Dichloro-1,4-benzoquinonediimine (BQDI)

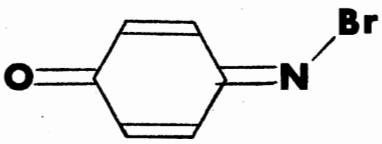
21: R=Cl, Chloro-(BQDI)

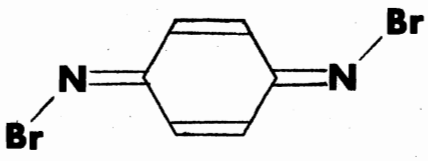
22: R=OCH_3, Methoxy-(BQDI)

23: R=CH_3, Methyl-(BQDI)

24: R=SO_3H,

25: R=NO_2, Nitro-(BQDI)





18: N-Bromo-1,4-benzoquinone-4-imine

19: N,N'-Dibromo-1,4-benzoquinonediimine

Figure 18. Structure of Quinonediimines

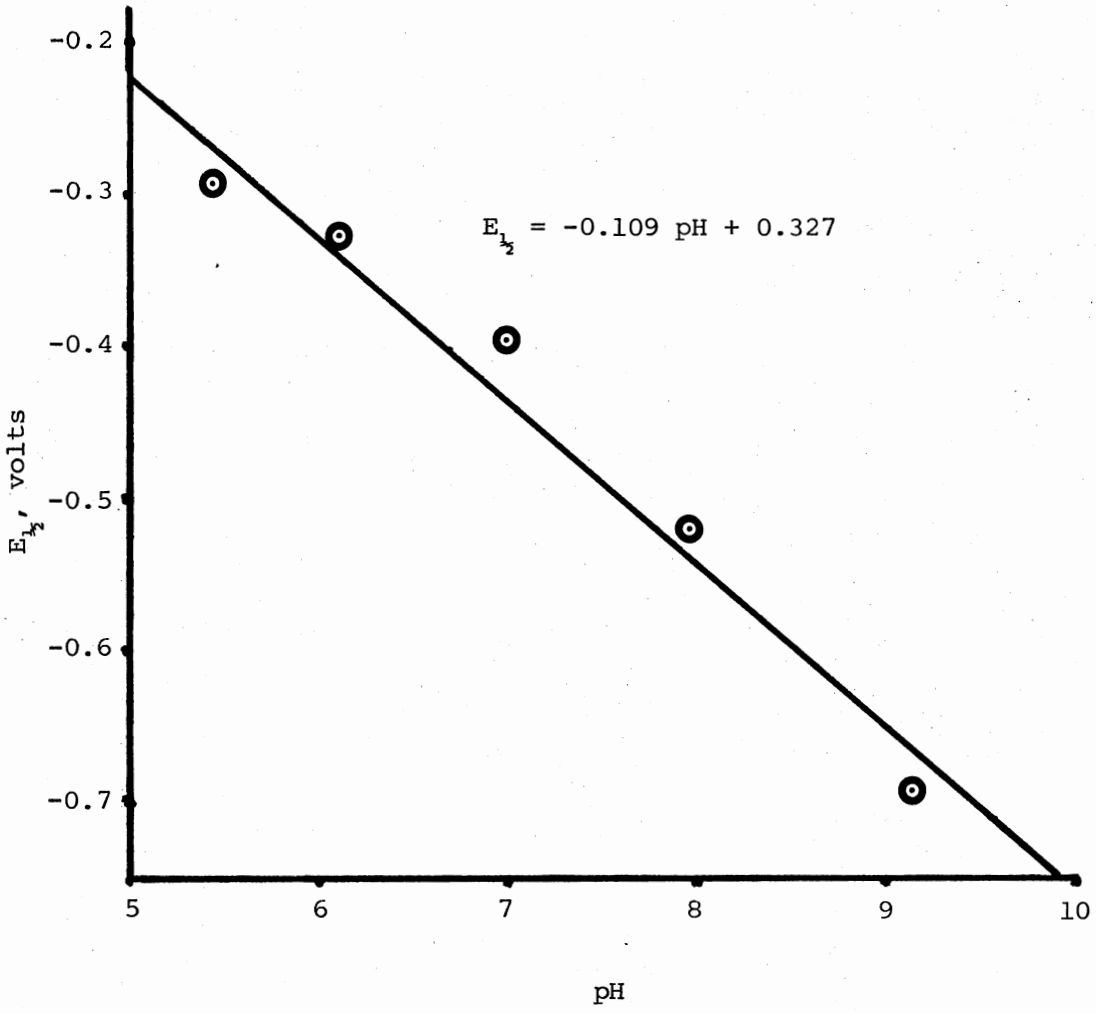


Figure 19. $E_{1/2}$ vs pH for Lapachol (1)

