Preparation and Cytotoxicity toward Cancer Cells of Mono(arylimino) Derivatives of *β*-Lapachone

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A regio- and stereospecific synthesis of monoarylimino *o*-quinones derived from *β*-lapachone (**1**) was achieved by treatment of the quinone with a slight excess of an arylamine in the presence of an excess of triethylamine/titanium tetrachloride 4:1. Imine formation occurred exclusively at position 6, giving the *Z* diastereomer, as determined by single-crystal X-ray analysis. In vitro tests for cytotoxicity in 55 human cancer cell cultures showed a substantial loss in activity for the *p*-nitrophenylimine (**5**), whereas the phenylimine (**2**), *p*-methylphenylimine (**3**), and *p*-methoxyphenylimine (**4**) retained (or bettered) most of the cytotoxicity and selectivity of the parent quinone. Preliminary in vivo testing in hollow fiber assays against a standard panel of 12 human tumor cell lines showed that although *β*-lapachone failed, compounds **2** and **3** had good scores with net cell kills.

Introduction

β-Lapachone (2,2-dimethyl-3,4,5,6-tetrahydro-2*H*-naphtho[1,2-*b*]oxino-5,6-dione, **1**) is an *o*-naphthoquinone present as a minor component, together with a variety of other naphthoquinones and anthraquinones, in the heartwood of the lapacho tree, *Tabebuia avellanedae* Lorentz ex. Griseb. (Bignoniaceae), and other *Tabebuia* trees native to Central and South America.[1,2] *β*-Lapachone has demonstrated activity against various cancer cell lines[3,4] and at lower doses is a radiosensitizer of several human cancer cell lines.[5] It gives rise to a variety of effects in vitro including the inhibition or activation of topoisomerase I in a distinct manner from that of other topoisomerase I inhibitors (e.g., camptothecin)[4,6] and the induction of topoisomerase IIα mediated DNA breaks.[7] In combination with taxol, *β*-lapachone has shown to be highly effective against established human ovarian and prostate tumors implanted in immunosuppressed mice.[8] Despite the above, its mechanism of action in vivo and its targets remain largely unknown. Recently, Pink et al. have presented evidence that supports the participation of NAD(P)H:quinone oxidoreductase (NQO1) in the activation process of *β*-lapachone, thus enhancing its toxicity.[9] NQO1 is overexpressed in a number of tumors including breast, lung, and colon cancers, compared with surrounding normal tissue, and may be exploited as an intracellular target.

Several synthetic analogues of *β*-lapachone, all of which retain the intact *o*-quinone moiety or eventually correspond to the isomeric *p*-quinones, have been tested for anticancer activity[7,10,11] and more recently as antipsoriatic agents;[12] however, to the best of our knowledge no attempts have been made to obtain analogues with modifications at the center of redox activity that may alter the redox cycling characteristics of the molecule. Iminoquinones show less facile redox cycling and radical generation than the corresponding quinones, and in some cases (e.g., 5-iminodaunorubicin) these properties have led to lessened toxicity as well as a better understanding of the mechanism of action of the parent quinone.[13] In particular, we were interested in mono(arylimino) derivatives in view of the increased potency and selective cytotoxicity toward cancer cells reported for one such compound derived from a *p*-naphthoquinone.[14] We now describe the synthesis, characterization, and cytotoxicity toward human cancer cells of a series of mono(arylimino) derivatives of *β*-lapachone.

