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Leishmanicidal activities and cytotoxicities of bisnaphthoquinone analogues and naphthol derivatives from Burman *Diospyros burmanica*

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ABSTRACT

A methanol extract of the wood of *Diospyros burmanica*, collected in Burma (Myanmar), was found to exhibit significant activity against *Leishmania major*. Subsequent chromatographically resolved fractionation led to the isolation of three novel bisnaphthoquinone analogues, burmanin A, B, and C (**1**–**3**), together with nine known compounds (**4**–**12**). The structure of **1** was confirmed by X-ray crystallography, and those of **2** and **3** by spectroscopic techniques, including 1D and 2D NMR. The inhibitory activities of the isolates were evaluated against the promastigote forms of *Leishmania major* and the murine macrophage-like cell line, RAW264.7.

1. Introduction

Leishmaniasis is a parasitic disease caused by protozoal species of the genus *Leishmania*.[1] *Leishmania* spp. are transmitted by sandflies (*Phlebotomus* spp.) with approximately twelve million people in 88 countries afflicted by the disease.[2] One of the causative agents of cutaneous leishmaniasis is Leishmania major. Thus far, pentavalent antimonials are the medicine of choice for this disease; however, the drug is extremely toxic and generally expensive,[3] and more economical drugs with lower toxicity have been long awaited.

We have previously examined the timber extracts of Myanmar *Diospyros ethretioides*, *Diospyros monatana*, and *Diospyros pendula* ex. HASSK, and found that they demonstrated no activity against *Leishmania* spp.[4]

We are currently studying the activity of the chemical components of Burman trees[5] and found that a methanol extract of the wood of *Diospyros burmanica* exhibited potent activity against *Leishmania major* [MLC (minimum lethal concentration): 6.25 μg/mL; MIC (minimum inhibitory concentration): 1.25 μg/mL].[4] Although, secondary metabolites of other *Diospyros* species such as *Diospyros melanoxylon*, *Diospyros decandra* and *Diospyros crasiflora* have been investigated previously,[6–18] the chemical compositions of *D. burmanica* have not been reported.

Timber from *D. burmanica* was shaved and the collected flakes were extracted with methanol. Fractionation of the methanol extract was performed by reversed-phase flash column chromatography, medium-pressure liquid chromatography (MPLC), and HPLC, yielding new bisnaphthoquinone analogues (**1**–**3**), four known naphthoquinones (**8**, **9**, **11**, **12**), and five known naphthols (**4**, **5**, **6**, **7**, **10**).

This report deals with the isolation and structural determination of the antileishmanial components of this plant. In addition, the cytotoxicity against the murine macrophage-like cell line, RAW264.7, was tested for each of the isolated compounds.

2. Results and discussion

2.1. Structure elucidation of new compounds

Burmanin A (**1**), $[\alpha]_D$,[†] was obtained as orange brown crystals. The obtained CD spectrum displayed a flat line. Its molecular formula

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[†] Although, many attempts were made to measure the $[\alpha]_D$ and CD spectra for the quinones **1**, **2**, and **3**, no reliable data were obtained because the observed values and spectra varied greatly each time. The same strange phenomena have been described for some sesquiterpene quinones.[23,24]

