

## Effects of 9,10-dihydroxy-4,4-dimethyl-5,8-dihydro-1(4H)-anthracenone derivatives on tumor cell respiration

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**Abstract**—A series of tricyclic hydroquinones, incorporating a carbonyl group in the *ortho* position relative to the phenol function, were tested as inhibitors of oxygen uptake against the TA3 mouse carcinoma cell line and its multidrug-resistant variant TA3-MTX-R. The title compound, which proved to be the most active one, also exhibited low micromolar dose-dependent growth inhibition of the human tumor U937 cell line (human monocytic leukemia). A tentative structure–activity relationship is proposed for these substances. A comparison between the cytotoxicities of the title compound and 4,4-dimethyl-5,8-dihydroxynaphthalene-1-one, with their activities as inhibitors of oxygen uptake by the TA3-MTX-R cell line, is presented. Also, the inhibition of oxygen uptake by 6-(4-methylpent-3-enyl)-1,4-naphthoquinone was determined and compared with its reported cytotoxicity toward P-388 (murine lymphocytic leukemia), A-549 (human lung carcinoma), HT-29 (human colon carcinoma), and MEL-28 (human melanoma) cells. The inhibition of oxygen uptake by TA3-MTX-R cells is useful as a quick test for preliminary screening of possible anticancer activity.

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### 1. Introduction

Phenolic compounds may stimulate or inhibit oxidative damage to biomolecules and it is believed that they can behave as either antioxidants or pro-oxidants.<sup>1–4</sup> Their ability to inhibit the growth and proliferation of certain malignant cells *in vitro* is strongly dependent on their structural characteristics.<sup>5–7</sup> The mechanism of phenol cytotoxicity has been associated with their pro-oxidative activity which can accelerate oxidative damage either to DNA or to proteins and carbohydrates, depending on the structure, dose, target molecule, and environment. This kind of compounds has been reported to display antiproliferative and cytotoxic properties in several tumor cell lines.<sup>5–10</sup> For example, some polyphenolic

antioxidants exhibited dose-dependent toxicity against human promyelocytic leukemia cells (HL-60), and their toxicity was suggested to be related to their pro-oxidant character.<sup>7</sup> Moreover, the inhibition of L1210 cancer cell growth has been described as a striking example of toxicity occurring via phenoxyl radicals.<sup>11</sup>

A comparative study of the cytotoxicity of phenols against melanotic human melanoma cell lines IRE 1 and IRE 2, and the lymphoma- and leukemia-derived cell lines RAJ I and K 562, has shown that monophenols and resorcinol are less toxic than di- (*ortho* and *para*) and triphenols. The major component of toxicity for up to 24 h is due to toxic oxygen species acting outside the cells and not due to cellular uptake of these phenols directly.<sup>12</sup> Nevertheless, many phenolic compounds appear to act by inhibiting mitochondrial electron transport<sup>13–15</sup> and/or by decoupling oxidative phosphorylation.<sup>16</sup> It may be hypothesized that in some cases these activities are due to catecholic or 1,4-hydroqui-

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none metabolites, or the corresponding semiquinone free radicals. This is justified because both the reduction of quinones and the oxidation of hydroquinones to afford semiquinones have been related to biological properties such as quinone cytotoxicity and antitumor activity.<sup>17–19</sup>

It seems noteworthy that a phenolic aryl ketone grouping is a common feature in many of these biologically active compounds, and it has been suggested as a critical feature for mutagenic activity.<sup>20</sup> In addition, we have demonstrated that compounds with this functionality are able to inhibit tumor cell respiration in the TA3 and multidrug-resistant TA3-MTX-R cell lines.<sup>21</sup> Because cancer cells have a lower rate of respiration than normal cells,<sup>22–25</sup> they may be expected to be more sensitive to mitochondrial inhibition.

