New Pyrano-4H-benzo[g]chromene-5,10-diones with Antiparasitic and Antioxidant Activities

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New pyranonaphthoquinone derivatives were synthesized and investigated for their activity against Trypanosoma brucei, Leishmania major, and Toxoplasma gondii parasites. The pentafluorophenyl derivative was efficacious against T. brucei with single digit micromolar EC_{50} values and against T. gondii with even sub-micromolar values. The 3-chloro-4,5-dimethoxyphenyl derivative showed an activity against amastigotes of Leishmania major parasites comparable to that of amphotericin B. In addition, antioxidant activities were observed for the bromophenyl derivatives, and their redox behavior was studied by cyclovoltammetry. Anti-parasitic and antioxidative activities of the new naphthoquinone derivatives appear uncorrelated.

Keywords: lawsone, neglected tropical diseases, pyran, biological activity.

Introduction

Infections of immune-compromised patients and newborn children with the world-wide occurring toxoplasmosis (caused by Toxoplasma gondii parasites) can lead to severe complications and, thus, efficient drugs for the treatment of toxoplasmosis are necessary. In addition, neglected tropical diseases (NTDs) pose an eminent danger to people living or working in affected territories. Human African trypanosomiasis (HAT, sleeping sickness) and leishmaniasis are NTDs which can ultimately lead to death of untreated patients. Trypanosoma brucei gambiense (T. b. gambiense, in West and Central Africa) and T. b. rhodesiense (in East Africa) are the two prevalent forms of trypanosomes which are responsible for sleeping sickness in humans while the Nagana disease of cattle (animal trypanosomiasis) is mainly caused by T. b. brucei. The diamidine pentamidine and the urea derivative suramin are drugs only applicable for patients with early stage sleeping sickness, late stages were treated with highly toxic arsenics such as melarsoprol for a long time. Meanwhile, the less toxic ornithine derivative eflornithine in combination with the nitrofuran nimexil (nimexil-methenamine combination treatment, NECT) has replaced melarsoprol for the treatment of late stage T. b. gambiense infections (g-HAT). The nitroimidazole fexinidazole was the first orally active drug which is applied for the treatment of stage 1 and stage 2 g-HAT patients. Leishmaniasis is
clinically subdivided into the cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL) and visceral leishmaniasis (VL) forms, which show different clinical outcomes. VL is caused by *Leishmania infantum* (*L. infantum*) and *L. donovani* protozoal parasites and eventually leads to death resulting into a severe medical problem in regions where VL is endemic, e.g., in South Asia, Africa, Latin America and the Mediterranean region.[7] The liposomal formulation of the natural polyene macrolide amphotericin B (AmBisome) and combinations of AmBisome with the phospholipid miltefosine are currently applied for the treatment of VL.[7] The skin damaging CL form, which is caused by various *Leishmania* species such as *L. major*, *L. tropica*, *L. mexicana*, *L. amazonensis* etc., is the most widespread form of leishmaniasis diseases with 0.7–1 million predominantly young patients every year.[8,9] Although CL is usually not lethal, it causes severe and disfiguring skin lesions and affected persons are often stigmatized.[10,11] Current treatment options for CL patients include pentavalent antimonials (sodium stibogluconate, meglumine antimoniate), miltefosine, amphotericin, and pentamidine. Recent works disclosed interesting preclinical results for benzoxaborole, nitroimidazoles and aminopyrazoles as well as for the antimonial drug activity enhancer D35 (a CpG oligonucleotide).[9,12] Aside of the toxicity of the currently applied drugs, the emergence of drug-resistant parasite forms poses a growing problem to the clinician and, thus, the search for new potent antiparasitic drugs is ongoing. The treatment of trypanosomiasis and leishmaniasis with natural products or with drugs derived from them appears promising.[3,13] Naphthoquinones represent a significant group of secondary metabolites of plants and lichens with a variety of biological activities such as antioxidant and trypanocidal activities.[3,13–15] The natural 2-hydroxy-1,4-naphthoquinone (lawsone, 1a) isolated from the Henna plant *Lawsonia inermis* showed biological activities including antibacterial effects and was applied as starting material for quinone drug candidates such as lapachol and atovaquone (ATO).[13,13–16] Modified lawsone derivatives with antitumor and antifungal activities are also known.[3,17,18] Quite a few lawsone derivatives were found efficacious against various parasites including *Plasmodium falciparum*, *Leishmania donovani*, *Trypanosoma cruzi* and *Toxoplasma gondii*.[3,19–23] Our own previous works have led to the identification of lawsone Mannich bases and naphthaquinone derivatives (1b–1d) with reasonable activities against *Trypanosoma brucei*, *Entamoeba histolytica*, *L. major* or *T. gondii* (Figure 1).[3,24,25] The fusion of the lawsone scaffold with heterocyclic rings such as pyrans can be another promising method in order to obtain new active compounds based on lawsone and various anti-tumor active derivatives as well as tumor cell senescence-inducing complexes were already identified (1e).[26,27] In continuation of these previous reports, we present new lawsone-derived pyranonaphthaquinone derivatives and their antioxidant and anti-parasitic activities against *T. brucei*, *L. major* and *T. gondii*.