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was established as $C_{24}H_{18}O_7Na$ by ESITOFMS (obsd *m/z* 441.0941, Calcd for $[M+Na]^+$ 441.0950). The IR spectrum showed absorptions for carbonyl (1653 cm^{-1}) and benzene (1574, 1540 cm^{-1}) groups. The UV maxima (EtOH) at 213 nm (logε 4.65), 257 (4.37), 312 (3.89), and 406 (3.72) suggested the presence of a naphthoquinone group. The ^1H NMR spectrum of **1** ($CDCl_3$) showed five aromatic proton signals at δ 7.92 (1H, d, J = 8.0 Hz, H-8′), 7.56 (1H, br s, H-5), 7.27 (1H, d, J = 8.0 Hz, H-7′), 7.13 (1H, br s, H-7), and 6.75 (1H, s, H-2); two methoxy proton signals at δ 4.01 (3H, s, H-11) and 3.90 (3H, s, H-12′); and two methyl groups at δ 2.48 (3H, s, H-12) and 2.09 (3H, s, H-11′). The ^{13}C NMR exhibited peaks due to four quinone carbonyls [δ 183.5 (C-4), 183.4 (C-1′), 183.1 (C-1), 182.5 (C-4′)]; three oxygenated aromatic quaternary carbons [δ 160.0 (C-8), 155.3 (C-6′), 146.2 (C-5′)]; eight aromatic quaternary carbons [δ 146.7 (C-6), 144.7 (C-2′), 142.1 (C-3), 140.9 (C-3′), 133.9 (C-10), 125.9 (C-9′), 124.0 (C-10′), 117.7 (C-9)], in addition to five aromatic methines [δ 140.1 (C-2), 125.1 (C-8′), 120.7 (C-5), 119.8 (C-7′), 118.6 (C-7)]; two methoxy carbons [δ 62.3 (C-12′), 56.5 (C-11)]; and two methyl carbons [δ 22.3 (C-12), 14.3 (C-11′)]. The HMBC spectrum of **1** showed the long-range $^1H/^{13}C$ correlations such as: Me-12/C-5, C-6, and C-7; Me-11/C-8; H-7/C-5, C-9, and C-12; H-5/C-4, C-7, and C-9; H-2/C-3, C-4, C-9, and C-3′; Me-12′/C-5′; Me-11′/C-3 (*), C-1′, C-2′, C-3′, and C-4′ (*); H-8′/C-1′, C-4′ (*), C-5′ (*), C-6′, and C-10′; H-7′/C-5′, C-6′, and C-9′ (The signals marked by (*) are due to $^4J_{HC}$). Owing to the presence of the unusual 4-bond long-range couplings, an unambiguous structure of this compound could not be determined using these methods. However, the compound gave crystals suitable for X-ray analysis, which led to the elucidation of structure **1** of burmanin A (Fig. 1) (The X-ray structure of **1** is shown in Fig. 2).

Compound **2**, burmanin B, $[\alpha]_D$,† was obtained as a brown amorphous solid. HRESITOFMS analysis of **2** showed the molecular ion at *m/z* 403.1196 $[M-H]^-$ (Calcd 403.1182) affording a molecular formula of $C_{24}H_{20}O_6$. The IR spectrum [quinone (1652 cm^{-1}) and benzene (1558, 1540 cm^{-1}) groups] and the UV spectrum [λ_{max} (EtOH) 214 nm (logε 5.39), 246 (5.16), 304 (4.63), and 403 (4.39)] are as a whole very similar to those of burmanin A (**1**). Comparing the molecular formula of **2** with that of **1**, there is one less oxygen and two extra hydrogens incorporated into the former, implying that **2** is a reduction-methylation product of **1**. By inspection of the NMR (CD_3OD) spectra, it appeared that compound **2** contained the same naphthoquinone moiety (A/B ring) as **1** [^{13}C of **2**: δ 185.7 (s) (C-1), 142.9 (d) (C-2), 148.6 (s) (C-3), 186.3 (s) (C-4), 121.5 (s) (C-5), 148.6 (s), (C-6), 119.9 (d) (C-7), 161.3 (s) (C-8), 118.7 (s) (C-9), 135.6 (s) (C-10), 56.8 (q) (C-11), 22.2 (q) (C-12)] [^1H of **2**: δ 6.80 (1H, s, H-2), 7.62 (1H, br s, H-5), 7.44 (1H, br s, H-7), 4.06 (3H, s, H-11), 2.55 (3H, s, H-12)]. No other carbonyl signals are present in the ^{13}C NMR spectrum, therefore, the quinone moieties in A′ ring of **1** must be changed to a reduction form in **2**. Correspondingly, a new aromatic proton appears at δ 6.76 (s; C-1′), in addition to two AB-type doublets (J = 8.0 Hz) at δ

