Synthesis and Biological Evaluation of Pyrazolylnaphthoquinones as New Potential Antiprotozoal and Cytotoxic Agents

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The importance of American trypanosomiasis (Chagas' disease) in human pathology is widely known. The prognosis of this disease is poor and the choice of effective medicines limited, thus study of new drugs is absolutely necessary. In this work, the activities of three new pyrazolylnaphthoquinones, heterocyclic naphthoquinones bearing 3-aminopyrazole rings, were evaluated on Trypanosoma cruzi, *the etiological agent of Chagas' disease. These activities were compared with those of three 5-aminoisoxazole analogues. In addition, since these compounds belong to a family of antiprotozoal and cytotoxic/antitumor agents, the activities of all six against* Plasmodium falciparum, Trypanosoma brucei rhodesiense, *and murine L-6 cells were also investigated. In the biological tests, five of the compounds showed significant in vitro trypanocidal activities against* T. cruzi, *with activities similar to that of benznidazole. Two of the 5-aminoisoxazole analogues also showed good activities, in one case highly selective, against the K1 and NF54 strains of* P. falciparum *($IC_{50} < 0.12\ \mu g\,mL^{-1}$). Three of the compounds were cytotoxic to murine L-6 cells ($IC_{50} = 0.21$–$0.50\ \mu g\,mL^{-1}$). The results suggested that the three pyrazolylnaphthoquinones and one of the 5-aminoisoxazole analogues could be starting points for lead optimization programs against* T. cruzi *and* P. falciparum, *respectively.*

KEYWORDS:

antimalarial agents · antiprotozoal agents · biological activity · heterocycles · quinones

Introduction

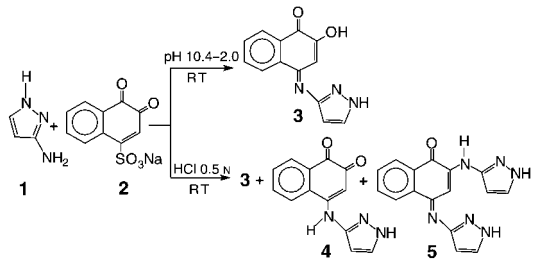
American trypanosomiasis (Chagas' disease) is caused by the hemoflagellate *Trypanosoma cruzi* and transmitted by reduviid bugs. It is the most lethal parasitic disease in the tropics and subtropics of North and South America and causes over one million cases of infection annually and more than 50 000 deaths per year. Currently, there are between 16 – 18 million chronically infected carriers of Chagas' disease, and more than 100 million people at risk.[1]

Despite its epidemiological importance, the chemotherapy of Chagas' disease is still an unsolved problem. In fact, there is neither a vaccine nor a recommended drug available to prevent the disease.[1] Moreover, once the infection has progressed to its later stages, no medication has proven to be effective. The drugs currently available, benznidazole and nifurtimox, although very helpful when given during the acute stage of infection, are ineffective in the chronic period and produce adverse side effects because of their toxicity.[2] More research to discover and develop new drugs to cure the chronic stage of the infection is therefore needed.

The broad spectrum of biological activities of quinones, either natural or synthetic, has been extensively studied, and ranges from antimicrobial[3] to antineoplastic properties.[4]

Our group's interest in quinone chemistry prompted us to give special attention to the synthesis[5] and biological properties of isoxazolylnaphthoquinones.[6] We found that several members of the series were active against *Trypanosoma cruzi*, although at higher concentrations than needed for other quinones.[6b]

In order to identify derivatives with the best activities, the influence of a different heterocyclic substituent on the activity of isoxazolylnaphthoquinones against *T. cruzi* was investigated. Here, the activities of three new heterocyclic naphthoquinones derived from 3(5)-aminopyrazole (**1**)—the pyrazolylnaphthoquinones **3** – **5** (Scheme 1)—were therefore evaluated in vitro



Scheme 1. *Reagents and conditions for the synthesis of pyrazolylnaphthoquinones **3** – **5**.*

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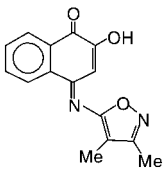
against *T. cruzi* and compared with those of three 5-amino-isoxazole analogues (**6** – **8**). In addition, since **3** – **8** belong to the quinone family, their antiprotozoal and cytotoxic activities against *Plasmodium falciparum*, *Trypanosoma brucei rhodesiense*, and murine L-6 cells were also tested.