Results and Discussion

Chemistry. Imine preparation is usually carried out by reaction of the carbonyl compound with an amine in acidic conditions and/or in the presence of a dehydrating agent; however, these methods are inefficient for hindered carbonyls. The condensation product of a nonsymmetrical *p*-quinone with aniline has been reported to occur readily with high regio- and stereoselectivity in an anhydrous medium in the presence of titanium tetrachloride.[14] Although *o*-quinones are easily rearranged to the corresponding *p*-quinones under acidic conditions, the use of a complexing agent such as titanium tetrachloride seemed to be appropriate to stabilize the *o*-quinone moiety; HF/6-31G** ab initio calculations supported the formation of a stable complex, with the quinone ring adopting a flat conformation in which C-6 appeared to be less hindered for attack by nucleophiles. Thus, the reaction of *β*-lapachone (**1**) with a 3-fold excess of aniline and titanium tetrachloride in dichloromethane gave a single product with complete disappearance of the starting material as determined

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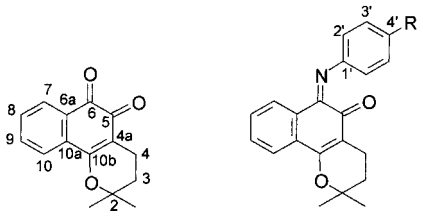

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2 R = H
3 R = CH_3
4 R = OCH_3
5 R = NO_2

by ^{1}H NMR analysis of the crude reaction mixture. However, a low yield (35%) of pure monoiminoquinone (**2**) with extensive recovery of β-lapachone resulted after isolation and purification, as a large part of the desired product was hydrolyzed during workup (Table 1, entry 1). The yield could be improved to 70% by using a 6-fold excess of aniline, although, still, β-lapachone was recovered after isolation and purification and formation of byproducts occurred mainly due to disubstitution, as determined by ^{1}H and ^{13}C NMR of the crude product (Table 1, entry 2). It was apparent that the main function of the excess aniline was to remove the titanium tetrachloride once imine formation was complete, thus "protecting" the product during isolation and purification; in that case, replacement of the excess aniline with a non-nucleophilic amine such as triethylamine would maintain a basic medium without contributing to the addition reaction. After several trials, a quantitative yield of iminoquinone **2** was obtained using only 1.15 equiv of aniline and a 4:1 ratio of triethylamine/titanium tetrachloride (Table 1, entry 3). Under these conditions, a titanium tetrachloride−triethylamine complex was formed at the end of the reaction and the pure iminoquinone could be easily recovered in high yield by extraction of the crude product with cyclohexane and filtration.

The position of the imine substituent in derivative **2** was established initially by ^{1}H and ^{13}C NMR by comparison with the spectra of β-lapachone.[15] Thus, H-7 showed a 0.17 ppm downfield shift while the rest of the signals remained practically unchanged, indicating that reaction had taken place at position 6 as predicted by the ab initio calculations. On the other hand, the moderate magnitude of this shift suggested that the new phenyl ring was oriented away from H-7 that is, a *Z* stereochemistry at the C−N double bond. In the ^{13}C NMR spectrum a normal carbonyl remained at 177.5 ppm, whereas the imino carbon was shifted upfield to 150.5 ppm. A COLOC experiment showed long-range correlations for the imino carbon with H-7 (150.5/8.23 ppm) and the carbonyl carbon with H-4 (177.5/2.40 ppm), thus confirming the substitution at C-6.[15]

The reaction was repeated with substituted anilines containing electron-donating or -withdrawing groups, namely, *p*-toluidine, *p*-methoxyaniline, and *p*-nitroaniline (Table 1, entries 4−6), yielding iminoquinones **3**−**5**. The nitro derivative **5** was sparingly soluble in cyclohexane and had to be recovered by recrystallization of the crude product from methanol, which resulted in a lower yield. Comparison of the ^{1}H NMR data of compounds **2**−**5** shows that hydrogens 3, 4, and 10 are virtually unaffected and almost identical to those in β-lapachone (**1**), whereas H-7 is shifted downfield by 0.15−0.18 ppm. Only small effects were observed in the ^{13}C NMR spectra except for C-6, when compared to **1**. Chemical shift differences among the iminoquinones were restricted to the additional aromatic ring supporting the fact that substitution occurred at the same position in all cases and that the stereochemistry of the imine double bond was also the same. Further structural confirmation was obtained for the nitro derivative **5**, which rendered crystals adequate for X-ray crystallography. The structure obtained showed the imine on C-6 with the *Z* stereochemistry. All iminoquinones synthesized were stable compounds, which could be stored for prolonged periods without noticeable decomposition.

