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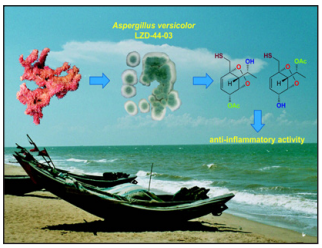

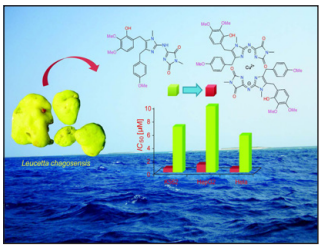

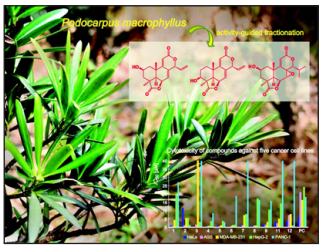

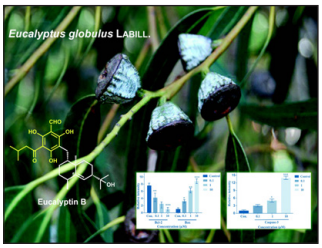

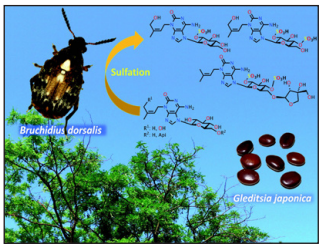

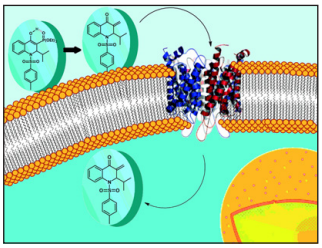

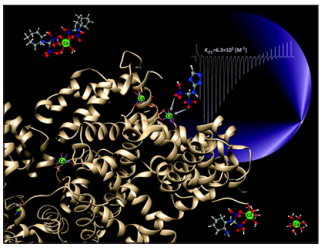

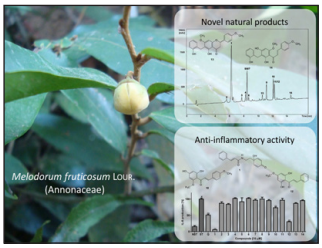

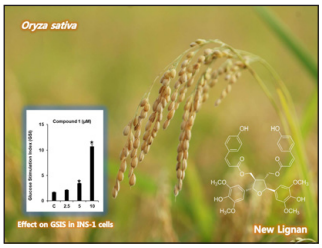

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New Pyrano-4*H*-benzo[*g*]chromene-5,10-diones with Antiparasitic and Antioxidant Activities

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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. Antiparasitic 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.[1] In addition, neglected tropical diseases (NTDs) pose an eminent danger to people living or working in affected territories.[2,3] 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*.[4,5] 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.[6] Meanwhile, the less toxic ornithine derivative eflornithine in combination with the nitrofuran nifurtimox (nifurtimox-eflornithine combination treatment, NECT) has replaced melarsoprol for the treatment of late stage *T. b. gambiense* infections (g-HAT).[6] The nitroimidazole fexinidazole was the first orally active drug which is applied for the treatment of stage 1 and stage 2 g-HAT.[5] The benzoxaborole derivative acoziborole is currently tested in phase 2/3 clinical trials with stage 2 g-HAT patients.[5] Leishmaniasis is

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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 anti-parasitic 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).[3,13–16] 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 naphthoquinone 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 pyranonaphthoquinone 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