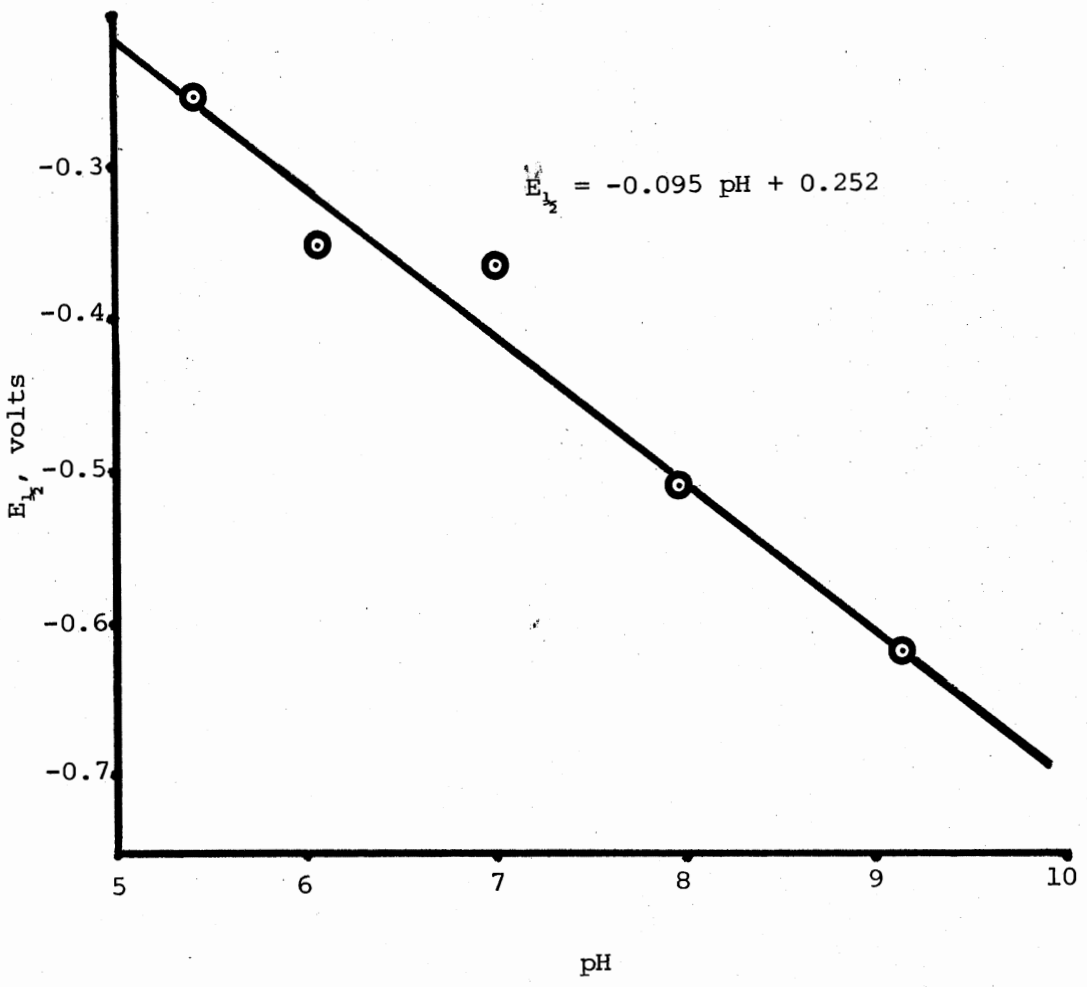


Figure 20. $E_{\frac{1}{2}}$ vs pH for Lapachol 1-oxime (4)

