Anomalous Electronic Absorption in Lapacholi–Alcohol Solutions

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An anomalous band at the red end of the absorption spectrum of lapachol (an anti-cancer compound) appears when the solvent used is a lighter molecular-weight alcohol, but not in purely aqueous solution. This band does not follow Beer's law, and is attributed to a weak transition of the structurally modified monomeric *ortho* naphthoquinone tautomer in these solvents. The "normal" *para* tautomer is considered to exist predominantly in the dimeric form at all concentrations above approximately 10^{-4} M. This interpretation is supported by spectroscopic data on structurally related compounds, as well as molecular-orbital calculations.

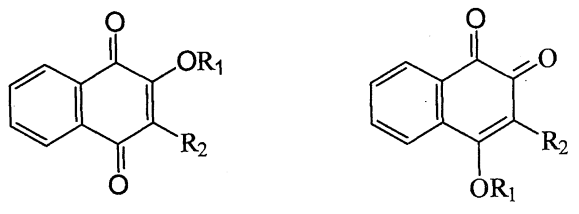
Lapachol (2-hydroxy-3-(3-methyl-2-butenyl)-1,4-naphthoquinone, Fig. 1) is a relatively abundant, natural hydroxynaphthoquinone that has been extracted from the heartwood of Tecoma[1a] trees and the root bark of Newbouldia-laevis,[1b] and used for its known anti-cancer, anti-viral, and anticoagulant activities[2] when taken orally. Although the origin of this activity is not completely understood, it is believed[3] that it derives from intercalation and reaction with DNA, inhibiting the replication of virus RNA.

For compounds that demonstrate biological activity it is important to know the molecular structure and how this varies when the solvent is modified, in order to understand such activity. What follows below is evidence for an important structural modification of lapachol (and its truncated form, lawsone) in the presence of a simple alcohol.

Experimental

The naphthoquinones studied were used as received without purification, because thin-layer chromatography, using solvent mixtures chloroform/methanol (3 : 1), tetrahydrofuran/methanol (3 : 1), and ethyl acetate/methanol (3 : 1), as well as gas chromatography all showed that only one compound was present. All naphthoquinones used were verified by elemental analysis and ^{1}H NMR. Solutions (having concentrations varying from 10^{-6} M to 10^{-3} M, M = mol dm^{-3}) were prepared in the following spectroscopic grade solvents: methanol, 2-propanol, hexane, chloroform, acetonitrile, dioxane, diethyl ether, and dimethyl sulfoxide (Grupo Química/Brasil), ethanol, acetone, and tetrahydrofuran (Merck/Brasil), 2-butanol (Analar/Brasil), *t*-butanol (Reagen/Brasil) and in 1-octanol (99.5%, Riedel). Absorption spectra were taken on a Cary 1-E UV-visible spectrophotometer.

Curve-fitting of the spectroscopic data was performed using EXCEL. Molecular-orbital calculations were carried out using lawsone (2-hydroxy-1,4-naphthoquinone, a de-alkylated lapachol, see Fig. 1) as the model compound and utilizing the CNDO-CI formalism[4] to calculate the excitation energies. The method was chosen because it is the spectroscopic version on CNDO/2, parameterized to calculate the energy gap between the ground and first excited singlet states. All one hundred and twenty singly excited configurations involving orbitals HOMO-11→HOMO and LUMO→LUMO+9 were considered in the configuration interaction calculation. The atomic coordinates used were those calculated to be of lowest energy for the ground state by the PM3-Pulay method using version 6.0 of the MOPAC[5] package. This was done because PM3 is considered to generate better bond distances and angles than the CNDO method. All calculations were carried out on a 486-DX$_2$ 66 microcomputer.



Para Ortho

Fig. 1. Lapachol (R_1 = H, R_2 = $CH_2CH{=}C(CH_3)_2$) *ortho* and *para*, Lawsone ($R_1 = R_2$ = H) *ortho* and *para*, α-Lapachone ($R_1{-}R_2$ = $C(CH_3)_2CH_2CH_2$) only *para*, β-Lapachone ($R_1{-}R_2$ = $C(CH_3)_2CH_2CH_2$) only *ortho* and 2-methoxylapachol (R_1 = CH_3, R_2 = $CH_2CH{=}C(CH_3)_2$) only *para*.

