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Trypanosoma cruzi: Activities of lapachol and α- and β-lapachone derivatives against epimastigote and trypomastigote forms

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Abstract—Derivatives of natural quinones with biological activities, such as lapachol, α- and β-lapachones, have been synthesized and their trypanocidal activity evaluated in vitro in *Trypanosoma cruzi* cells. All tested compounds inhibited epimastigote growth and trypomastigote viability. Several compounds showed similar or higher activity as compared with current trypanocidal drugs, nifurtimox and benznidazole. The results presented here show that the anti-*T. cruzi* activity of the α-lapachone derivatives can be increased by the replacement of the benzene ring by a pyridine moiety. Free radical production and consequently oxidative stress through redox cycling or production of electrophilic metabolites are the potential biological mechanism of action for these synthetic quinones.
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1. Introduction

Chagas disease is one of the most important endemic diseases caused by *Trypanosoma cruzi*, which affects 16–18 million people in large areas of Latin America.[1,2] The drugs used for the treatment of this disease are nifurtimox, a nitrofuran derivative, and benznidazole, a nitroimidazole derivate. Both drugs present severe side effects. Furthermore, nifurtimox is no longer used in several countries because of its toxicity.[3–5] The need of effective drugs, without adverse effects, has stimulated the search for new compounds with potential clinical utility.

Many natural and synthetic naphthoquinones have been tested against *T. cruzi* parasites as possible anti-chagasic agents. Among natural naphthoquinones, lapachol (**1**), β-lapachone (**2**), and its α-isomer (**3**) have demonstrated trypanocidal activities.[6–9] Compound **2** inhibited parasite motility progressively and also the growth of epimastigote cultures at a concentration of 0.8 μg/mL. This was one of the first natural product-derived molecules that showed evidence of oxidative stress generated in parasites.[10–13] Interestingly, compounds derived from β-lapachone and with an imidazole ring linked to the naphthopyrane moiety showed enhanced activity compared with **2**.[14] On the other hand, an oxyran derivative of α-lapachone has been described as potent trypanocidal agent.[15]

In the search for new trypanocidal agents, in this work, we evaluate the trypanocidal activity of lapachol, α- and β-lapachone derivatives through inhibition of *T. cruzi* epimastigote growth and trypomastigote viability. We also investigate biological activity, including parasite free radical production and respiration inhibition of the parasite.

Trypanocidal activity of all compounds was tested against *T. cruzi* epimastigote growth. The quinones were incorporated into the medium at different concentrations. Growth inhibition until day 10 was evaluated in comparison to control at day 5. Nifurtimox and benznidazol were used as the reference trypanocidal drugs. To establish the relative efficacy of the quinones

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Table 1. Effect of lapachol derivatives upon culture growth and oxygen uptake in *T. cruzi* epimastigotes

Compound	IC_{k50}[a]	Respiration[b]	Oxygen redox cycling[c]
Control	—	33.28 (100)	2.2 ± 0.15 (100)
Lapachol (**1**)	31.3 ± 0.01	16.21 (51.8)	1.8 ± 0.30 (82)
β-Lapachone (**2**)	0.21 ± 0.01	32.52 (103.9)	2.0 ± 0.40 (91)
α-Lapachone (**3**)	24.7 ± 0.36	33.65 (107.5)	2.2 ± 0.40 (100)
4	3.75 ± 1.36	30.30 (96.8)	2.2 ± 0.20 (100)
6	35.2 ± 0.07	31.49 (100.6)	2.2 ± 0.20 (100)
7	24.5 ± 0.29	43.44 (138.8)	3.8 ± 0.20 (173)
8	1.93 ± 0.07	31.49 (100.6)	2.2 ± 0.10 (100)
10	26.2 ± 1.14	29.86 (95.4)	2.1 ± 0.20 (96)
11	36.3 ± 0.15	44.60 (142.5)	3.7 ± 0.15 (168)
12	>100	39.13 (125)	2.4 ± 0.30 (109)
18	0.19 ± 0.02	10.39 (33.2)	1.2 ± 0.15 (55)
19	20.1 ± 0.04	76.15 (243.3)	5.6 ± 0.30 (254)
20	38.1 ± 0.04	31.36 (100.2)	2.2 ± 0.15 (100)
21	22.2 ± 0.07	42.41 (135.5)	3.7 ± 0.20 (168)
Nifurtimox	9.5 ± 0.10	37.69 (120.4)	2.5 ± 0.15 (113)
Benznidazole	20.6 ± 0.10	18.09 (57.8)	1.8 ± 0.10 (82)

[a] IC_{kc50}, drug concentration needed to lower the growth constant (kc) by 50%.