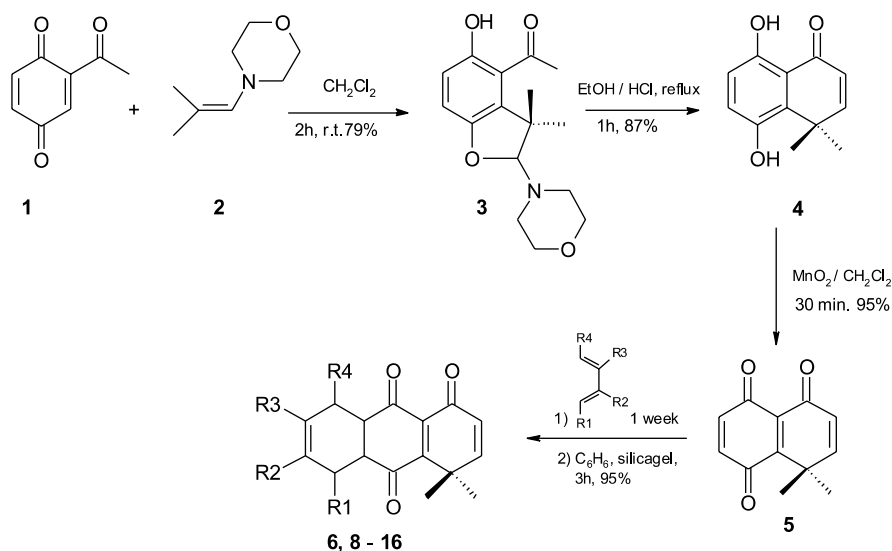
In our previous report, we showed that compounds incorporating a carbonyl group at position *ortho* with regard to a phenol function inhibit tumor cell respiration. We also suggested that the phenoxyl radicals derived from these compounds remain inside the tumor cells at levels sufficient to inhibit oxygen uptake.<sup>21</sup> In that analysis, the intramolecular hydrogen bond of the hydroxyl proton *ortho* to a carbonyl group was seen as a factor weakening the O–H bond, as is also the case in hydrogen bond-acceptor solvents.<sup>26</sup> However, during the past few years, the interest in the effect of intramolecular interactions on the reactivity of phenolic functions has increased considerably. Experimental studies have shown that phenolic hydrogens involved in intramolecular H-bonding are actually less reactive toward peroxy radicals than free hydroxyl groups. It has also been shown that their reactivity is less affected by H-bond-acceptor solvents, and that the stabilization of phenol is lost in the phenoxyl radical, so that the energy needed to abstract the hydrogen atom is greater than in non-H-bonded phenols.<sup>27</sup> Conjugated carbonyl groups would also be expected to increase the oxidation potential,<sup>28</sup> thus hindering free radical formation by electron

transfer. However, structure–activity relationships based on the stability of free radicals derived from our earlier series of phenolic compounds<sup>21</sup> strongly suggest that such free radicals are generated in the tumor cell and are able to disrupt mitochondrial oxygen uptake.

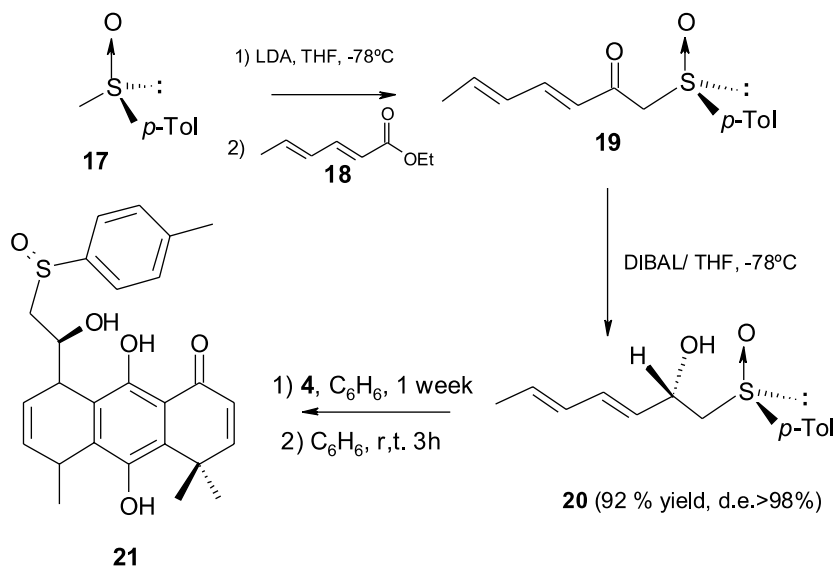
In our previous paper,<sup>21</sup> we reported that 4,4-dimethyl-5,8-dihydroxynaphthalene-1-one (**4**) and a series of derivatives inhibit mitochondrial respiration at low micromolar to sub-micromolar concentrations in the TA3 and TA3-MTX-R cell lines. Considering that alkylation of the hydroquinone moiety should stabilize the semiquinone free radical presumably involved in the inhibition of cellular respiration, we screened another set of analogues of **4** that incorporate a third ring in the molecular structure, blocking the free positions of the aromatic ring. Some members of this series have shown antifungal activity against *Botrytis cinerea*.<sup>29</sup> The results for these compounds as inhibitors of mitochondrial respiration are reported here. Besides, in order to study the effect of the title compound in a human tumor cell line, we tested it against human monocytic leukemia U937 cells. This cell line has proved useful as a model for studying the mechanism of cell death induced by various compounds which alter the redox state of the cells.<sup>30,31</sup>