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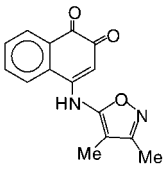

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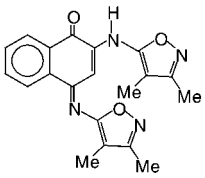

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Results

Chemistry

Preliminary experiments investigating the reaction between **1** and sodium-1,2-napthoquinone-4-sulfonate (**2**) showed that the reaction pathway was governed by the medium.

Basic, neutral, and mildly acidic conditions

The synthesis of **3** (Scheme 1) proceeded readily in buffer phosphate (pH 10.4), in distilled water (pH 7.0), and in citrate – phosphate buffer (pH 4.0 and 2.0). The reaction was monitored by TLC (in different adsorbent and solvent systems) and paper electrophoresis, which showed that **3** was a chromatographically and electrophoretically homogeneous product.

Acidic conditions

When the reaction between **1** and **2** was carried out in aqueous HCl (0.5 N) at room temperature, a mixture of **3** (20 %), **4** (5 %), and **5** (40 %) was obtained (Scheme 1). Compounds **4** and **5** were also found to be chromatographically and electrophoretically homogeneous products.

The structures of **3** – **5** were verified by analytical and spectroscopic data obtained by MS, FTIR spectroscopy, and ^{1}H, ^{13}C, and 2D NMR spectroscopy. The following trends in the ^{1}H NMR spectra (DMSO-d_6) of **3** – **5** were observed:

1) The chemical shift of the NH-pyrazole proton was large ($\delta \approx$ 12.5, exchangeable by D_2O), which is consistent with literature data.[7]
2) The pyrazole protons appeared as doublets, with the H-5′ proton resonance at lower field than its H-4′ counterpart, as observed in other pyrazole derivatives.[8]
3) Spectra obtained at different concentrations (0.04 M and 0.16 M) did not show duplicated signals, fractional integrations, or concentration effects, which suggests that no tautomeric mixtures or self-association were present in DMSO.

Biological and cytotoxic activities

The results of the biological evaluation of **3** – **5** against *T. cruzi*, *T. b. rhodesiense*, and *P. falciparum* are presented in Table 1, which also contains data for the isoxazolylnaphthoquinones **6** – **8** for comparison.

Table 1. *In vitro antiprotozoal and cytotoxic activities of* ***3*** *–* ***8*** *against* T. cruzi, T. b. rhodesiense, P. falciparum, *and murine* L*-6 cells.*[a]

Compound	*T. cruzi*	*T. b. rhodesiense*	*P. falciparum* K1	*P. falciparum* NF54	Cytotoxicity L-6 cells
Standard	0.56[b]	0.0061[c]	0.067[d] 0.0015[e]	0.003[d] 0.0028[e]	-
3	0.87	1.2	2.31	2.95	1.35
4	0.69	0.4	1.11	1.46	0.21
5	0.43	0.4	0.84	1.36	0.21
6	4.56	3.64	8.78	>10	4.5
7	0.65	0.47	0.11	0.16	0.5
8	12.85	0.92	0.12	0.19	51

[a] The figures reported are IC_{50} values in $\mu g\,mL^{-1}$. IC_{50} = the concentration required to give 50% inhibition. Standards: [b] benznidazole, [c] melarsoprol, [d] chloroquine, [e] artemisinin.

Compounds **3** – **5** exhibited good inhibitory activity against *T. cruzi*, with IC_{50} values in a range comparable to that of benznidazole (IC_{50}: 0.56 $\mu g\,mL^{-1}$). It is noteworthy that **3** – **5** are more active than **6** (IC_{50}: 4.56 $\mu g\,mL^{-1}$), the prototype of this group of drugs, which was previously[6c] found to be active against *T. cruzi* in vitro and in vivo without inducing toxic effects on Balb/c mice even at doses of 1300 $mg\,kg^{-1}$. It is also remarkable that **4** and **7**, which are 1,2-quinones, were more potent than the quinone imines **3** and **6**, which suggests that different mechanisms of action may be involved.[9]

Activity against *T. b. rhodesiense* was less pronounced and far weaker than the activity of the standard drug melarsoprol. However, **4**, **5**, and **7** were more active than the other derivatives tested, with IC_{50} values $< 0.5\ \mu g\,mL^{-1}$ (Table 1).

In terms of their antimalarial activity, **3** – **6** were found to be inactive against the *P. falciparum* strains K1 (resistant to chloroquine and pyrimethamine) and NF54 (sensitive to all known drugs). Their IC_{50} values were in a range (0.84 to $> 10\ \mu g\,mL^{-1}$) outside the threshold set for activity (0.5 $\mu g\,mL^{-1}$). In contrast, **7** and **8** showed good antimalarial activity, with IC_{50} values below 0.2 $\mu g\,mL^{-1}$. Compound **8** is of particular interest because it has a highly selective activity against *P. falciparum*, shows no cytotoxicity, and is also structurally distinct from existing antimalarial agents (Table 1).