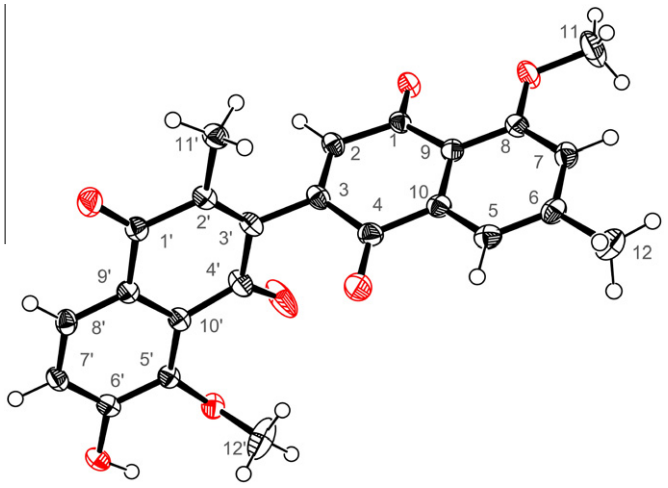

Figure 2. ORTEP drawing of **1** as determined by X-ray analysis.

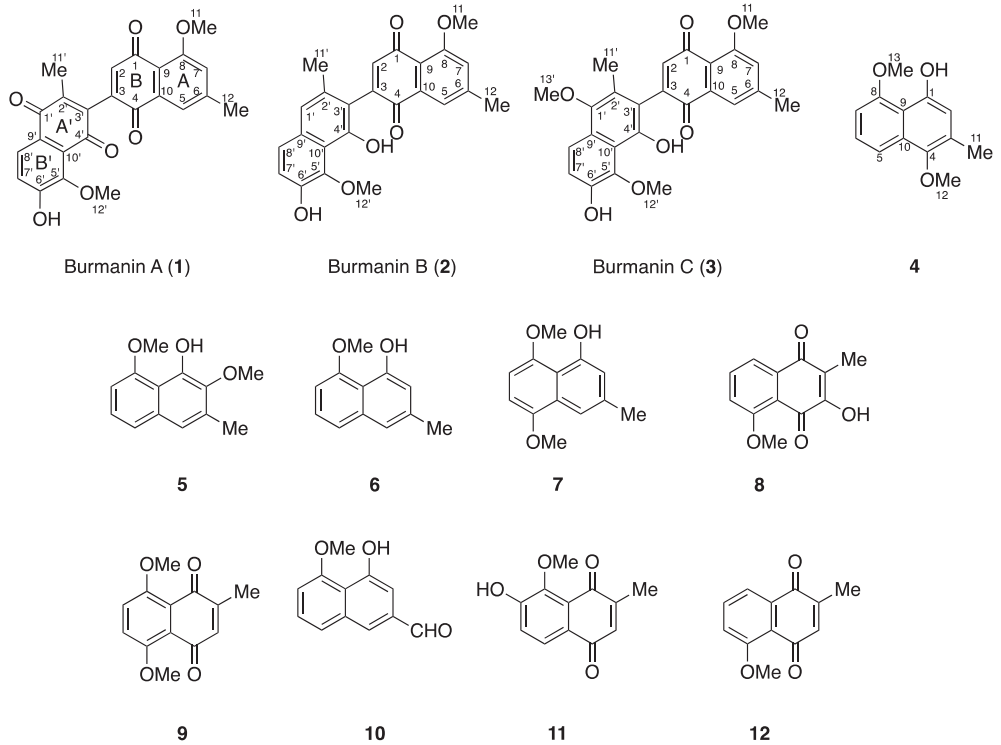

Figure 1. Compounds isolated from *Diospyros burmanica*. Compounds **1–3** are new and **4–12** are known.