**Results and Discussion**

Compounds 2a–2o were prepared by Knoevenagel reaction of malononitrile and the respective aryl aldehyde under basic conditions followed by Michael addition of lawsone (1a) and ring-closure to the pyran ring in a multi-component one-pot reaction.

![Figure 1. Lawsone (1a), selected anti-parasitic derivatives (1b–1d), and anti-tumor active pyran (1e).](image-url)
Compounds 2a–2o were obtained as yellow, brown or red-orange solids in moderate yields. The compounds 2a–2o were tested against T. gondii parasites and their activities were compared with those against non-malignant Vero cells (Table 1). Except for 2a and 2c, all test compounds exhibited distinct activity against T. gondii. Compound 2h showed the highest activity in the sub-micromolar range (EC$_{50}$ = 0.7 μM) and a reasonable selectivity (SI = 19.1). Compounds 2g and 2o showed similar selectivities (SI = 19.6 for 2g, 22.1 for 2o). Considerably higher selectivities were observed for 2d (SI = 34.5), 2f (SI = 34.9), and 2l (SI = 41.4). Although the selectivity of these compounds is lower when compared with the selectivity of the approved drug ATO, they were less toxic than ATO against Vero cells and, thus, they can be less toxic alternatives to ATO treatment.

Next, compounds 2a–2o were investigated against L. major promastigotes and amastigotes and their activities were compared with those against non-malignant Vero cells (Table 2). Compound 2b showed excellent activity against L. major amastigotes (EC$_{50}$ = < 0.5 μM) and, thus, 2b was at least comparably active when compared with the approved drug amphotericin B (AmB) in this regard. However, 2b was also quite toxic to Vero cells (i.e., it was almost as toxic as doxorubicin) leading to a relatively low selectivity when compared with AmB.[28] In addition, compounds 2a, 2e, 2h, 2i, and 2m showed activities against the amastigotes with EC$_{50}$ values below 10 μM. Compounds 2d (SI = 4.22) and 2o (SI = 5.26) revealed the highest selectivities for L. major amastigotes. Compound 2h also showed moderate activity against L. major promastigotes (EC$_{50}$ = 15.3 μM). However, the activity of the test compounds 2a–2o against promastigotes was distinctly lower when compared with their activities against amastigotes.

Selected compounds were also tested against T. b. brucei (Table 3). Pentamidine served as positive control here.[29] Compound 2h showed the highest activities against the T. b. brucei cells followed by 2f and 2a. The highest selectivity was observed for 2f (SI = 12.8). Compounds 2b, 2c, and 2m were inactive at doses up to 10 μM.

The antioxidant activities of selected compounds 2a–2f, 2h, 2m, and 2n were evaluated using the 1,1-diphenyl-2-picrylhydrazil (DPPH) assay (Table 4). The radical derivative DPPH is a radical scavenger and functions as a trap for other radicals and, thus, it is the functional compound of common antioxidant assays.[30–32] Compounds 2a and 2c showed distinctly higher antioxidant activities when compared with the other test compounds. Both compounds were also

Table 1. Antitoxoplasmal activity of compounds 2a–2o as EC$_{50}$ (effective concentration that causes 50% inhibition for T. gondii in μM)\[a\], while IC$_{50}$ is the inhibition concentration for 50% of the Vero (African green monkey kidney epithelial in μM). ATO was used as positive control.

| Compound | EC$_{50}$ (T. gondii) | IC$_{50}$ (Vero) | SI (Vero/T. gondii)$^{[b]}$ |
|----------|------------------------|-----------------|-----------------------------|
| 2a       | 131 ± 13               | 4.1 ± 0.6       | 0.03                        |
| 2b       | 1.7 ± 0.2              | 0.7 ± 0.08      | 0.43                        |
| 2c       | 10.6 ± 1.7             | 6.0 ± 0.72      | 0.57                        |
| 2d       | 1.7 ± 0.18             | 59.8 ± 7.4      | 34.5                        |
| 2e       | 2.5 ± 0.3              | 17.1 ± 2.3      | 6.92                        |
| 2f       | 2.2 ± 0.25             | 76.6 ± 8.7      | 34.9                        |
| 2g       | 1.8 ± 0.22             | 35.8 ± 4.4      | 19.6                        |
| 2h       | 0.7 ± 0.11             | 13.7 ± 1.5      | 19.1                        |
| 2i       | 2.8 ± 0.32             | 17.4 ± 2.4      | 6.26                        |
| 2j       | 4.5 ± 0.46             | 45.1 ± 6.2      | 9.94                        |
| 2k       | 3.1 ± 0.38             | 30.3 ± 3.9      | 9.92                        |
| 2l       | 1.3 ± 0.5              | 54.6 ± 6.2      | 41.4                        |
| 2m       | 1.4 ± 0.42             | 9.1 ± 1.4       | 6.44                        |
| 2n       | 1.4 ± 0.53             | 17.9 ± 2.1      | 12.64                       |
| 2o       | 2.8 ± 0.35             | 62.7 ± 6.0      | 22.1                        |
| ATO      | 0.07 ± 0.004           | 9.5 ± 1.54      | 136                         |
| Doxorubicin | –                      | 0.4 ± 0.01$^{[c]}$ | –                           |

[a] Values are the average of three repeated reading for each test ± SE. Which were obtained from concentration-response curves by measuring the percentage of vital cells relative to untreated group after 3 days of incubation. [b] Selectivity index (SI) calculated by dividing IC$_{50}$ over EC$_{50}$ of the corresponding values. [c] Value is taken from ref. 28.
more active than the known antioxidant ascorbic acid. There seems to be no or just a marginal correlation between antioxidant activity and anti-parasitic activity of the test compounds.