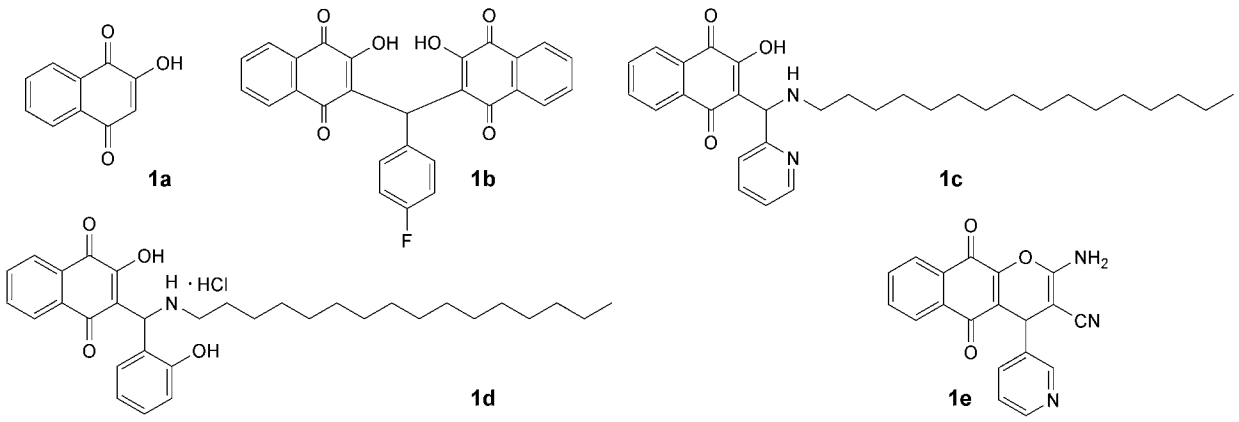
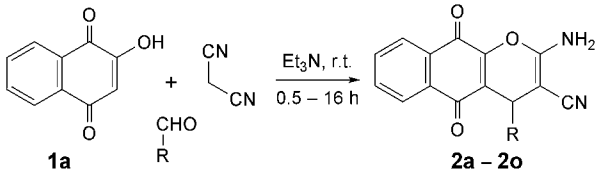


Figure 1. Lawsone (**1a**), selected anti-parasitic derivatives (**1b**–**1d**), and anti-tumor active pyran (**1e**).



	R	Yield [%]		R	Yield [%]
2a	3-Br-4,5-$(MeO)_2$–C_6H_2	56	**2i**	3,4-Cl_2–C_6H_3	34
2b	3-Cl-4,5-$(MeO)_2$–C_6H_2	47	**2j**	4-MeS–C_6H_4	75
2c	3,5-Br_2-4-MeO–C_6H_2	50	**2k**	3-F-4-MeS–C_6H_3	61
2d	2-F–C_6H_4	60	**2l**	3-SF_5–C_6H_4	41
2e	3,4-F_2–C_6H_3	42	**2m**	3-CN–C_6H_4	31
2f	3,5-F_2–C_6H_3	45	**2n**	4-CN–C_6H_4	45
2g	2,4,5-F_3–C_6H_2	31	**2o**	4-HC≡C–C_6H_4	32
2h	2,3,4,5,6-F_5–C_6	31			

Scheme 1. Synthesis of compounds **2a**–**2o**.

(*Scheme 1*).[25,26] 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.

Table 1. Antitoxoplasmal activity of compounds **2a**–**2o** as EC_{50} (effective concentration that causes 50% inhibition for *T. gondi* 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 ± 0.1	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.

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 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
2 l	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 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} (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

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 mV (i_{pa} −0.889 μA) and −556 mV (i_{pc} 1.792 μA) indicative of a three-electron transfer redox couple with $E_{1/2} = -457$ mV. While **2c** and **2h** showed similar redox couples with almost the same △E values, these compounds displayed another irreversible cathodic peak between −1004 mV and 1050 mV with different current values (i_{pc} 0.3104 μA in case of **2c**). Such a redox behavior was reported for other lawsone derivatives and the redox couple at $E_{1/2} = -457$ 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 $2e^-$ reduction to the catechol form (NSQ→CAT).[33–35] The shift of this redox couple towards a more negative potential when compared to the parent lawsone compound can be attributed to the changes occurring in the electron density based

on the bromine atoms of **2a** and **2c**. The electron distributions in redox active ligands were correlated with a preferential reduction of certain ligands.[36–38] The π-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 μA and i_{pa} −1.4905 μA) was probably due to the electron withdrawing effects of the fluorine atom and, thus, a fast electron transfer was facilitated (*Figure S1*).[36] Compound **2h** showed a redox couple at $E_{1/2} = -428.5$ mV with high current values (i_{pc} 1.9638 μA and i_{pa} −1.0944 μA).

