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Synthesis and antileishmanial activity of novel buparvaquone oxime derivatives

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Abstract—Novel oxime derivatives (**2**, **3** and **5**) of buparvaquone (**1**) and *O*-methyl-buparvaquone (**4**) were synthesized and their in vitro activities against *Leishmania donovani*, the causative agent of visceral leishmaniasis (VL), were determined. Buparvaquone-oxime (**2**) was also studied as a bioreversible prodrug structure of buparvaquone (**1**). Buparvaquone-oxime (**2**) released buparvaquone (**1**) in vitro when it was incubated with induced rat liver microsomes, which suggests that the oxime-structure is a useful prodrug template for developing novel prodrugs of buparvaquone and other hydroxynaphthoquinones. Moreover, the formation of NO_2^-, formed via oxidation of NO, was confirmed during the bioconversion. The release of NO from buparvaquone-oxime (**2**) may provide an additional therapeutic effect in the treatment of leishmaniasis. Buparvaquone-oxime (**2**) and buparvaquone-*O*-methyl-oxime (**3**) demonstrated moderate activity against amastigotes of the *Leishmania* species that causes VL. However, the studied oximes (**2**, **3**) most probably did not release buparvaquone (**1**) and NO during the present in vitro experiment. Further in vivo studies are needed to verify the biological activity of buparvaquone-oximes in the treatment of leishmaniasis.

1. Introduction

Leishmaniasis is a widespread parasitic disease that is caused by protozoan parasites of the genus *Leishmania* in tropical and subtropical areas in both the old and new worlds.[1] This disease occurs in two major forms; cutaneous (CL) and visceral leishmaniasis (VL). The parasitic protozoa *Leishmania donovani* is the causative agent of VL, and species that cause CL are *L. major*, *L. tropica* and *L. aethiopica L. braziliensis*, *L. panamensis*, *L. amazonensis* and *L. mexicana*.[2] The most widely used treatment for leishmaniasis over the past 50 years has been pentavalent antimonial compounds.[1,2] These drugs are effective, but they have several disadvantages such as the need for parenteral administration at high dosages, long duration of therapy (several weeks), considerable toxicity, resistance and variable efficacy. Although new drugs are on clinical trial,[2] there remains an urgent need for improved antileishmanials.

Some hydroxynaphthoquinones, for example buparvaquone, have demonstrated activity against *L. donovani* and some other *Leishmania* species, both in vitro and in vivo as oral, subcutaneous and topical administration, over 20 years ago.[3–6] In our previous paper, buparvaquone (**1**) the high activity against species that cause CL was confirmed.[7] The antiprotozoal activity of hydroxynaphthoquinones has also been demonstrated for other parasite species; for example, *Theilaria Parva*,[4] *Eimeria tenella*,[4] *Plasmodium falciparum*,[4] *Trypanosoma cruzi*,[8] *Trypanosoma bruce*.[8] The efficacy of hydroxynaphthoquinones varies significantly in different species, and the mechanism of action of hydroxynaphthoquinones remains partly unclear. In *Plasmodium* it has been proposed that these compounds inhibit electron transfer at complex III of the mitochondrial respiratory chain.[6,9] The other proposed mode of action for hydroxynaphthoquinones involves the

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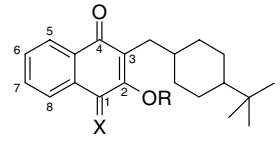
ability of the drug to form free radicals during interaction with the parasite's respiratory chain.[5,10] In the presence of oxygen, the semiquinone radical reduces oxygen to superoxide radicals.[10] Besides the production of superoxide (O_2^-), the generation of nitric oxide (NO) by activated macrophages is an important mechanism to eliminate intracellular *Leishmania* parasites.[11–13] NO is normally produced by inducing nitric oxide synthases (iNOS) in the macrophage, which catalyzes the oxidation of L-arginine by NADPH and O_2, with the subsequent formation of L-citrulline and NO.[14] In addition to NO biosynthesis by the oxidation of L-arginine, many studies have shown that various compounds such as alkyl- and aryl-aldoximes, ketoximes, amidoximes and guanidoximes containing a C=NOH structure can be oxidized by liver microsomal cytochrome P450 with the formation of nitrogen oxides, including NO, and the corresponding compound containing a carbonyl group.[15–18]

