Note

A New Bis-indole Alkaloid, Spermaocymine A, and an Anthraquinone from *Spermacoce ocymoides*

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A phytochemical study on *Spermacoce ocymoides* has led to the isolation of a novel bis-indole alkaloid, spermaocymine A (2), together with the known alkaloid 4-methyl-borreverine (1), as well as an anthraquinone, 8-hydroxy-2-(hydroxymethyl)-1-methoxyanthracene-9,10-dione (3). The structures of the isolated compounds were elucidated by analyzing spectroscopic and spectrometric data, including one-dimensional (1D)- and 2D-NMR and high resolution (HR)-MS. Newly isolated alkaloid 2 was a C-3,14-stereoisomer of 1, the first natural stereoisomer of related bis-indoles containing an indeno[1,2-*b*]indole skeleton with an epiminoethano bridge. When 1–3 were assayed against five tumor cell lines including multi-drug resistant cells, compound 1 exhibited potent antiproliferative activity with IC_{50} values of 6.2–11.5 μM.

Key words *Spermacoce ocymoides*, bis-indole alkaloid, anthraquinone, antiproliferative activity

Introduction

The genus *Spermacoce* (synonym of *Borreria*) belonging to the family Rubiaceae is widespread in tropical areas. Several species have been used as traditional folk medicines to treat malaria, diarrhea, fever, headache, hemorrhage, urinary and respiratory infections, gum and eye inflammation, and skin diseases.[1)] In prior phytochemical studies of *Spermacoce* (*Borreria*), two characteristic bis-indole alkaloids, borreverine[2)] and spermacoceine,[3)] were isolated from *B. verticillata*. Both alkaloids include a unique 3-substituted indole connected to an indeno[1,2-*b*]indole skeleton with an epiminoethano bridge (Fig. 1). The same skeleton is found in only two other alkaloids, 4-methyl borreverine (**1**),[4)] which is also reported as auricularine,[5)] and 15′-hydroxy-14′,15′-dihydroborreverine. They were isolated from specific plants, *Flindersia fournieri* (Rutaceae)[4)] and *Hedyotis auricularia* (Rubiaceae).[5)] Several related alkaloids without an epiminoethano bridge were also isolated from *F. fournieri*.[4)] These natural bis-indole alkaloids have the same relative configurations (3*R**, 14*R**, 2′*R**, 3′*R**, 7′*S**). Borreverine and unnatural 3′-*epi*-borreverine were synthesized through Diels–Alder dimerization of the prenylated dihydropyrido[3,4-*b*]indole.[6)] Biological investigations of borreverine reported antiplasmodial[7)] and antibacterial activities.[8)]

Despite the presence of such unique natural products in the genus *Spermacoce* (*Borreria*), a thorough phytochemical study of *S. ocymoides* has not been performed. Only some terpenoids and fatty acids were reported to be isolated.[9)] Our continuing phytochemical study of unexplored tropical rainforest plants prompted the elucidation of chemical constituents in *S. ocymoides* (Burm.f.) DC., synonym of *B. ocymoides*,[10)] collected in South Sulawesi, Indonesia.

Results and Discussion

Isolation and Structure Elucidation We isolated a new bis-indole alkaloid, spermaocymine A (**2**), 8-hydroxy-2-(hydroxymethyl)-1-methoxyanthracene-9,10-dione (**3**) (Fig. 2), and the known alkaloid **1**[4,5)] (Fig. 1) from the MeOH extract of the aerial parts of *S. ocymoides*. Although **1** was isolated and identified previously, its detailed spectroscopic data including

