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Monoarylhydrazones of α-lapachone: synthesis, chemical properties and antineoplastic activity

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The biological activities of the naphthoquinones lapachol, extracted from trees of the genus *Tabebuia* and its cyclization products α and β-lapachone, have been intensively studied. Giving continuity to the research about new derivatives obtained from the reaction of these naphthoquinones with amino-containing reagents, a series of arylhydrazones of α-lapachone was synthesized and their antineoplastic activity was evaluated. This new structure is based on the great electrophilicity of 1,4-quinoidal carbonyl groups towards reagents containing nitrogen as nucleophilic centers, such as arylhydrazines. The products were assayed by the National Cancer Institute (NCI, USA) and their binding to DNA, redox properties and QSAR studies were also determined.

1. Introduction

Quinones are compounds that have in their molecular skeleton an α,β-bisdienonic ring system that confers to them capacity of reversible chemical reactivity of oxireduction. This characteristic is of great importance in several biological processes. Among several naturally occurring quinones, the naphthoquinones emerge, because they are widely distributed in the plant kingdom and they are involved in oxidative processes such as photosynthesis and electron transfer reactions [1].

The biological activity of the naphthoquinone lapachol (**1**), extracted from the heartwood of the trees of genus *Tabebuia (Bignonaceae)*, and its cyclization product β-lapachone (**2**) have been intensively studied but not α-lapachone (**3**) and its derivatives. Although some of these quinones were already isolated and characterized in the 19th century, only in the last decades their biological activities have been investigated. This group of quinones presents activity against several microorganisms [2–5] and HIV [6]. These naphthoquinones exhibit antitumor effect [7–10] and β-lapachone is a potent inhibitor of DNA repair system [11–12]. The proposed mechanism of action of β-lapachone involves apoptosis [13–15], reactive oxygen intermediates [16] and topoisomerase inhibition [17, 18].

Taking in consideration the diversity of pharmacological effects, the easy access to natural sources of precursors of these quinones from South American typical flora, and the synthetic alternative routes already known by quinoidal carbonyl towards nucleophylic agents [19–22]; we took α and β-lapachones as starting points for medicinal chemistry studies.

Several synthetic analogues of α and β-lapachones retain the intact *o*- and *p*-quinone moiety. We attempted to obtain analogues with modifications at the center of redox activity that may alter the redox characteristics of these molecules: the monoarylhydrazones would not show less facile redox cycling and radical generation than the corresponding quinones so they could exhibit selective cytotoxicity towards human tumor cell lines.

2. Investigations, results and discussion

2.1. Synthesis of the compounds

Initially, lapachol (**1**) was extracted from the heartwood of *Tabebuia* sp. employing an improved large-scale method. From this starting material, β-lapachone (**2**) and α-lapachone (**3**) can be synthesized through a cyclization reaction, employing sulphuric acid or hydrochloric acid and acetic acid, respectively.

In this first step of a larger research project, we focused our interest on the synthesis of compounds possessing a 1,4-quinoidal carbonyl group, so α-lapachone was prepared in order to study its chemical reactivity.

First of all, the formation of an imine derivative was intended by means of reaction with aniline. Even with the use of concentrated sulphuric acid, anhydrous magnesium sulphate or tin tetrachloride as catalysts, the expected imine product was not obtained, but a mixture of coloured compounds difficult to purify. However, the literature describes the preparation of the imine derivative synthesized from the condensation reaction of a non-symmetrical *p*-quinone with aniline, with high regio- and stereoselectivity, in an anhydrous solvent and titanium tetrachloride [23]. More recently, another author reported the reaction of β-lapachone with anilines and titanium tetrachloride to give the corresponding iminoquinone [24], but this procedure does not work when it is used to prepare α-lapachone derivatives.