All the compounds except for **8** showed some degree of cytotoxicity for the L-6 cells. Examination of the data in Table 1 shows that **3** – **5** were more cytotoxic than **6** – **8**. Compounds **4** and **5** have to be considered cytotoxic (IC_{50}: 0.21 $\mu g\,mL^{-1}$). Compound **7** was slightly less cytotoxic (IC_{50}: 0.50 $\mu g\,mL^{-1}$) than **4** and **5**, but its good antiparasitic activities could also lack specificity. In contrast, **8** showed no cytotoxicity for the L-6 cells (IC_{50}: 51 $\mu g\,mL^{-1}$).

Discussion

These results provide evidence of in vitro activity of the compounds under investigation against *T. cruzi* and *P. falciparum*. On the basis of their promising activity against *T. cruzi*, compounds **3** – **5** could have potential as leads, provided that their cytotoxicity can be circumvented. Likewise, the highly selective activity of **8** against *P. falciparum* warrants investigation of its potential as an antiplasmodial agent, which should be taken as the starting point for a lead optimization program against this parasite. Previous studies[3] have reported the susceptibility of *P. falciparum* to naphthoquinones, but there are no previous descriptions of antiplasmodial activity of **8**.

Modification of the heterocyclic substituent on the C-2 and N positions of **6** – **8**, studied here, indicated that such substitution can increase the antiprotozoal and cytotoxic effect in vitro. If the activity of **3** – **8** against L-6 cells is compared with the trypanocidal activity, the correlation is highly significant. In this context, it was previously observed[6a, b] that some isoxazolylnaphthoquinones affected *T. cruzi* in a similar way to *o*-quinones, which inhibit growth and DNA synthesis in *T. cruzi* and stimulate oxygen uptake and superoxide anion generation by the parasites epimastigotes, although direct inhibition of enzyme reactions could also take place. These results support the idea that the effects of **6** and **7** were mostly connected with the *o*-quinone function. In contrast, it seems that the degree of cytotoxic and antiprotozoal activity in **3** – **5** was mainly dependent on the heterocyclic moiety. In fact, **3** was more cytotoxic than **6**, while **5** showed cytotoxicity, quite the opposite of **8**, which is not cytotoxic. These findings offer some insight into the design of more efficacious drugs by suggesting that the trypanocidal and the cytotoxic activities might be separated by molecular modification. On the other hand, the cytotoxic action of **3** – **5** raises some interesting questions concerning their potential as antitumor molecules, since it is known that several therapeutic drugs, such as lapachona,[10] azaserine, and mechloroethamide,[11] have simultaneously shown antiparasitic and anticancer activities, though through different mechanisms. Thus, further studies are necessary to evaluate the possible correlation between the antiprotozoal and the cytotoxic activity of this family of compounds.

Experimental Section

Melting points (m.p.s) were determined by differential scanning calorimetry (DSC) on a MDSC 2920 differential scanning calorimeter (TA Instruments, Inc.) Yields of solids refer to crude products. IR (KBr), mass, and NMR spectra were recorded on Nicolet 5-SXC FTIR, Finningan 3300, and Bruker AC 200 (equipped with an Aspect 3000 computer) spectrometers, respectively. ^{13}C NMR spectra (broad-band decoupled and destortionless enhancement by polarization transfer (DEPT)) were taken with approximately 0.16 M solutions; the center of the solvent peak was used as an internal standard, which was related to tetramethylsilane (TMS) [$\delta = 39.50$ ppm for DMSO-d_6]. The ^{1}H and ^{13}C NMR signals were assigned by comparing chemical shifts of model substances described in the literature [3(5)-aminopyrazole (also run in DMSO-d_6 in order to obtain values suitable for comparison), pyrazole,[7] pyrazole derivatives,[8] and isoxazolylnaphthoquinones[5]], and also by selective proton decoupling and 2D NMR spectroscopy [HETCOR experiment]. The NMR and FTIR spectra were processed by use of the Mestre-C [Magnetic Resonance Companion, J.C. Cobas, J. Cruces, and F.J. Sardina (1996 – 1998)] and OMNIC 1.0 programs, respectively. High-resolution mass spectra (HRMS) were obtained at the McMaster Center for Mass Spectrometry, Canada. 3-Aminopyrazole (98% purity) was purchased from Aldrich, Co. Compounds **6** – **8** were synthesized and purified by a reported procedure.[5a]