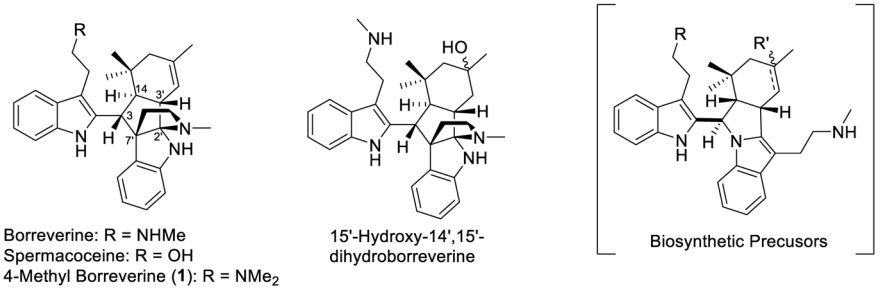

Fig. 1. Previously Isolated Unique Bis-indole Alkaloids and Their Biosynthetic Precursors

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^{13}C-NMR were not reported. Thus, the ^{1}H- and ^{13}C-NMR data of both **1** and newly isolated **2** are shown in comparison in Table 1 as well as in the supplementary materials (Supplementary Figs. S1–S17).

Compound **2** was obtained as a colorless solid with a positive response to Dragendorff reagent. The molecular formula, $C_{33}H_{42}N_4$, was determined based on a protonated molecular ion peak at *m/z* 495.3471 $[M+H]^+$, (calcd 495.3488) by high resolution (HR)-MS. The ^{1}H-NMR data of **2** (Table 1) revealed the presence of geminal methyl groups at δ_H 0.39 (s, 3H, CH_3-16) and 0.93 (s, 3H, CH_3-17), a vinyl methyl at δ_H 1.57 (s, 3H, CH_3-17′), a tertiary *N*-methyl at δ_H 2.43 (s, 3H), a dimethyl amino at δ_H 2.23 (s, 6H), four methylenes at δ_H 2.38–2.83 (m, 4H, H-5 and H-6) and at δ_H 1.51–2.83 (m, 4H, H-5′ and H-6′), three methines at δ_H 3.40 (d, J = 12.2 Hz, 1H, H-3), 2.88 (m, 1H, H-3′) and 2.49 (dd, J = 5.8, 12.2 Hz, 1H, H-14), an olefinic proton at δ_H 5.20 (br s, 1H, H-14′), eight aromatic protons [δ_H 6.52 (d, J = 7.5 Hz, 1H, H-12′), 6.55 (t, J = 7.5 Hz, 1H, H-10′), 6.78 (d, J = 7.5 Hz, 1H, H-9′), 6.97 (t, J = 7.5 Hz, 1H, H-11′), 7.09 (dd, J = 7.6, 8.3 Hz, 1H, H-10), 7.15 (dd, J = 7.6, 8.3 Hz, 1H, H-11), 7.34 (d, J = 8.3 Hz, 1H, H-12), 7.58 (d, J = 7.6 Hz, 1H, H-9)] and an amine proton at δ_H 8.09 (s, 1H, H-1′). The ^{13}C-NMR data (Table 1) revealed 33 carbon signals including three methyls (δ_C 24.3, 28.1, and 29.4), five methylenes

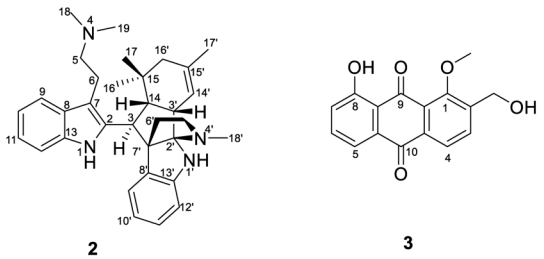

Fig. 2. Structures of Novel Compounds **2** and **3** Isolated from the Aerial Parts of *S. ocymoides*