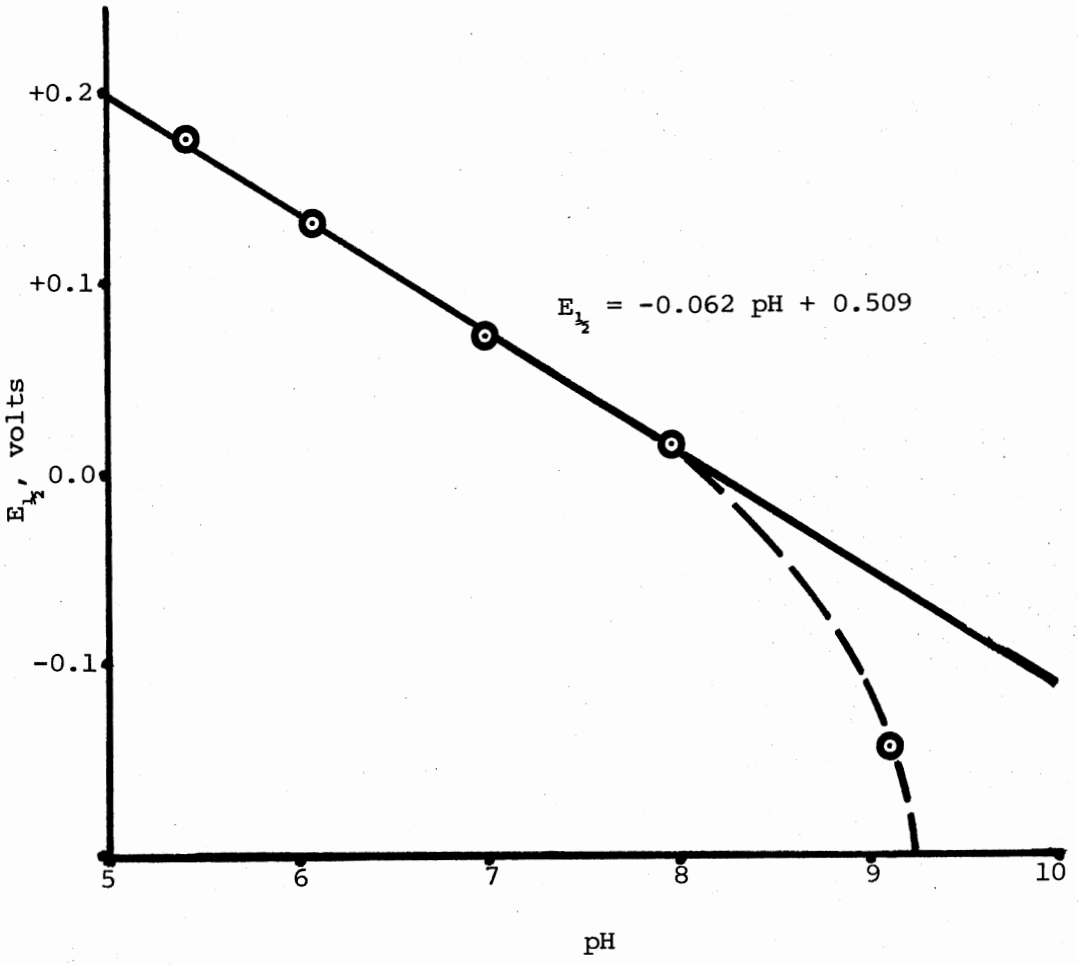


Figure 21. $E_{1/2}$ vs pH for N-chloro-1,4-benzoquinone-4-imine (16)

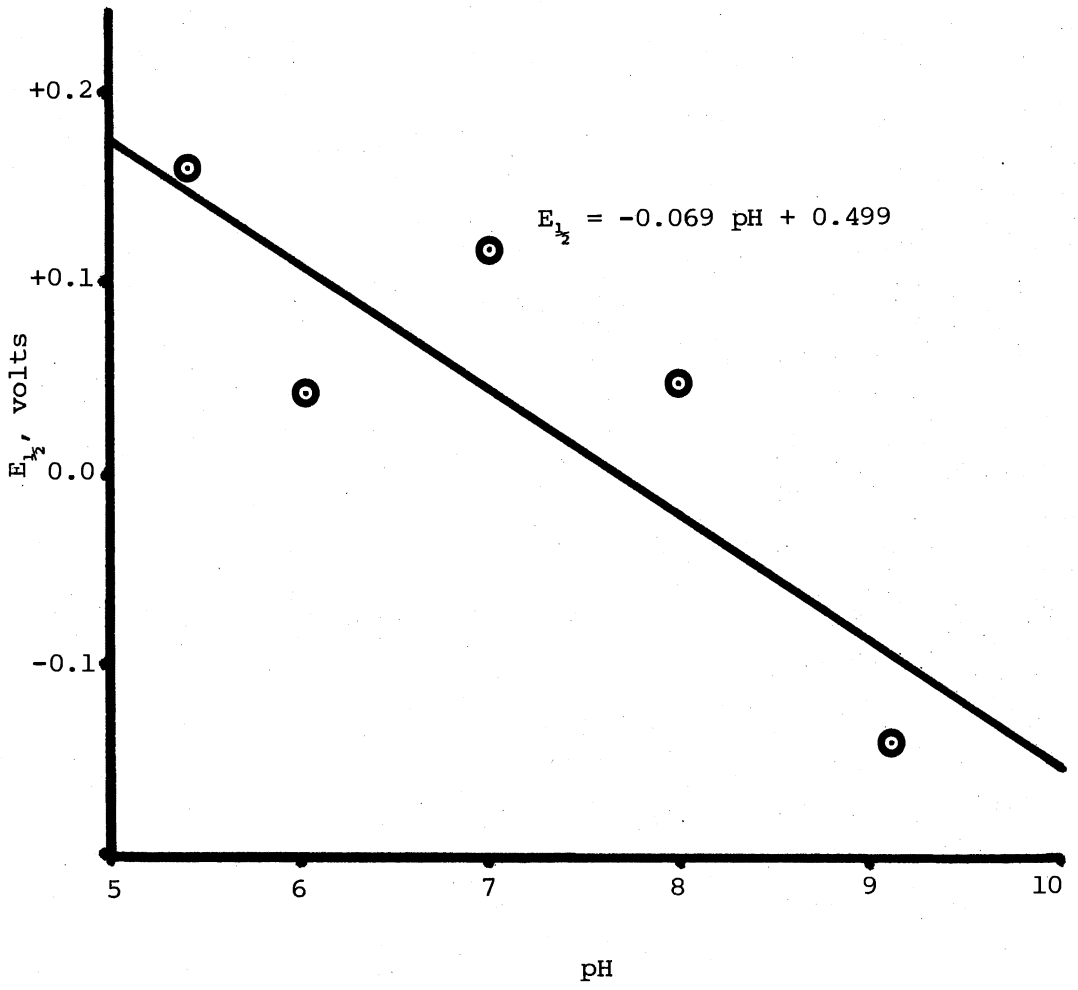


Figure 22. $E_{\frac{1}{2}}$ vs pH for N,N'-dichloro-1,4-benzoquinonediimine (17)