2-Hydroxy-*N*-[3(5)-pyrazolyl]-1,4-naphthoquinone-4-imine (3): A solution of **1** (0.083 g, 1 mmol) in water (10 mL) was added to a solution of **2** (0.260 g, 1 mmol) in phosphate buffer (pH 10.4, 50 mL). The reaction mixture was stirred for 30 min at room temperature. After that, the mixture was filtered off, washed with water, and dried to give **3** (0.169 g, 71% yield). The crude product was recrystallized from dimethyl sulfoxide (DMSO)/water. M.p. (DSC): 264.6 °C (dec.); ^{1}H NMR (200 MHz, DMSO-d_6, 25 °C, TMS): $\delta = 6.35$ (d, 1H; H-4′), 6.87 (br s, 1H; H-3), 7.70 (td, 1H; H-6), 7.83 (d, 1H; H-5′), 7.86 (td, 1H; H-7); 8.03 (dd, 1H; H-5), 8.38 (dd, 1H; H-8), 9.88 (br s, 1H; 2-OH, disappears with D_2O), 12.84 (br s, 1H; NH, disappears with D_2O) ppm; ^{13}C NMR (200 MHz, DMSO-d_6, 25 °C, TMS): $\delta = 98.8$ (C-4′), 102.9 (C-3), 123.6 (C-8), 127.9 (C-5), 129.5 (C-9 and C-5′), 130.8 (C-10 and C-6), 133.9 (C-7), 147.2 (C-3′), 151.5 (C-2), 176.6 (C-4), 180.8 (CO) ppm; IR (KBr): $\tilde{\nu} =$ 3237/3138 (NH, OH), 1693 (CO), 1606/1532 (C=N) cm^{-1}; MS (70ev): *m/z* (%): 239 (80) $[M]^+$, 240 (100) $[M+1]^+$, 212 (50)$[M-27]^+$, 27 (18); HRMS: *m/z* found: 239.067 $[M]^+$; $C_{13}H_9N_3O_2$ calcd: 239.0694.

***N*-(3(5)-Pyrazolyl)-1,2-naphthoquinone-4-amine (4) and 2-*N*-(3(5)-pyrazolylamino)-1,4-naphthoquinone-4-imine (5)**: A solution of **1** (1.66 g, 20 mmol) in water (50 mL) was added to a solution of **2** (2.60 g, 10 mmol) in aqueous HCl (0.5 N, 70 mL). The mixture was stirred for 30 min at RT and then extracted three times with ethyl acetate. The organic extracts were collected and then extracted with aqueous NaOH (0.05 N), washed with water, dried with Mg_2SO_4, and evaporated under reduced pressure to give a deep red crude product, which was purified by radial preparative chromatography on silica gel. Compound **4** was eluted with benzene/ethyl acetate (4:1) and **5** with benzene/ethyl acetate (3:2). Both solids were recrystallized from absolute methanol.

Compound **4:** M.p. (DSC): 251.7 °C; ^{1}H NMR (200 MHz, DMSO-d_6, 25 °C, TMS): $\delta = 6.38$ (d, 1H; H-4′), 7.40 (s, 1H; H-3), 7.68 (d, 1H; H-5′), 7.77 (m, 1H; H-6), 7.85 (m, 1H; H-7), 7.95 (m, 1H; H-5), 8.05 (m, 1H; H-8), 9.54 (br s, 1H; 4-NH, disappears with D_2O), 12.52 (br s, 1H; NH, disappears with D_2O) ppm; ^{13}C NMR (200 MHz, DMSO-d_6, 25 °C, TMS): $\delta = 96.9$ (C-4′), 106.5 (C-3), 125.2 (C-5), 126.1 (C-8), 129.1 (C-9$^{\#}$), 130.4 (C-5′), 132.6 (C-10$^{\#}$), 132.5 (C-6), 134.8 (C-7), 143.3 (C-3′), 148.7 (C-4), 181.9 (CO), 182.6 (CO) ppm ($^{\#}$: interchangeable); IR (KBr): $\tilde{\nu} = 3263$ (NH), 1676/1628 (CO), 1608/1533 (C=N) cm^{-1}; MS (70ev): *m/z* (%): 240 (23) $[M+1]^+$, 213 (25)$[M+1-27]^+$, 68 (33), 44 (100); HRMS: *m/z* found: 239.069 $[M]^+$; $C_{13}H_9N_3O_2$ calcd: 239.0694.