Table 1. ^{1}H- and ^{13}C-NMR Spectroscopic Data of **2** and **1**

Position	**2**		**1**	
	δ_H (J in Hz)[a]	δ_C[a]	δ_H (J in Hz)[b]	δ_C[b]
2		135.56		133.8
3	3.40 d (12.2)	45.8	3.44 d (12.2)	47.7
5a	2.38 m	60.2	2.57 m	61.2
5b	2.67 m		2.67 m	
6	2.83 m	23.6	2.98 m	23.3
7		110.6		110.8
8		128.5		128.1
9	7.58 d (7.6)	118.8	7.57 d (8.7)	118.3
10	7.09 dd (7.6, 8.3)	119.1	7.05 overlapped	118.9
11	7.15 dd (7.6, 8.3)	121.3	7.05 overlapped	121.1
12	7.34 d (8.3)	110.7	7.03 overlapped	110.6
13		135.60		135.1
14	2.49 dd (5.8, 12.2)	51.2	1.88 t (12.2)	53.9
15		32.8		32.7
16	0.39 s	29.4	0.32 s	20.9
17	0.93 s	28.1	0.89 s	28.9
18	2.23 s	45.5	2.39 s	45.7
19	2.23 s	45.5	2.39 s	45.7
2′		96.6		93.6
3′	2.88 m	46.1	2.72 m	47.9
5′a	2.34 m	53.3	2.73 m	55.2
5′b	2.83 m		2.91 m	
6′a	1.51 dd (4.1, 11.3)	36.2	1.95 dd (5.9, 12.8)	39.7
6′b	2.28 m		2.29 m	
7′		68.2		69.9
8′		135.0		132.1
9′	6.78 d (7.5)	118.1	5.91 d (6.4)	118.6
10′	6.55 t (7.5)	118.2	6.37 t (6.4)	118.7
11′	6.97 t (7.5)	127.9	6.98 t (7.6)	128.3
12′	6.52 d (7.5)	107.2	6.64 d (7.6)	109.7
13′		150.3		151.9
14′	5.20 br s	123.6	5.58 br s	126.5
15′		133.9		138.5
16′a	1.89 d (17.9)	40.3	1.72 d (16.9)	48.2
16′b	1.43 d (17.9)		1.58 d (16.9)	
17′	1.57 s	24.3	1.68 s	23.7
18′	2.43 s	33.9	2.54 s	34.9
NH	8.09 br s		6.87 s	

a) ^{1}H-NMR: 600 MHz, ^{13}C-NMR: 150 MHz in $CDCl_3$. *b*) ^{1}H-NMR: 400 MHz, ^{13}C-NMR: 100 MHz in $CDCl_3$.

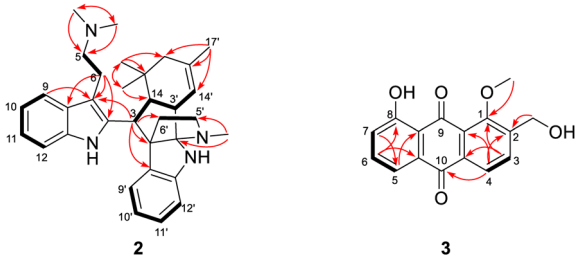

Fig. 3. ^{1}H–^{1}H COSY (Bold Lines) and Selected Key HMBC (Arrows) Correlations of Compounds **2** and **3**