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 (δ) are given in parts per million (ppm) downfield from Me_4Si 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.[3]

2-Amino-4-(3-bromo-4,5-dimethoxyphenyl)-5,10-dihydro-5,10-dioxo-4*H*-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_3N 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, 1655, 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; EI-MS: 468 (100) [M^+], 466 (97) [M^+], 437 (11), 435 (11), 387 (72), 251 (92). Anal. calc. for $C_{22}H_{15}BrN_2O_5$ (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-4*H*-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_3N 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 H_2O/MeCN 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, 56.2, 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; EI-MS: 424 (34) [M^+], 422 (100) [M^+], 387 (63), 251 (77). Anal. calc. for $C_{22}H_{15}ClN_2O_5$ (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-4*H*-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_3N 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, 1183, 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), 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. EI-MS: 518 (40) [M^+], 516 (72) [M^+], 514 (40) [M^+], 437 (16), 435 (15), 251 (100). Anal. calc. for $C_{21}H_{12}Br_2N_2O_4$ (516.15): C 48.87, H 2.34, N 5.43; found: C 48.69, H 2.24, N 5.30.

2-Amino-4-(2-fluorophenyl)-5,10-dihydro-5,10-dioxo-4*H*-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_3N 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, 1364, 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. EI-MS: 346 (100) [M^+], 302 (6), 251 (87), 223 (7), 173 (7), 105 (5), 76 (5). Anal. calc. for $C_{20}H_{11}FN_2O_3$ (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-4*H*-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_3N 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, 3256, 3195, 3073, 2206, 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, 56.9, 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. EI-MS: 364 (100) [M^+], 320 (6), 251 (91), 223 (8). Anal. calc. for $C_{20}H_{10}F_2N_2O_3$ (364.31): C 65.94, H 2.77, N 7.69; found: C 66.02, H 2.61, N 7.80.

2-Amino-4-(3,5-difluorophenyl)-5,10-dihydro-5,10-dioxo-4*H*-naphtho[2,3-*b*]pyran-3-carbonitrile (**2f**). 3,5-Difluorobenzaldehyde (142 mg, 1.0 mmol) and malononitrile (70 mg, 1.0 mmol) were dissolved in MeCN (5 mL) and three drops of Et_3N 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: 165 mg (0.45 mmol, 45%). Orange-red solid. M.p. 270 °C. IR: 3395, 3315, 3249, 3211, 3190, 3090, 2200, 1663, 1622, 1594, 1580, 1463, 1449, 1404, 1363, 1338, 1311, 1303, 1290, 1242, 1206, 1178, 1124, 1077, 1040, 1026, 1005, 991, 963, 944, 863, 838, 817, 772, 737, 728, 710, 682, 674. ^{1}H-NMR (300 MHz, (D_6)DMSO)): 4.70 (1 H, s), 7.0–7.2 (3 H, m), 7.41 (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, 56.6, 102.2–102.9 (m), 110.8-111.1 (m), 119.0, 120.2, 125.8, 126.0, 130.8, 131.0, 134.1, 134.4, 148.1–148.3 (m), 149.6, 158.3, 162.4 (dd, J = 13.1 Hz, 247 Hz), 176.7, 182.6. EI-MS: 364 (96) [M^+], 251 (100). Anal. calc. for $C_{20}H_{10}F_2N_2O_3$ (364.31): C 65.94, H 2.77, N 7.69; found: C 66.00, H 2.63, N 7.78.

2-Amino-5,10-dihydro-5,10-dioxo-4-(2,4,5-trifluorophenyl)-4*H*-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_3N 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 collected, washed with MeCN and dried in vacuum. Yield: 120 mg (0.31 mmol, 31%); Orange solid. M.p. 274–275 °C. IR: 3411, 3318, 3220, 3194, 3059, 2208, 1660, 1640, 1626, 1606, 1593, 1515, 1427, 1412, 1369, 1332, 1316, 1302, 1266, 1245, 1208, 1185, 1158, 1142, 1094, 1070, 1019, 950, 899, 874, 846, 816, 761, 741, 715, 670, 620. ^{1}H-NMR (300 MHz, $CDCl_3$/(D_6)DMSO)): 4.85 (1 H, s), 6.46 (2 H, s), 6.8–6.9 (1 H, m), 7.0–7.1 (1 H, m), 7.6–7.7 (2 H, m), 7.8–7.9 (1 H, m), 8.0–8.1 (1 H, m). ^{13}C-NMR (75.5 MHz, $CDCl_3$/(D_6)DMSO)) δ 30.7, 56.4, 105.1–105.7 (m), 117.1–117.4 (m), 118.0, 120.5, 125.9, 126.0, 129.9, 130.6, 133.5, 134.1, 148.5, 158.5, 176.3, 181.8. EI-MS: 382 (100) [M^+], 251 (92). Anal. calc. for $C_{20}H_9F_3N_2O_3$ (382.30): C 62.84, H 2.37, N 7.33; found: C 62.75, H 2.29, N 7.24.