[b] Oxygen consumption expressed as nanoatoms-gram of oxygen/min/mg protein when drug concentration used was equal to IC_{k50}. Numbers in parentheses correspond to percentage of control.

[c] Oxygen consumption expressed as nanoatoms-gram of oxygen/min/mg protein when drug concentration used was equal to IC_{k50} and 20 mM KCN. Numbers in parentheses correspond to percentage of control with KCN. The results are mean values of three different experiments. See Section 4.

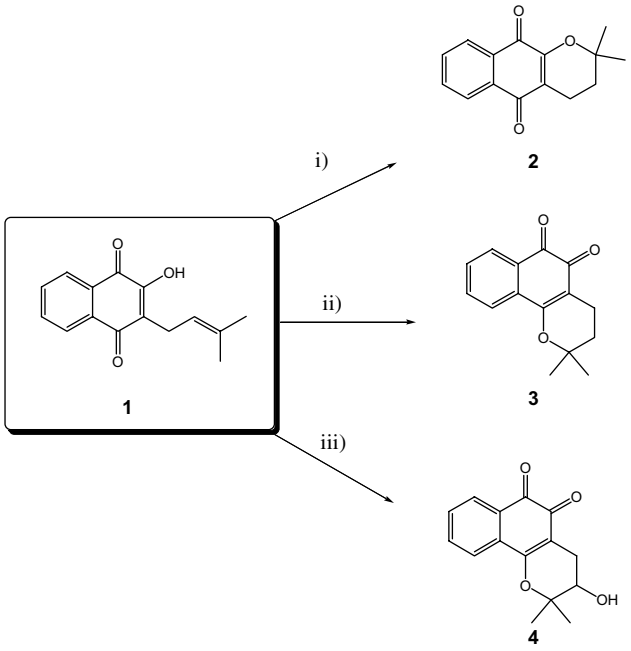
in vitro compared with the standard drugs, the IC_{k50} was determined. IC_{k50} is defined as the drug concentration needed to decrease the growth constant (k) by 50%.

The anti-trypanosomal activity of quinones has been attributed to oxygen radical formation and consequently strong oxidative stress.[16–19] To evaluate this possibility, oxygen uptake experiments with and without cyanide were undertaken. Respiration is a composite value where oxygen uptake depends upon mitochondrial reactions as well as extra-mitochondrial reactions such as redox cycling.[20] Thus, cyanide addition inhibits oxygen consumption in the respiratory chain and allows possible redox cycling induced by the drug to be observed more clearly. The effect of quinones on *T. cruzi* respiration was studied measuring oxygen consumption by epimastigotes at different concentrations of these compounds. The results are presented in Table 1.

2. Results and discussion

2.1. Chemistry

Lapachones **2** and **3** and its hydroxylic derivative **4** were obtained from the commercially available lapachol (**1**) by treatment with different acid conditions[21] and *meta*-chloroperbenzoic acid (MCPBA),[22] respectively (Scheme 1). Pyranonaphthoquinones **7**–**8** were prepared from the easily available Michael adduct **6**[23] by reduction with sodium borohydride followed by cyclization in acid conditions, without isolation of the respective