Results and Discussion

Lapachol in methanol, ethanol or 2-propanol at room temperature shows three broad, featureless, absorption (Fig. 2) bands, centered at 330, 390, and 490 nm (in ethanol). The bands at 330 and 390 nm follow Beer's law over the entire range studied (until 10^{-3} M) with apparent values of $\varepsilon_{330} = 2.8\times10^3$ cm^{-1} M^{-1} and $\varepsilon_{390} = 1.4\times10^3$ cm^{-1} M^{-1} in ethanol, as shown in Table 1. The band at 490 nm is considerably weaker ($\varepsilon_{\text{apparent}} = 6.3\ (\pm.1)\times10^2$ cm^{-1} M^{-1} at low concentrations) and decidedly does not follow Beer's law (Fig. 3). The increase of absorption vs. concentration for this band tapers off at concentrations above approximately 2×10^{-5} M and reaches a maximum at approximately 2×10^{-4} M. This red-shifted band was not observed at any

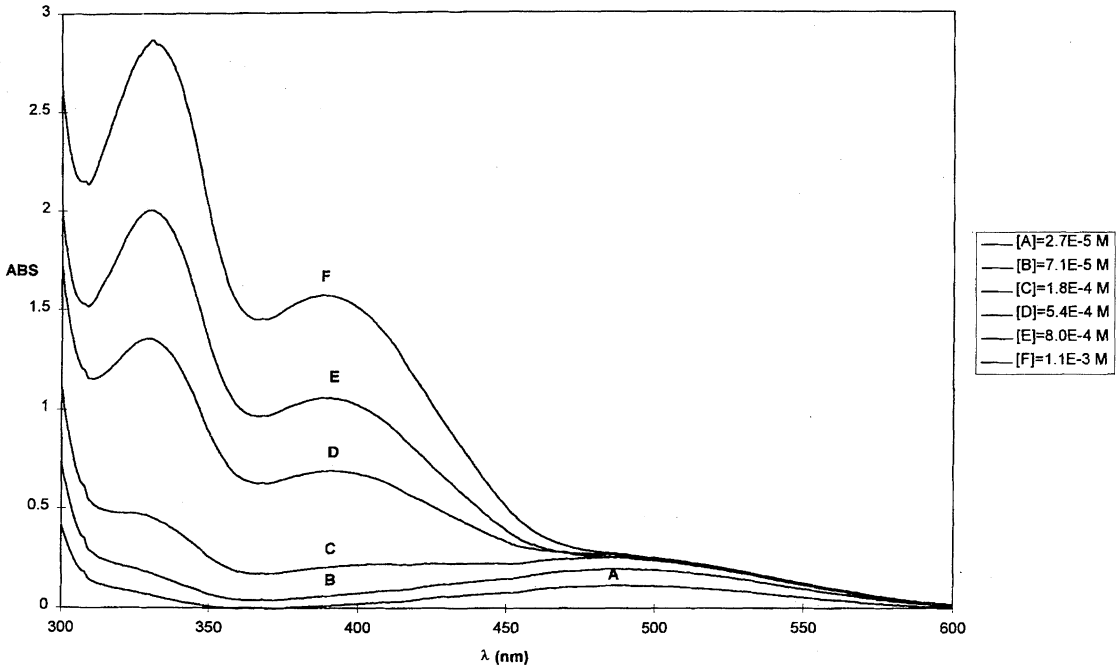


Fig. 2. Absorption spectra of lapachol in ethanol at various concentrations.

Table 1. Experimental Absorption Maxima (in nm) for Naphthoquinones

Solvent	Lapachol	Lawsone	α-Lapachone	β-Lapachone	2-Methoxylapachol
Ethanol	330 (2.77) 390 (1.44) 490 (110[a])	330 (3.22) 370 (1.1[b]) 460 (3.30[c])	330 (2.07) 390 (0.64)	330 (1.78) 430 (2.12)	330 (1.78) 390 (0.56)
n-Hexane	330 (3.33) 380 (1.71)	330 (1.5) 370 (0.28[b])	330 (1.93) 390 (0.33)	330 (0.65) 430 (1.26)	

$\varepsilon/10^3$ ($cm^{-1}\,M^{-1}$) in parentheses. a) See text. b) Value approximate. Not well separated from band at 330 nm. c) $\varepsilon_{apparent}$ at low concentration.

concentration in the other solvents listed above, the first band for lapachol-hexane solutions, for example, having a maximum at 380 nm. Those measurements in an aqueous solution were somewhat prejudiced by the limited solubility of lapachol in water; however, it was possible to detect the first absorption band of the anion, generated by adding NaOH to the solution, with a maximum at 475 nm.