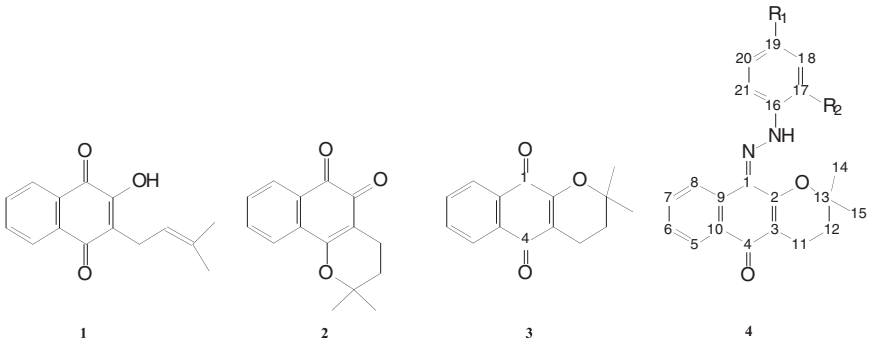

Table 1: Physical properties and NSC (Code) of monoarylhydrazones of α-lapachones

Compd.	NSC (Code)	R_1	R_2	Yield (%)	M. p. (°C)	Formula
4a	716759	−Cl	−H	74	180–182	$C_{21}H_{19}N_2O_2Cl$
4b	716758	$-OCH_3$	−H	89	179–182	$C_{22}H_{22}N_2O_3$
4c	716761	$-SO_2NH_2$	−H	57	268–270	$C_{21}H_{21}N_3O_4S$
4d		$-CF_3$	−H	77	157–160	$C_{22}H_{19}N_2O_2F_3$
4e	716763	$-CH_3$	−H	84	152–154	$C_{22}H_{22}N_2O_2$
4f		−COOH	−H	67	296–298	$C_{22}H_{20}N_2O_4$
4g	716757	−F	−H	64	175–177	$C_{21}H_{19}N_2O_2F$
4h	716760	$-NO_2$	−H	76	218–219	$C_{21}H_{19}N_3O_4$
4i		$-NO_2$	$-NO_2$	78	258–260	$C_{21}H_{18}N_4O_6$
4j		$-OCH_3$	$-NO_2$	75	238–239	$C_{22}H_{21}N_3O_5$
4k		−H	−Cl	80	180–182	$C_{21}H_{19}N_2O_2Cl$
4l		−H	$-CF_3$	77	162–164	$C_{22}H_{19}N_2O_2F_3$
4m		−H	$-CH_3$	69	159–162	$C_{22}H_{22}N_2O_2$
4n		−H	−F	71	170–171	$C_{21}H_{19}N_2O_2F$
4o	713843	−H	−H	78	175–178	$C_{21}H_{20}N_2O_2$
4p		−H	$-NO_2$	78	229–230	$C_{21}H_{19}N_3O_4$

It was for the above reasons that we decided to synthesize arylhydrazones. Thus, α-lapachone reacted easily with 2,4-dinitrophenyl-hydrazine (DNPH) in methanol, at room temperature, with sulphuric acid as catalyst, to give a unique product in excellent yield. The nitrogen elemental analysis and ^{1}H NMR spectroscopical data confirmed the monophenylhydrazone of α-lapachone, **4i** (Table 1). Notably, a singlet signal at δ 16.67 was found at a higher value than expected for a hydrazone hydrogen atom. Molecular modeling studies [25] of this structure showed that such a hydrogen atom is situated at 2.12 Å to the pyrane oxygen atom and at 2.06 Å to the 2-nitro substituent, so the formation of a double hydrogen bond can be established (Fig. 1b). The deprotection that it exerts on the hydrazone hydrogen atom justifies that δ value; so the condensation reaction occurs stereoselectively on the carbonyl group at C1 and this was also confirmed by ^{13}C NMR data. The difference of reactivity between C1 and C4 atoms could be explained through molecular electrostatic potential (MEP) calculations of α-lapachone, where C1 resulted more reactive than C4 in a 3 : 1 ratio, owing to the inductive effect produced by the pyranic oxygen atom (Fig. 1a), [25].