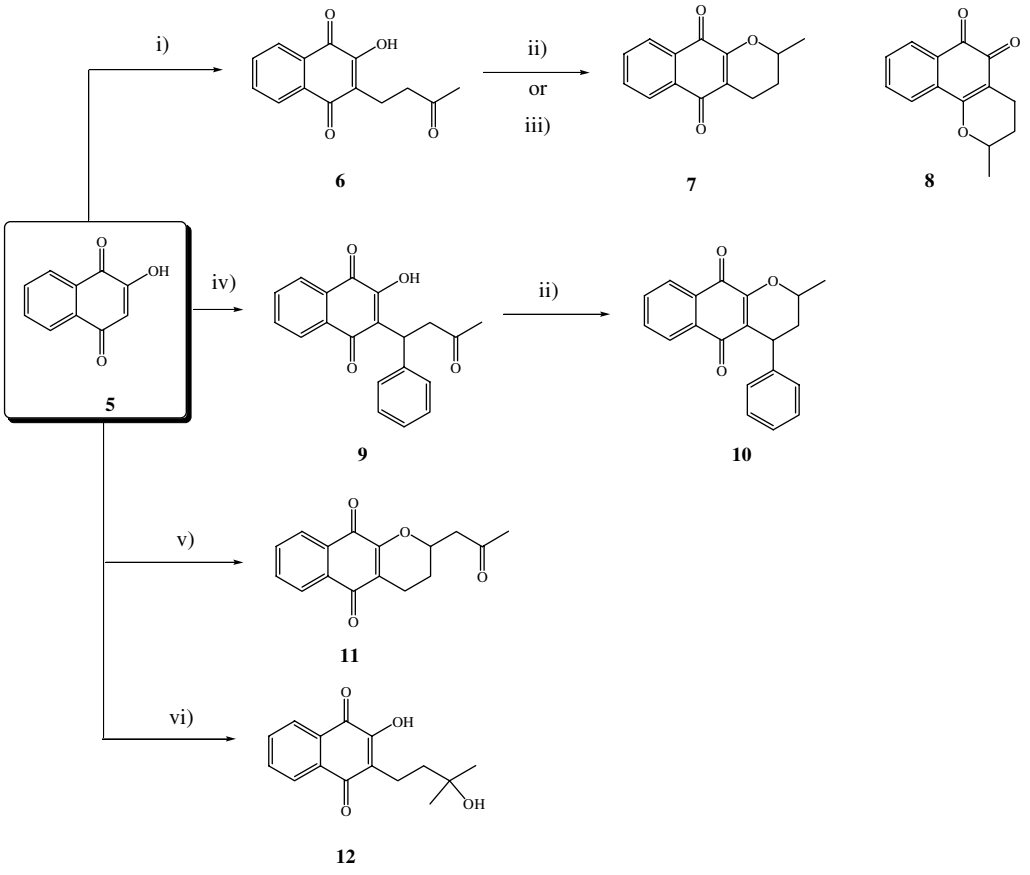
Scheme 1. Synthesis of α- and β-lapachone and derivative **4**. Reagents and conditions: (i) H_2SO_4, rt, 30 min, 80%; (ii) HCl, 50 °C, 3 h, 70%; (iii) CH_2Cl_2, rt, MCPBA, 24 h, (26%).

alcohol intermediate, in 64–75% yields (Scheme 2). In the present study, the new α-lapachone derivative **10** was synthesized through Michael adduct **9**,[24] prepared by reaction of 2-hydroxy-1,4-naphthoquinone **5** with benzalacetone in refluxing quinoline for 3 h in 35% yield (Scheme 1). Reduction of compound **9** with $NaBH_4$ for 3 h in ethanol and further treatment with HCl under reflux for 2 more hours gave the pyranonaphthoquinone **10** in 59% yield as a mixture of diastereoisomers. On the other hand, compounds **11**, **12** were synthesized from **5** according to our previously described procedures.[25,26] Finally, derivatives **18**–**21** were easily prepared in a single step by a Diels–Alder reaction of the pyranonaphthoquinone **13** and 1-substituted electron-rich dienes of type **14**–**17**, followed by aromatization of the resulting adducts (Scheme 3).[27–29]

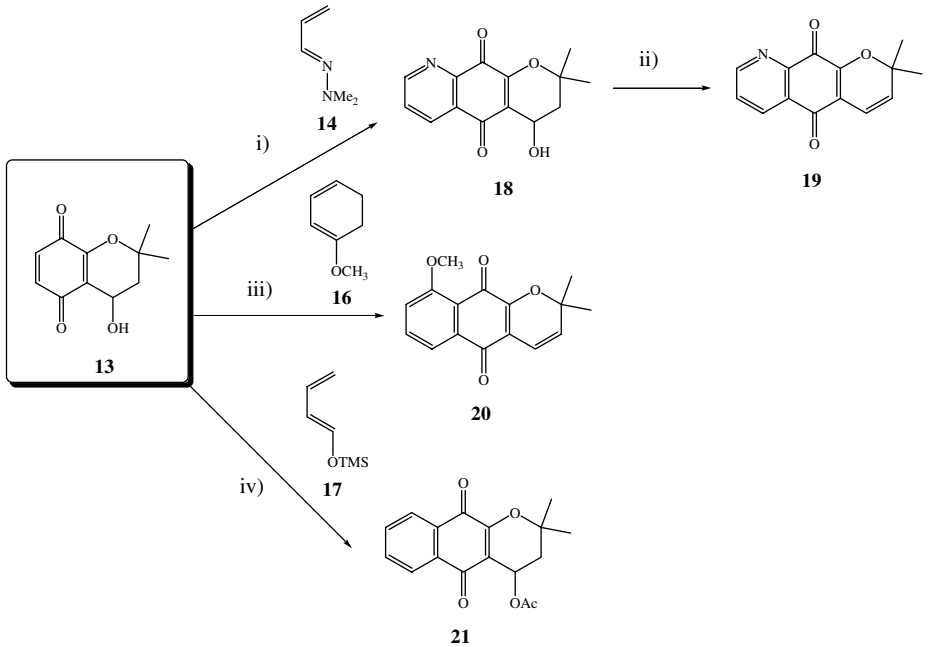
2.2. Biological evaluation

Table 1 shows the effect of the quinones tested on the growth of Tulahuén strain *T. cruzi* epimastigotes, at IC_{k50} concentration. It is evident from these data that all tested compounds inhibit parasite growth, the lapachol-derived naphthoquinone **12** being the least active of the series. However, those compounds with IC_{k50} values higher than 20 μM are equipotent when compared with nifurtimox and benznidazole, and thus devoid of pharmacological interest.