As can be seen in Table 1, lawsone shows the same behavior as lapachol. α-Lapachone (3,4-dihydro-2,2-dimethyl-2*H*-naphtho[2,3-*b*]pyran-5,10-dione, Fig. 1) as well as 2-methoxylapachol (two model compounds for lapachol with the quinonyl oxygens locked into the para position) show no sign of a band with a maximum above 400 nm. β-Lapachone (3,4-dihydro-2,2-dimethyl-2*H*-naphtho[1,2-*b*]pyran-5,6-dione, a model compound for lapachol with the quinonyl oxygens locked into the ortho position (Fig. 1)) does not have an absorption band at 390 nm, but shows a band with a maximum at 430 nm (in ethanol) and a tail which extends out well past 500 nm.

The PM3-calculated relative energies are shown in Table 2. The resulting calculated equilibrium constants were obtained by assuming that the PV contributions in these condensed solutions are trivial, as is the entropy term for the *ortho–para* tautomerization. The entropy term for dimerization was taken to be -12.8 cal mol^{-1} deg^{-1}, the same as found[6] for benzoic acid dimerization in benzene. The temperature considered was 298 K. The CNDO-CI calculated results for lawsone predict a red shift of the lowest energy absorption band (Table 3) in the *ortho* tautomer, relative to the *para* tautomer, of some 100 nm. In addition, both an anionic form and a dimeric form (calculated to be most stable in a totally planar, doubly hydrogen-bonded, *para* form, Fig. 4) were calculated to absorb to the blue of the *ortho* form.

The spectroscopic results of compounds which are unable to tautomerize (the compound frozen into the *ortho* form and not containing a dissociable proton absorbing almost 40 nm to the red of the two compounds frozen into the *para* isomer) are obviously consistent with the band at 490 nm, being due to the tautomeric *ortho* form of lapachol (and lawsone) in the three smaller alcohols. This is an hypothesis that has been raised[7] previously to explain the observation of fused naphtho[1,2-*d*]-1,3-oxazoles as reaction products in the con-