2-Amino-5,10-dihydro-5,10-dioxo-4-(2,3,4,5,6-pentafluorophenyl)-4*H*-naphtho[2,3-*b*]pyran-3-carbonitrile (**2h**). Pentafluorobenzaldehyde (196 mg, 1.0 mmol) and malononitrile (70 mg, 1.0 mmol) were dissolved in MeCN (3 mL) and five drops of Et_3N 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 collected, washed with MeCN and dried in vacuum. Yield: 131 mg (0.31 mmol, 31%). Orange solid. M.p. 281–282 °C. IR: 3427, 3286, 3174, 2202, 1669, 1635, 1520, 1505, 1412, 1366, 1330, 1302, 1247, 1207, 1154, 1118, 1079, 1041, 1024, 992, 954, 937, 824, 790, 768, 719, 704, 648, 610. ^{1}H-NMR (300 MHz, (D_6)DMSO)) δ 5.13 (1 H, s), 7.66 (2 H, s), 7.8–8.0 (3 H, m), 8.0–8.1 (1 H, m). ^{13}C-NMR (75.5 MHz, (D_6)DMSO)) δ 26.8, 52.6, 115.8, 118.7–118.9 (m), 125.9, 126.2, 129.7, 130.4, 130.9, 135.3, 138.4, 143.0, 146.4, 149.3, 159.5, 176.6, 182.2. EI-MS: 418 (100) [M^+], 251 (75). Anal. calc. for $C_{20}H_7F_5N_2O_3$ (418.28): C 57.43, H 1.69, N 6.70; found: C 57.30, H 1.62, N 6.61.

2-Amino-4-(3,4-dichlorophenyl)-5,10-dihydro-5,10-dioxo-4*H*-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_3N 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. 283–285 °C. IR: 3396, 3328, 3196, 2203, 1658, 1638, 1605, 1593, 1471, 1411, 1362, 1333, 1294, 1243, 1207, 1177, 1157, 1127, 1093, 1079, 1029, 1017, 973, 948, 872, 834, 822, 793, 772, 750, 738, 728, 715, 682, 659, 617, 583. ^{1}H-NMR (300 MHz, (D_6)DMSO)): 4.69 (1 H, s), 7.36 (1 H, dd, J = 8.8 Hz, 2.2 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)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, 134.5, 144.8, 149.4, 158.3, 176.8, 182.6. EI-MS: 398 (15) [M^+], 396 (23) [M^+], 251 (61), 57 (100). Anal. calc. for $C_{20}H_{10}Cl_2N_2O_3$ (397.21): C 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-4*H*-naphtho[2,3-*b*]pyran-3-carbonitrile (**2j**). 4-Methylsulfanylbenzaldehyde (152 mg, 1.0 mmol) and malononitrile (70 mg, 1.0 mmol) were dissolved in MeCN (5 mL) and three drops of Et_3N were added. The reaction mixture was stirred at room temperature for 30 min. The formed precipitate was dissolved by heating the reaction mixture and 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: 280 mg (0.75 mmol, 75%). Orange-red solid. M.p. 266 °C. IR: 3398, 3319, 3248, 3211, 2194, 1691, 1655, 1638, 1604, 1593, 1494, 1409, 1365, 1336, 1303, 1238, 1208, 1179, 1162, 1097, 1077, 1041, 1026, 986, 986, 956, 945, 846, 833, 800, 771, 741, 720, 686. ^{1}H-NMR (300 MHz, (D_6)DMSO)): 2.43 (3 H, s), 4.58 (1 H, s), 7.19 (2 H, d, J = 8.6 Hz), 7.26 (2 H, d, J = 8.6 Hz), 7.33 (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)): 14.6, 36.0, 57.3, 119.3, 121.8, 125.8, 126.1, 128.3, 130.6, 131.0, 134.1, 134.5, 148.8, 158.3, 176.9, 182.6. EI-MS: 374 (100) [M^+], 327 (45), 251 (69). Anal. calc. for $C_{21}H_{14}N_2O_3S$ (374.41): C 67.37, H 3.77, N 7.48; found: C 67.22, H 3.66, N 7.37.