These findings led us to investigate various oxime derivatives of buparvaquone as novel antileishmanials. The present study describes the synthesis of buparvaquone-oxime (**2**), buparvaquone-*O*-methyloxime (**3**) and *O*-methyl-buparvaquone-oxime (**5**). The oxidation of buparvaquone-oxime (**2**), which contains a C=N–OH group, was studied in induced rat liver microsomes, to investigate the possible bioconversion of this derivative (**2**) to the corresponding active metabolite, buparvaquone (**1**), thus making the formation of NO possible. Therefore, to further investigate the effects of the oxime function on the activity of these compounds were determined against the amastigote forms of *L. donovani* in macrophages in vitro.

2. Results and discussion

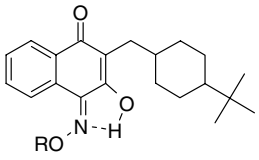
2.1. Chemistry

The structures of buparvaquone-oxime (**2**), buparvaquone-*O*-methyloxime (**3**) and *O*-methyl-buparvaquone-oxime (**5**) are presented in Scheme 1. The compounds were prepared according to the methods described by Strenge and Rademacher.[19,20] Buparvaquone was treated with hydroxylamine hydrochloride or with *O*-methylhydroxylamine hydrochloride to afford the oximes **2** and **3** in high yield. Compound **5** was synthesized by the previous method using methylated buparvaquone as a starting compound, which was prepared as described earlier.[21] The synthesized compounds were



1: X = -O, R = -H (Buparvaquone)
2: X = -NOH, R = -H
3: X = -NOCH$_3$, R = -H
4: X = -O, R = -CH$_3$
5: X = -NOH, R = -CH$_3$

Scheme 1.



Scheme 2. The predominant *Amphi* conformer (*E*-isomer) of **2** (R = H) and **3** (R = CH$_3$).

purified by flash chromatography, and in all cases the products were obtained as mixtures of *E*- and *Z*-isomers at the C=N double bonds. The pure geometrical isomers were not isolated, as the literature suggests that the *E*- and *Z*-forms are in a pH and temperature dependent equilibrium.[22–25] However, *E*- and *Z*-isomers of each compound (**2**, **3** and **5**) can be easily verified by the ^{1}H and ^{13}C NMR chemical shifts from the downfield shift of the C-8 proton for the major isomers of **2**, **3** and **5**, due to the aromatic ring current.[26] Experimental and theoretical investigations on the structure of 2-hydroxy-3-methyl-1,4-naphthoquinone show that it exists predominantly as the *E*-isomer (*amphi* conformer) by forming a N···H–O intramolecular hydrogen bond.[27] Correspondingly, the same *amphi* conformer is suggested for compounds **2** and **3** (Scheme 2). Steric factors may explain the preference of the *E*-isomer for **5**. The HMBC NMR spectrum (the H–C long range correlation) confirmed that the reaction of buparvaquone with hydroxyl- and methoxyamine regio-selectively produced the oximes only at the C-1 carbonyl, which has been previously shown for the naphthoquinone lapachol oxime.[28] Hydrogen bonding is also a logical explanation for the regio-selective reaction at the C-1 carbonyl (Scheme 2).