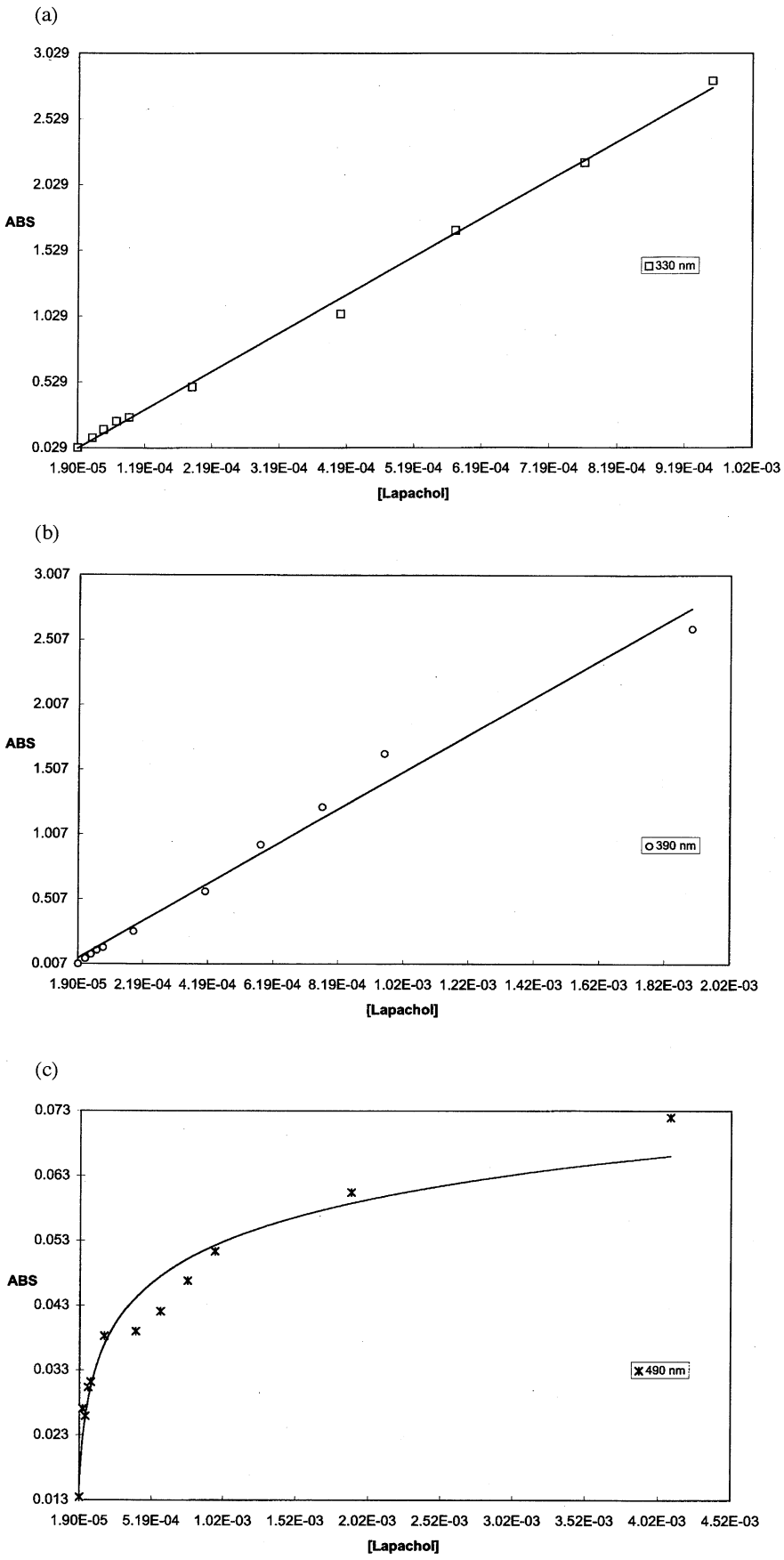


Fig. 3. Plots of Abs. vs. [Lapachol] at 330 nm, 390 nm, and 490 nm. Solvent = Ethanol.

Table 2. PM3 Calculated Relative Stabilities and Equilibrium Constants for Lawsone

Total energy (kcal/mol)			Equilibrium constants	
Para	*Ortho*	Dimer-2E (*Para*)	K_t	K_d
(0)	2.925	−1.69	139	0.027

Table 3. Calculated (CNDO-CI) Singlet State Electronic Transition Wavelengths (in nm) for Lawsone

Ortho	*Para*	Anion	Dimer
569	470	534	399
388	439	456	372
316	278	360	284
276	277	334	
229	251	283	

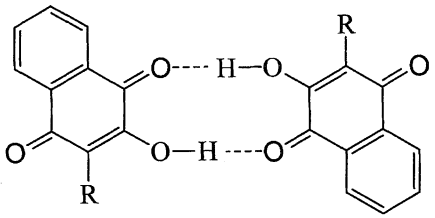


Fig. 4. Proposed planar dimer of *para* lawsone and lapachol.

densation of lapachol with alkyl amines, but not proven. It is interesting to consider why 1) the phenomenon is observed only in the smaller alcohols, and 2) at total concentrations above 5×10^{-5} M (lapachol in ethanol) the slope of the curve $Abs_{490\,nm}$ vs. total concentration for the band attributed to the ortho quinone decreases, and eventually becomes zero.

Regarding the first point, if one considers that tautomerization requires a proton transfer, one can rationalize the necessity for polar solvent molecules to solvate the proton. However, that lapachol in DMSO does not exhibit this anomalous band suggests that high polarity is not sufficient, and that the solvent needs to have the capability of both an electron donor and a proton donor in H-bonds. This would explain the tautomerization results in methanol, ethanol, and 2-propanol. However, the absence of tautomerization in the larger alcohols (2-butanol, *t*-butanol and 1-octanol) suggests that more than one solvent molecule is necessary to solvate the proton efficiently, and that the bulkier alcohols are unable to converge on the proton. This is consistent with previous[8] results that show that proton transfer in 7-hydroxyquinoline mediated by alcohols requires at least three solvent molecules. It is also consistent with the fact that, as pure liquids, smaller alcohols are stronger acids than larger alcohols, due to greater solvation of the ions by the former. The absence of tautomerization in an aqueous solution can be understood in terms of proton and anion solvation being favored over molecular solvation in this solvent, leading to an ion-pair structure rather than the proton-transferred *ortho* structure. This is consistent with the fact that, to the best of our knowledge, excited-state intramolecular proton transfer has never been reported in aqueous solution.