Table 1. Reaction of β-Lapachone (**1**) with Arylamines[a]

arylamine	β-lapachone/ arylamine ratio	β-lapachone/ triethylamine ratio	products (yield %)[b]
aniline	1:3		**1** (60%), **2** (35%)
aniline	1:6		**1** (15%), **2** (70%)[c]
aniline	1:1.15	1:6	**2** (100%)
p-toluidine	1:1.15	1:6	**3** (98%)
p-methoxyaniline	1:1.15	1:6	**4** (84%)
p-nitroaniline	1:1.15	1:6	**5** (46%)[d]

[a] β-lapachone/$TiCl_4$ ratio 1:1.5 in all cases. [b] Yields correspond to isolated products. [c] Ca. 10% of a byproduct tentatively characterized by NMR as the 5,6-diimine was also formed. [d] Product was isolated by recrystallization from methanol.

Biological Results. The iminoquinones **2**−**5** were examined for antiproliferative activity against the human cancer cell lines in the National Cancer Institute screen, in which the activity of each compound was evaluated with ∼55 different cancer cell lines of diverse tumor origins. The GI_{50} values obtained with selected cell lines, along with the corresponding mean graph midpoint (MGM) values, are summarized in Table 2. The mean graph midpoints are based on a calculation of the average GI_{50} for all of the cell lines tested (∼55) in which values below and above the test range (10^{-4}−10^{-8} M) are taken as the minimum (10^{-8} M) and maximum (10^{-4} M) drug concentrations used in the screening test.[16] Data for β-lapachone (**1**) are included for comparison.[17]

Derivatives **2** and **3** showed activities similar to those of β-lapachone, with especially high selectivity for certain human melanoma and breast tumor cells (see Supporting Information). Introduction of polar substituents on the additional ring as in **4** and **5** decreased selectivity (evidenced by a diminished range of LC_{50} values pertaining to the MGM). This deleterious effect and an overall loss of activity were particularly intense for the electron-withdrawing nitro substituent, rendering derivative **5** considerably less cytotoxic.

Compounds **2** and **3** were selected for preliminary in vivo testing using the hollow fiber assay at the NCI against a standard panel of 12 human tumor cell lines.[18] Table 3 shows the results obtained compared to β-lapachone (**1**) using the point system adopted by the NCI, where a value of 2 is assigned for each compound dose that results in a ≥50% reduction in viable cell mass (results of individual cell lines are not reported due to the statistical nature of the assay). Interestingly, although β-lapachone completely failed in this assay, the aryliminoquinones **2** and **3** gave net cell kills (i.e., reduction of the viable cell mass below the level present

Table 2. Cytotoxicity of β-Lapachone (**1**) and Analogues **2**−**5** toward Human Cancer Cells

	cytotoxicity (GI_{50}, μM)[a]									
compd	leukemia CCRF-CEM	lung NCI-H23[b]	colon COLO 205[b]	CNS SF-295[b]	melanoma LOXIMVI[b]	ovarian OVCAR-5[b]	renal SN12C	prostate DU-145	breast MDA-MB-435[b]	MGM[c]
1[d]	1.41	0.76	1.78	1.54	0.31	1.82	1.45	1.74	21.4	1.12
2	1.55	1.23	1.55	1.86	0.22	1.81	1.12	1.56	0.20	1.17
3	2.56	0.92	1.80	1.26	0.30	2.03	0.46	1.76	0.19	0.89
4	1.54	1.17	1.02	0.88	0.89	12.3	0.90	0.87	0.096	1.15
5	19.6	18.3	16.8	14.9	7.59	14.5	15.9	16.8	15.8	15.5

[a] Cytotoxicity GI_{50} values are the concentrations corresponding to 50% growth inhibition. [b] Cell line used in the hollow fiber assay (Table 3). [c] MGM is the mean graph midpoint for growth inhibition of all human cancer cell lines successfully tested. [d] Data for β-lapachone (NSC 629749) were taken from ref 17.