Table 1
^{1}H NMR spectroscopic data for **1**–**3**

Position	**1**[a] δ_H (J in Hz)	**2**[b] δ_H (J in Hz)	**3**[b] δ_H (J in Hz)
1			
2	6.75, s	6.80, s	6.84, s
3			
4			
5	7.56, s	7.62, br s	7.60, br s
6			
7	7.13, s	7.44, br s	7.38, br s
8			
9			
10			
11	4.01, s	4.06, s	4.03, s
12	2.48, s	2.55, s	2.52, s
1′		6.76, s	
2′			
3′			
4′			
5′			
6′			
7′	7.27, d (8.0)	7.08, d (8.0)	7.22, d (8.0)
8′	7.92, d (8.0)	7.20, d (8.0)	7.74, d (8.0)
9′			
10′			
11′	2.09, s	2.22, s	2.17, s
12′	3.90, s	4.07, s	4.04, s
13′			3.84, s
OH(1)			
OH(6′)	1.63, br s		

[a] Measured at 400 MHz; obtained in $CDCl_3$; J values (Hz) are given in parentheses. Assignments are based on ^{1}H –^{1}H COSY, HSQC, and HMBC spectroscopic data.
[b] Measured at 400 MHz; obtained in CD_3OD.

Table 2
^{13}C NMR spectroscopic data for **1**–**3**

Position	**1**[a] δ_C, mult.	**2**[b] δ_C, mult.	**3**[b] δ_C, mult.
1	183.1, C	185.7, C	185.9, C
2	140.1, CH	142.9, CH	142.0, CH
3	142.1, C	148.6, C	146.7, C
4	183.5, C	186.3, C	185.9, C
5	120.7, CH	121.5, CH	121.4, CH
6	146.7, C	148.6, C	148.5, C
7	118.6, CH	119.9, CH	119.7, CH
8	160.0, C	161.3, C	161.2, C
9	117.7, C	118.7, C	118.5, C
10	133.9, C	135.6, C	135.6, C
11	56.5, CH_3	56.8, CH_3	56.8, CH_3
12	22.3, CH_3	22.2, CH_3	22.2, CH_3
1′	183.4, C	113.3, C	147.6, C
2′	144.7, C	133.7, C	124.0, C
3′	140.9, C	122.4, C	117.9, C
4′	182.5, C	154.5, C	147.6, C
5′	146.2, C	141.9, C	142.3, C
6′	155.3, C	146.0, C	146.7, C
7′	119.8, CH	120.7, CH	120.9, CH
8′	125.1, CH	123.4, CH	120.3, CH
9′	125.9, C	130.7, C	125.8, C
10′	124.0, C	118.1, C	118.4, C
11′	14.3, CH_3	20.4, CH_3	13.6, CH_3
12′	62.3, CH_3	62.1, CH_3	62.1, CH_3
13′			61.7, CH_3

[a] Measured at 100 MHz; obtained in $CDCl_3$; J values (Hz) are given in parentheses. Assignments are based on ^{1}H –^{1}H COSY, HSQC, and HMBC spectroscopic data.
[b] Measured at 100 MHz; obtained in CD_3OD.

7.08 and 7.20 (H-7′ and H-8′, respectively). The benzylic methyl proton (δ 2.22) at C-2′ shows HMBC correlations with three ^{13}C singlets at δ 113.3 (C-1′), 133.7 (C-2′), and 122.4 (C-3′), suggesting that the new aromatic proton [δ 6.76 (s)] is located at C-1′ as this proton correlates with the carbon signal at δ 113.3 in the HSQC spectrum. The following HMBC correlations were also observed: Me-12 (δ 2.55)/C-5, C-6, and C-7; H-11 (δ 4.06)/C-8; H-12′ (δ 4.07)/C-5′; H-1′ (δ 6.76)/C-3′, C-4′,‡ and C-10′; H-2 (δ 6.80)/C-1, C-3, C-8,‡ C-9, and C-3′; H-7′ (δ 7.08)/C-5′, C-6′, and C-9′; H-8′ (δ 7.20)/ C-5′,‡ C-6′, and C-10′; H-7 (δ 7.44)/C-5, C-8, and C-9; H-5 (δ 7.62)/C-4, C-7, and C-9. Among the HMBC cross peaks, that appearing from H-2 to C-3′ was essential to fix the combination sites of the A–B ring with A′–B′ ring. Thus, structure **2** was elucidated for burmanin B.