Compounds **4**, **6**, **8–16** were obtained according to Scheme 1.<sup>32–34</sup>

Compound **21** was synthesized by the Diels–Alder reaction between the enantiomerically pure diene **20** and quinone **5**, as shown in Scheme 2. The synthesis of diene **20** was achieved by condensation of the anion derived from (*SR*)-methyl-*p*-tolylsulfoxide<sup>35</sup> (**17**) with ethyl sorbate (**18**). The obtained  $\beta$ -ketosulfoxide (**19**) was stereoselectively reduced to (*2S,SR*)-1-*p*-tolylsulfinyl-3,5-heptadien-2-ol (**20**) with DIBAL.<sup>35</sup> The Diels–Alder adduct was transformed to the corresponding hydroquinone **21** following the procedure described in Scheme 1. The regiochemistry of **21** was assessed by HMQC and



Scheme 1.



Scheme 2.

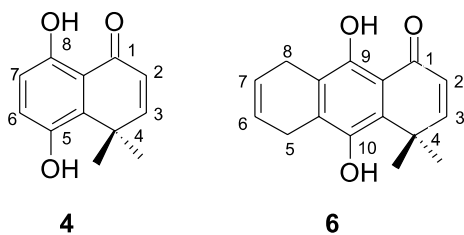
HMBC NMR experiments, but the stereochemistry of the ring stereogenic centers has not yet been determined.

## 2. Results and discussion

The reported activities of **4** and 9,10-dihydroxy-4,4-dimethyl-5,8-dihydro-1(4*H*) anthracenone, **6**, against the TA3 mouse carcinoma cell line and its multidrug-resistant variant TA3-MTX-R,<sup>21</sup> are shown in Table 1.

Substituting positions C6 and C7 of hydroquinone **4** by incorporation of a third ring raises the activity by a factor of 15 in the TA3 cell line and 26-fold in the TA3-MTX-R subline. To examine the possible antitumor activity of these compounds, we investigated their effect on the growth of the human U937 cell line. Exponentially dividing cells were treated with increasing concentrations of **4** (21.58–75.66  $\mu\text{M}$ ) and **6** (3.37–18.80  $\mu\text{M}$ ) for 48–96 h. Both compounds caused a dose-dependent and time-dependent inhibition of cell growth reaching maximal  $\text{IC}_{50}$  values between days 3 and 4, depending on the concentration of **4** or **6** (Table 2). The dose-dependent growth inhibition of the U937 cell line caused by com-

Table 1. Inhibition of oxygen uptake by naphthalenone **4** and anthracenone **6**



Compound	$\text{IC}_{50}$ (mM) TA3	$\text{IC}_{50}$ (mM) TA3-MTX-R
<b>4</b>	$1.25 \pm 0.014$	$1.83 \pm 0.07$
<b>6</b>	$0.08 \pm 0.01$	$0.07 \pm 0.01$

Table 2. Growth inhibition of the U937 cell line at 72 h by compounds **4** and **6**.