The cell-independent redox properties of lawsone derivatives 2a to 2o were studied by cyclic voltammetry (Figure S1–S7). 2a gave rise to a pair of peaks at $-358 \text{ mV} (i_{\text{pa}} = -0.889 \text{ \mu A})$ and $-556 \text{ mV} (i_{\text{pc}} = 1.792 \text{ \mu A})$ indicative of a three-electron transfer redox couple with $E_{1/2} = -457 \text{ mV}$. While 2c and 2h showed similar redox couples with almost the same $\Delta E$ values, these compounds displayed another irreversible cathodic peak between $-1004 \text{ mV}$ and $1050 \text{ mV}$ with different current values ($i_{\text{pc}} = 0.3104 \text{ \mu A}$ in case of 2c). Such a redox behavior was reported for other lawsone derivatives and the redox couple at $E_{1/2} = -457 \text{ mV}$ can be assigned to the conversion of naphthoquinone (NQ) to naphthosemiquinone (NSQ) (NQNSQ), while the irreversible peak at 1050 mV can be assigned to the following $2e^- \text{ reduction to the catechol form (NSQ---CAT).}$

### Table 2. Antileishmanial activity of compounds 2a–2o as EC$_{50}$ (effective concentration that causes 50% inhibition for *L. major* amastigotes and promastigotes in μM)$^a$, AmB was used as positive control.

| Compound | EC$_{50}$ promastigotes | EC$_{50}$ amastigotes | SI Vero/promastigotes$^b$ | SI Vero/amastigotes$^b$ |
|----------|-------------------------|-----------------------|---------------------------|-------------------------|
| 2a       | 49.2 ± 5.2              | 8.4 ± 1.1             | 0.08                      | 0.49                    |
| 2b       | 34.1 ± 4.4              | < 0.5                 | 0.02                      | > 1.5                   |
| 2c       | 22.3 ± 3.1              | 35.8 ± 4.3            | 0.27                      | 0.17                    |
| 2d       | 146 ± 16.5              | 14.2 ± 1.6            | 0.41                      | 4.22                    |
| 2e       | 37.9 ± 4.7              | 7.4 ± 0.9             | 0.45                      | 2.31                    |
| 2f       | 86.5 ± 9.1              | 31.3 ± 3.4            | 0.89                      | 2.45                    |
| 2g       | 25.6 ± 3.6              | 11.3 ± 0.9            | 1.40                      | 3.19                    |
| 2h       | 15.3 ± 1.9              | 5.7 ± 0.8             | 0.89                      | 2.38                    |
| 2i       | 35.8 ± 4.0              | 9.8 ± 1.1             | 0.49                      | 1.77                    |
| 2j       | 98.0 ± 10.1             | 34.2 ± 4.7            | 0.46                      | 1.32                    |
| 2k       | 46.4 ± 5.2              | 24.5 ± 3.9            | 0.65                      | 1.24                    |
| 2l       | 84.1 ± 9.3              | 22.2 ± 2.8            | 0.65                      | 2.46                    |
| 2m       | 29.4 ± 3.6              | 7.9 ± 1.0             | 0.31                      | 1.15                    |
| 2n       | 37.9 ± 4.5              | 26.6 ± 2.6            | 0.47                      | 0.67                    |
| 2o       | 41.2 ± 5.0              | 11.9 ± 2.1            | 1.52                      | 5.26                    |
| AmB      | 0.83 ± 0.09             | 0.47 ± 0.06           | 9.6                       | 16.4                    |

$^a$ Values are the average of three repeated readings for each test ± SE. Which were obtained from concentration-response curves by measuring the percentage of vital cells relative to untreated group after 3 days of incubation. $^b$ Selectivity index (SI) calculated by dividing IC$_{50}$ (from Table 1) over EC$_{50}$ of the corresponding values.

### Table 3. Antitrypanosomal activity of compounds 2a–2c, 2f, 2h and 2m as EC$_{50}$ (effective concentration that causes 50% inhibition for *T. b. brucei* in μM)$^a$, pentamidine was used as positive control.

| Compound | IC$_{50}$ (T. b. brucei) | SI Vero/T. b. brucei$^b$ |
|----------|--------------------------|--------------------------|
| 2a       | 7.6                      | 0.54                     |
| 2b       | > 10                     | –                        |
| 2c       | > 10                     | –                        |
| 2f       | 6.0                      | 12.8                     |
| 2h       | 4.9                      | 2.8                      |
| 2m       | > 10                     | –                        |
| Pentamidine | 0.000042$^c$ | –                        |

$^a$ Values are the means of at least three independent experiments (SD ± 15%). They were derived from concentration–response curves obtained by measuring the percentage of vital cells relative to untreated controls after 72 h. $^b$ Selectivity index calculated from the corresponding IC$_{50}$ values for the Vero cells and the IC$_{50}$ values for *T. b. brucei*. $^c$ Value is taken from ref. [29].