Table 3. In Vivo Hollow Fiber Assay Data for Compounds **1**−**3**[a]

compd	ip[b]	sc[b]	cell kill[c]
1	0	0	no
2	2	8	yes
3	2	12	yes

[a] Each compound was assessed by intraperitoneal injection at two dose levels against a standard panel of 12 human tumor cell lines (for details see ref 18). [b] A value of 2 is assigned for each compound dose that results in ≥50% reduction in viable cell mass of intraperitoneal (ip) or subcutaneous (sc) implants. [c] Reduction in the viable cell mass below the level present at the start of the experiment for at least one cell line in either implant site.

at the start of the experiment) and were active in four and six of the distant site combinations (intraperitoneal drug/subcutaneous culture), respectively.

Conclusion

Although β-lapachone cytotoxicity toward cancer cells in vitro is well documented, in vivo data appear to be less conclusive.[9] This may be ascribed in part to (redox?) activation/inactivation mechanisms operating in the whole organism. The change in redox cycling characteristics inherent to the aryliminoquinone structures described above and the fact that this change does not necessarily affect overall activity and selectivity of the parent quinone (e.g., compounds **2** and **3**)[19] may constitute an alternative approach in the search for new anticancer agents derived from this interesting natural product.

Experimental Section

Melting points were determined in a Fisher-Johns apparatus and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AC-200 (200.13 and 50.32 MHz) NMR spectrometer. Multiplicity determinations (DEPT) and 2D spectra (COSY 45, HETCOR, and COLOC) were obtained using standard Bruker software. Chemical shifts are given in parts per million downfield from TMS as internal standard. EIMS were collected on a VG Trio-2 mass spectrometer at 70 eV by direct inlet. IR spectra (1% KBr dispersion) were measured on a Nicolet Magna 550 FT IR spectrophotometer. Ab initio calculations were performed with the Gaussian 98W software package.[20] Single-crystal X-ray measurements were performed on an R3m Siemens four-circle diffractometer, with graphite monochromated Mo Kα radiation. The structure was solved by direct methods with SHELXS97[21] and refined (hydrogen atoms included) by full-matrix least squares in F^2 using SHELXL97.[22] Molecular plots were drawn with XP, in the SHELXLTL-PC package.[23] TLC analysis was performed on an Si gel 60 F254 (0.2 mm thick). β-Lapachone (mp 155−156 °C) was obtained by acid cyclization of lapachol isolated from the heartwood of *Tabebuia avellanedae*[24] and identified by spectroscopic methods (NMR and MS).

General Procedure for the Synthesis of Aryliminoquinones 2—5. To a vigorously stirred solution of β-lapachone (**1**, 1 g, 4.13 mmol) in dry dichloromethane (50 mL) at room temperature under an N_2 atmosphere was added a 1.0 M solution of titanium tetrachloride in dichloromethane (2.07 mL, 2.07 mmol). To the resulting violet solution was added a solution of the aromatic amine (4.75 mmol) in dichloromethane (15 mL) followed immediately by dry triethylamine (3.45 mL, 24.8 mmol). Two portions of 1.0 M solution of titanium tetrachloride in dichloromethane (2.06 mL, 2.06 mmol each) were added at 10 min intervals, and then the reaction mixture was poured over 100 mL of cold water and extracted with dichloromethane. The organic layer was dried with $MgSO_4$ and evaporated in vacuo. The solid residue was suspended in cyclohexane, filtered, and washed thoroughly with cyclohexane; the combined filtrates were evaporated in vacuo to yield pure aryliminoquinone.