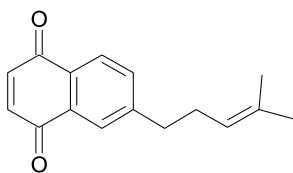
Compound	$\text{IC}_{50}$ ( $\mu\text{M}$ )
<b>4</b>	40.39
<b>6</b>	7.96

pounds **4** and **6** (Table 2) showed a similar trend to that observed for the inhibition of oxygen uptake by the TA3 and TA3-MTX-R mouse carcinoma cell lines. Considering that the cytotoxicity test takes several days, and the oxygen uptake test (5 separate determinations) takes about 3 h, the latter assay can be used as a quick test for preliminary screening of possible anticancer activity.

The results obtained with these two compounds support the hypothesis that, as suggested in our previous paper,<sup>21</sup> an increase in the stability of the free radical derived from the hydroquinone substrate, due now to annulation at C6 and C7, leads to increased activity. However, improved cell penetration resulting from the greater lipophilicity of **6** cannot be disregarded.

Further confirmation of the validity of the oxygen uptake assay as a preliminary screen of cytotoxicity was obtained by testing the activity of 6-(4-methylpent-3-enyl)-1,4-naphthoquinone (**7**) as an inhibitor of cellular respiration of the mouse TA3-MTX-R cell line. Molecule **7** is the most cytotoxic member of a series of terpenylquinone and hydroquinone derivatives,<sup>36</sup> which we synthesized according to the published procedure. The reported activities ( $\text{IC}_{50}$ ) of this compound against the following cell lines are given in parentheses: P-388 (murine lymphocytic leukemia: 0.4  $\mu\text{M}$ ), A-549 (human lung carcinoma: 1.0  $\mu\text{M}$ ), HT-29 (human colon carcinoma: 1.0  $\mu\text{M}$ ), and MEL-28 (human melanoma: 0.4  $\mu\text{M}$ ).<sup>35</sup> We found that compound **7** is highly active as an inhibitor of oxygen uptake in the mouse TA3-MTX-R cell line with an  $\text{IC}_{50}$  value of  $7.40 \pm 0.03 \mu\text{M}$ . Thus, the  $\text{IC}_{50}$  for oxygen uptake inhibition is about an order of

magnitude greater than the IC<sub>50</sub> for cytotoxicity, as found for compounds **4** and **6**.



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With the goal of obtaining a preliminary structure–activity relationship and to confirm that inhibition of oxygen uptake is not due to free radical generation outside the cells, we tested a series of compounds bearing substituents on the additional ring. This series consists of tricyclic hydroquinones bearing one or two substituents at positions C5 to C8. In the monomethylated series, the partition coefficient, log  $P_{oct}$ , has a value of about 3.16 (vs 2.98 for the parent compound: ChemOffice) and should remain approximately constant because only the position of the substituent changes. Therefore, assuming passive transport, any change in activity should reflect some selectivity in the compound's interaction in the interior of the cell.

All derivatives were less active than the parent hydroquinone **6**, in both cell lines, and with the exception of **9**, all were more potent (Table 3) than the reference compound **4** (Table 2). Substitutions are better tolerated at some positions than others. Thus, compound **9**, bearing the methyl group at C6, is the least potent in the monomethylated series, being about three times less active than hydroquinones **8**, **10**, and **11**, which bear the methyl group at positions C5, C7 or C8, respectively. Comparison between the activities of compounds **9** and **12** suggests that introduction of a second methyl group at C7 may offset the unfavorable effect of the methyl group at C6, since in both cell

lines the activity of compound **12** is 1.5 to 2 times greater than that of **4**, in spite of the further increase in lipophilicity.

A comparison among the disubstituted compounds **13–16** shows that replacement of a methyl group by a hydroxymethyl or an acetoxymethyl group, at C8 or C5 leads to compounds with very similar activities. The introduction of a bulky substituent at C8, however, as in compound **21**, abolishes the activity.

In summary, 9,10-dihydroxy-4,4-dimethyl-5,8-dihydro-1(4*H*)-anthracenone (**6**) inhibits TA3 and methotrexate-resistant TA3-MTX-R tumor cell respiration with IC<sub>50</sub> values below 10<sup>-4</sup> M. This represents a potency more than one order of magnitude better than that of the original prototype (**4**). Compound **6** also inhibits the growth of the human tumor U937 cell line at low micromolar concentrations. Lipophilicity does not seem to be an important factor in determining the potency of a set of analogues of **6**, as oxygen uptake inhibitors in TA3 and TA3-MTX-R cells. Increased steric bulk, particularly near C6 and to a lesser extent near C8, may also be an unfavorable feature.