2.2. Microsomal oxidation of buparvaquone oxime

Treatment of rats with known inducers of cytochrome P450, such as phenobarbital (PB), dexamethasone (DEX) and 3-methylcholanthrene (3-MC), results in increased titers of cytochrome P450 in liver microsomes. The ability of the buparvaquone-oxime (**2**) derivative, containing a C=NOH group, to undergo a P450 dependent oxidative cleavage to buparvaquone (corresponding C=O group) required the incubation of buparvaquone-oxime (**2**) with rat liver microsomes in

Table 1. Formation of buparvaquone and NO_2^- during incubations of buparvaquone-oxime (**2**) in the presence of NADPH (mean; $n = 2$) and rat liver microsomes (at 37 °C) from untreated rats or from rats treated with either 3-methylcholanthrene (3-MC), dexamethasone (DEX) or phenobarbital (PB), or without microsomes (blank)

Microsomes	Formation of buparvaquone[a]	Formation of NO_2^-[a]
Without (blank)	Nd[b]	Nd[b]
Untreated	3.9	Nd[b]
3-MC	7.5	0.5
DEX	11.8	1.8
PB	10.6	0.1

[a] Results are expressed as nmol of product (5 mg of protein)$^{-1}$ (24 h)$^{-1}$.
[b] Nd = not detectable.

the presence of NADPH (Table 1). Microsomes from rats treated with the inducers had a higher ability to oxidize buparvaquone-oxime (**2**) to buparvaquone than the untreated microsomes. The metabolite of the oxidation was verified to be buparvaquone (**1**) by HPLC with an authentic buparvaquone standard. The formation of buparvaquone did not occur in the absence of microsomes and, thus, the data suggests that the reaction is catalyzed by microsomal oxidizing enzymes. Also, the chemical degradation of buparvaquone-oxime (**2**) to buparvquone was not observed during five days of chemical stability determinations at pH 7.4 (data not shown). Poor stability in aqueous solution does not limit the use of the oxime structure in the case of buparvaquone, in contrast to studies with other oximes.[29] Moreover, the formation of NO_2^- was observed (Table 1) during the incubation with rat liver microsomes induced by the known inducers, as earlier reported for other oxime structures.[15,16,18] Thus, NO is apparently released during the bioconversion of buparvaquone-oxime (**2**) to buparvaquone (**1**). A major limitation of these studies was the poor aqueous solubility of buparvaquone-oxime (**2**) (Table 2) and buparvaquone.[7] Although 2% (v/v) ethanol was used as a co-solvent, buparvaquone-oxime (**2**) was studied as a suspension in the incubations. Due to an even lower aqueous solubility of buparvaquone-*O*-methyloxime (**3**) and *O*-methyl-buparvaquone-oxime (**5**) (Table 2), their oxidation in microsomes were not investigated at all.

2.3. In vitro antileishmanial activity

The in vitro activity of buparvaquone-oxime (**2**), buparvaquone-*O*-methyloxime (**3**) and *O*-methyl-buparvaquone-oxime (**5**) against intracellular amastigotes *L. donovani* are shown in Table 3. The ED_{50} values for amastigotes indicate that oximes **2**, **3** and **5** had lower activity than buparvaquone (**1**), whose high antileishmanial activity against *Leishmania* spp. have been shown in earlier studies.[5–7] However, the antileishmanial activity of **2** and **3** were comparable to the activity of sodium stibogluconate (Sb^v), the standard control compound, whereas *O*-methyl-buparvaquone-oxime (**5**) was almost inactive. It was likely that buparvaquone-oxime (**2**), buparvaquone-*O*-methyloxime (**3**) and *O*-methyl-buparvaquone-oxime (**5**) did not release buparvaquone (**1**) and NO during the in vitro experiments, and thus they demonstrated only moderate in vitro activity against *Leishmania*. Thus, further in vivo activity studies, which enable the oximes to release buparvaquone (**1**) and NO via a P450 dependent oxidative cleavage, are needed to verify the biological activity of various buparvaquone-oximes against *Leishmania*.