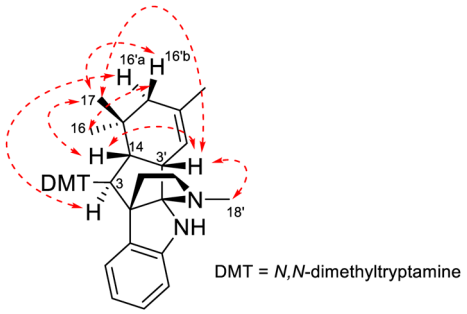

Fig. 4. Key NOESY Correlations (Dotted Arrows) of Compound **2**

(δ_C 23.6, 36.2, 40.3, 53.3 and 60.2), three methines (δ_C 45.8, 46.1, and 51.2), an *N*-methyl (δ_C 33.9), and two *N*-geminal methyls (δ_C 45.5) which were observed as equivalent signals. Two olefinic carbons and 14 aromatic carbons were detected in the region δ_C 107.7–150.3. The ^{1}H–^{1}H correlation spectroscopy (COSY) spectrum revealed the following proton–proton cross-peaks, an olefinic proton (δ_H 5.20, brs, H-14′) with H-3′ (δ_H 2.88, m), H-3 (δ_H 3.40, d, J = 12.0 Hz) with H-14 (δ_H 5.8, dd, J = 12.4 Hz), and H-14 with H-3′ and all aliphatic protons (H-5, H-6, H-5′ and H-6′). The aromatic protons (H-9, H-10, H-11, H-12, H-9′, H-10′, H-11′ and H-12′) were also assigned based on the ^{1}H–^{1}H COSY spectrum. In the heteronuclear multiple bond connectivity (HMBC) spectrum of **2**, the *N*-dimethyl protons correlated with C-18/C-19/C-5 and H-6 correlated with C-7/C-8/C-2 linked to the indole moiety (Fig. 3). Identically, H-3 correlated with C-6′/C-7′/C-8′ and C-7. The positions of the remaining methyl groups were also determined based on HMBC correlations.

These data were close to those of the known **1**; however, the nuclear Overhauser effect spectroscopy (NOESY) correlations of **2** (Fig. 4) indicated different configurations at H-3, H-3′, and H-14 in **2** from those in **1**. The key correlations of H-3′ with H-14 and H-18′ suggested a β-orientation for H-3′ and H-14, putting these hydrogens on the same side as the bridged *N*-methyl pyrrolidine. The additional correlation between H-3′ with H-17 indicated a β-orientation for pseudoaxial H-17. Since H-16′b was correlated with H-16 and H-17, H-16′b should be oriented on a β-pseudoequatorial, and consequently H-16′a was on a α-pseudoaxial. The correlation between H-16′a and H-3 indicated an α-orientation for H-3. The above spectroscopic evidence strongly suggested that compound **2** (spermaocymine A) is a new bis-indole alkaloid with (3*S**, 14*S**, 2′*R**, 3′*R**, 7′*S**) configurations, a C-3,14-stereoisomer of **1**. It should be noted that this compound is the first natural stereoisomer of related bis-indole alkaloids with an epimino-ethano bridge.

Table 2. ^{1}H- and ^{13}C-NMR Spectroscopic Data of **3**

Position	**3**	
	δ_H (J in Hz)[a]	δ_C[a]
1		158.5
2		145.2
3	8.06 d (7.9)	133.8
4	8.10 d (7.9)	123.3
5	7.73 dd (1.4, 7.3)	118.4
6	7.76 t (7.9)	136.4
7	7.32 dd (1.4, 7.9)	124.2
8		162.1
9		188.9
10		181.9
11		133.1
12		117.1
13		124.1
14		134.3
OCH_3	3.94 s	61.3
CH_2OH	4.86 d (5.5)	58.6
OH	4.52 t (5.5)	

a) ^{1}H-NMR: 400 MHz, ^{13}C-NMR: 100 MHz in acetone-d_6.

Compound **2** exhibited a slightly negative optical rotation but no circular dichroism (CD) absorption. Since all other related compounds, except auricularine isolated from *H. auricularia*,[5] were reported as racemates, it is highly possible that **2** is also a racemate.

Compound **3** was obtained as a yellow solid. The molecular formula, $C_{16}H_{12}O_5$, was determined based on a protonated molecular ion peak at *m/z* 285.0761 $[M+H]^+$, (Calcd 285.0763) by HR-MS. The ^{1}H-NMR data of **3** (Table 2) revealed the presence of a methoxy at δ_H 3.94 (s, 3H), a methylene connected to an OH at δ_H 4.86 (d, J = 5.5 Hz, 2H), and five aromatic protons at δ_H 7.32 (dd, J = 1.4, 7.9 Hz, 1H, H-7), 7.73 (dd, J = 1.4, 7.3 Hz, 1H, H-5), 7.76 (t, J = 7.9 Hz, 1H, H-6), 8.06 (d, J = 7.9 Hz, 1H, H-3), and 8.10 (d, J = 7.9 Hz, 1H, H-4), which corresponded to three contiguous protons and two contiguous protons on two respective phenyls. The ^{13}C-NMR spectrum showed 16 carbon signals including a hydroxymethyl at δ_C 58.6, a methoxy at δ_C 61.3, two carbonyls at δ_C 181.9 and 188.9, and 12 aromatic carbons at δ_C 117.1, 118.4, 123.3, 124.1, 124.2, 133.1, 133.8, 134.3, 136.4, 145.2, 158.5, and 162.1. These signals were characteristic of an anthraquinone skeleton substituted with a hydroxymethyl, a methoxy, and a hydroxy group.