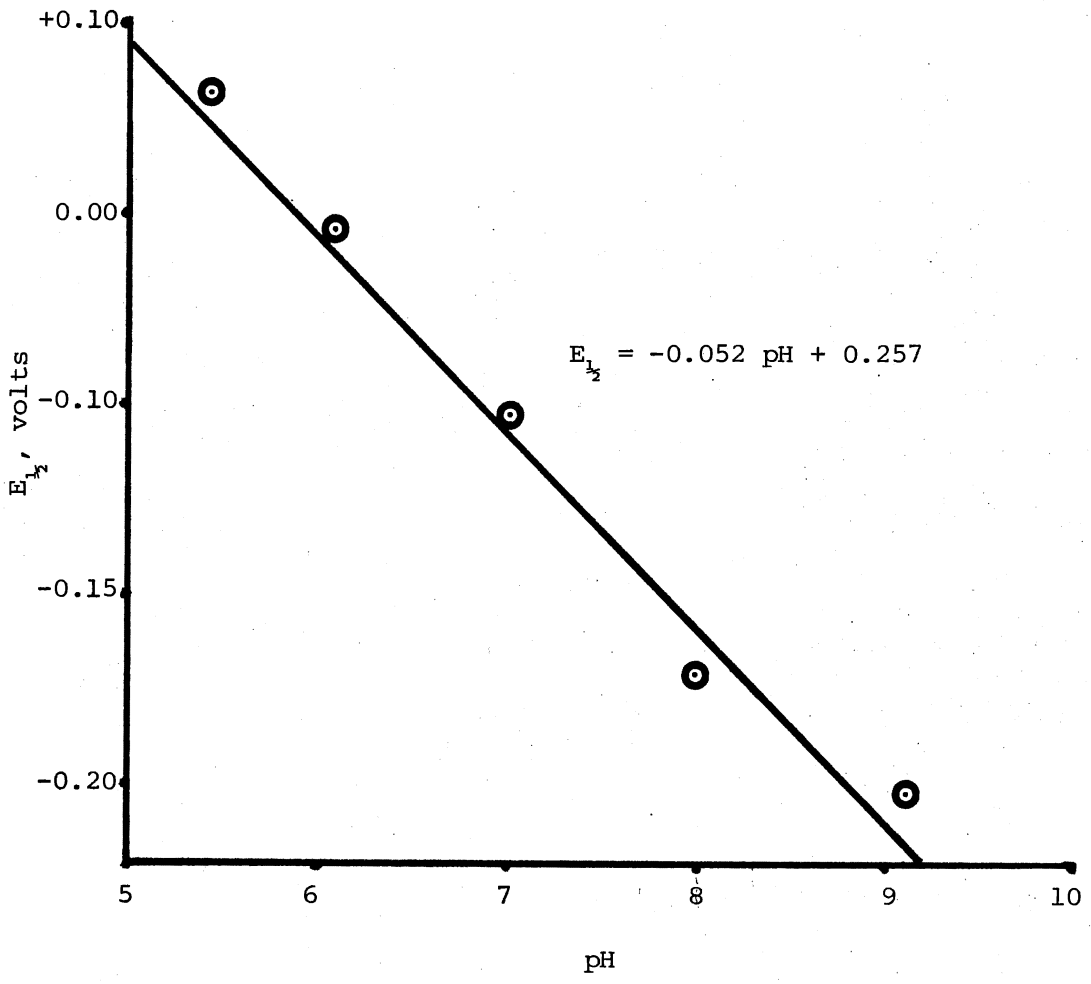


Figure 23. $E_{\frac{1}{2}}$ vs pH for N-bromo-1,4-benzoquinone-4-imine (18)