Table 2. Aqueous solubility of buparvaquone-oxime (**2**), buparvaquone-*O*-methyloxime (**3**), *O*-methyl-buparvaquone-oxime (**5**) and buparvaquone (BPQ) in 0.185 M borate buffer (pH 7.4) at room temperature (mean ± SD; $n = 3$)

Compound	Aqueous solubility (μg/mL)
BPQ	0.03 ± 0.01[a]
2	5.42 ± 0.46
3	<0.03
5	Nd[b]

[a] From Ref. 7.
[b] Not determined.

Table 3. The in vitro activity of buparvaquone-oxime (**2**), buparvaquone-*O*-methyloxime (**3**), *O*-methyl-buparvaquone-oxime (**5**) against *L. donovani* HU3 amastigotes

Compound	% Inhibition (μg/mL)				ED_{50} (μg/mL)
	30	10	3	1	
BPQ	—	—	—	—	0.14[a]
2	86.9	12.2	0.0	—	17.52
3	86.1	1.9	0.0	—	21.16
5	3.6	0.0	—	—	>30
Sb^v (control)[b]	66.1	39.7	11.4	—	15.89

[a] From Ref. 7.
[b] Sodium stibogluconate (Pentostam).

3. Conclusion

Novel buparvaquone-oxime (**2**), buparvaquone-*O*-methyloxime (**3**) and *O*-methyl-buparvaquone-oxime (**5**) were synthesized as novel antileishmanials. Compound **2** underwent an enzymatic oxidative cleavage to buparvaquone and, thus, is a prodrug of buparvaquone (**1**). In addition, the release of NO during bioconversion may provide an additional therapeutic effect in the treatment of leishmaniasis. Moreover, the present study reports moderate in vitro activity for the studied compounds (**2** and **3**) against intracellular *L. donovani* amastigotes. The lower in vitro activity of the oxime compounds compared to buparvaquone (**1**) is most probably due to their slow conversion to buparvaquone (**1**) during the in vitro experiment, and thus further in vivo studies are needed to demonstrate the advantage of the novel buparvaquone-oximes in the treatment of leishmaniasis.

4. Experimental section

4.1. General procedures

^1H and ^{13}C NMR spectra were recorded on a Bruker Avance DRX500 spectrometer (Bruker, Rheinstetter, Germany) operating at 500.13 and 125.76 MHz, respectively, using TMS as an internal reference for both ^1H and ^{13}C. Elemental analyses were carried out on a ThermoQuest CE Instruments EA 110-CHNS-O elemental analyser. Mass spectra were acquired by a LCQ quadrupole ion trap mass spectrometer with an electrospray ionisation source (Finnigan, San Jose, CA). The samples were diluted with methanol (20 μg/mL) and 5 μL of samples were injected. TLC analyses were run on Kieselgel 60 F_{254} plates (DC-Alufolien, Merck), and column chromatography was performed with silica gel 60 (Merck) (0.063–0.200 mm). All solvents and reagents were from commercial suppliers and used as received.