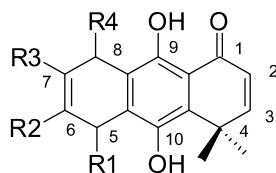
### 3. Experimental

#### 3.1. Chemicals

The <sup>1</sup>H and <sup>13</sup>C NMR spectra were performed at 300.13 and 75.47 MHz, respectively, using CDCl<sub>3</sub> as solvent. The chemical shifts are reported as ppm downfield from TMS for <sup>1</sup>H NMR and relative to the central CDCl<sub>3</sub> resonance (77.0 ppm) for <sup>13</sup>C NMR. Melting points are uncorrected.

Tris-HCl was from Sigma. Hydroquinones **4**,<sup>32</sup> **7**,<sup>35</sup> **6**, **8–12**,<sup>33</sup> **13–16**<sup>34</sup> were synthesized following the described

Table 3. Inhibitory activities of the anthracenone derivatives



Compound	R1	R2	R3	R4	IC <sub>50</sub> (mM) TA3	IC <sub>50</sub> (mM) TA3-MTX-R
<b>6</b>	H	H	H	H	0.08 ± 0.01	0.07 ± 0.01
<b>8</b>	CH <sub>3</sub>	H	H	H	0.40 ± 0.02	0.60 ± 0.02
<b>9</b>	H	CH <sub>3</sub>	H	H	1.75 ± 0.35	1.80 ± 0.4
<b>10</b>	H	H	CH <sub>3</sub>	H	0.50 ± 0.01	0.70 ± 0.021
<b>11</b>	H	H	H	CH <sub>3</sub>	0.55 ± 0.02 <sup>a</sup>	0.61 ± 0.02 <sup>a</sup>
<b>12</b>	H	CH <sub>3</sub>	CH <sub>3</sub>	H	0.86 ± 0.01	1.20 ± 0.07
<b>13</b>	CH <sub>3</sub>	H	H	CH <sub>2</sub> OH	0.42 ± 0.02	0.45 ± 0.02
<b>14</b>	CH <sub>2</sub> OH	H	H	CH <sub>3</sub>	0.29 ± 0.02	0.28 ± 0.02
<b>15</b>	CH <sub>2</sub> OAc	H	H	CH <sub>3</sub>	0.39 ± 0.02	0.48 ± 0.02
<b>16</b>	CH <sub>3</sub>	H	H	CH <sub>2</sub> OAc	0.51 ± 0.14	0.57 ± 0.01 <sup>a</sup>
<b>21</b>	CH <sub>3</sub>	H	H	CH(OH)R <sup>b</sup>	Inactive	Inactive

<sup>a</sup> Only two measurements.

<sup>b</sup> R = CH<sub>2</sub>-SO-*p*-Tol.

procedures. The new compounds (**20** and **21**) were synthesized as follows:

**3.1.1. (2*S*,*SR*)-1-(*p*-tolylsulfinyl)-3,5-heptadien-2-ol (**20**).** (*R*)-Methyl-*p*-tolylsulfoxide (500 mg, 3.24 mmol) in dry THF (5 ml) was added to a solution of LDA (3.2 mmol) in THF (5 ml) at  $-78^{\circ}\text{C}$ . The mixture was allowed to warm up to  $0^{\circ}\text{C}$  and was kept at this temperature for 30 min. After cooling again to  $-78^{\circ}\text{C}$ , and after a few minutes, a solution of ethyl sorbate (300 mg, 2.14 mmol) in dry THF (10 ml) was slowly added. The mixture was stirred for 2 h, and saturated  $\text{NH}_4\text{Cl}$  solution (10 ml) was added. The organic phase was separated, and the aqueous layer was extracted with  $\text{CH}_2\text{Cl}_2$  (4  $\times$  10 ml). The organic solutions were dried with anhydrous  $\text{MgSO}_4$  and the solvent was removed under reduced pressure. The residue was purified by silica gel column chromatography eluting with EtOAc–hexane (1:1). Yield, 478 mg (90%) of (*SR*)-1-(*p*-tolylsulfinyl)-3,5-heptadien-2-one (**19**).  $^1\text{H}$  NMR  $\delta$ : 1.86 (d, 3H,  $J = 6.0$  Hz); 2.39 (s, 3H); 3.86 (d, 1H,  $J = 13.5$  Hz); 4.07 (d, 1H,  $J = 13.5$  Hz); 6.05–6.34 (m, 3H); 7.09 (dd, 1H,  $J_1 = 10$  Hz,  $J_2 = 15.6$  Hz); 7.29 (d, 2H,  $J = 8.1$  Hz); 7.53 (d, 2H,  $J = 8.1$  Hz).  $^{13}\text{C}$  NMR  $\delta$ : 19.02; 21.47; 66.89; 124.22; 127.23; 130.02; 130.11; 139.91; 142.09; 143.07; 146.33; 190.92. mp  $75.7$ – $77.5^{\circ}\text{C}$ . IR (KBr) 3429; 3026; 2985; 2906; 1680; 1630; 1588; 1041;  $814\text{ cm}^{-1}$  HRMS found (M+H) 249.0941  $\text{C}_{14}\text{H}_{16}\text{O}_2\text{S}$  requires M+H 249.0949.

Compound **20** (110 mg, 0.44 mmol) in dry THF (2.5 ml) was cooled to  $-78^{\circ}\text{C}$  and a solution of DIBAL (0.6 ml, 1.0 M in hexane) was added dropwise. After 45 min, methanol (0.5 ml) was added and the mixture was allowed to reach room temperature. The solvent was removed, and the residue was suspended in a saturated  $\text{NH}_4\text{Cl}$  solution (5 ml) and stirred for 15 min. The product was extracted with EtOAc, dried over anhydrous  $\text{MgSO}_4$ , and the solvent was removed. Purification by silica gel column chromatography with an EtOAc–hexane mixture (1:0.7) (v/v) afforded 101 mg (92%).  $^1\text{H}$  NMR  $\delta$ : 1.74 (d, 3H,  $\text{CH}_3$ ,  $J = 6.5$  Hz); 2.43 (s, 3H,  $\text{CH}_3$ -Ar); 2.71 (dd, 1H,  $J_1 = 13.5$  Hz,  $J_2 = 1.4$  Hz, *CHH*-SOTol); 3.06 (dd, 1H,  $J_1 = 13.3$  Hz,  $J_2 = 9.8$  Hz, *CHH*-SOTol); 4.71 (m, 1H, *CH*-OH); 5.50 (dd, 1H,  $J_1 = 15$  Hz,  $J_2 = 6$  Hz, H3); 5.72 (dq, 1H,  $J_1 = 7$  Hz,  $J_2 = 14$  Hz, H6); 6.00 (dd, 1H,  $J_1 = 14$  Hz,  $J_2 = 10$  Hz, H5); 6.22 (dd, 1H,  $J_1 = 15$  Hz,  $J_2 = 10$  Hz, H4); 7.35 (d, 2H,  $J = 8$  Hz); 7.53 (d, 2H,  $J = 8$  Hz).  $^{13}\text{C}$  NMR  $\delta$ : 18.12; 21.40; 61.18; 67.52; 124.01; 129.74; 130.09; 130.31; 131.72; 139.48; 141.66. mp  $139^{\circ}\text{C}$ . IR (KBr) 3353; 2912; 1658; 1494; 1011;  $811\text{ cm}^{-1}$  HRMS found (M–OH) 233.1000  $\text{C}_{14}\text{H}_{18}\text{O}_2\text{S}$  requires M–OH 233.1000.