The most active compound among lapachone derivatives was pyranoquinolinequinone **18**, an α-lapachone derivative. This result is very interesting because normally α-lapachones have a weak trypanocidal activity,[15,17,30] indicating that nitrogen substitution in the aromatic ring increases the trypanocidal activity of these



Scheme 2. Synthesis of α- and β-lapachone derivatives **7**–**12**. Reagents and conditions: (i) pyridine, MVK, reflux, 9 h, 90%; (ii) 1—EtOH, $NaBH_4$, reflux, 1 h; 2—H_2SO_4 20%, reflux, 2 h, 64% for **7** and X% for **10**; (iii) 1—EtOH, $NaBH_4$, reflux, 1 h; 2—HCl 10%, reflux, 3 h, 75%; (iv) quinoline, benzalacetone, reflux, 3 h, 35%; (v) Ref. 21; (vi) Ref. 24.



Scheme 3. Synthesis of α-lapachone derivatives **18**–**21**. Reagents and conditions: (i) 1—CH_2Cl_2, **14**, rt, 1,5 h; 2—SiO_2, 10 min, Ag_2O, rt, 5 h, 33%; (ii) toluene, PTSA, reflux, 4 h, 68%; (iii) 1—MeOH, **15**, rt, 1 h; 2—THF, NaH, 0 °C, 30 min; 3—xilene, reflux, 45 min, 79%; (iv) 1—CH_2Cl_2, **17**, rt, 24 h; 2—SiO_2, chromatography, 90%.

compounds. A similar result was obtained in aza-naphthofuranoquinones, as previously reported.[31] β-Lapachone derivatives **4** and **8** were less active than β-lapachone **2**, indicating that a hydroxyl group at C-3 and one

methyl group at C-2 diminish the activity compared with β-lapachone.

Compound **18**, an α-lapachone derivative, is the most potent of this series, having an IC_{k50} lower than current anti-chagasic drugs (50 times and 108 times lower than nifurtimox and benznidazole, respectively). β-Lapachone **2** and its derivatives **8** and **4** showed IC_{k50} values 4–50 times lower than nifurtimox. Comparing compounds **2** and **18** it is noticeable that *p*-quinone **18** shows higher activity than β-lapachone **2**,[17,30] which is observed for the first time. These results suggest that trypanocidal activity may be increased in an *o*-quinone system if the benzene ring is replaced by a pyridine moiety.

The ability of these compounds to produce free radicals in the parasite is a well-known mechanism for the trypanocidal activity of quinones.[16–19] To evaluate this possibility, we conducted experiments of oxygen uptake where cellular respiration was measured at the IC_{k50} concentrations. Quinone addition might produce an increase (redox cycling), a decrease or no alteration in oxygen uptake. The effect of the studied quinones upon *T. cruzi* epimastigote cellular respiration at the IC_{k50} values can be seen in Table 1.

From our analysis of the results, three effects of the quinones upon oxygen uptake can be distinguished: (i) nifurtimox and quinones **7**, **11**, **19**, and **21** increase oxygen uptake. Also, nifurtimox and quinones **7**, **11**, **19**, and **21** present high oxygen redox cycling (Fig. 1), indicating that reactive oxygen species such as superoxide anion and hydroxyl radicals are generated, inducing oxidative stress; (ii) α- and β-lapachone **2**, and quinones **4**, **6**, **8**, and **20** do not inhibit cellular respiration neither produce redox cycling. The trypanocidal action of these compounds is probably mediated by the production of electrophilic metabolites, which bind to and inactivate *T. cruzi* macromolecules. Figure 1 shows a proposal of the metabolic pathways for a molecule with a *p*-quinone nucleus in *T. cruzi* epimastigotes, including the bioreductive process, redox cycling, and generation of electrophilic metabolites[19]; (iii) lapachol and compounds **10** and **18** diminish oxygen uptake and probably inhibit cellular respiration.