4.2. Chemistry

4.2.1. 3-(*trans*-4-*tert*-Butyl-cyclohexylmethyl)-2-hydroxy-[1,4]naphthoquinone-1-oxime (2). To a solution of buparvaquone (**1**) (1.00 g, 3.06 mmol) and hydroxylamine hydrochloride (0.53 g, 5.66 mmol) in ethanol (100 mL) was added sodium acetate (0.76 g, 9.19 mmol) in water (2 mL) and the mixture was refluxed for 3 days. The solvent was removed under reduced pressure, and a crude product was purified by silica gel column chromatography (petroleum ether/ethyl acetate, 3:1) affording **2** (5:1 mixture of *E*/*Z* isomers) ($R_f = 0.35$) as a yellow solid (0.92 g, 2.69 mmol, 88.1%): mp (decomposed). ^{1}H NMR (d_6-DMSO) *E*-isomer δ 0.80 (9H, s, –C(CH_3)$_3$), 0.82–1.01 (5H, m, CHC(CH$_3$)$_3$, H$_{ax}$), 1.46 (1H, br m, CH–CH$_2$), 1.69 (4H, m, H$_{eq}$), 2.39 (2H, d, $J = 7.2$ Hz, CH_2–CH), 7.61 (1H, td, $J = 7.5, 1.3$ Hz, H-6), 7.67 (1H, td, $J = 7.7, 1.6$ Hz, H-7), 8.09 (1H, dd, $J = 7.6, 1.6$ Hz, H-5), 8.97 (1H, d, $J = 8.1$ Hz, H-8), 9.61 (1H, br s, OH), 13.57 (1H, br s, OH); *Z*-isomer δ 7.56 (1H, td, $J = 7.5, 1.4$ Hz, H-6), 7.60 (1H, td, $J = 7.6, 1.6$ Hz, H-7), 7.98 (1H, dd, $J = 7.6, 1.6$ Hz, H-5), 8.08 (1H, d, $J = 7.8$ Hz, H-8), (other signals overlap with the *E*-isomer). ^{13}C NMR (d_6-DMSO) *E*-isomer δ 26.8, 27.3, 29.9, 32.0, 33.3, 37.1, 47.5, 116.1, 125.8, 125.9, 129.2, 130.3, 130.4, 132.0, 139.7, 158.7, 183.3; *Z*-isomer δ 29.3, 32.0, 33.3, 36.9, 47.5, 122.1, 125.2, 129.3, 130.7, 131.4, (other signals overlap with the *E*-isomer). ESI-MS: 340.7 (M−1). Anal. Calcd ($C_{21}H_{27}NO_3 \cdot 0.1H_2O$) C: 73.31, H: 7.97, N: 4.10; found C: 73.48, H: 7.99, N: 4.08.

4.2.2. 3-(*trans*-4-*tert*-Butyl-cyclohexylmethyl)-2-hydroxy-[1,4]naphthoquinone-1-(*O*-methyloxime) (3). To a solution of **1** (3.01 g, 9.22 mmol) and *O*-methyl-hydroxylamine hydrochloride (1.95 g, 23.35 mmol) in ethanol (200 mL) was added sodium acetate (2.28 g, 27.80 mmol) in a mixture of water (5 mL) and refluxed for 5 days. The solvent was removed under vacuum. 50 mL of water was added, and the product was extracted with DCM (3 × 75 mL). The combined organic layers were dried with sodium sulfate and evaporated to give a yellow solid as a 20:1 mixture of *E*/*Z* isomers (3.2 g, 9.00 mmol, 97.6%): mp 150 °C. ^{1}H NMR ($CDCl_3$) *E*-isomer δ 0.80 (9H, s, –C(CH_3)$_3$), 0.82–1.10 (5H, m, CHC(CH$_3$)$_3$, H$_{ax}$), 1.55 (1H, br m, CH–CH$_2$), 1.75 (4H, m, H$_{eq}$), 2.50 (2H, d, $J = 7.3$ Hz, CH_2–CH), 4.28 (3H, s, OCH_3), 7.57 (2H, m, H-6, H-7), 7.66 (1H, s, OH), 8.26 (1H, m, H-5), 8.74 (1H, m, H-8); *E*-isomer: δ 7.52 (2H, m, H-6, H-7), 8.12 (2H, m, H-5, H-8), 9.55 (1H, s, OH) (other signals overlap with the *E*-isomer). ^{13}C NMR ($CDCl_3$, *E*-isomer) δ 27.3, 27.6, 30.5, 32.4, 33.8, 37.6, 48.1, 65.0, 118.3, 125.8, 127.1, 129.6, 131.2, 131.5, 132.0, 139.8, 155.9, 184.5. ESI-MS: 354.5 (M−1). Anal. Calcd ($C_{22}H_{29}NO_3$) C: 74.33, H: 8.22, N: 3.94; found C: 74.10, H: 8.26, N: 3.90.