### Table 4. Inhibitory concentrations IC$_{50}$ of ascorbic acid (positive control) and test compounds 2a–2f, 2h, 2m, and 2n when tested for their antioxidant activities (DPPH assay).

| Compound | IC$_{50}$ [μM] |
|----------|----------------|
| Ascorbic acid | 20 |
| 2a | 3.75 |
| 2b | 22 |
| 2c | 2.25 |
| 2d | 28 |
| 2e | 16 |
| 2f | 21 |
| 2h | 25 |
| 2m | 20 |
| 2n | 30 |

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on the bromine atoms of 2a and 2c. The electron distributions in redox active ligands were correlated with a preferential reduction of certain ligands.\textsuperscript{36–38} The $\pi$-orbital of the benzosemiquinone-type radical ligand of 2c having a large overlap area might contribute to its greater ROS producing effects and, thus, showed the highest antioxidant activity compared to the other test compounds. In case of 2d, a large gain in the current ($i_{pc}$ 1.3047 $\mu$A and $i_{pa}$ −1.4905 $\mu$A) was probably due to the electron withdrawing effects of the fluorine atom and, thus, a fast electron transfer was facilitated (Figure S1).\textsuperscript{38} Compound 2h showed a redox couple at $E_{1/2} = −428.5$ mV with high current values ($i_{pc}$ 1.9638 $\mu$A and $i_{pa}$ −1.0944 $\mu$A).

Conclusions

New lawsone-derived compounds were identified as anti-parasitic and antioxidant agents. The high anti-parasitic activities of 2h against T. brucei and T. gondii warrant a further investigation of its effects on these parasites and other protozoal parasites such as Plasmodium species. The highest antioxidant activities, i.e., the strongest suppression of radical formation, were observed for compounds 2a and 2c, which showed relatively weak activities in the anti-parasitic assays (except for 2a against T. brucei) and so there is apparently no strong correlation between antioxidant and anti-parasitic activities of the test compounds.

Experimental Section

General

Starting materials and pure solvents were purchased from common providers and used without further purification. IR spectra were measured on a PerkinElmer Spectrum One FT-IR spectrophotometer equipped with an ART sampling unit. NMR spectra were measured on a Bruker Avance 300 spectrometer and chemical shifts (d) are given in parts per million (ppm) downfield from Me$_4$Si as internal standard. Coupling constants (J) are given in Hz. Mass spectra were measured on a Varian MAT 311A (EI). Elemental analyses were carried out with a Perkin–Elmer 2400 CHN elemental analyzer.\textsuperscript{31}

2-Amino-4-(3-bromo-4,5-dimethoxyphenyl)-5,10-dihydro-5,10-dioxo-4H-naphtho[2,3-b]pyran-3-carbonitrile (2a). 3-Bromo-4,5-dimethoxybenzaldehyde (245 mg, 1.0 mmol) and malononitrile (70 mg, 1.0 mmol) were dissolved in MeCN (5 mL) and three drops of Et$_3$N were added. The reaction mixture was stirred at room temperature for 30 min. 2-Hydroxy-1,4-naphthoquinone (174 mg, 1.0 mmol) was added, and the reaction mixture was stirred at room temperature for 16 h. The formed precipitate was collected, washed with MeCN and n-hexane and dried in vacuum. Yield: 262 mg (0.56 mmol, 56%). Orange solid. M.p. 250–251°C. IR: 3392, 3319, 3256, 3222, 3201, 3017, 2989, 2940, 2825, 2201, 1666, 1653, 1637, 1607, 1591, 1567, 1488, 1460, 1442, 1403, 1363, 1316, 1302, 1278, 1241, 1226, 1206, 1177, 1136, 1077, 1037, 1001, 949, 866, 819, 769, 747, 732, 721, 712, 680. $^1$H-NMR (300 MHz, (D$_6$)DMSO)): 3.70 (3 H, s), 3.81 (3 H, s), 4.61 (1 H, s), 7.01 (1 H, s), 7.11 (1 H, s), 7.36 (2 H, s), 7.8–7.9 (3 H, m), 8.0–8.1 (1 H, m). $^{13}$C-NMR (75.5 MHz, (D$_6$)DMSO)): 36.2, 56.2, 57.1, 60.0, 112.2, 116.7, 119.3, 120.8, 123.1, 125.8, 126.0, 130.8, 131.1, 134.1, 134.4, 141.2, 144.6, 149.3, 153.3, 158.3, 176.8, 182.7; El-MS: 468 (100) [M$^+$], 466 (97) [M$^+$], 437 (11), 435 (11), 387 (72), 251 (92). Anal. calc. for C$_{12}$H$_{13}$BrN$_2$O$_5$ (467.28): C 56.55, H 3.24, N 6.00; found: C 56.41, H 3.16, N 5.88.