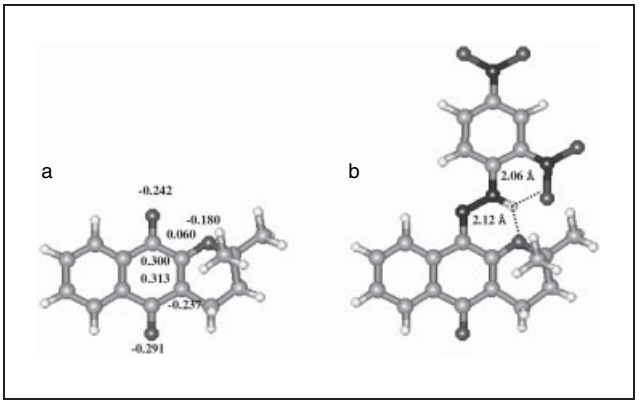

Fig. 1: a) α-Lapachone, electrostatic potencial, b) 2,4 dinitrophenyhidrazone of α-lapachone, hydrogen bonds

These results prompted us to develop a new α-lapachone monoarylhydrazone series, so a careful selection of the substituent groups of arylhydrazines was planned and these reagents were synthesized according to the literature [26]. These substituents involved some electronic, steric and lipophilic effects that could modulate the reactivity of the arylhydrazines as well as the biologic activity of the products, then structure-activity relationship (SAR) and QSAR studies could be carried out.

In order to improve the yields when less reactive arylhydrazines were used, it was necessary to increase the temperature and the adding of just calcined anhydrous magnesium sulphate; then the achieved yields were good enough for all members of the series. The physical data of these 16 terms (compounds **4a**–**4p**) are depicted in Table 1.

Table 2: DNA affinity assay

Compd. (NSC Code)	DNA Affinity (a_{24}/a_0)
4a (716759)	1.00
4b (716758)	0.45
4c (716761)	0.86
4o (713843)	1.00
m-AMSA	0.54
Mitoxantrone	0.00
Bis-benzimide	0.57

Table 3: *In vitro* antitumor activity on 60 human cell lines

Compound NSC (Code)	Log_{10} GI_{50}[a]		Log_{10} TGI_{50}[b]		Log_{10} LC_{50}[c]	
	MG-MID[d]	Range	MG-ID	Range	MG-ID	Range
3b (716758)	−4.75	1.32	−4.34	0.61	−4.09	0.31
3c (716761)	−4.99	1.20	−4.48	1.14	−4.14	0.54
3e (716763)	−4.68	1.38	−4.28	0.85	−4.09	0.42
3g (716757)	−4.54	1.05	−4.12	0.68	−4.03	0.34
3o (713843)	−4.55	0.80	−4.14	0.51	−4.02	0.22

[a] GI_{50}: log_{10} of molar concentration for 50% growth inhibition of tumor cells; [b] TGI_{50}: log_{10} of molar concentration that produces a total growth inhibition; [c] LC_{50}: log_{10} of molar concentration that produces cytotoxic effect in 50% of tumor cell; [d] Meangraph-Midpoint: Parameter giving averaged activity on all cell lines.

2.2. Binding to DNA

The compounds were tested for their ability to union with DNA, using the DNA binding assay as described previously [27]. The binding capacity of these compounds was tested by measuring the hypochromic and bathochromic effects of their absorbance in the UV spectra. The typical procedure was enhanced by means of a slow rotation of DNA-drug mixture stirring, in a 5 : 1 ratio during 24 h, and it was validated by repeating assays with well known intercalating agents (*m*-AMSA and mitoxantrone) and a compound which binds closely in the minor group, bis-benzimide. The degree of interaction was expressed by means of the ratio between the final absorbance area after 24 h (a_{24}) and that of the compounds at the same concentration (a_0), centered at the maximal absorbance, λ_{max}. Values of 1 or greater indicate a total lack of affinity and value 0 shows that the whole compound was bound to DNA. Under such experimental conditions all these compounds exhibited a bathochromic effect and data ranged from 0.86 to 1.00 thus indicating low affinity and no affinity (Table 2), except for compound **4b** that scored 0.45, a similar value shown by *m*-AMSA and bis-benzimide.
A correlation between degree of affinity and antineoplastic activity was not observed, so a different mechanism of action is suggested, like an inhibition of specific enzymes.