Table 2 shows the activity of β-lapachone derivatives upon trypomastigote forms of the parasite. These forms are found in mammalian blood and are the target for anti-chagasic drugs. A viability test such as MTT reduction was carried out using drug concentrations equivalent to IC_{k50} values for epimastigotes. Concentrations used (Table 1) were in the range of 0.19–100 μM, and the value of the MTT test ranged from 3 to 30 percent compared with control. Derivatives **4**, **8**, and **18** showed stronger activity against *T. cruzi* than nifurtimox or benznidazole. These results indicate that mitochondrial

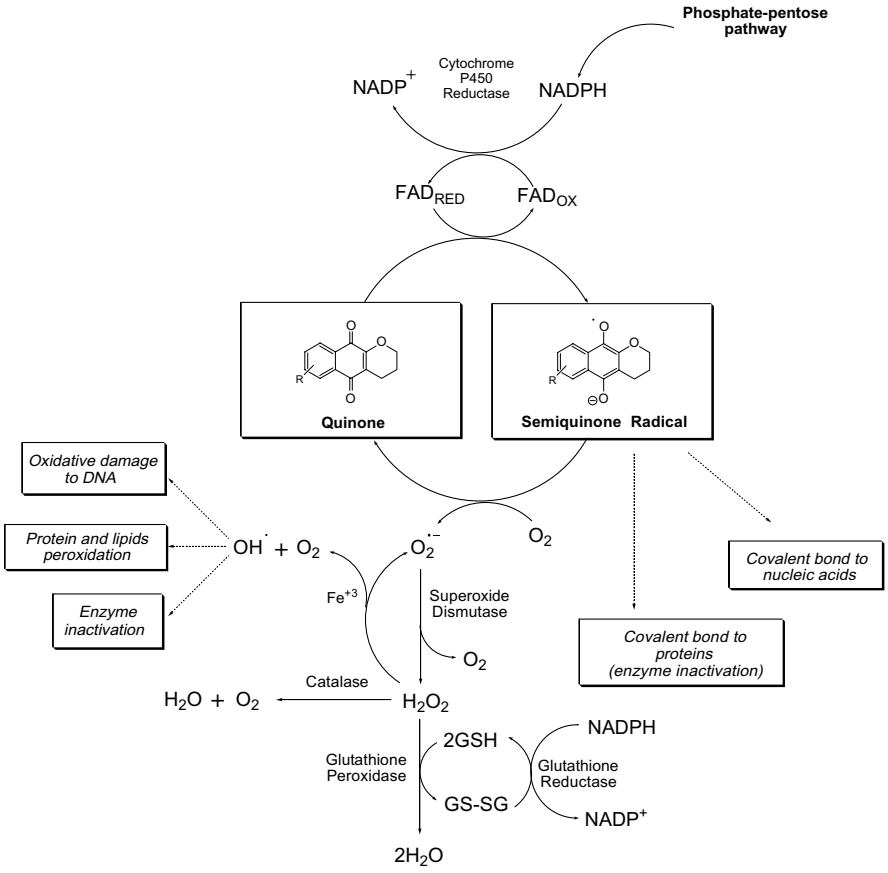


Figure 1. Schematic representation of *p*-quinone metabolism. Redox cycling and production of electrophilic metabolites.

Table 2. Effect of lapachol derivatives upon *T. cruzi* trypomastigote viability[a]

Compound	Viability (% of control)
Control	100
Lapachol (**1**)	41.7
β-Lapachone (2)	4.8
α-Lapachone (3)	2.9
4	30.4
7	15.6
8	40.3
12	112.6
18	8.8
19	44.8
20	26.7
21	32.9
Nifurtimox	66.3

[a] Drug concentrations used were the IC_{k50} values from Table 1. See Section 4.

reductase damage is very important in cytotoxicity experiments.

3. Conclusion

The most active compound against epimastigote and trypomastigote forms of *T. cruzi* was the aza-α-lapachone derivative **18**. The results presented herein indicate that compound **18** is a potential lead compound for the design of new drugs for Chagas disease. This result may also be important for the design of new β-lapachone derivatives with a nitrogen isosteric modification on the aromatic ring in order to improve the anti-*T. cruzi* activity.

4. Experimental

4.1. Chemistry

4.1.1. General remarks. Melting points were determined with a Meltemp apparatus and are not corrected. IR spectra were recorded on a Bruker Model Vector 22 spectrophotometer using KBr discs. ^{1}H and ^{13}C NMR spectra were obtained on Bruker ACP-200 and AM-400 instruments, using tetramethylsilane as internal reference. Column chromatography was performed on silica gel Merck 60 (70–230 mesh). High-resolution mass spectrum was obtained using a Thermo Finnigan Model MAT95XP instrument.

4.1.2. Synthetic procedures. Compounds **11**–**13**[25,26] and **18**–**21**[25–29] were prepared according to our previously described procedures and their spectroscopic data have been already reported.