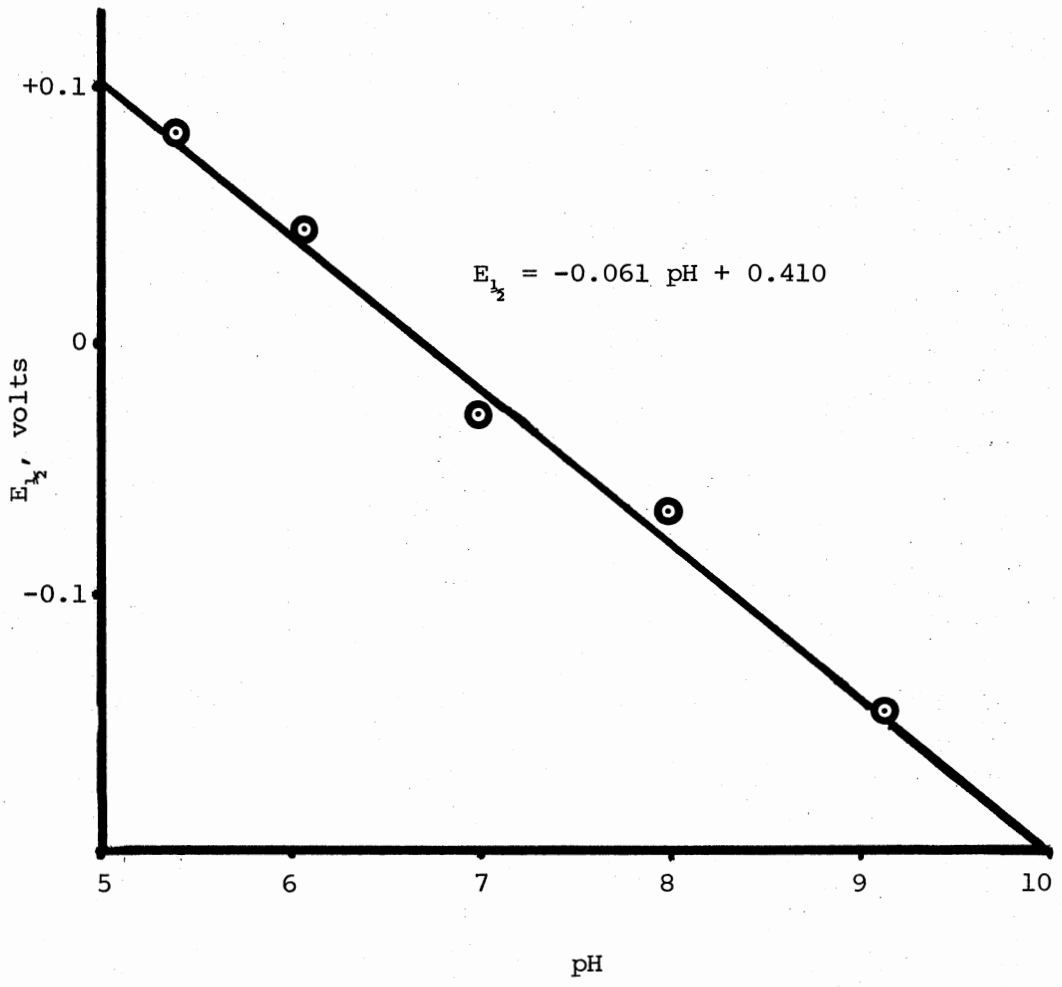


Figure 24. $E_{\frac{1}{2}}$ vs pH for N,N-dimethylindoaniline (20)

the pH increases, the concentration of hydrogen ions decreases and the nature of the reduction changes. The shift to more negative potentials indicates that reduction becomes less favorable as the pH increases.[50]

Linear plots are observed for the pH range studied for N-bromo-1,4-benzoquinone-4-imine (Figure 23), lapachol-1-oxime (Figure 20) and N,N-dimethylindoaniline (Figure 24). A linear plot is also observed for N,N'-dichlorobenzoquinonediimine (Figure 22) but the correlation coefficient is lower, indicating greater deviation from linearity. Although there appears to be a slight deviation from linearity as the pH increases in the case of lapachol (Figure 19), it is not significant enough to preclude a linear relationship between $E_{\frac{1}{2}}$ and pH. In the case of N-chloro-1,4-benzoquinone-4-imine (Figure 21) the plot is linear until pH 9.10. At this pH, there is a break in the slope of the curve and the actual half-wave potential observed is more negative than predicted from the first four data points. Deviations from linearity in the plots of pH vs $E_{\frac{1}{2}}$ indicate the reduction mechanism at the surface of the electrode is changing as the pH increases.

Useful information can be obtained from plots of pH versus $E_{\frac{1}{2}}$. A dependence of the half-wave potential upon the pH indicates the reduction mechanism involves protons, either in the electron transfer step or in a preprotonation reaction. The change in $E_{\frac{1}{2}}$ may be due to the effects of acidity upon acid-base equilibria. There are many possible mechanisms for a reduction which is pH dependent, a typical one is given below:

$$Ox + \underline{p}H^{+} + ne \rightleftarrows RH_{\underline{p}}$$

where Ox is the oxidized species, RH is the reduced form, n is the number of electrons and p is the number of protons. The half-wave potential

can be described by the following equation:

$$E_{\frac{1}{2}} = E_s^o - \frac{0.059}{n} \log \left(- \frac{k_o}{k_{RH_p}}\right) - \left(\frac{0.059}{n} p\right) pH$$

where $E_{\frac{1}{2}}$ is the half-wave potential, E_s^o is the standard potential of the half-reaction under the given conditions, k_o and k_{RH_p} are rate constants for the diffusion of the oxidized and reduced form of the molecule to and from the surface of the electrode, n is the number of electrons and p is the number of protons involved in the reaction.

From the above equation it can be seen that, if all conditions are kept constant, and the pH is varied, a plot of $E_{\frac{1}{2}}$ versus pH will be a straight line with a slope of $- \frac{0.059p}{n}$. This is true until the mechanism changes, as the pH varies, changes in the mechanism usually arise from the change in protonation of the species initially reduced.[51]

In their proposed mechanism for reduction of quinone dioxime, Bonastre and Castetbon postulated the reduction of the dianion of 1,4-benzoquinone dioxime at pH values greater than 12, and the diprotonated form at pH values less than 6.[22] Changes in the slope of the plot of $E_{\frac{1}{2}}$ versus pH were observed at these pH values. A similar reaction is probably occurring with the compounds of this study which show deviations from linearity.

There are many types of polarographic currents. The two most common are diffusion and kinetic currents. A diffusion current is limited by the rate of diffusion of the electroactive species to the electrode, kinetic currents are limited by the rate of a chemical reaction at the surface of the electrode, quite often a protonation reaction. These two types can be distinguished from each other by varying experimental

parameters such as h, the height of the mercury column above the electrode, pH, and concentration of the electroactive species. A diffusion current varies linearly with the square root of h, is independent of pH, and increases with increasing concentration of the electroactive specie, while a kinetic current does not vary with h, is very often a function of pH, and varies with concentration. The effects of pH and h upon the height of the waves were not determined and as a result no conclusion can be made concerning the type of current observed in this study.

The half-wave potentials for a series of twelve quinonoid compounds shown in Figure 18 were determined at pH 9.10. At this pH the reduction wave of the compound is sufficiently negative to overcome any masking by the residual current of the electrolyte. Various electrolytes of pH 9.10 were tested but they masked the wave to varying degrees. Among those solutions tested were a (1) 2-amino-2-hydroxymethyl-1,3-propanediol and sodium hydroxide electrolyte and (2) an electrolyte of K_3PO_4, Na_2HPO_4, and sodium hydroxide. In all of these electrolytes, the potential at which the background electrolyte is reduced occurs too close to the potentials of the compounds being studied to accurately determine their potentials. In the acetate electrolyte used, the residual current rises at a potential of +0.25 volts and remains constant until hydrogen begins to be reduced at about -1.75 volts. Since variations in the anion of the sodium or potassium salt change the potential of the residual electrolyte, reduction of the anion is probably the reason for the variation observed in the potential of the electrolytes tested. By determining the $E_{\frac{1}{2}}$ values in the same electrolyte the basis for comparison of the potentials is more valid.