2.3. Antineoplastic activity

The seven compounds **4a, 4b, 4c, 4e, 4g, 4h, 4o**, selected by the NCI (National Cancer Institute), were tested using a one dose (10^{-4} M) primary anticancer *in vitro* assay against tumor in the 3-cell line panel consisting of MCF7 (breast), NCI-H460 (lung) and SF-268 (CNS) and five of them showed marked activity against all three cell types. Compounds **4b, 4c, 4e, 4g** and **4o**, which passed the first criterion of activity, were further evaluated at five concentrations in 10-fold dilutions (10^{-4}–10^{-8} M), against the panel of 60 human tumor cell lines, belonging to nine cancer types [28]. The meangraph-midpoint (MG-MID) values, which indicate the average sensitivity of all cell lines to each tested compound, are also given in each case (Table 3). These results are of special interest because there is a great difference between the cytostatic activity and the cytotoxic activity in this series.
Tables 4 and 5 show that compounds **4b** (NSC 716758) and **4c** (NSC 716761) are particularly active against a number of solid hard-to-treat tumors, such as CNS, melanoma, ovarian, renal, breast and colon cancers. Based on the screening results, compound **4c**, with a 4-sulphonamide group, exhibited cytostatic activity values (GI_{50}) between 1.12 10^{-5} and 1.82 10^{-6} M, comparable to drugs in clinical trials.

Table 4: Antitumor activity of compound 3b in most sensitive tumor cell lines

Tumor	Cell lines	GI_{50}
CNS	SF-268	−4.82
	SF-295	−4.83
MELANOMA	SK-MEL-5	−4.91
	LOX IMVI	−4.79
	UACC-62	−4.84
OVARIAN	IGROV1	−4.82
	OVCAR-3	−4.83
	OVCAR-4	−4.74
RENAL	A498	−4.82
	ACHN	−4.86
	RXF 393	−4.91
BREAST	MCF7	−4.91
	MDA-N	−4.81
	T-47D	−5.60
COLON	HCC-2998	−4.92
	HCT-15	−4.86
	HT29	−4.87
	KM12	−4.87

2.4. Quantitative structure activity relationship

QSAR studies were performed by the classical Hansch method [29]. The physicochemical parameters of lipophilicity π, electronic effect σ, steric terms L and B_1, and molar refractivity MR, were extracted from tables [29, 30]. The descriptors calculated lipophilicity coefficient clog P,

Table 5: Antitumor activity of compound 3c in most sensitive tumor cell lines

Tumor	Cell lines	GI_{50}
CNS	SNB-75	−5.36
	U251	−5.06
MELANOMA	LOX IMVI	−5.36
	MALME-3M	−5.48
	SK-MEL-5	−5.74
OVARIAN	OVCAR-4	−5.24
	SK-OV-3	−5.38
	OVCAR-8	−4.97
RENAL	786-0	−5.13
	A498	−5.63
	RXF 393	−5.27
	SN12C	−4.95
BREAST	MDA-MB-231/ATCC	−4.91
	HS 578T	−5.50
	MDA-N	−5.55
	MDA-MB-435	−4.97
LEUKEMIA	CCRF-CEM	−4.95
	SR	−5.28
COLON	HCC-2998	−5.28
	KM12	−4.98

Table 6: Physicochemical parameters, antineoplastic activity and correlation coefficient values

Cpd.	R_1	$-\log GI^{50}$ MG-MID	π	σ	L	clog P	TR	B_1	Parachor	MR (cm^3/mol)	M.DIP (Debye)	Vol. Å^3	Hyd.E. (Kcal/mol)	Refrac. (Å^3)	Polarizab. (Å^3)
3b	−OCH3	4.75	−0.02	−0.27	−0.27	4.97	4.97	1.35	760.40	7.87	3.850	1047.69	−6.41	106.20	40.35
3c	−SO2NH2	4.99	−1.82	0.57	0.57	1.94	1.94	2.11	798.40	12.28	4.395	1095.86	−10.64	114.07	40.37
3e	−CH3	4.68	0.56	−0.17	−0.17	3.52	3.52	1.52	741.20	5.65	4.476	1023.26	−3.50	105.77	39.71
3g	−F	4.54	0.14	0.06	0.06	3.19	3.19	1.35	710.30	0.92	3.347	982.35	−4.44	10094	37.79
3o	−H	4.55	0.00	0.00	0.00	5.22	5.22	1.00	710.10	1.03	4.154	972.05	−4.77	99.73	37.88
R^2			0.68	0.37	0.67	0.35	0.65	0.81	0.99	0.97	0.28	0.97	0.80	0.98	0.73