The positions of the three functional groups were determined from HMBC correlations (Fig. 3); the HMBC spectrum showed cross-peaks between an aromatic proton (H-4) with C-2/C-10/C-13, a methoxy proton at δ_H 3.94 with C-1, and a hydroxymethyl proton at δ_H 4.86 with C-2. Regarding the two carbonyls on the quinone skeleton, δ_C 188.9 was shifted to slightly lower field than δ_C 181.9, suggesting the presence of a hydroxy at C-5 or C-8 of the anthraquinone. The aromatic proton at δ_H 7.73 exhibited cross-peaks with C-7, C-10, and C-12. H-6 at δ_H 7.76 and H-7 at δ_H 7.32 were associated with C-8/C-11 and C-5/C-12, respectively. These data indicated that the OH was located at C-8. Accordingly, the structure of **3** was established 8-hydroxy-2-(hydroxymethyl)-1-methoxyanthracene-9,10-dione.

Antiproliferative Activity Although the antimalarial

Table 3. Antiproliferative Activity of Compounds **1**–**3**

Compounds	Cell lines ($IC_{50}\mu M$)[a]				
	A549	MDA-MB-231	MCF-7	KB	KB-VIN
1	10.2 ± 2.2	6.2 ± 0.1	8.8 ± 0.5	11.5 ± 1.3	7.4 ± 1.2
2	28.4 ± 5.1	17.1 ± 2.1	16.2 ± 0.4	22.3 ± 4.3	15.9 ± 0.8
3	39.2 ± 5.2	>40	37.4 ± 1.7	>40	>40
PXL[b] (*n*M)	10.5 ± 0.1	7.9 ± 0.2	10.9 ± 0.6	7.0 ± 0.0	>2000

[a] Antiproliferative activity stated as IC_{50} values for each cell line, the concentration of compound that caused 50% reduction relative to untreated cells evaluated by the SRB assay ($n = 6$). [b] Paclitaxel.

activities of borreverine were investigated,[7] the antitumor effects of related compounds have not been reported. The isolated bis-indole alkaloids **1** and **2** together with anthraquinone **3** were evaluated for their antiproliferative activity against five human tumor cell lines, A549 (lung cancer), MCF-7 (breast cancer: estrogen receptor (ER) positive, and HER2 negative), MDA-MB-231 (breast cancer: ER, progesterone receptor (PR), and HER2 negative), KB (cervical cancer cell line HeLa derivative) and KB-VIN (vincristine resistant KB subline: P-glycoprotein (P-gp)-overexpressing) (Table 3). Compound **1** showed potent cytotoxicity against all tested cell lines including the KB-VIN multidrug-resistant (MDR) cell line with IC_{50} values of 6.2–11.5 μM. This finding suggested that **1** is not a substrate of P-gp. Since compound **2** was less active than **1**, the stereochemistry around C-3, C-14, and C-3′ might be important for the activity.

Conclusion

As part of our continuing phytochemical study of unexplored plants found in tropical rainforests, a thorough investigation of *S. ocymoides* yielded a novel bis-indole alkaloid, spermaocymine A (**2**) and 8-hydroxy-2-(hydroxymethyl)-1-methoxyanthracene-9,10-dione (**3**) along with the known alkaloid **1**. Spermaocymine A (**2**) was identified as the first natural stereoisomer of related bis-indole alkaloids with an epiminoethano bridge. We have also reported the detailed NMR data of known compound **1**, which were not described previously. The evaluation of the antiproliferative activities of **1**–**3** against human tumor cell lines revealed that **1** was potent against all tested cell lines including the KB-VIN MDR cell line with IC_{50} values of 6.2–11.5 μM.