Burmanin C (**3**), $[\alpha]_D$,† λ_{max} (EtOH) 214.5 nm (logε 4.54), 234 (4.61), 330.5 (3.74), and 370 (3.54), was obtained as a dark brown amorphous solid. The HRESITOFMS of **3** gave a deprotonated molecular ion peak at m/z 433.1256 $[M-H]^-$ (Calcd 433.1287), consistent with a molecular formula of $C_{25}H_{22}O_7$. The IR spectrum showed absorptions of carbonyl (1653 cm^{-1}) and benzene (1574, 1540 cm^{-1}) groups. The ^{1}H NMR spectrum (CD_3OD) of **3** was very similar to that of burmanin B (**2**), except for the lack of the aromatic proton singlet (H-1′) found in **2** and the presence of three methoxy signals (δ 4.04, 4.03, 3.84). In both the ^{1}H and ^{13}C NMR spectra of **3**, the signals due to A, B, and B′ rings of **2** are also observed (Tables 1 and 2), and thus, the substituents of A′ ring of **3** must be different from those of **2**. The position of the new methoxy group was determined to be C-1′ by the HMBC cross peak from Me-11′ (δ_H 2.17) to C-1′ (δ_C 147.6) that was correlated with Me-13′ protons (δ_H 3.84) through $^3J_{CH}$. The structure of **3** was finally confirmed by the following HMBC correlations: Me-11′ (δ 2.17)/C-1′, C-2′, C-3′, and C-9′;‡ Me-12 (δ 2.52)/C-5, C-6, and C-7; Me-13′ (δ 3.84)/C-1′; H-11 (δ 4.03)/ C-8; H-12′ (δ 4.04)/C-5′; H-2 (δ 6.84)/C-1, C-3, C-8,‡ C-9, and C-3′; H-7′ (δ 7.22)/C-5′, C-6′, and C-9′; H-7 (δ 7.38)/C-5, and C-9; H-5 (δ 7.60)/C-4, C-7, C-9; H-8′ (δ 7.74)/C-1′, C-5′,‡ C-6′, and C-10′.

‡ J_{CH} interpretable as W-type C–H long-range couplings except for the three observed between H-1′ and C-4′, H-8′ and C-5′ in **2**, and H-8′ and C-5′ in **3**.

2.2. Identification of known compounds

Compounds **6**,[19] **7**,[20] **8**,[15,21] **10**,[13,14] and **12**[15,16] were identified by comparison of the obtained spectroscopic data with those reported in the literature. The spectral properties of compounds **4**,[15] **5**,[17] **9**,[22] and **11**,[18] however, are inadequately described in the literature, and so their structures were fully characterized by spectroscopic analyses in the present study (see Supplementary data), and the resultant structures were found to accurately correspond to those previously reported.

2.3. Biological activity

The activities of burmanins A–C (**1**–**3**) and the known compounds (**4**–**12**) were tested against the promastigote form of *L. major*. Results are shown in Table 3. Among the tested samples, burmanin A (**1**) showed the most potent inhibitory activity (IC_{50} 0.053 ± 2.7 × 10^{-3} μM), with the activity of dimeric analogues **2** and **3** being almost as strong. Compound **12**, the monomeric naphthoquinone, has been shown inhibitory activity (IC_{50} 3.3 ± 0.19 μM). In contrast, the other monomeric naphthoquinones (**9** and **11**) and naphthols (**4**, **5**, **6**, **7**, and **10**) exhibited much weaker activities (>38 μM).