At pH 9.10 all compounds except nitro-N,N'dichloro-1,4-benzoquinonediimine gave well defined waves. In the case of N,N'-dibromo-1,-4-benzoquinonediimine the height of the wave increased upon standing. The $E_{\frac{1}{2}}$ value obtained from this solution is the same as the one obtained from a freshly prepared solution. The compound has not undergone any chemical reaction but more of it has probably dissolved. The nature of the wave of the nitro substituted compound will be discussed later. N,N-Dimethylindoaniline, lapachol, and lapachol-1-oxime all showed waves which rose out of the residual current. All the N-halo, mono- and diimines have similar waves which appeared to rise out of a previous wave which is itself masked by the residual current of the electrolyte. Figure 25 shows a typical wave.

The effect of substituents upon the $E_{\frac{1}{2}}$ were determined for a series of N,N'-dichloro-1,4-benzoquinonediimines at a pH of 9.10. Table V summarizes the results and offers some comparisons with data in the literature. As would be expected, addition of a chlorine atom or sulfonic group to the ringe increases the $E_{\frac{1}{2}}$ value. The electron-withdrawing inductive effect of chlorine and the sulfonic group reduces electron density of the quinonoid system and therefore shifts the potential to larger values. The negative shift of the potential of the methoxy substituted compound is also easily explained by looking at the inductive and mesomeric effects of methoxy group. It is a very weakly electron-withdrawing group but its mesomeric effect, its ability to back donate unshared p electrons into the system of the quinonoid ring, is large. The mesomeric effect is more powerful than the opposing inductive effect and the net result is an increase in the electron density of the quinonoid system. A decrease in the $E_{\frac{1}{2}}$ value would be expected and is

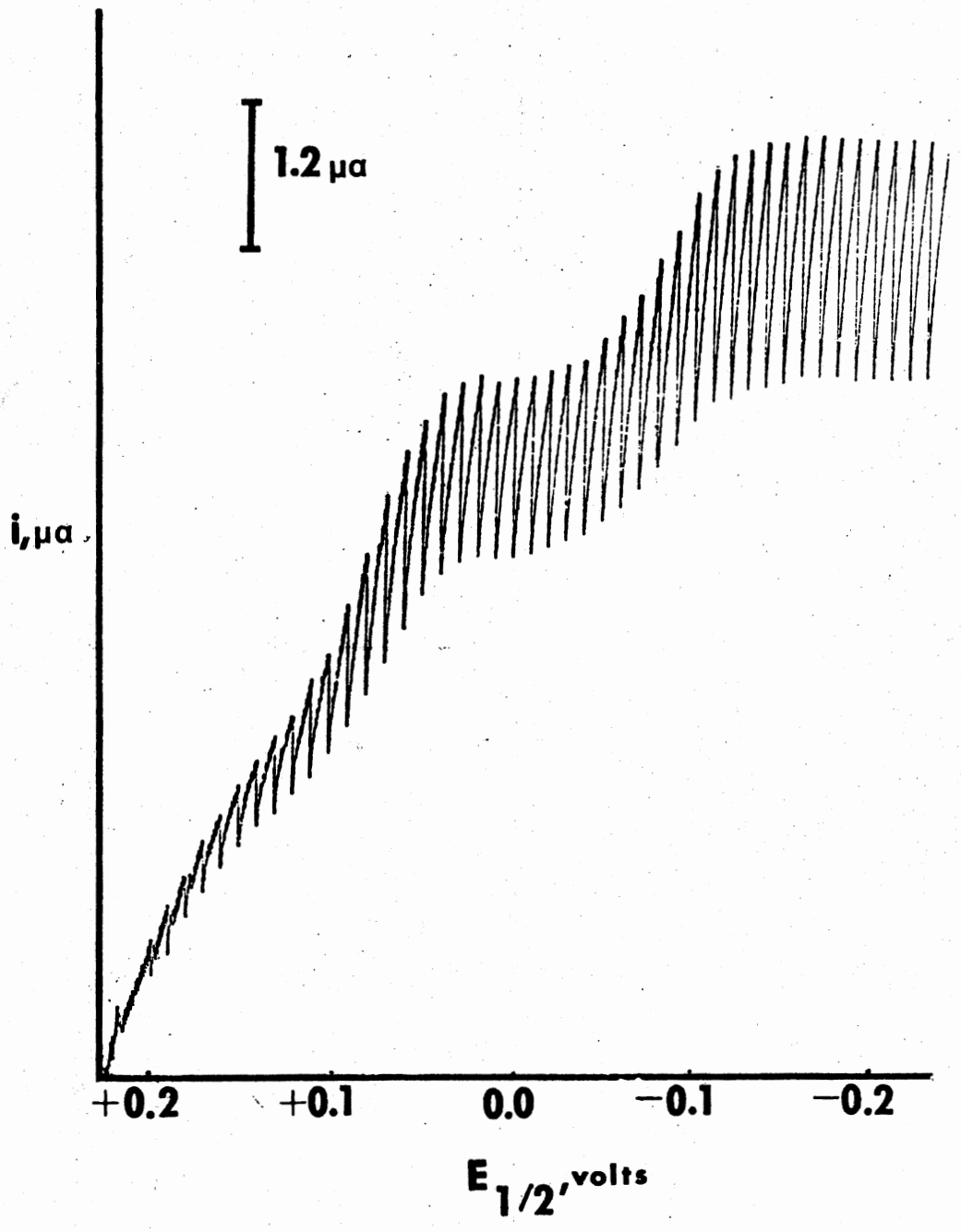
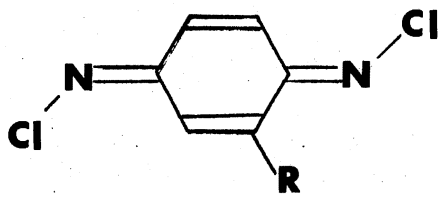