2-Amino-4-[3-fluoro-4-(methylthio)phenyl]-5,10-dihydro-5,10-dioxo-4*H*-naphtho[2,3-*b*]pyran-3-carbonitrile (**2k**). 3-Fluoro-4-methylsulfanylbenzaldehyde

(170 mg, 1.0 mmol) and malononitrile (70 mg, 1.0 mmol) were dissolved in MeCN (5 mL) and three drops of Et_3N 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. ^{1}H-NMR (300 MHz, (D_6)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). ^{13}C-NMR (75.5 MHz, (D_6)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. EI-MS: 392 (100) [M^+], 345 (28), 251 (82). Anal. calc. for $C_{21}H_{13}FN_2O_3S$ (392.40): C 64.28, H 3.34, N 7.14; found: C 64.09, H 3.26, N 7.05.

2-Amino-5,10-dihydro-5,10-dioxo-4-[3-(pentafluoro-λ^6-sulfanyl)phenyl]-4*H*-naphtho[2,3-*b*]pyran-3-carbonitrile (**2 l**). 3-Pentafluorothiobenzaldehyde (232 mg, 1.0 mmol) and malononitrile (70 mg, 1.0 mmol) were dissolved in MeCN (5 mL) and three drops of Et_3N 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. ^{1}H-NMR (300 MHz, (D_6)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). ^{13}C-NMR (75.5 MHz, (D_6)DMSO)): 36.5, 56.8, 119.0, 120.5, 124.5, 125.0, 125.8, 126.1, 129.9, 130.7, 131.0, 131.9, 134.2, 145.4, 149.5, 152.9, 158.3, 176.8, 182.6. EI-MS: 454 (100) [M^+], 326 (51), 251 (100). Anal. calc. for $C_{20}H_{11}F_5N_2O_3S$ (454.37): C 52.87, H 2.44, N 6.17; found: C 52.72, H 2.34, N 6.11.

2-Amino-4-(3-cyanophenyl)-5,10-dihydro-5,10-dioxo-4*H*-naphtho[2,3-*b*]pyran-3-carbonitrile (**2m**). 3-Cyanobenzaldehyde (131 mg, 1.0 mmol) and malononitrile (70 mg, 1.0 mmol) were dissolved in MeCN (3 mL) and three drops of Et_3N 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 1 h. The formed precipitate was collected, washed with MeCN and *n*-hexane and dried in vacuum. Yield: 110 mg (0.31 mmol, 31 %). Red solid. M.p. 257 °C. IR: 3425, 3329, 3284, 3256, 3222, 3190, 2235, 2201, 1668, 1656, 1637, 1602, 1589, 1484, 1411, 1370, 1336, 1301, 1243, 1195, 1180, 1160, 1140, 1091, 1074, 7036, 1022, 955, 928, 815, 774, 756, 727, 716, 696. ^{1}H-NMR (300 MHz, (D_6)DMSO)): 4.73 (1 H, s), 7.41 (2 H, s), 7.5–7.6 (1 H, m), 7.7–7.8 (2 H, m), 7.8–7.9 (4 H, m), 8.0–8.1 (1 H, m). ^{13}C-NMR (75.5 MHz, (D_6)DMSO)): 36.2, 56.8, 111.6, 118.7, 119.1, 120.4, 125.8, 126.0, 129.7, 130.8, 130.9, 131.4, 132.8, 134.1, 134.5, 145.3, 149.6, 158.3, 176.8, 182.6. EI-MS: 353 (94) [M^+], 251 (100). Anal. calc. for $C_{21}H_{11}N_3O_3$ (353.34): C 71.39, H 3.14, N 11.89; found: C 71.31, H 3.22, N 11.78.