In regard to the question of why the *ortho* quinone structure (O) does not follow Beer's law, with its absorption tapering off upon attaining a concentration above 5×10^{-5}, it is obvious that the experimental result obtained here is not explicable by means of a two-component system, which could only generate monotonic curves of $Abs_{490\,nm}$ vs. concentration. A system that considers only tautomerization, for example, would predict a Beer's law behavior. Slightly more complicated explanations that involve only two species, such as ester or anion formation, can be discarded also. (The possibility of a lapachol–alcohol complex was tested by varying the concentration of ethanol in ethanol/diethyl ether solutions between 0 and 70%. All absorption spectra were identical with those of lapachol in pure ether, arguing against this hypothesis.) We are thus forced to consider a more complex explanation, such as the following system, in which there is

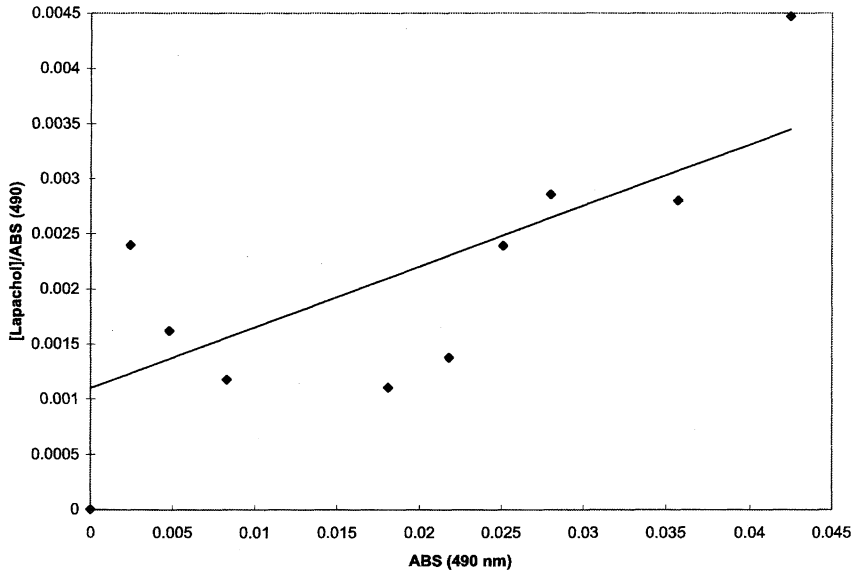


Fig. 5. Plot of [Lapachol]/Abs at 490 nm vs. Abs at 490 nm. Solvent = Etanol.

both an *ortho–para* tautomeric equilibrium:

$$O \rightleftharpoons P \qquad K_t \tag{1}$$

and an equilibrium in the para tautomer between the monomeric (P) and dimeric (P_2) forms

$$2\ P \rightleftharpoons P_2 \qquad K_d \tag{2}$$

The total concentration can be expressed as

$$[C] = [O] + [P] + 2[P_2]. \tag{3}$$

At equilibrium, setting $\alpha \equiv K_t + 1$ and $\beta \equiv 4\ K_d\ K_t^2$, one can obtain

$$[O] = \{(\alpha^2 + 2\beta[C])^{1/2} - \alpha\}/\beta. \tag{4}$$

Considering that the experimental data are $Abs_{490\,nm}$ vs. [C] and assuming that only the *ortho* form absorbs at 490 nm for a 1 cm path length absorption cell, $Abs_{490\,nm} = \varepsilon$ [O], where ε refers to the ortho form. Thus, Eq. 4 becomes

$$Abs_{490\,nm} = \{\varepsilon/\beta\}\{(\alpha^2 + 2\beta[C])^{1/2} - \alpha\}. \tag{5}$$

It can be shown that Eq. 5 is capable of generating a curve that behaves like the experimental data by considering the tangent of the $Abs_{490\,nm}$ vs. [C] curve. This is given by

$$d\,Abs_{490\,nm}/d[C] \equiv \Delta = \varepsilon(\alpha^2 + 2\beta[C])^{-1/2}. \tag{6}$$