2-Amino-4-(3-chloro-4,5-dimethoxyphenyl)-5,10-dihydro-5,10-dioxo-4H-naphtho[2,3-b]pyran-3-carbonitrile (2b). 3-Chloro-4,5-dimethoxybenzaldehyde (200 mg, 1.0 mmol) and malononitrile (70 mg, 1.0 mmol) were dissolved in MeCN (5 mL) and three drops of Et$_3$N were added. The reaction mixture was stirred at room temperature for 30 min. 2-Hydroxy-1,4-naphthoquinone (174 mg, 1.0 mmol) was added, and the reaction mixture was stirred at room temperature for 16 h. The formed precipitate was collected, washed with MeCN and n-hexane and dried in vacuum. Yield: 200 mg (0.47 mmol, 47%). Orange solid. M.p. 306–307°C. IR: 3393, 3323, 3256, 3222, 3200, 2991, 2941, 2827, 2202, 1668, 1655, 1638, 1592, 1572, 1492, 1448, 1428, 1417, 1404, 1364, 1341, 1331, 1318, 1303, 1282, 1242, 1228, 1207, 1179, 1161, 1094, 1079, 1046, 1028, 1001, 949, 868, 851, 844, 818, 7973 784, 771, 752, 733, 722, 697, 682, 631, 602; $^1$H-NMR (300 MHz, (D$_6$)DMSO) ): 3.71 (3 H, s), 3.81 (3 H, s), 4.62 (1 H, s), 6.9–7.0 (2 H, m), 7.35 /2 H, s), 7.8–7.9 (3 H, m), 8.0–8.1 (1 H, m). $^{13}$C-NMR (75.5 MHz, (D$_6$)DMSO)): 36.2, 56.0, 57.1, 60.1, 111.5, 119.2, 120.4, 120.7, 125.8, 126.0, 126.8, 130.8, 131.0, 134.1, 134.4, 140.6, 143.6, 149.3, 153.4, 158.3, 176.8, 182.6; El-MS: 442 (100) [M$^+$], 422 (63), 251 (77). Anal. calc. for C$_{12}$H$_{13}$ClN$_2$O$_5$ (422.82): C 62.50, H 3.58, N 6.63; found: C 62.29, H 3.46, N 6.46.
2-Amino-4-(3,5-dibromo-4-methoxyphenyl)-5,10-dihydro-5,10-dioxo-4H-naphtho[2,3-b]pyran-3-carbonitrile (2c). 3,5-Dibromo-4-methoxybenzaldehyde (293 mg, 1.0 mmol) and malononitrile (70 mg, 1.0 mmol) were dissolved in MeCN (5 mL) and three drops of Et$_3$N were added. The reaction mixture was stirred at room temperature for 30 min. 2-Hydroxy-1,4-naphthoquinone (174 mg, 1.0 mmol) was added and the reaction mixture was stirred at room temperature for 16 h. The formed precipitate was collected, washed with MeCN and n-hexane and dried in vacuum. Yield: 260 mg (0.50 mmol, 50%). Orange-red solid. M.p. 267 °C. IR: 3414, 3323, 3281, 3253, 3211, 3194, 2201, 1658, 1638, 1606, 1594, 1547, 1471, 1417, 1401, 1362, 1334, 1300, 1262, 1203, 118, 1100, 1074, 1040, 1025, 991, 948, 800, 782, 733, 717, 695, 668; $^1$H-NMR (300 MHz, CDCl$_3$/(D$_6$DMSO)): 3.74 (3 H, s), 4.60 (1 H, s), 6.85 (2 H, s), 7.40 (2 H, s), 4.60 – 4.70 (1 H, m), 0.7.7 – 7.8 (2 H, m), 7.9 – 8.0 (1 H, m), 8.0 – 8.1 (1 H, m). $^{13}$C-NMR (75.5 MHz, CDCl$_3$/(D$_6$DMSO)): 35.3, 56.8, 59.9, 117.6, 118.4, 121.5, 125.9, 126.0, 129.9, 130.7, 131.7, 133.6, 134.2, 140.9, 148.1, 152.6, 158.6, 176.4, 181.9. El-MS: 516 (72) [M$^+$], 514 (40) [M$^+$], 346 (100) [M$^+$]. Anal. calc. for C$_{21}$H$_{13}$Br$_2$N$_2$O$_4$ (516.15): C 48.87, H 2.63, N 7.78; found: C 48.89, H 2.69, N 7.79.

2-Amino-4-(3,5-difluorophenyl)-5,10-dihydro-5,10-dioxo-4H-naphtho[2,3-b]pyran-3-carbonitrile (2d). 2-Fluorobenzaldehyde (124 mg, 1.0 mmol) and malononitrile (70 mg, 1.0 mmol) were dissolved in MeCN (5 mL) and three drops of Et$_3$N were added. The reaction mixture was stirred at room temperature for 30 min. 2-Hydroxy-1,4-naphthoquinone (174 mg, 1.0 mmol) was added and the reaction mixture was stirred at room temperature for 90 min. The formed precipitate was collected, washed with MeCN and n-hexane and dried in vacuum. Yield: 207 mg (0.60 mmol, 60%). Orange solid. M.p. 250 – 251 °C. IR: 3401, 3318, 3253, 3215, 3190, 2197, 1685, 1664, 1634, 1601, 1579, 1488, 1453, 1407, 1346, 1328, 1303, 1247, 1227, 1207, 1174, 1150, 1098, 1076, 1025, 951, 845, 779, 756, 745, 714, 671. $^1$H-NMR (300 MHz, (D$_6$DMSO)): 4.91 (1 H, s), 7.1 – 7.5 (6 H, m), 7.8 – 7.9 (3 H, m), 8.0 – 8.1 (1 H, m). $^{13}$C-NMR (75.5 MHz, (D$_6$DMSO)): 30.4, 56.0, 115.3 – 115.6 (m), 119.1, 120.9, 124.8, 125.8, 126.1, 129.1 – 129.2 (m), 130.2 – 130.9 (m), 134.2 – 134.6 (m), 149.5, 158.0, 159.7 (d, J = 246 Hz), 176.8, 182.5. El-MS: 346 (100) [M$^+$], 302 (6), 251 (87), 223 (7), 173 (7), 105 (5), 76 (5). Anal. calc. for C$_{20}$H$_{11}$F$_2$N$_2$O$_3$ (346.32): C 69.36, H 3.20, N 8.09; found: C 69.44, H 3.12, N 7.96.