4.2.3. 3-(*trans*-4-*tert*-Butyl-cyclohexylmethyl)-2-methoxy-[1,4]naphthoquinone (4). Compound **1** (2.00 g, 6.10 mmol) and anhydrous potassium carbonate (3.50 g, 24.4 mmol) were suspended in 100 mL of dry acetone, and dimethyl sulfate (2.3 mL, 24.4 mmol) was added dropwise. The mixture was refluxed for 4 h and evaporated to dryness under high vacuum. The crude product was purified by column chromatography (petroleum ether/ethyl acetate 10:1) affording **4** ($R_f = 0.42$) as a yellow oil (2.04 g, 6.0 mmol 98.7%): ^{1}H NMR ($CDCl_3$) δ 0.81 (9H, s, –C(CH_3)$_3$), 0.82–1.10 (5H, m, CHC(CH$_3$)$_3$, H$_{ax}$), 1.49 (1H, br m, CH–CH$_2$), 1.74 (4H, m, H$_{eq}$), 2.50 (2H, d, $J = 7.3$ Hz, CH_2–CH), 4.11 (3H, s, OCH_3), 7.69 (2H, m, H-6, H-7), 8.05 (2H, m, H-5, H-8).

4.2.4. 3-(*trans*-4-*tert*-Butyl-cyclohexylmethyl)-2-methoxy-[1,4]naphthoquinone-1-oxime (5). Compound **5** was prepared as a product of compound **2** from **4** (2.18 g, 6.4 mmol) to give a brown oil, which was purified by column chromatography (petroleum ether/ethyl acetate 20:1) yielding **5** ($R_f = 0.25$) as a viscose oil (10:1 mixture of *E*/*Z* isomers) (0.3 g, 0.84 mmol, 13.1%). ^{1}H NMR (d_6-DMSO) *E*-isomer δ 0.79 (9H, s, –C(CH_3)$_3$), 0.82–1.00 (5H, m, CHC(CH$_3$)$_3$, H$_{ax}$), 1.41 (1H, br m, CH–CH$_2$), 1.69 (4H, m, H$_{eq}$), 2.40 (2H, d, $J = 7.2$ Hz, CH_2–CH), 3.92 (3H, s, OCH_3), 7.62 (1H, td, $J = 7.5, 1.2$ Hz, H-6), 7.72 (1H, td, $J = 7.4, 1.5$ Hz, H-7), 8.08 (1H, dd, $J = 7.8, 1.5$ Hz, H-5), 8.93 (1H, d, $J = 7.9$ Hz, H-8), 13.47 (1H, br s, OH); *Z*-isomer δ 3.76 (3H, s, OCH_3), 7.55 (1H, td, $J = 7.3, 1.2$ Hz, H-6), 7.65 (1H, td, $J = 7.3, 1.6$ Hz, H-7), 7.95 (1H, dd, $J = 7.9, 1.4$ Hz, H-5), 8.04 (1H, d, $J = 7.9$ Hz, H-8) (other signals overlap with the *E*-isomer). ^{13}C NMR (d_6-DMSO) *E*-isomer δ 27.3, 27.6, 31.2, 32.4, 34.0, 37.6, 48.1, 62.0, 126.9, 127.0, 129.2, 130.1, 130.7, 131.2, 132.6, 141.6, 161.0, 185.7; *Z*-isomer δ 37.7, 48.1, 61.1, 123.4, 126.3, 129.4, 130.2, 131.3, 132.3, 133.1, 142.7, 156.4, 185.6 (other signals overlap with the *E*-isomer). ESI-MS: 356.4 (M+1). Anal. Calcd ($C_{22}H_{29}NO_3 \cdot 0.5$EtOAc) C: 75.16, H: 9.08, N: 3.51; found C: 75.14, H: 7.37, N: 3.73.