π, σ and MR values obtained from ref. [28].
L and B_1 values obtained from ref. [29].
Clog P, M. Dipolar, Volumen, Hydratation Energy, Refractivity and Polarizability were calculated using HyperChem® 7.0 [25]
RT: retention time, were determinated by HPLC-RP 18 column.
Parachor values obtained from ChemSketch® 2.0 [30]

Table 7: Autocorrelation matrix of the physicochemical descriptors used to develop eqs. (2) and (4)

	π	L	RT	B_1	MR	Vol.	Hyd.E.	Refrac.	Polarizab.	Parachor
π	1									
L	**0.2383**	1								
RT	0.6076	0.6606	1							
B_1	0.5962	0.4659	0.6829	1						
MR	0.5299	0.7795	0.6044	0.7360	1					
Vol.	0.5475	0.8048	0.6780	0.7826	0.9928	1				
Hyd.E.	0.9498	0.4268	0.7136	0.6006	0.6775	0.6935	1			
Refrac.	0.6116	0.6629	0.6481	0.8877	0.9580	0.9685	0.7028	1		
Polarizab.	**0.1842**	0.8403	**0.3671**	0.4602	0.8622	0.8362	**0.3355**	0.7330	1	
Parachor	0.5957	0.7617	0.6493	0.7598	0.9953	0.9934	0.7380	0.9663	0.8147	1

dipolar moment Dip.M., volume, hydration energy Hyd.E., refractivity and polarizability were calculated employing HyperChem® 7.0 [25], while Parachor was calculated using ChemSketch® 2.0 [31]. The retention time RT was determined by reverse-phase HPLC and it is proportional to an experimental lipophilicity coefficient [32]. The values corresponding to the descriptors mentioned above were linearly correlated with the cytostatic activity data, $-\log GI_{50}$ MG-MID, using the statistical package of Microsoft Excel 2000. The square correlation coefficients R^2 obtained are shown in Table 6 and most of these values are acceptable for a linear fit. The autocorrelation matrix of such parameters is given in Table 7. Those descriptors whose R^2 are less than 0.4, are reasonable orthogonal equations, such as π and L ($R^2 = 0.24$); π and polarizability ($R^2 = 0.18$), RT and polarizability ($R^2 = 0.37$) and Hyd.E. and polarizability ($R^2 = 0.34$). The multiple regression linear (MRL) analysis was applied in these four couples of descriptors in order to perform QSAR studies:

$$-\log GI_{50} = -0.113(\pm 0.049)\cdot\pi + 4.293(\pm 0.171)\cdot L + 0.124(\pm 0.056) \quad (1)$$

$n = 5;\quad R^2 = 0.91;\quad R = 0.82;\quad s = 0.08$

$F_{(0.05;\,2;\,2)} = 19.0;\quad F = 10.016$

$$-\log GI_{50} = -0.113(\pm 0.016)\cdot\pi + 1.277(\pm 0.457) \times \text{Polarizab} \pm 0.087(\pm 0.012) \quad (2)$$

$n = 5;\quad R^2 = 0.99;\quad R = 0.98;\quad s = 0.03$

$F_{(0.05;\,2;\,2)} = 19.0;\quad F = 89.770$

$$-\log GI_{50} = -0.029(\pm 0.021)\cdot RT + 1.656(\pm 1.941) \times \text{Polarizab} + 0.082(\pm 0.048) \quad (3)$$

$n = 5;\quad R^2 = 0.86;\quad R = 0.72;\quad s = 0.1$

$F_{(0.05;\,2;\,2)} = 19.0;\quad F = 6.078$

$$-\log GI_{50} = -0.039(\pm 0.011)\cdot \text{Hyd.E} + 1.649(\pm 0.866) \times \text{Polarizab} + 0.072(\pm 0.023) \quad (4)$$

$n = 5;\quad R^2 = 0.97;\quad R = 0.93;\quad s = 0.05$

$F_{(0.05;\,2;\,2)} = 19.0;\quad F = 27.843$

The figures in parenthesis show the confidence intervals of the regression constants at 95% level, n is the number of terms, R is the multiple regression coefficient, R^2 is adjusted R square, s is the standard deviation of the regression, F(k, n − k − 1) is the variance ratio with k and n − k − 1 degrees of freedom where k is the number of independent variables.