2-Amino-4-(3,4-difluorophenyl)-5,10-dihydro-5,10-dioxo-4H-naphtho[2,3-b]pyran-3-carbonitrile (2e). 3,4-Difluorobenzaldehyde (142 mg, 1.0 mmol) and malononitrile (70 mg, 1.0 mmol) were dissolved in MeCN (5 mL) and three drops of Et$_3$N were added. The reaction mixture was stirred at room temperature for 30 min. 2-Hydroxy-1,4-naphthoquinone (174 mg, 1.0 mmol) was added and the reaction mixture was stirred at room temperature for 16 h. The formed precipitate was collected, washed with MeCN and n-hexane and dried in vacuum. Yield: 151 mg (0.42 mmol, 42%). Orange solid. M.p. 260 °C. IR: 3403, 3331, 3290, 3195, 3073, 2026, 1664, 1656, 1639, 1620, 1606, 1594, 1581, 1517, 1438, 1412, 1363, 1332, 1302, 1284, 1274, 1244, 1204, 1179, 1153, 1117, 1096, 1076, 1039, 1021, 972, 950, 933, 900, 882, 865, 838, 820, 799, 790, 772, 763, 752, 730, 713, 673. $^1$H-NMR (300 MHz, (D$_6$DMSO)): 4.67 (1 H, s), 7.2 – 7.5 (5 H, m), 7.8 – 7.9 (3 H, m), 8.0 – 8.1 (1 H, m). $^{13}$C-NMR (75.5 MHz, (D$_6$DMSO)): 35.8, 69.5, 116.6 – 117.5 (m), 119.1, 120.7, 124.6, 125.8, 126.0, 130.7, 131.0, 134.1, 134.5, 141.4 – 141.5 (m), 146.8 – 147.8 (m), 149.3, 150.0 – 151.1 (m), 158.2, 176.8, 182.6. El-MS: 304 (100) [M$^+$], 320 (6), 251 (91), 223 (8). Anal. calc. for C$_{20}$H$_{10}$F$_2$N$_2$O$_3$ (364.31): C 65.94, H 2.77, N 7.69; found: C 66.02, H 2.61, N 7.80.
2-Amino-5,10-dihydro-5,10-dioxo-4-(2,4,5-trifluorophenyl)-4H-naphtho[2,3-b]pyran-3-carbonitrile (2g). 2,4,5-Trifluorobenzaldehyde (160 mg, 1.0 mmol) and malononitrile (70 mg, 1.0 mmol) were dissolved in MeCN (3 mL) and five drops of Et$_3$N were added. The reaction mixture was stirred at room temperature for 30 min. Lawsone (174 mg, 1.0 mmol) was added and the reaction mixture was stirred at room temperature for 30 min. The formed precipitate was washed with MeCN and dried in vacuum. Yield: 135 mg (0.34 mmol, 34%). Orange-red solid. M.p. 128.8 – 128.9 °C. IR: 3398, 3319, 3248, 3211, 2194, 1691, 1655, 1638, 1604, 1593, 1471, 1411, 1362, 1333, 1294, 1243, 1207, 1177, 1157, 1127, 1093, 1079, 1029, 1017, 973, 948, 872, 834, 799, 772, 750, 738, 728, 715, 682, 659, 617, 583. $^1$H-NMR (300 MHz, (D$_6$)$_2$DMSO)): 4.69 (1 H, s), 7.36 (1 H, d, J = 8.8 Hz), 7.40 (2 H, s), 7.64 (1 H, dd, J = 8.3 Hz, 2.4 Hz), 7.42 (2 H, s), 7.57 (1 H, d, J = 8.3 Hz), 7.64 (1 H, d, J = 2.4 Hz), 7.8 – 7.9 (3 H, m), 8.0 – 8.1 (1 H, m). $^{13}$C-NMR (75.5 MHz, (D$_6$)$_2$DMSO)): 35.8, 56.7, 119.1, 120.4, 125.8, 126.0, 128.3, 129.7, 129.8, 130.6, 130.8, 131.0, 131.1, 134.1, 143.4, 144.8, 149.4, 158.3, 176.8, 182.6. El-MS: 398 (15) [M$^+$], 396 (23) [M$^{2+}$], 251 (61), 57 (100). Anal. calc. for C$_{20}$H$_{16}$Cl$_2$N$_2$O$_3$: 60.48, H 2.54, N 7.05; found: C 60.32, H 2.48, N 7.11.