4.2.5. Preparation of hepatic microsomes. Wistar rats (150–200 g) were treated ip for 4 days with either phenobarbital (80 mg/kg/day in 0.9% NaCl solution), 3-methylcholanthrene (20 mg/kg/day in olive oil), dexamethasone (50 mg/kg/day in olive oil) or 0.5 mL olive oil (control rats).[16] Microsomes were prepared as previously reported[30] and stored at −80 °C until use. Protein concentrations were determined by using the Biorad Protein Assay (BioRad, Hercules, USA), and cytochrome P450 contents were determined as reported earlier.[31]

4.2.6. Enzymatic incubations in microsomes. A stock solution of buparvaquone-oxime (**2**) was prepared in ethanol. 20 μL of the stock solution (final substrate concentration of 0.95 mM), $MgCl_2$ solution (5 mM), liver microsomes (protein concentration of 4.4–6.3 mg/mL) and 50 mM phosphate buffer pH 7.4 (total volume 1 mL) were incubated for 5 min at 37 °C before adding NADPH solution (final concentration of 4 mM). For the blank sample, liver microsomes were replaced by the same volume of water. Reactions were incubated in a water bath at 37 °C for 24 h, and then stopped either by mixing 1 mL of ice-cold acetonitrile with the sample for

HPLC analysis, or by heating the sample 5 min at 100 °C for NO_2^- assays. The samples were kept on ice, centrifuged for 10 min at 11,000 rpm and the supernatant was analysed by HPLC.

4.2.7. Determination of nitrite. For the determination of nitrite (NO_2^-), enzymatic incubations in microsomes were performed as described above, except nitrate reductase (60 mU from *Aspergillus niger*) was added after 23 h of incubation medium to reduce available nitrate (NO_3^-) to nitrite (NO_2^-). The incubation was continued for 1 h after the addition of nitrate reductase. Proteins were precipitated by heating, which also eliminated the bleaching effect of NADPH on the Griess reaction. The samples were centrifuged for 15 min at 11,000 rpm. The concentration of nitrite in the supernatants were determined by the Griess reaction, where 240 μL of sulfanilamide (1.8% m/V) solution in 1 M HCl solution) and 160 μL of *N*-(1-naphthyl)ethylenediamine dihydrochloride (0.3% m/V) solution in 1 M HCl solution were added to 800 μL of the supernatants before measurement at an absorbance of 548 nm.[32] For the standard curve, solutions of known nitrite concentrations (0.1–40 μM sodium nitrite solution) were prepared into the mixture of 50 mM phosphate buffer pH 7.4 and 5 mM MgCl solution, and the absorbance was measured after treatment with Griess reagents. Quantitations of total NO_2^- were obtained from the resulting standard curve.

4.2.8. Aqueous solubility. The aqueous solubilities of buparvaquone oximes (**2** and **3**) were determined in borate buffer (0.185 M) at pH 7.4, as reported earlier[7]. The solutions were shaken for 72 h, and filtered solutions were analysed by HPLC.

4.2.9. In vitro antileishmanial activity. The assay for intracellular antileishmanial activity is described in detail elsewhere.[7] In the present study infected macrophages were maintained in a 3-fold dilution series, with quadruplicate cultures at each concentration (30–10–3–1 μg/mL), for 5 days.

4.2.10. HPLC analysis. The HPLC system consisted of a Beckman System Gold Programmable Solvent Module 126, a Beckman System Gold Detector Module 166 with variable wavelength (set at 250 nm for buparvaquone and 291 nm for its oxime derivatives), and a Beckman System Gold Autosampler 507e. For sample separations, an Agilent Zorbax® SB-phenyl column (150 × 4.6 mm, 5 μM) was used. The mobile phase used consisted of 50% (v/v) 0.02 M phosphate buffer solution (pH 2.5) in acetonitrile at a flow rate of 2.0 mL/min.

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