Figure 25. Polarogram of Methyl-N,N'-Dichloro-1,4-Benzoquinonediimine in Solution V

TABLE V

HALF-WAVE POTENTIALS AND SUBSTITUENT EFFECTS IN SOLUTION V (pH 9.10) FOR SOME SUBSTITUTED N,N'-DICHLORO-1,-4-BENZOQUINONE-1,4-DIIMINES



Compound R	$E_{\frac{1}{2}}$, Volts	$\Delta E_{\frac{1}{2}}$, Volts	$\Delta E_{\frac{1}{2}}$, (lit.) Volts
H	-0.140	---	---
Cl	-0.082	+0.058	+0.040(17)
OCH_3	-0.180	-0.040	-0.080(25)
CH_3	-0.117	+0.023	+0.002(17)
SO_3H	-0.037	+0.103	---
NO_2	-0.659	-0.519	---

All $E_{\frac{1}{2}}$ vs. S.C.E.

Temperature = 25^{o}.

observed. Electronic effects do not predict the positive shift of the $E_{\frac{1}{2}}$ value observed with the methyl substituent. In their polarographic study of 1,4-benzoquinonedioximes, Bonastre and Castetbon determined that 2,6-dimethyl-1,4-benzoquinonedioxime has a half-wave potential 95 mv more positive than its parent compound.[22] The authors attribute this to a distortion of the coplanar structure of the molecule because of steric hindrance and hence a decrease in the conjugation and stability of the molecule. Whether steric effects are important in the present study can not be stated with certainty as only one methyl group is present in the ring and the imine substituent could and probably does adopt a configuration anti to the methyl group.

The wave obtained upon electrolysis of the nitro-N,N'-dibromo-1,4-benzoquinonediimine is different from that of other compounds in the same series. There is no well defined rise in current as seen in other polarograms, however the height of the wave does increase with increased concentration. The potential is constant as the concentration is increased but the wave does not become better defined. The potential is much more negative (519 mv) than the parent compound. This shift is not expected when the strong inductive effect of the nitro group is considered. This fact, coupled with the appearance of the wave, suggest a different reduction mechanism is in operation: quite possibly the nitro group is undergoing reduction. Controlled potential electrolysis followed by isolation of the products might help elucidate the electrode reaction. In addition, determination of the pH dependencies of the height of the wave should shed light upon the mechanism. This type of information is useful in determining the sequences of steps in the reduction, i.e., where the protonation reactions occur in relation to the

electron transfer reactions.

The N-bromo-1,4-benzoquinone-4-imines behave differently. Both the N-bromo-1,4-benzoquinonemono- and diimines had very small reduction waves if a wave was present. Waves were detected at all pH values for the monoimine. For the corresponding diimine, waves were detected at pH 7.97 and pH 9.10. At lower pH values no cathodic wave was observed. However, there appears to be an anodic wave at +0.109 volts vs SCE whose potential does not change as the pH is varied. As no attempts to isolate the products of electrolysis were made nothing can be said about species responsible for this wave. Both the N-bromomono- and diimines are less stable than their chloro counterparts, they change colors when exposed to sunlight. The observed differences in behavior might be due to rapid chemical transformation in the alcoholic electrolyte. Alternatively, the N-bromoimines might have undergone chemical changes as a result of exposure to light.

Figure 26 and Table VI shows the relationship between the observed half-wave potential and the biological activity, T/C, %. The equation which best describes this relationship is given below:

$$(\%\ T/C - 100)\ (E_{\frac{1}{2}}) = 12.8$$

Included in these data is the half-wave potential of the nitro compound even though the polarographic wave appears to be the result of a different electrode mechanism.

The results do not show any direct linear relationship between the half-wave potential and biological activity. The data seems to indicate that a potential of at least -0.25 volts vs. SCE is required for significant biological activity. All compounds with $E_{\frac{1}{2}}$ values greater