2-Amino-5,10-dihydro-4-(4-(methylthio)phenyl)-5,10-dioxo-4H-naphtho[2,3-b]pyran-3-carbonitrile (2j). 4-Methylsulfonylbenzaldehyde (152 mg, 1.0 mmol) and malononitrile (70 mg, 1.0 mmol) were dissolved in MeCN (5 mL) and three drops of Et$_3$N were added. The reaction mixture was stirred at room temperature for 30 min. The formed precipitate was washed with MeCN and $n$-hexane and dried in vacuum. Yield: 131 mg (0.31 mmol, 31 %). Orange-red solid. M.p. 128.3, 130.6, 131.0, 134.1, 143.4, 144.8, 149.4, 158.3, 176.8, 182.6. El-MS: 398 (15) [M$^+$], 251 (61), 57 (100). Anal. calc. for C$_{20}$H$_{16}$Cl$_2$N$_2$O$_3$: 60.48, H 2.54, N 7.05; found: C 60.32, H 2.48, N 7.11.

2-Amino-4-(3,4-dichlorophenyl)-5,10-dihydro-5,10-dioxo-4H-naphtho[2,3-b]pyran-3-carbonitrile (2i). 3,4-Dichlorobenzaldehyde (175 mg, 1.0 mmol) and malononitrile (70 mg, 1.0 mmol) were dissolved in MeCN (5 mL) and three drops of Et$_3$N were added. The reaction mixture was stirred at room temperature for 30 min. 2-Hydroxy-1,4-naphthoquinone (174 mg, 1.0 mmol) was added and the reaction mixture was stirred at room temperature for 16 h. The formed precipitate was collected, washed with MeCN and $n$-hexane and dried in vacuum. Yield: 135 mg (0.34 mmol, 34%). Orange-red solid. M.p. 125.8, 126.0, 128.3, 129.7, 129.8, 130.6, 130.8, 131.0, 131.1, 134.1, 143.4, 144.8, 149.4, 158.3, 176.8, 182.6. El-MS: 398 (15) [M$^+$], 396 (23) [M$^{2+}$], 251 (61), 57 (100). Anal. calc. for C$_{20}$H$_{16}$Cl$_2$N$_2$O$_3$: 60.48, H 2.54, N 7.05; found: C 60.32, H 2.48, N 7.11.
(170 mg, 1.0 mmol) and malononitrile (70 mg, 1.0 mmol) were dissolved in MeCN (5 mL) and three drops of Et₃N were added. The reaction mixture was stirred at room temperature for 30 min. 2-Hydroxy-1,4-naphthoquinone (174 mg, 1.0 mmol) was added and the reaction mixture was stirred at room temperature for 16 h. The formed precipitate was collected, washed with MeCN and n-hexane and dried in vacuum. Yield: 240 mg (0.61 mmol, 61%). Orange-red solid. M.p. 244 – 245 °C. IR: 3402, 3320, 3251, 3212, 3071, 3046, 2937, 2896, 2195, 1691, 1663, 1665, 1639, 1603, 1593, 1563, 1481, 1412, 1363, 1336, 1300, 1279, 1238, 1207, 1179, 1153, 1096, 1065, 1040, 1026, 954, 931, 881, 838, 822, 803, 776, 761, 736, 719, 695, 685, 619, 561, 553. H-NMR (300 MHz, (D₆)DMSO)): 2.45 (3 H, s), 4.64 (1 H, s), 7.1 – 7.3 (3 H, m), 7.36 (2 H, s), 7.8 – 7.9 (3 H, m), 8.0 – 8.1 (1 H, m). 13C-NMR (75.5 MHz, (D₂)DMSO): 14.0, 35.8, 57.0, 114.2 – 114.5 (m), 119.1, 120.9, 123.7, 124.0, 124.4, 125.8, 126.0, 127.5, 130.7, 131.0, 134.1, 134.4, 142.9, 149.2, 158.3, 158.9 (d, J = 242 Hz), 176.8, 182.6. El-MS: 392 (100) [M+]. 2-Amino-4-(3-cyanophenyl)-5,10-dihydro-5,10-dioxo-4H-naphtho[2,3-b]pyran-3-carbonitrile (2l). 3-Pentafluorothiobenzaldehyde (232 mg, 1.0 mmol) and malononitrile (70 mg, 1.0 mmol) were dissolved in MeCN (5 mL) and three drops of Et₃N were added. The reaction mixture was stirred at room temperature for 30 min. 2-Hydroxy-1,4-naphthoquinone (174 mg, 1.0 mmol) was added and the reaction mixture was stirred at room temperature for 16 h. The formed precipitate was collected, washed with MeCN and n-hexane and dried in vacuum. Yield: 185 mg (0.41 mmol, 41%). Orange-red solid. M.p. 283 °C. IR: 3413, 3340, 3256, 3222, 3194, 3076, 2201, 1656, 1637, 1591, 1484, 1435, 1412, 1361, 1336, 1300, 1252, 1243, 1203, 1185, 1161, 1112, 1095, 1072, 1022, 945, 881, 839, 823, 805, 793, 780, 731, 718, 699, 687, 678. H-NMR (300 MHz, (D₂)DMSO)): 4.84 (1 H, s), 7.43 (2 H, s), 7.5 – 7.6 (1 H, m), 7.6 – 7.7 (1 H, m), 7.7 – 7.9 (5 H, m), 8.0 – 8.1 (1 H, m). 13C-NMR (75.5 MHz, (D₂)DMSO)): 36.5, 56.8, 119.0, 120.5, 124.5, 125.0, 125.8, 126.1, 129.9, 130.7, 130.1, 131.9, 134.2, 145.4, 149.5, 152.9, 158.3, 176.8, 182.6. El-MS: 454 (100) [M+]. 2-Amino-4-(3-cyanophenyl)-5,10-dihydro-5,10-dioxo-4H-naphtho[2,3-b]pyran-3-carbonitrile (2m). 3-Cyanobenzaldehyde (131 mg, 1.0 mmol) and malono-
reaction mixture was stirred at room temperature for 30 min. 2-Hydroxy-1,4-naphthoquinone (174 mg, 1.0 mmol) was added and the reaction mixture was stirred at room temperature for 1 h. The formed precipitate was collected, washed with MeCN and n-hexane and dried in vacuum. Yield: 114 mg (0.32 mmol, 32%). Orange-red solid. M.p. > 395 °C. IR: 3401, 3322, 3296, 3215, 2193, 1693, 1662, 1639, 1601, 1593, 1529, 1505, 1414, 1365, 1336, 1301, 1241, 1208, 1182, 1161, 1095, 1073, 1042, 1018, 963, 947, 849, 799, 779, 755, 744, 720, 693. 1H-NMR (300 MHz, (D$_6$)DMSO): 4.14 (1 H, s), 4.64 (1 H, s), 7.3 – 7.4 (6 H, m), 7.8 – 7.9 (3 H, m), 8.0 – 8.1 (1 H, m). 13C-NMR (75.5 MHz, (D$_6$)DMSO): 57.0, 80.8, 83.2, 119.2, 120.5, 121.4, 125.8, 126.1, 128.1, 130.7, 131.0, 131.9, 134.2, 134.5, 144.5, 149.1, 158.4, 176.8, 182.6. EI-MS: 352 (100) [M$^+$], 251 (81). Anal. calc. for C$_{35}$H$_3$N$_2$O$_3$: C 74.99, H 3.31, N 7.82.