2-Amino-4-(4-cyanophenyl)-5,10-dihydro-5,10-dioxo-4*H*-naphtho[2,3-*b*]pyran-3-carbonitrile (**2n**). 4-Cyanobenzaldehyde (131 mg, 1.0 mmol) and malononitrile (70 mg, 1.0 mmol) were dissolved in MeCN (3 mL) and three drops of Et_3N 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 1 h. The formed precipitate was collected, washed with MeCN and *n*-hexane and dried in vacuum. Yield: 160 mg (0.45 mmol, 45 %). Red solid. M.p. 279–280 °C. IR: 3402, 3322, 3284, 3249, 3218, 3194, 2231, 2203, 1675, 1662, 1639, 1602, 1505, 1411, 1363, 1340, 1331, 1303, 1244, 1203, 1178, 1159, 1095, 1074, 1037, 1020, 949, 842, 797, 777, 752, 740, 721, 679. ^{1}H-NMR (300 MHz, (D_6)DMSO)): 4.74 (1 H, s), 7.44 (2 H, s), 7.56 (2 H, dd, J = 8.4 Hz, 1.8 Hz), 7.78 (2 H, dd, J = 8.4 Hz, 1.8 Hz), 7.8–7.9 (3 H, m), 8.0–8.1 (1 H, m). ^{13}C-NMR (75.5 MHz, (D_6)DMSO)): 36.6, 56.5, 109.9, 118.7, 119.0, 120.7, 125.8, 126.1, 128.9, 130.7, 130.9, 132.6, 134.2, 134.5, 149.0, 149.5, 158.4, 176.7, 182.5. EI-MS: 353 (95) [M^+], 251 (100). Anal. calc. for $C_{21}H_{11}N_3O_3$ (353.34): C 71.39, H 3.14, N 11.89; found: C 71.29, H 3.20, N 11.81.

2-Amino-4-(4-ethynylphenyl)-5,10-dihydro-5,10-dioxo-4*H*-naphtho[2,3-*b*]pyran-3-carbonitrile (**2o**). 4-Ethynylbenzaldehyde (130 mg, 1.0 mmol) and malononitrile (70 mg, 1.0 mmol) were dissolved in MeCN (3 mL) and three drops of Et_3N 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 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. $>395\,^{\circ}C$. 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. ^{1}H-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). ^{13}C-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 $C_{22}H_{12}N_2O_3$ (352.35): C 74.99, H 3.43, N 7.95; found: C 75.10, H 3.31, N 7.82.

Toxoplasma gondii *Cell Line, Culture Conditions and Assay*

Vero cells (ATCC® CCL81™, USA) were applied for the cultivation of *T. gondii* tachyzoites of the RH strain. The Vero cells in 96-Well plates were cultured in RPMI 1640 medium (5×10^3 cells/well in 200 μL) with 10% heat inactivated fetal bovine serum (FBS, Invitrogen, USA) in a humidified 5% CO_2 atmosphere at 37 °C. The inhibition assay with the *T. gondii* parasites in Vero cells was carried out as described previously.[25,39]

Leishmania major *Cell Isolation and Culture Conditions*

Promastigotes of *L. major* were isolated in February 2016 and maintained in Schneider's Drosophila medium (Invitrogen, USA), which was supplemented with 10% FBS and antibiotics, in a tissue culture flask with weekly transfers at 26 °C. Promastigotes were cryopreserved in liquid nitrogen at concentrations of 3×10^6 parasite/mL. The virulence of *L. major* parasites was maintained by passing in female BALB/c mice via injection of 1×10^6 stationary-phase promastigotes in the hind footpads. *L. major* amastigotes were isolated from the treated mice after 8 weeks. The transformation of the isolated amastigotes into promastigotes was achieved by culturing at 26 °C in Schneider's medium supplemented with 10% FBS and antibiotics. Amastigote-derived promastigotes with less than five *in vitro* passages were used for the infection of mice. Male and female BALB/c mice were obtained from Pharmaceutical College, King Saud University, Saudi Arabia, and maintained in specific pathogen-free facilities.[40,41] Concerning the mentioned laboratory animals, we followed all the instructions and rules designed by the Scientific Research Deanship, Qassim University, Saudi Arabia, under the permission/accreditation number 10124-cosao-2020-1-3-I.

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).[46,47] The free radical scavenging kinetics for standard antioxidant viz. ascorbic acid and the test compounds were calculated using the following formula:

$$\text{DPPH scavenging effect } [\%] = [(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}] \times 100$$

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}.[49,50]

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_4NClO_4 as supporting electrode. Ferrocene served as internal standard.[27] 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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