Compound **5:** M.p. (DSC): 270.2 °C (dec.); ^{1}H NMR (200 MHz, DMSO-d_6, 25 °C, TMS): $\delta = 6.27$ (d, 1H; H-4′$^{\#}$), 6.37 (d, 1H; H-4′′$^{\#}$), 7.66 (s, 2H; H-3 and H-5′), 7.77 (td, 1H; H-6), 7.84 (td, 1H; H-7), 8.16 (dd, 1H; H-5), 8.43 (d, 1H; H-5′′$^{\#}$), 8.50 (dd, 1H; H-8), 9.34 (br s, 1H; 2-NH, disappears with D_2O), 12.48 (br s, 1H; NH, disappears with D_2O), 12.96 (br s, 1H; NH, disappears with D_2O) ppm ($^{\#}$: interchangeable); ^{13}C NMR (200 MHz, DMSO-d_6, 25 °C, TMS) $\delta = 96.0$ (C-4′ and C-4′′), 101.4 (C-3), 124.7 (C-5′′ and C-8), 125.9 (C-5), 128.7 (C-10$^{\#}$), 128.9 (C-5′), 129.9 (C-9$^{\#}$), 130.3 (C-6), 133.4 (C-3′and C-7), 135.1 (C-3′′), 139.1 (C-2), 149.2 (C-4), 180.6 (CO) pm ($^{\#}$: interchangeable); IR (KBr): $\tilde{\nu} = 3296$ (NH), 1656 (CO), 1605/1533 (C=N) cm^{-1}; MS (70ev): *m/z* (%): 304 (94) $[M]^+$, 305 (100) $[M+1]^+$, 277 (11) $[M-27]^+$, 83 (42), 41 (67), 27 (33); HRMS: *m/z* found: 304.104; $C_{16}H_{12}N_6O$ calcd: 304.107.

Biological screening tests:

T. cruzi: Rat skeletal myoblasts (L-6 cells) were seeded in 96-well microtiter plates at 2000 cells/well/100 μL in RPMI 1640 medium with 10% fetal bovine serum and L-glutamine (2 mM). After 24 hours, 5000 trypomastigotes of *T. cruzi* (Tulahuen strain C2C4 containing the β-galactosidase (*lac Z* gene)) were added (100 μL per well) with serial drug dilutions. The plates were incubated at 37 °C in 5% CO_2 for 4 days. After 96 hours, the substrate (CPRG/Nonidet) was added to the wells. The color reaction, which developed during the following 2–4 hours, was read photometrically at 540 nm. Optical density values were expressed as percentages of the control, and IC_{50} values were calculated from the sigmoidal inhibition curve.

T. b. rhodesiense: Minimum essential medium (MEM; 50 μL), supplemented according to Baltz et al.[12] with 2-mercaptoethanol and 15% heat-inactivated horse serum, was added to each well of a 96-well microtiter plate. Serial drug dilutions were added to the wells, followed by a trypanosome suspension (*T. b. rhodesiense* STIB 900, 50 μL). After incubation for 72 hours at 37 °C under a 5% CO_2 atmosphere, Alamar Blue (10 μL) was added to each well, and incubation was continued for a further 2–4 hours. The plate was then read with a Millipore Cytofluor 2300 instrument by use of an excitation wavelength of 530 nm and an emission wavelength of 590 nm.[13] Fluorescence development was expressed as percentage of the control and the IC_{50} values were determined.

P. falciparum: Antiplasmodial activity was determined with the *P. falciparum* strains K1 and NF54. A modification of the [^{3}H]-hypoxanthine incorporation assay[14] was used. Briefly, infected human red blood cells were exposed to serial drug dilutions in microtiter plates for 72 hours. Viability was assessed by measuring the incorporation of [^{3}H]-hypoxanthine during the final 24 hours of incubation by liquid scintillation counting. Counts were expressed as percentages of the control and presented as sigmoidal inhibition curves. IC_{50} values were calculated by linear interpolation with selection of values above and below the 50% mark, according to Hills et al.[15]

Cytotoxicity for L-6 cells: The determination of the cytotoxicity was performed with L-6 rat skeletal myoblast cells according to a previously reported procedure.[16] Briefly, L-6 cells were seeded in 96-well microtiter plates at a density of 10^5 mL^{-1} in MEM supplemented with 10% heat-inactivated fetal bovine serum. A three-fold serial dilution ranging from 90 to 0.123 μg mL^{-1} in test medium was added. The plates were incubated as described for the antitrypanosomal assay. After 70 hours, Alamar Blue (10 μL) was added to each well and incubation was continued for a further 2–4 hours. The plate was then processed in the same way as described for *T. b. rhodesiense*.

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