The leishmania protozoan parasite dwells and multiplies within mammalian macrophages. For a compound to be a candidate for antileishmanial drug, it is required both high leishmanicidal activity and low cytotoxicity. Therefore, the cytotoxicities of **1**–**12** were tested against the murine macrophage-like cell line, RAW264.7. Burmanin A–C (**1**–**3**) exhibited only weak toxicities with IC_{50} values 24 ± 1.7, 31 ± 0.45, and 22 ± 0.64 μM, respectively.

Table 3
In vitro leishmanicidal activity, cytotoxicity and selectivity index of compounds **1**–**12**

Compound	Activity[a] (IC_{50} ± SD)		SI[b]
	L. major	RAW264.7	
1	$0.053 \pm 2.7 \times 10^{-3}$	24 ± 1.7	453
2	$0.18 \pm 5.4 \times 10^{-3}$	31 ± 0.45	172
3	$0.15 \pm 11 \times 10^{-3}$	22 ± 0.64	147
4	>100	>100	–
5	45 ± 1.4	>100	>2.22
6	50 ± 6.8	>100	>2.00
7	38 ± 5.6	>100	>2.63
8	>100	>100	–
9	NT[c]	>100	–
10	>100	>100	–
11	93 ± 2.2	96 ± 2.9	1.03
12	3.3 ± 0.19	45 ± 1.4	13.6
AmB [d]	$0.035 \pm 1.9 \times 10^{-3}$	–	–
MG132 [e]	–	0.63 ± 0.030	–

[a] Concentration in μM (n = 3).
[b] Selectivity index. Ratio of cytotoxicity (IC_{50}) to leishmanicidal activity (IC_{50}).
[c] NT = not tested.
[d] AmB = Amphotericin B. Positive control for antileishmanial assay.
[e] Positive control for cytotoxicity assay.

3. Conclusion

The selectivity index (SI = $IC_{50(RAW264.7)}IC_{50(L.\ major)}$) for burmanins A–C (**1–3**) was introduced to compare the cytotoxicity and the leishmanicidal activity. As is shown in Table 3, burmanins A–C (**1–3**) exhibited SI values, **1**: 453, **2**: 172, **3**: 147. These values are much larger than the reported ones of natural monomeric naphthoquinones determined from amastigotes of GFP-transfected *L. major* versus BMMΦ (SI = 1.2–7.0).[25] The present findings demonstrate that the new dimeric naphthoquinones (**1**–**3**) can be the potential lead compounds for drugs to cure the parasitic diseases caused by *L. major*.

4. Materials and methods

4.1. General experimental procedures

The optical rotations were measured using a JASCO 1010 polarimeter. IR spectra were measured on a JASCO FT/IR-6300 spectrophotometer. UV spectra were taken on a JASCO International V-530 spectrophotometer. The 1D- and 2D NMR spectra were obtained on Bruker AVANCE 700 MHz, 400 MHz, and Varian Unity INOVA 500 MHz spectrometers. ESITOFMS were measured on a JASCO International Q-TOF Micro mass spectrometer. For MPLC, reversed-phase material (Ultrapak, Yamazen Co., Ltd) was used. ODS-flash column chromatography was carried out on a Cosmosil 75C18-OPN (Nacalai Tesque Co., Ltd). For HPLC, columns of Shiseido Capcell pak C18 MG 5 μm 20 × 250 mm and C18 UG120 5 μm 10 × 250 mm, Cosmosil MS-II C18 5 μm 20 × 250 and Intact Unison UK-C18 3 μm 10 × 250 mm, and the HPLC system of JASCO Co., Ltd, were used. TLC was conducted on pre-coated silica gel 60 F_{254} (Merck) and/or RP-18 F_{254}s (Merck) and the spots were detected by heating after spraying with *p*-methoxybenzaldehyde-H_2SO_4 reagent.

4.2. Plant material

The wood of *Diospyros burmanica* was produced and kindly donated by the Ministry of Forestry of Myanmar in November 2004, and identified by Dr. Nyan Tun, a taxonomist at the Institute of Forestry, Forest Department, Ministry of Forestry, Union Myanmar. A voucher specimen was deposited at Tokushima Bunri University, Kagawa, Japan (voucher # MY310706).