Equations (1) and (3) do not fit to F-test because their observed F is less than critical F when $\alpha = 0.05$. Contrary, eqs. (2) and (4) fit F-test, so the antineoplastic activity linearly depends on π and polarizability, and Hyd.E. and polarizability, respectively. These parameters were evaluated with t-test to determine their contribution in the MRL. In eq. (2), π parameter has $|t| = 6.89$; polarizability, $|t| = 2.79$; and the independent term, $|t| = 7.42$. Since critical t (0.05, 2) = 2.92, π is the most contributory parameter. Furthermore, in eq. (4), Hyd.E. has $|t| = 3.71$; polarizability $|t| = 1.90$ and the independent term, $|t| = 3.13$; so in this case Hyd.E. is the most contributory parameter.

In summary, the QSAR studies show that lipophilicity, π, and Hyd.E. play a significant role in eliciting antineoplastic activity in this class of compounds. This findings will help us to synthesize more effective analogous with judicious modifications of the substituents at the *para* position aryl moiety.

2.4. Cyclic voltammetry

It is known that the mechanism of action of α and β-lapachones with antibacterial, antineoplastic and trypanomicidal activities is related to their capacity of generating

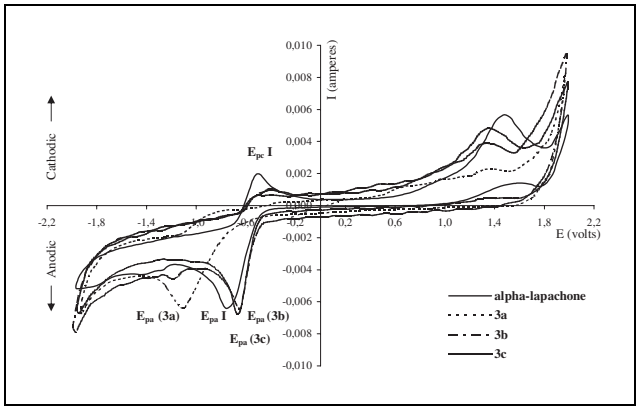

Fig. 2: Cyclovoltammograms of α-lapachone and derivatives

redox cycling with reactive oxygen species which can damage DNA [33]. We could expect that the synthesized monarylhydrazones would show a different redox pattern. The cyclovoltammetric studies of **4a**, **4b** and **4c**, exhibited a change in their shape comparing with the α-lapachone cyclovoltagramm. Fig. 2 depicts the cyclovoltammograms of these compounds; the anodic peaks at $E_{pa} = -0.765$ V and the cathodic peak at $E_{pc} = -0.482$ V arrange a *quasi* reversible pattern for α-lapachone. On the other hand, for derivatives **4a**, **4b** and **4c**, the cathodic peak diminishes, so this change is important to perform an irreversible redox cycling and disqualifies these compounds like oxyradical generation inductors. Coincidentally, the anodic peak potential (E_{pa}) for the active compounds **4b** and **4c** are quite close to α-lapachone, but this does not occur for compound **4a**, which is inactive. This implies that a critical (E_{pa}) value is required in order to interact with some specific enzymes like topoisomerases.

It was proposed that the inhibitory effect of β-lapachone of human DNA topoisomerase occurs when naphtoquinone binds directly to the enzyme to prevent DNA unwinding by topoisomerase I [34]. It was also suggested that the cytotoxic action of naphthoquinone derives, in part, from alkylation of exposed thiol residues on topoisomerase II-DNA complexes. Other authors have suggested a small change in the voltammogram for α-lapachone when this stabilized adduct has formed [35].