**3.1.2. 9,10-Dihydroxy-8-[1-hydroxy-2-(*p*-tolylsulfinyl)-ethyl]-4,4,5-trimethyl-5,8,-dihydro-4*H*-anthracen-1-one (**21**).** 50 mg (0.2 mmol) of diene **20** was added to a solution of 42 mg (0.2 mmol) of quinone **5** in benzene (15 ml). The solution was kept in the dark at room temperature for 10 days. Evaporation of the solvent gave 90 mg (98%) of a mixture of cycloadducts. The

mixture of cycloadducts was redissolved in benzene and stirred overnight at room temperature with silica-gel (1 g). The mixture was filtered and the solid washed with methanol. Evaporation of the solvent gave a mixture of anthracenones (88 mg, 98%). By column chromatography it was possible to separate the major compound **21** (49 mg, 50%).  $^1\text{H}$  NMR  $\delta$ : 1.35 (d, 3H,  $J = 7$  Hz, 5-Me), 1.58 (s, 3H, 4- $\text{CH}_3$ ), 1.64 (s, 3H, 4- $\text{CH}_3$ ), 2.42 (s, 3H,  $\text{CH}_3$ -Ar), 2.86 (dd, 1H,  $J_1 = 2.2$  Hz,  $J_2 = 13.4$  Hz, *CHH*-SOTol), 3.18 (dd, 1H,  $J_1 = 10.4$  Hz,  $J_2 = 13.4$  Hz, *CHH*-SOTol), 3.42 (m, 2H, H-5 and –OH), 3.82 (m, 1H, H-8), 4.52 (m, 1H, 8-*CHOH*), 4.61 (m, 1H, –OH), 5.94 (dd, 1H,  $J_1 = 4.7$  Hz,  $J_2 = 10$  Hz, H-7), 6.12 (dd, 1H,  $J_1 = 5.0$  Hz,  $J_2 = 10.0$  Hz, H-6), 6.2 (d, 1H,  $J = 10$  Hz, H-2), 6.82 (d, 1H,  $J = 10$  Hz, H-3), 7.3 (d, 2H,  $J = 8$  Hz), 7.51 (d, 2H,  $J = 8$  Hz), 13.25 (s, 1H, OH).  $^{13}\text{C}$  NMR  $\delta$ : 21.43, 21.56, 25.06, 25.27, 30.45, 38.18, 40.56, 60.45, 68.36, 113.07, 122.01, 123.71, 123.94, 124.15, 129.97(2), 130.00, 132.90, 133.17, 137.69, 139.73, 141.50, 142.83, 154.17, 161.14, 191.13. mp  $234$ – $236^{\circ}\text{C}$ . IR (KBr) 3430.7, 2924.6, 1598.2,  $1423.2\text{ cm}^{-1}$  Anal. Calcd for  $\text{C}_{26}\text{H}_{28}\text{O}_5\text{S}$ : C, 69.00; H, 6.24; S, 7.08. Found: C, 68.25; H, 6.41; S, 6.39.

### 3.2. Cell respiration

Oxygen uptake was measured polarographically at  $25^{\circ}\text{C}$  with a Clark electrode No. 5331 (Yellow Springs Instruments) and using a YSI model 53 monitor linked to a 100 mV single channel Goerz RE 511 recorder. The 2.0 ml reaction mixture contained 150 mM NaCl, 3 mM KCl, and 10 mM Tris–HCl, pH 7.4, plus 5 mM glutamine as substrate and 2.5 mg protein/mL of either TA3 ascites tumor cells derived from mouse mammary adenocarcinoma or their multidrug-resistant variant TA3.MTX-R,<sup>37</sup> as described before.<sup>21</sup>

### 3.3. Cell viability assay

Cell viability was measured using the trypan blue dye exclusion test. A total of  $1.5 \times 10^5$  cells/well was seeded onto a flat-bottomed 24-well plate, treated with DMSO (0.1%) or increasing doses of compound **4** or **6** in 0.1% DMSO, and incubated for 72 h. The results were expressed as a percentage of the control cells treated with 0.1% DMSO alone, which was always taken as 100%, and were representative of three independent experiments. The  $\text{IC}_{50}$  value was obtained adjusting the dose–response curve to a sigmoidal model ( $a + (b - a) / 1 + 10^{(x - c)}$ ), where  $c = \log \text{IC}_{50}$ . The human U937 myeloid-derived cancer cell line was obtained from the American Type Culture Collection (Manassas, VA). U937 cells were grown in RPMI 1640 (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (HyClone Laboratories) maintained at  $37^{\circ}\text{C}$  in a 5%  $\text{CO}_2$  atmosphere.

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