4.1.3. 3,4-Dihydro-2,2-dimethyl-2*H*-naphtho[1,2-*b*]pyran-5,6-dione (2). A mixture of lapachol **1** (50 mg, 0.21 mmol) and concentrated sulfuric acid (20 mL) was stirred at room temperature for 0.5 h. The reaction mixture was poured into ice and extracted with chloroform (2× 25 mL). The combined organic layers were washed with a saturated solution of sodium bicarbonate, water, dried over magnesium sulfate, and the solvent was evaporated. The residue was purified by column chromatography on silica gel using chloroform as eluent to afford the quinone **2** (40 mg, 80%), mp 153–154 °C (lit.[32] 153–154 °C). IR υ_{max} cm^{-1}: 1700, 1645 (CO). ^{1}H NMR ($CDCl_3$): 1.40 (s, 6H, Me), 1.82 (t, 2H, J = 7.0 Hz, H-3), 2.57 (t, 2H, J = 7.0 Hz, H-4), 7.60 (m, 2H, H-7 and H-8), 8.00 (m, 2H, H-6 and H-9). ^{13}C ($CDCl_3$): 16.2, 26.8 (2C), 31.6, 79.3, 112.3, 124.1, 128.6, 130.1, 130.7, 132.6, 134.8, 162.1, 178.6, 179.9.

4.1.4. 3,4-Dihydro-2,2-dimethyl-2*H*-naphtho[2,3-*b*]pyran-5,10-dione (3). A mixture of lapachol **1** (50 mg, 0.21 mmol) and 12 N hydrochloric acid (20 mL) was stirred at 70 °C for 3 h. After cooling to room temperature, ice water (20 mL) was added and the reaction mixture was extracted with chloroform (2× 25 mL). The combined organic layers were washed with a saturated solution of sodium bicarbonate, water, dried over magnesium sulfate, and the solvent was evaporated. The residue was purified by column chromatography on silica gel using chloroform as eluent to afford the quinone **3** (45 mg, 90%), mp 113–114 °C (lit.[32] 115–116 °C). IR υ_{max} cm^{-1}: 1682, 1615 (CO). ^{1}H NMR ($CDCl_3$): 1.44 (s, 6H, Me), 1.83 (t, 2H, J = 6.6 Hz, H-3), 2.63 (t, 2H, J = 6.6 Hz, H-4), 7.66 (m, 2H, H-7 and H-8), 8.10 (m, 2H, H-6 and H-9). ^{13}C ($CDCl_3$):16.8, 26.5 (2C), 31.4, 78.2, 120.2, 126.0, 126.3, 131.2, 132.1, 132.9, 133.8, 154.6, 180.0, 184.4.

4.1.5. 3,4-Dihydro-3-hydroxy-2,2-dimethyl-2*H*-naphtho[1,2-*b*]pyran-5,6-dione (4). To a solution of lapachol **1** (100 mg, 0.42 mmol) in dichloromethane (20 mL) was added *m*-chloroperbenzoic acid (90 mg, 0.52 mmol) and the mixture was stirred at room temperature for 24 h. The reaction mixture was washed with a saturated solution of sodium bicarbonate, water, dried over magnesium sulfate, and the solvent was evaporated. The residue was purified by column chromatography on silica gel using chloroform/ethyl acetate (2:1) as eluent to afford quinone **4** (45 mg, 42%), mp 203–205 °C (lit.[33] 204–206 °C). IR υ_{max} cm^{-1}: 1700, 1630 (CO). ^{1}H NMR ($CDCl_3$): 1.45 (s, 3H, Me), 1.53 (s, 3H, Me), 1.90 (s, 1H, OH), 2.64 (dd, 1H, J = 26 and 5.7 Hz, H-4), 2.82 (dd, 1H, J = 26 and 5.3 Hz, H-4), 3.91 (dd, 1H, J = 5.7 and 5.3 Hz, H-3), 7.75 (m, 4H). ^{13}C ($CDCl_3$): 21.0, 22.6, 23.2, 69.0, 79.7, 110.0, 124.3, 128.8, 130.1, 131.0, 132.0, 135.0, 161.2, 178.6, 179.4.