3. Experimental

3.1. Material and methods

Melting points were determined with an Electrotermal 9100 SERIES-Digital apparatus and are uncorrected. IR spectra were recorded with a Bruker IFS25 FT Spectrum from KBr discs. UV spectra were measured with a Jasco V-570 UV/VIS/NIR spectrophotometer. ^{1}H NMR (400 MHz) and ^{13}C NMR (100.6 MHz) spectra were obtained with a Bruker Advance 400 spectrometer, with tetramethylsilane as internal standard at room temperature. Chemical shifts (δ) are reported in ppm.

The chromatographic system was performed using Chromatotron® and silica gel Merck 60 PF-254. The HPLC chromatograms were obtained in KONIK-500-A SERIES chromatograph, using ODS Hypersil column (5 μm, 200 × 4.6 mm) Hewlett Packard. The cyclic voltammetry experiments were carried out with an EQMAT-S1 potentiostat controlled by EQSOFT software. All the elemental analysis results were in an acceptable range.

3.2. Synthesis

3.2.1. Extraction of lapachol (**1**)

The sawdust of lapacho wood (500 g) was treated with benzene (2 l) for 24 h at RT. The benzenic extract was filtered and extracted with Na_2CO_3 5% until achieving a lightly red colored aqueous phase.

The alkaline solution was acidified with conc. HCl until pH 1 and lapachol was precipitated. The solid product was filtered off, to give 5 g of crude lapachol (1% yield) and it was recrystallized from benzene, m.p. 138–140 °C (Lit. 141–143 °C) [36].

3.2.2. Synthesis of α*-lapachone* (**3**)

Lapachol (500 mg, 2.07 mmol) was heated with 5 ml of AcOH and 1.25 ml of conc. HCl at 95 °C for 1.5 h. Then the mixture was quenched over ice-water and the solid product was filtered off to give 450 mg of crude α-lapachone, m.p. 100–104 °C. It was recrystallized from a mixture of AcOH and chromic acid 7% (1 : 1), m.p. 117 °C (Lit. 117 °C) [37].

3.2.3. Synthesis of monoarylhydrazones of α*-lapachone* (**4a**–**4p**)*. General method*

To a solution of α-lapachone (242 mg, 1 mmol) in 15 ml dry MeOH, the corresponding substituted arylhydrazine chlorhydrate (1.05 mmol) was added [26]. The mixture was stirred at RT for 10 min. Then anh. $MgSO_4$ (60 mg, 0.5 mmol) was added and the reaction was heated under reflux for 2 h. The solvent was removed under reduced pressure and the residue was extracted with CH_2Cl_2 and filtered.

The organic solution was evaporated under reduced pressure. Some products were isolated by preparative centrifugally accelerated radial TLC (Chromatotron®) and eluted with ethyl acetate/hexane (7 : 3). Other products were purified by recrystallization from methylene chloride/methanol. The purity of all compounds was checked by HPLC, using ODS column and acetonitrile/water (70 : 30) with flow of 2 ml/min. Melting points and yields of the obtained compounds are summarized in Table 1.

3.2.3.1. 10-{Aza[(4-chlorophenyl)amino]methylene}-2,2-dimethyl-3,4-dihydrobenzo[*g*]2*H*-chromen-5-one **(4a)**

Orange solid. IR (KBr, cm^{-1}): 3050, 2960, 2920, 1600, 1580, 1550, 1480, 1380, 1300, 1250, 830, 750. UV/VIS (CH_2Cl_2, nm), λ_{max}: 242, 457. ^{1}H NMR (Cl_3CD): δ [ppm] 1.48 (6 H, s), 1.90 (2 H, t), 2.68 (2H, t), 7.36–7.54 (6 H, m), 7.96 (1 H, d), 8.36 (1 H, d), 16.28 (1 H, s) (NH). ^{13}C NMR (Cl_3CD): δ [ppm] 16.89 (C_{11}), 26.95 ($C_{14,15}$), 31.47 (C_{12}), 79.00 (C_{13}), 113.76 (C_3), 115.16 ($C_{17,21}$), 122.95 (C_8), 124.95 (C_9), 125.57 (C_5), 127.37 (C_6), 127.50 (C_{10}), 129.05 (C_{19}), 129.52 ($C_{18,20}$), 131.25 (C_7), 134.46 (C_{16}), 141.88 (C_1), 155.79 (C_2), 183.07 (C_4).