Leishmania major Cell Assays

Cell assays with L. major promastigotes and amastigotes were carried out as described previously.$^{[41–48]}$

Trypanosoma Cell Line and Culture Conditions

Cultivation of the T. b. brucei bloodstream form cell strain Lister 427 was carried out in HMI-9 medium, pH 7.5, supplemented with 10% FBS at 37°C in a humidified 5% CO$_2$ atmosphere.$^{[3,42]}$

Alamar Blue (AB) Assay

Viable cells after treatment with drug candidates were identified via the AB assay.$^{[42–45]}$ Pink resorufin is formed in intact cells from the irreversible reaction of the blue dye resazurin and NADH. T. b. brucei cells (8000/well) were seeded on 96-well microplates, test compounds (dissolved in DMSO) were added and the cells were incubated for 72 h (5% CO$_2$, 95% humidity, 37°C). AB reagent (10 μL of 500 μM resazurin sodium salt in PBS) was added and the cells were incubated for additional 4 h at 37°C. Fluorescence (extinction at 544 nm, emission at 590 nm) was determined on an Omega FLUOstar (BMG Labtech) fluorescence plate reader. The IC$_{50}$ values were determined with the Quest Graph™ IC$_{50}$ Calculator (AAT Bioquest Inc.).$^{[47]}$

In Vitro Cytotoxicity Assay

MTT assay with Vero cells was carried out for cytotoxicity evaluation of compounds. The assay was carried out as reported previously.$^{[47,48]}$

Antioxidant Assay

Aliquots of eight concentrations (1, 5, 10, 20, 25, 50, 100, and 150 μM) of test compounds dissolved in methanol were added to eight test tubes. Compounds were accurately dissolved in methanol to achieve the required concentrations by dilution techniques. 5 mL of 0.004% 1,1-diphenyl-2-picrylhydrazil (DPPH) solution was given to each test tube using a micropipette. The solutions were kept at room temperature for 30 minutes to complete the reaction. DPPH was added...
to a blank test tube containing only methanol. After 30 min the absorbance was measured with a double beam spectrophotometer (JASCO V-630) at 517 nm. IC$_{50}$ values were calculated from the plot of inhibition (In %) vs. concentration (in μM). The free radical scavenging kinetics for standard antioxidant viz. ascorbic acid and the test compounds were calculated using the following formula:

$$\text{DPPH scavenging effect (\%)} = \left( \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \times 100$$

The biological activities of the ligands were examined in terms of DPPH radical scavenging activities where the count fraction causing 50% inhibition of DPPH is called IC$_{50}$.

**Cyclovoltammetry**

Cyclic voltammograms were recorded of test compounds dissolved in DMSO. Bioanalytical System BASi EPSILON Model instrument with X-Y recorder was applied with a three-electrode framework consisting of a glassy carbon working anode, Ag$^+$/AgCl as reference electrode, a platinum wire as auxiliary electrode in 0.1 M Et$_4$NClO$_4$ as supporting electrode. Ferrocene served as internal standard. The direction of feed potential was from anode to cathode.

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**Author Contribution Statement**

B. B. prepared the compounds and wrote the article. I. S. N., T. A. K. and J. J. carried out the antiparasitic assays. A. S. and P. S. S. carried out the antioxidant assay and the cyclovoltammetry experiments. W. S. K., K. A., K. E. and R. S. provided the material, supervised the work and proofread the article.

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