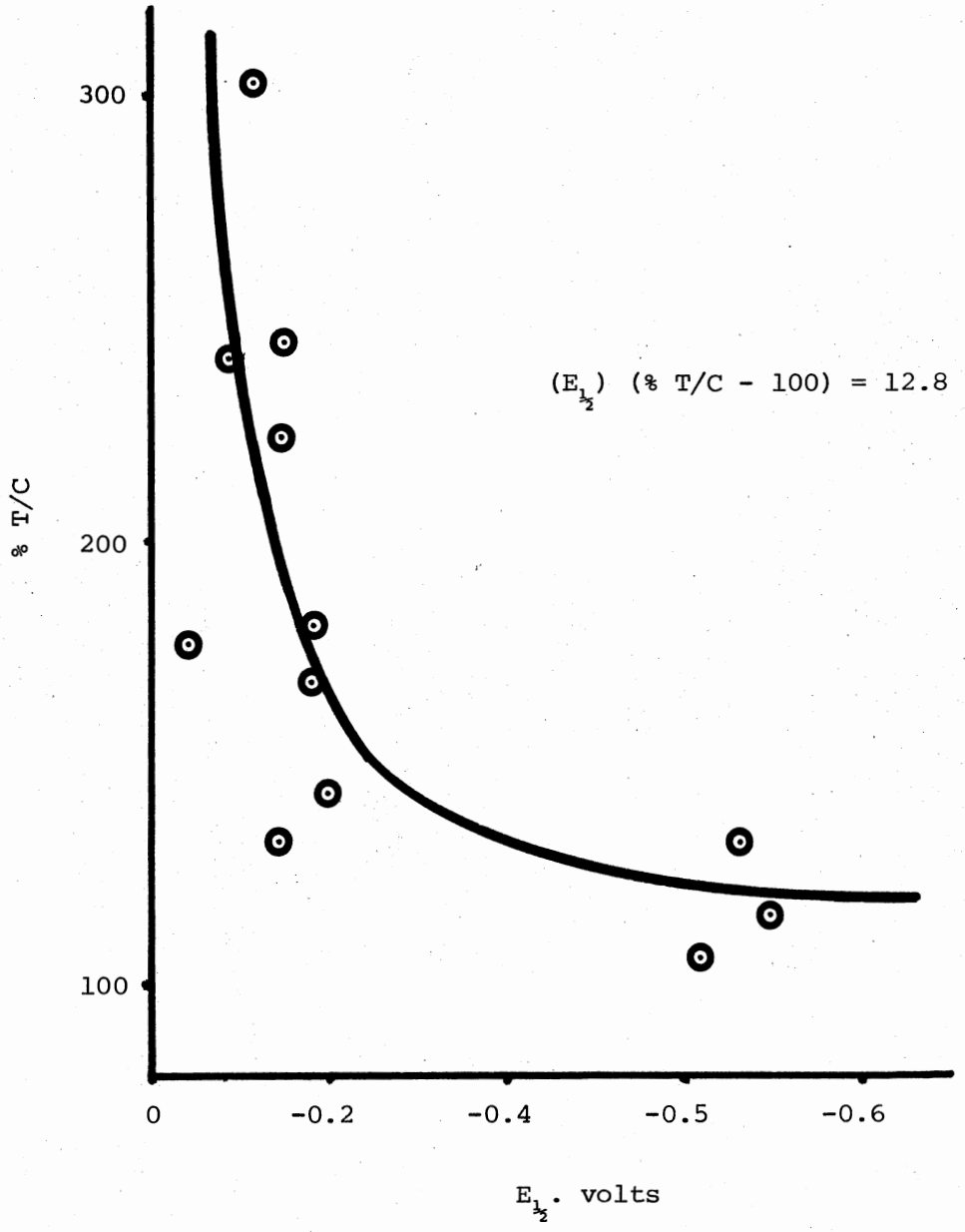


Figure 26. % T/C vs $E_{\frac{1}{2}}$

TABLE VI

BIOLOGICAL ACTIVITIES AND HALF-WAVE POTENTIALS AT pH 9.10 OF SOME QUINONOID COMPOUNDS

Compound	$E_{\frac{1}{2}}$, Volts	T/C, %
1	-0.695	116
4	-0.618	106
20	-0.147	132
18	-0.202	145
16	-0.147	246
19	-0.175	169
17	-0.140	224
21	-0.082	242
22	-0.180	182
23	-0.117	307
24	-0.037	176
25	-0.659	132

All $E_{\frac{1}{2}}$ values vs S.C.E.

Temperature = 25°.

than -0.25 volts vs. SCE have significant activities. Other factors are obviously important in determining how effective these compounds are against the SA 180 ascitic tumor system in Swiss white mice. No prediction of biological activity can be made when $E_{\frac{1}{2}}$ values fall above -0.25 volts vs. SCE. According to the data, a half-wave potential less than -0.5 volts vs. SCE precludes any significant biological activity. The validity of the results should be tested by biological screening of compounds with known $E_{\frac{1}{2}}$ values more negative than -0.25 volts vs. SCE, the region with fewer data points in Figure 26. All of these points were obtained in solution of pH 9.10 which were 50% in methanol. As most of the pH dependencies of the $E_{\frac{1}{2}}$ values of the parent compounds were linear, extrapolation to physiological pH of 7.2 - 7.4 appears valid. Although no studies were performed to determine solvent effects, the assumption is made that the relative values of the half-wave potentials will be the same in aqueous solutions or that they will change in a consistent manner by a constant small value.

Since the primary objective of this study was to obtain half-wave potentials of these quinonoid compounds little work was done to elucidate the mechanism of reduction. Further work on these compounds should include the determination of the nature of the waves observed, whether they are kinetic or diffusion controlled, whether they are reversible or irreversible. Determination of the pH dependence of the wave height would yield useful information concerning the role of protons in electrode reaction. Controlled potential electrolysis followed by isolation of the products should also yield useful information. By isolating electrolysis products perhaps some insight could be gained into the mechanism of activity in biological systems.

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Thesis: A STUDY OF SOME QUINONEIMINES AS POTENTIAL ANTI-TUMOR AGENTS. I. PREPARATION OF SOME DERIVATIVES OF LAPACHOL, A NATURALLY OCCURRING NAPHTHOQUINONE. II. A POLAROGRAPHIC STUDY OF SOME QUINONEIMINES

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