4.3. Isolation of compounds

The shaved timber of *D. burmanica* (390 g) was soaked in MeOH and extracted at 40 °C for 4 h three times. The MeOH extract was concentrated under reduced pressure to give a residue (14 g), which was then treated with H_2O. The resultant substance was partitioned between hexane (2 g) and 90% MeOH. The 90% MeOH extract was diluted with H_2O up to 60%, and then the aqueous mixture was partitioned between chloroform (13 g) and 60% MeOH (1.6 g). A sample of the chloroform extract (5 g) was subjected to silica gel column chromatography and eluted with hexane–acetone (9:1–6:4) giving eight fractions (Fraction 1-1-1-8). Fraction 1-1 (85 mg) was subjected to ODS-flash column chromatography, eluted with MeOH–distilled H_2O (7:3–9:1), to give five fractions (Fraction 2-1-2-5). Fraction 1-2 (148.8 mg) was subjected to ODS-flash column chromatography, eluted with MeOH–distilled H_2O (7:3–95:5), to give seven fractions (Fraction 3-1-3-7). Fraction 2-2 (21.8 mg), fraction 2-4 (19 mg), fraction 3-2 (14.1 mg) and fraction 3-4 (6.6 mg) were combined and separated by HPLC [acetonitrile–distilled H_2O (1:1)] to afford **4** (8.1 mg, 0.0021% from dried timber) **5** (7.5 mg, 0.0019%), **6** (2.6 mg, 0.0007%), **7** (7.5 mg, 0.0019%), and **8** (2.5 mg 0.0006%). Fraction 1-4 (411.5 mg) was subjected to ODS-flash column chromatography, eluted with MeOH–distilled H_2O (6:4–100:0), to give nine fractions (Fraction 4-1-4-9). Fraction 4-3 (82.5 mg) was separated by HPLC [acetonitrile–distilled H_2O (1:1)] to afford **9** (7.2 mg, 0.0018%), **10** (1.2 mg 0.0003%), and **11** (2.0 mg, 0.0005%). Fraction 1-5 (707.8 mg) was subjected to ODS-flash column chromatography with MeOH–distilled H_2O (1:1–9:1) to give nine fractions (Fraction 5-1-5-9). Fraction 5-3 (53.9 mg) mainly consisting of **12** was also purified in a similar manner (39.3 mg, 0.0101%). Combined fractions of 1-6 and 1-7 (729.5 mg) were subjected to ODS-flash column chromatography, eluted with MeOH–distilled H_2O (7:3–100:0), to give twelve fractions (Fraction 6-1-6-12). Fraction 6-5 (52.7 mg) was separated by HPLC [acetonitrile–distilled H_2O (1:1)] to afford new bisnaphthoquinones, burmanin A (**1**) (11.7 mg, 0.0030%), burmanin B (**2**) (3.7 mg, 0.0009%), and burmanin C (**3**) (35.9 mg, 0.0092%).

4.4. Burmanin A (1)

Orange brown crystals; mp 183.5 °C; ^1H NMR ($CDCl_3$, 400 MHz) see Table 1; ^{13}C NMR ($CDCl_3$, 100 MHz) see Table 2.

4.5. X-ray analysis of burmanin A (1)

Crystal size, 0.50 × 0.40 × 0.30 mm; molecular formula, $2(C_{24}H_{18}O_7)$, CH_2Cl_2, crystal system, monoclinic; space group, *C2/c*; unit cell dimensions (*a*, *b*, *c*), 27.836(1) Å, 10.2236(6) Å, 20.353(1) Å; α = 90, β = 133.908(1), γ = 90, volume, 4173.0(4) Å^3; Z = 4; density, 1.467 g cm^{-3}; absorption coefficient, 0.230 mm^{-1}; F(000) = 1912.0; diffractometer used, Rigaku RAXIS-RAPID; radiation (λ) Mo Kα (0.71073 Å); 2θ max 55.0°; reflections collected, 20455; independent reflections, 4754; observed reflections, 3859 [R(int) = 0.050]; final R indices, R = 0.0700 (obsd data), *WR2* = 0.2040 (indept data); goodness of fit, 1.406; T = 123(1) K. The structure was solved by direct methods and refined by full matrix least-squares on F^2.[26]

4.6. Burmanin B (2)

Brown amorphous; ^1H NMR (CD_3OD, 400 MHz) see Table 1; ^{13}C NMR (CD_3OD, 100 MHz) see Table 2.