2,2-Dimethyl-(*Z*)-6-phenylimino-3,4,5,6-tetrahydro-2*H*-naphtho[1,2-*b*]oxin-5-one (2). Iminoquinone **2** was isolated in 100% yield as a brown-red solid that was recrystallized from *n*-hexane: mp 136−138 °C; IR (KBr) 2980, 2931, 1647, 1587, 1389, 1172, 748, 689 cm^{-1}; ^{1}H NMR δ 8.23 (m, 1H, H-7), 7.81 (m, 1H, H-10), 7.45 (m, 2H, H-8,9), 7.32 (t, J = 7.8 Hz, 2H, H-3′), 7.05 (tt, J = 7.6 and 1.1 Hz, 1H, H-4′), 6.74 (dd, J = 7.6 and 1.2 Hz, 2H, H-2′), 2.40 (t, J = 6.6 Hz, 2H, H-4), 1.76 (t, J = 6.6 Hz, 2H, H-3), 1.42 (s, 6H, 2-CH_3); ^{13}C NMR δ 177.5 (C-5), 161.2 (C-10b), 153.1 (C-1′), 150.5 (C-6), 132.6 (C-10a), 131.0 (C-8), 130.0 (C-9), 129.1 (C-6a), 128.7 (C-3′), 126.8 (C-7), 123.1 (C-10), 122.7 (C-4′), 115.6 (C-2′), 111.9 (C-4a), 78.3 (C-2), 31.5 (C-3), 26.6 (2-CH_3), 16.0 (C-4); EIMS, m/z 317 (M^+, 32), 302 (6), 261 (20), 232 (67), 77, (53), 41 (100). Anal. Calcd for $C_{21}H_{19}O_2N$: C, H, N.

2,2-Dimethyl-(*Z*)-6-(4-methylphenylimino)-3,4,5,6-tetrahydro-2*H*-naphtho[1,2-*b*]oxin-5-one (3). Iminoquinone **3** was isolated in 98% yield as a dark brown solid that was recrystallized from *n*-hexane: mp 138−140 °C; IR (KBr) 2980, 2933, 1647, 1568, 1384, 1311, 939, 775 cm^{-1}; ^{1}H NMR δ 8.21 (dd, J = 9.0 and 2.3 Hz, 1H, H-7), 7.83 (dd, J = 9.0 and 2.4 Hz, 1H, H-10), 7.49 (m, 2H, H-8,9), 7.14 (d, J = 8.2 Hz, 2H, H-3′), 6.67 (d, J = 8.2 Hz, 2H, H-2′), 2.42 (t, J = 6.6 Hz, 2H, H-4), 2.34 (s, 3H, 4′-CH_3), 1.78 (t, J = 6.6 Hz, 2H, H-3), 1.43 (s, 6H, 2-CH_3); ^{13}C NMR δ 177.7 (C-5), 161.2 (C-10b), 150.8 (C-1′), 150.5 (C-6), 132.9 (C-4′), 132.5 (C-10a), 131.0 (C-8), 130.0 (C-9), 129.8 (C-6a), 129.4 (C-3′), 126.8 (C-7), 123.1 (C-10), 116.1 (C-2′), 112.1 (C-4a), 78.3 (C-2), 31.8 (C-3), 26.7 (2-CH_3), 20.9 (4′-CH_3), 16.1 (C-4); EIMS, m/z 331 (M^+, 45), 316 (77), 260 (54), 246 (47), 232 (50), 65 (48), 41 (100). Anal. Calcd for $C_{22}H_{21}O_2N$: C, H, N.