4.1.6. 2-Hydroxy-3-(3-oxobutyl)-1,4-naphthoquinone (6). To a solution of 2-hydroxy-1,4-naphthoquinone **5** (1.0 g, 5.74 mmol) in a pyridine/*tert*-butanol mixture (1:10, 25 mL) was added 3-buten-2-one (1.0 mL) and the mixture was heated at reflux for 12 h. The reaction mixture was cooled, acidified with 1:1 hydrochloric acid, and evaporated under reduced pressure. The residue was washed with water and dried to yield quinone **5** (1.35 g, 96%), mp 149–150 °C (lit.[23] 149–145.5 °C). IR υ_{max} cm^{-1}: 1712, 1660, 1650 (CO). ^{1}H NMR ($CDCl_3$): 2.19 (s, 3H, Me), 2.80 (m, 4H), 7.65 (s, 1H, OH), 7.70 (m, 2H, H-6 and H-7), 8.09 (m, 2H, H-5 and H-8). ^{13}C ($CDCl_3$): 17.9, 29.6, 41.7, 122.8, 126.2, 126.8, 129.5, 132.8, 133.1, 134.9, 153.5, 181.1, 184.6, 208.2.

4.1.7. 3,4-Dihydro-2-methyl-2*H*-naphtho[2,3-*b*]pyran-5,10-dione (7). To a solution of **5** (50 mg, 0.21 mmol) in ethanol (10 mL) was added sodium borohydride (50 mg, 1.56 mmol) and the mixture was heated at reflux for 1 h. After cooling to room temperature, aqueous sulfuric acid (20%, 30 mL) was added dropwise and the mixture was heated at reflux for 2 h. The reaction mixture was cooled and extracted with dichloromethane (2× 25 mL). The combined organic layers were washed with a saturated solution of sodium bicarbonate, water, dried over magnesium sulfate, and the solvent was evaporated. The residue was purified by column chromatography on silica gel using chloroform as eluent to afford the quinone **7** (30 mg, 64%), mp 121–122 °C (lit.[32] 122.5 °C). IR υ_{max} cm^{-1}: 1675, 1650 (CO). ^1H NMR ($CDCl_3$): 1.50 (d, 3H, $J = 6.4$ Hz, Me), 1.85 (m, 2H, H-3), 2.6 (m, 2H, H-4), 4.31 (m, 1H, H-2), 7.66 (m, 2H, H-7 and H-8), 8.06 (m, 2H, H-6 and H-9). ^{13}C ($CDCl_3$): 18.3, 20.5, 27.2, 74.4, 121.2, 126.0, 126.3, 131.0, 132.0, 133.0, 133.9, 155.4, 179.8, 184.3.

4.1.8. 3,4-Dihydro-2-methyl-2*H*-naphtho[1,2-*b*]pyran-5,6-dione (8). To a solution of **6** (50 mg, 0.21 mmol) in ethanol (10 mL) was added sodium borohydride (50 mg, 1.56 mmol) and the mixture was heated at reflux for 1 h. After cooling to room temperature the reaction mixture was diluted with water (30 mL), acidified with 10% HCl, and extracted with dichloromethane (2× 20 mL). To the combined organic phase, dried with anhydrous magnesium sulfate, were added five drops of boron trifluoride-etherate and heated at reflux for 1 h. After evaporation of the solvent, the residue was purified by column chromatography on silica gel using chloroform as eluent to afford quinone **8** (35 mg, 75%), mp 163–164 °C (lit.[25,26] 164 °C). IR υ_{max} cm^{-1}: 1670, 1645 (CO). ^1H NMR ($CDCl_3$): 1.54 (d, 3H, $J = 6.3$ Hz, Me), 1.84 (m, 2H, H-2), 2.60 (m, 2H, H-4), 4.38 (m, 1H, H-4), 7.55 (m, 2H, H-8 and H-9), 7.80 (m, 1H, H-10), 8.06 (m, 1H, H-7). ^{13}C ($CDCl_3$): 18.0, 20.7, 27.7, 75.3, 113.7, 124.0, 128.7, 130.0, 130.7, 132.3, 134.6, 162.9, 178.7, 180.0.

4.1.9. 2-Hydroxy-3-(1-phenyl-3-oxobutyl)-1,4-naphthoquinone (9). To a solution of 2-hydroxy-1,4-naphthoquinone **5** (1.0 g, 5.74 mmol) in quinoline (25 mL) was added 4-phenyl-3-buten-2-one (1.0 g, 6.85 mmol) and the mixture was heated at reflux for 3 h. The solvent was removed at reduced pressure and the residue was purified by column chromatography on silica gel using chloroform as eluent to afford quinone **9** (0.65 g, 35%), mp 143–144°C (lit.[24] 143–144 °C). IR υ_{max} cm^{-1}: 1712, 1660, 1650 (CO). ^1H NMR ($CDCl_3$): 2.16 (s, 3H, Me), 3.23 (dd, 1H, $J = 18$ and 8 Hz, CH_2), 3.75 (dd, 1H, $J = 18$ and 10 Hz, CH_2), 4.96 (dd, 1H, $J = 10$ and 6 Hz, CH), 7.36 (m, 5H), 7.64 (s, 1H, OH), 7.69 (m, 2H, H-6 and H-7), 8.06 (m, 2H, H-5 and H-8). ^{13}C ($CDCl_3$): 30.0, 36.4, 46.3, 124.6, 125.6, 126.1, 126.8, 127.0, 127.7, 128.6 (2C), 129.2 (2C), 132.9, 135.1, 141.7, 152.6, 181.7, 184.3, 207.1.