4.7. Burmanin C (3)

Dark brown amorphous; ^{1}H NMR (CD_3OD, 400 MHz) see Table 1; ^{13}C NMR (CD_3OD, 100 MHz) see Table 2.

4.8. Bioassay methods

4.8.1. Culture media and reagents

Dulbecco's modified Eagle's medium (DMEM) was supplemented with 10% heat-inactivated fetal calf serum, L-glutamine (2 mM), non-essential amino acids (100 μM), penicillin (100 units/mL), sodium pyruvate (1 mM), and streptomycin (100 μg/mL) (DM). *Leishmania* growth medium consisted of Medium-199 supplemented with 10% fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin (M199). Assay reagent for *Leishmania* promastigotes was TetraColor One [a mixture of WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-*2H*-tetrazolium, monosodium salt) and 1-methoxy-PMS (1-methoxy-5-methylphenazinium methyl sulfate)] (Seikagaku Biobusiness Corp.) XTT assay kit (Roche) was used for RAW264.7 cells. The experiments were performed in triplicate.

4.8.2. Cultivation of *Leishmania promastigotes*

Leishmania major organisms were maintained by animal passage and cryopreserved in liquid nitrogen. Promastigotes were cultured in M199 at 27 °C, 5% CO_2 in a humidified incubator. The cultures were passaged every 3–4 days.

4.8.3. Cell culture

The murine macrophage-like cell line, RAW264.7 (TIB-71), was obtained from ATCC, and the cells were grown in DM at 37 °C in 5% CO_2. The cells were grown on tissue culture-treated plastic (Nunc, Thermo-Fisher Sciences) in DM medium and sub-cultured at 70% confluency. For detachment, the medium was replaced with cold Ca^{2+}/Mg^{2+}-free phosphate-buffered saline and the cells were scraped. Subcultivation ratio was 1:3–1:6.

4.8.4. Leishmanicidal assay

The leishmanicidal effects of the samples were assessed using an improved version of the MTT assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] as follows. Cultured *L. major* promastigotes were seeded at a density of 4×10^5 cells per 50 μL of medium into 96-well microplates. After this, 50 μL samples of the test compounds, at different concentrations, dissolved in a mixture of DMSO and culture medium, were added to each well. Each concentration was tested in triplicate. The microtiter plate was incubated at 27 °C in 5% CO_2 for 48 h. Tetra Color One (10 μL) [a mixture of WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-*2H*-tetrazolium, monosodium salt) and 1-methoxy-PMS (1-methoxy-5-methylphenazinium methyl sulfate)] was added to each well and the plates were incubated at 27 °C in 5% CO_2 for 6 h. Optical density values (test wavelength 450 nm, reference wavelength 630 nm) were measured using a Viento® XS Multi-spectrophotometer (Dainippon pharmaceutical). Leishmanicidal activities were expressed as a minimum lethal concentration (MLC) and a minimum inhibitory concentration (MIC). The IC_{50} (50% inhibitory concentration) values for compounds were estimated from the produced graphs. Amphotericin B was used as a positive control.

4.8.5. XTT assay of RAW264.7 cells

Compounds were assayed for cytotoxicity against the murine macrophage-like cell line, RAW 264.7, using the XTT method.[27–30] The IC_{50} is the concentration of agent that reduced cell growth by 50% under the experimental conditions. MG132 (carbobenzoxy-L-leucyl-L-leucyl-L-leucinal) was used as positive control.[31]

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.06.055.

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