3.2.3.2. 10-{Aza[(4-methoxyphenyl)amino]methylene}-2,2-dimethyl-3,4-dihydrobenzo[*g*]2*H*-chromen-5-one **(4b)**

Red solid. IR (KBr, cm^{-1}): 3052, 2965, 2920, 2830, 1620, 1580, 1550, 1480, 1380, 1300, 1250, 830, 750. UV/VIS (CH_2Cl_2, nm), λ_{max}: 262, 463. ^{1}H NMR (Cl_3CD): δ [ppm] 1.52 (6 H, s), 1.92 (2 H, t), 2.69 (2 H, t), 3.84 (3 H, s, OCH_3), 6.93–7.32 (4 H, m), 7.40–8.40 (4 H, m), 11.86 (1 H, s, NH), disappears after deuteration. ^{13}C NMR (Cl_3CD): δ [ppm] 16.79 (C_{11}), 26.75 ($C_{14,15}$), 31.44 (C_{12}), 79.00 (C_{13}), 113.74 (C_3), 115.20 ($C_{17,21}$), 120.43 (OCH_3), 121.95 (C_8), 124.76 (C_9), 145.57 (C_5), 127.32 (C_6), 127.66 (C_{10}), 128.05 (C_{19}), 129.33 ($C_{18,20}$), 131.35 (C_7), 134.26 (C_{16}), 142.88 (C_1), 155.89 (C_2), 18217 (C_4).

3.2.3.3. 4-{[Aza(2,2-dimethyl-5-oxo(3,4-dihydrobenzo[*g*]2*H*-chromen-10-ylidene)) methyl] amino} benzenesulfonamide (**4c**)

Orange solid. IR (KBr, cm^{-1}): 3320, 3055, 3260, 2960, 2920, 1620, 1570, 1554, 1480, 1350, 1300, 1250, 1160, 833, 750. UV/VIS (CH_2Cl_2, nm), λ_{max}: 243, 428. ^{1}H NMR (DMSO-d_6): δ [ppm] 1.49 (6 H, s), 1.94 (2 H, t), 2.67 (2 H, t), 7.35 (2 H, s, SO_2NH_2), 6.82–7.78 (8 H, m), 16.45 (1 H, s, NH).

3.3. DNA affinity assay

DNA solution: Calf thymus DNA (12.5 mg) was slowly magnetically stirred in 5 ml Tris-HCl buffer (10 mM, pH 7.4) for 24 h at 4 °C. 0.6 ml were taken from this solution and diluted to 25 ml with the same buffer.

The test compound solution was prepared at a 10^{-4} M concentration using a minimal volume of EtOH or DMF and then diluted adding water to a concentration of 2×10^{-5} M. A 3.0 ml sample of this solution was mixed with 3.0 ml of the DNA solution. The mixture was slowly rotated during 24 h and then, its UV spectra were recorded at 20 °C using a 1 cm quartz cell.

3.4. Cyclic voltammetry

Cyclic voltammetry studies were carried out using a glassy carbon working electrode ($A = 3.03$ cm^2), a platinum auxiliary electrode, and a Ag/AgCl reference electrode and methanol of electrochemical purity. The potentials were scanned from −2000 to +2000 mV employing scan rates of 0.2 mV/s. The glassy carbon electrode was polished intensively with aluminium oxide on a smooth polishing cloth and degreased in methanol prior to each electrochemical measurement. All the solutions examined by electrochemical techniques were first deaerated for at least 10 min with nitrogen, after which a continuous stream of nitrogen was passed over the solution during the measurements. Lithium perchlorate 0.1 M was using as support electrolyte.

3.5. Antineoplastic activity

The compounds were evaluated for antiproliferative properties according to NCI *in vitro* protocols. They were assayed *in vitro* against a panel consisting of 60 human tumor cell lines, derived from nine cancer types (melanoma, leukemia, lung, colon, brain, ovarian, renal, prostate and breast cancers). The results are displayed in Table 3, 4 and 5.

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