4.1.10. 3,4-Dihydro-2-methyl-4-phenyl-2*H*-naphtho[2,3-*b*]pyran-5,10-dione (10). Quinone **10** was prepared from compound **9** (50 mg, 0.16 mmol) according to the procedure for the preparation of **7**, yielding pure product **10** (28 mg, 59%), mp 210–211 °C. IR υ_{max} cm^{-1}: 1682, 1652 (CO). ^1H NMR ($CDCl_3$): 1.38 (d, 3H, $J = 6.3$ Hz, Me), 2.05 (2H, m, CH_2), 4.34 (2H, m, H-2 and H-4), 7.36 (5H, m, arom H), 7.65 (2H, m, arom H), 8.06 (2H, m, arom H). ^{13}C ($CDCl_3$): 114.6, 117.4, 124.5, 126.3, 126.9, 126.7, 127.8, 128.6, 128.8, 130.4, 131.0, 132.2, 135.0, 143.6, 163.9, 178.1,179.6. HRMS calcd for $C_{20}H_{16}O_3$ 304.10995. Found: 304.10922.

4.2. Biology

4.2.1. Epimastigote culture and growth inhibition assays. *Trypanosoma cruzi* epimastigotes, Tulahuén strain, were cultured, at an initial density of 3×10^6 parasites/mL, at 28 °C in monophasic Diamond's culture medium, supplemented with 4 μM Hemin, bovine fetal calf serum at a final concentration of 5% v/v, and sodium penicillin 100 mg/mL and streptomycin 100 mg/mL. The assayed drugs were dissolved in 1% dimethylsulfoxide (DMSO). All measurements were carried out in triplicate. Epimastigote growth was followed daily by nephelometry for 10 days. The nephelometry measurements were directly proportional to the number of parasites in suspension. No toxic effect was attributable to DMSO at the final concentration of 1%. Since parasite growth is exponential, the data were analyzed according to a first order equation and the respective growth constant (k), corresponding to the slope, was calculated for controls and parasites treated with different drug concentrations. The IC_{k50} is defined as the drug concentration needed to lower the k by 50%.

4.2.2. Trypomastigotes. VERO cells were infected with MF strain *T. cruzi* metacyclic trypomastigotes from 15 days old epimastigote cultures.[34] Subsequently, the trypomastigotes harvested from this culture were used to reinfect further VERO cell cultures at 1×10^6 parasites per 25 cm^2 density. VERO cell cultures infected with trypomastigotes were incubated at 37 °C in humidified air and 5% CO_2 for 5–7 days. After that time, the culture medium was collected, centrifuged at 3000g for 5 min, and the trypomastigote-containing pellet was resuspended in RPMI supplemented with fetal bovine serum 5% and penicillin–streptomycin at a final density of 1×10^7 parasites/mL. 210×10^6 trypomastigotes are equivalent to 1 mg of protein or 12 mg of wet weight.

4.2.3. Oxygen uptake and redox cycling. Epimastigotes were harvested on the 4th or 5th day of culture by centrifugation at 500g for 10 min, followed by washing and re-suspension in 0.05 M sodium phosphate buffer (0.107 M NaCl; 0.05 M KH_2PO_4; pH 7.4). Respiration measurements were carried out polarographically with a Clark 5331 electrode (Yellow Spring Instruments) in a 53 YSI model oxygraph. The chamber volume was 1.0 mL and the temperature 28 °C. The amount of parasites used was equivalent to 1 mg of protein. The drugs were added at epimastigote IC_{k50} values. To evaluate drug-induced oxygen redox cycling, mitochondrial respiration was inhibited with 20 mM KCN.

4.2.4. Trypomastigote viability assay. Viability assays were performed using the MTT reduction method as described previously.[35] 1×10^7 trypomastigotes were incubated in FBS-RPMI culture medium at 37 °C during 24 h with or without the drugs studied. An aliquot of the parasite suspension was extracted and incubated in a 96-flat bottom well plate and MTT was added at 0.5 mg/mL final concentration, incubated at 28 °C during 4 h and then made soluble with 10 % SDS–0.1 mM HCl and incubated overnight. Formazan formation was measured at 570 nm with reference wavelength at 690 nm in a multiwell reader (Labsystems MultoskanMS, Finland).

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