At high concentrations

$$\lim_{[C]\to\infty} \Delta = 0$$

i.e., the curve levels off. Equation 5 can be transformed into a linear form for treating the data, i.e.,

$$[C]/Abs_{490\,nm} = (\beta/2\varepsilon^2)Abs_{490\,nm} + \alpha/\varepsilon \tag{7}$$

A plot of $[C]/(Abs_{490\,nm})$ vs. $(Abs_{490\,nm})$ should generate a straight line having a slope of $(S=\beta/2\ \varepsilon^2)$ and intercept at $(I=\alpha/\varepsilon)$. It is obvious that only two parameters (of the three α, β, and ε) can be obtained by fitting the absorption data to Eq. 7. The values found (see Fig. 5), considering only concentrations up to 2×10^{-4} M (at higher concentrations the tail of the second band, at 390 nm, tends to cover up the first band), were $S=0.055\pm0.020$ and $I=0.0011\pm0.0005$. Using the definition of I and assuming $K_t=139$ (Table 2), yields $\varepsilon=1.3(\pm.6)\times10^5$ $cm^{-1}\,M^{-1}$. An alternative method to evaluate ε is given by

$$\varepsilon = \varepsilon_{apparent}[C]/[O]. \tag{8}$$

Assuming that at low total concentrations there is no dimerization, $[C]/[O]=\alpha$ and Eq. 8 yields $\varepsilon=8.8(\pm.1)\times10^4$ $cm^{-1}\,M^{-1}$, within the error bars of the first value of 1.3×10^5 $cm^{-1}\,M^{-1}$. Both of these values of ε depend on the value of K_t chosen, ε being proportional to K_t for $K_t \gg 1$.

Comparing the values of ε calculated here to the value of $\varepsilon=2.12\times10^3$ for the first band of β-lapachone might lead one to conclude that the calculated ε value for the *ortho* form of lapachol is too high, implying that the K_t value is also too high. However, the fact that 2-methoxylapachol shows no sign of a band with a maximum above 400 nm indicates that methylation occurs almost exclusively on the *para* isomer, thus being consistent with a high value of K_t. In addition, the spectra at the lower concentrations in Fig. 2, where the band at 490 nm is considerably more intense than the band at 390 nm, indicate that $\varepsilon_{490\,nm} \gg \varepsilon_{390\,nm}$.

From the definition of S one can obtain

$$K_d = (S/2)(\varepsilon/K_t)^2. \tag{9}$$

Using the average of the two values of ε generates the value $K_d=1.7(\pm0.9)\times10^4$, which is comparable to the experimental values extrapolated to 25 °C of 6.1×10^2, 2.8×10^3, and 1.2×10^4 for benzoic acid[6] in benzene, carbon tetrachloride, and cyclohexane, respectively. One can note that for $K_t \gg 1$ Eq. 9 becomes $K_d=S/2I^2$, i.e., the calculated value for K_d becomes independent of the values attributed to ε and K_t. Although the value of K_d calculated here differs considerably from the PM3 calculated value of 0.027 in Table 2, the former is much more consistent with the spectroscopic evidence (Fig. 3) that supports the idea of large scale dimerization at the higher concentrations. It is not surprising that the calculated energy of dimerization is not correctly accounted for within the PM3 framework, considering that the parameterization is specifically for completely covalent systems. These equilibrium constants suggest that the *para* form is more stable than the *ortho* form and that the dimeric form of the *para* isomer predominates over the monomeric form at all total concentrations higher than, approximately, 10^{-4} M, which, in turn, decreases the fraction of molecules in the *ortho* form. It is worth pointing out that β-lapachone has been shown[9] to inhibit DNA cleavage, whereas no such activity has been reported for α-lapachone, suggesting that the tautomerization described here might be essential to the biological activity of lapachol.

It is interesting to note that the PM3 calculated energy splitting between the equivalent individual monomeric first excited singlet (S_1) states in the dimer is minimal (37 cal mol^{-1}) suggesting that the coupling through the two H-bonds is calculated to be weaker in the S_1 state than in the ground state. This is consistent with the fact (Fig. 2) that no new absorption bands are observed at the highest concentrations used here, where the model predicts substantial dimerization.

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