2,2-Dimethyl-(*Z*)-6-(4-methoxyphenylimino)-3,4,5,6-tetrahydro-2*H*-naphtho[1,2-*b*]oxin-5-one (4). Iminoquinone **4** was isolated in 84% yield as a dark blue solid that was recrystallized from *n*-hexane: mp 133−135 °C; IR (KBr) 2980, 2929, 1645, 1599, 1498, 1387, 1244, 831, 783 cm^{-1}; ^{1}H NMR δ 8.21 (m, 1H, H-7), 7.81 (m, 1H, H-10), 7.48 (m, 2H, H-8,9), 6.85 (m, 4H, H-2′,3′), 3.80 (s, 3H, OCH_3), 2.43 (t, J = 6.6 Hz, 2H, H-4), 1.78 (t, J = 6.6 Hz, 2H, H-3), 1.43 (s, 6H, 2-CH_3); ^{13}C NMR δ 177.7 (C-5), 161.2 (C-10b), 156.4 (C-4′), 150.9 (C-6), 145.6 (C-1′), 133.1 (C-10a), 130.7 (C-8), 130.0 (C-9), 129.7 (C-6a), 126.8 (C-7), 123.2 (C-10), 118.8 (C-2′), 114.1 (C-3′), 112.1 (C-4a), 78.2 (C-2), 55.4 (OCH_3), 31.9 (C-3), 26.7 (2-CH_3), 16.1

(C-4); EIMS, *m*/*z* 347 (M^+, 11), 316 (11), 291 (15), 248 (20), 159 (28), 57 (42), 41 (100). Anal. Calcd for $C_{22}H_{21}O_3N$: C, H, N.

2,2-Dimethyl-(*Z*)-6-(4-nitrophenylimino)-3,4,5,6-tetrahydro-2*H*-naphtho[1,2-*b*]oxin-5-one (5). Iminoquinone **5** was isolated in 46% yield upon crystallization of the crude solid residue from methanol. The red solid was recrystallized from *n*-hexane/ethyl acetate (1:1): mp 215−217 °C; IR (KBr) 2984, 2933, 1605, 1504, 1333, 1109, 775, 580 cm^{-1}; ^1H NMR δ 8.24 (m, 1H, H-7), 8.23 (d, J = 8.9 Hz, 2H, H-3′), 7.85 (m, 1H, H-10), 7.55 (m, 2H, H-8,9), 6.75 (d, J = 8.9 Hz, 2H, H-2′), 2.40 (t, J = 6.7 Hz, 2H, H-4), 1.81 (t, J = 6.7 Hz, 2H, H-3), 1.45 (s, 6H, 2-CH_3); ^{13}C NMR δ 177.1 (C-5), 162.4 (C-10b), 160.2 (C-1′), 150.3 (C-6), 142.9 (C-4′), 132.1 (C-8), 131.5 (C-10a), 130.5 (C-9), 130.3 (C-6a), 127.5 (C-7), 125.3 (C-3′), 123.7 (C-10), 115.5 (C-2′), 111.5 (C-4a), 79.0 (C-2), 31.7 (C-3), 26.7 (2-CH_3), 16.0 (C-4); EIMS, *m*/*z* 362 (M^+, 23), 345 (40), 315 (10), 289 (11), 231 (30), 69 (63), 55 (35), 41 (100). Anal. Calcd for $C_{21}H_{18}O_4N_2$: C, H, N.

Crystallographic Data and Data Collection Parameters for Compound 5. Red prismatic crystals were recrystallized from methanol: mp 215−217 °C; $C_{21}H_{18}N_2O_4$, M = 362.37, triclinic, space group P-1 (no 2); cell constants a = 8.0374(14) Å, b = 10.8815(14) Å, c = 11.014(3) Å; α = 67.528-(14)°; β = 84.503(17)°; γ = 82.971(12)°; V = 882.2(3) $Å^3$, D_c (Z = 2) = 1.364 g cm^{-3}; crystal dimensions 0.30 × 0.14 × 0.08 mm, reflections measured, 3432; reflections unique, 3122; reflections observed [$I > 2\sigma(I)$], 2109; R = 0.047 and R_w^2 = 0.107.

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Supporting Information Available: Displacement elipsoid diagram and complete crystallographic data (in cif format) for compound **5**, HF/6-31G** calculated structure and relevant data for β-lapachone−$TiCl_4$ complex, and mean graphs of the biological evaluations performed at the NCI for compounds **2**−**5**. This material is available free of charge via the Internet at http://pubs.acs.org.

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