Experimental

General Experimental Procedures Optical rotations were measured on a JASCO P-2200 digital polarimeter in $CHCl_3$. NMR spectra were recorded on JEOL JNM-ECS400 and JNM-ECA600 NMR spectrometers with tetramethyl silane as an internal standard, and chemical shifts are stated as δ values. HR-MS data were obtained from JMS-700 (FAB) mass spectrometer. Analytical and preparative TLC were performed on precoated silica gel 60 F_{254} plates (0.25 or 1 mm thickness; Merck, Germany) and NH_2 silica gel F_{254} (0.5 mm; Wako, Japan). Column chromatography (CC) was performed with silica gel 60N (spherical, 63–210 μm, neutral, Kanto Chemical, Japan). Analytical and reversed-phase preparative TLC (PTLC) was conducted on Silica gel 60 RP-18 F254S (0.25 mm, Merck).

Plant Material *S. ocymoides* (Burm.f.) DC. aerial parts were collected at Lengkese Village, Parigi Subdistrict, Gowa, South Sulawesi, Indonesia in April 2017 and authenticated by Djoko Santoso, Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada, Indonesia. A voucher specimen was deposited in the Pharmacognosy-Phytochemistry Laboratory, Hasanuddin University (2017_AR_FFUH_01).

Extraction and Isolation Compounds The air-dried aerial parts of *S. ocymoides* (125 g) were powdered and extracted three times (2.0 L for each time) with MeOH at room temperature for 48 h. The volatile solvent was evaporated to afford a crude MeOH extract (15.4 g). The MeOH extract in EtOAc (100 mL) was subjected to ultrasound irradiation for 15 min to separate EtOAc soluble and insoluble parts. The EtOAc insoluble part (10.9 g) was further partitioned between *n*-BuOH and H_2O. The *n*-BuOH extract was subjected to CC on silica gel eluted with $CHCl_3$–MeOH and MeOH 100% to give eight fractions (F_1A–F_1H). Fraction F_1D (16.2 mg) was separated by NH_2 silica gel PTLC developed with *n*-hexane–acetone to give **2** (1.7 mg). Fraction F_1G was separated into soluble and insoluble parts by addition of MeOH. The MeOH soluble part was subjected to NH_2 silica gel PTLC developed with $CHCl_3$–MeOH to give **1** (1.8 mg). The EtOAc soluble portion (4.1 g) was partitioned between *n*-hexane and MeOH–H_2O (9 : 1). The MeOH–H_2O extract (1.6 g) was subjected to CC on silica gel eluted with *n*-hexane–EtOAc, MeOH, and acetone to give ten fractions (F_2A–F_2J). Fraction F_2D (99.5 mg) was separated by reversed-phase medium pressure liquid chromatography (MPLC) eluted with MeOH–H_2O, MeOH, and acetone to give ten fractions (F_2D1–F_2D10). Fraction F_2D6 (9.1 mg) was applied to a reversed-phase preparative HPLC eluted with MeOH–H_2O to obtain four subfractions (F_2D6A–F_2D6D). The addition of H_2O to subfraction F_2D6B (1.3 mg) yielded **3** (0.5 mg) as insoluble material.

Spermaocymine A (2) Colorless solid; ^{1}H-NMR ($CDCl_3$, 600 MHz) and ^{13}C-NMR ($CDCl_3$, 150 MHz), see Table 1; HR-MS *m/z* 495.3471 $[M+H]^+$, (Calcd for $C_{33}H_{43}N_4$, 495.3488).

8-Hydroxy-2-(hydroxymethyl)-1-methoxyanthracene-9,10-dione (3) Yellow solid; ^{1}H-NMR (Acetone-d_6, 400 MHz) and ^{13}C-NMR (Acetone-d_6, 100 MHz), see Table 2; HR-MS *m/z* 285.0761 $[M+H]^+$, (calcd for $C_{16}H_{13}O_5$, 285.0763).

Antiproliferative Activity Assay The antiproliferative activity assay was carried out by the sulforhodamine B (SRB) assay using A549, MDA-MB-231, MCF-7, KB, and KB-VIN as previously presented.[11]

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials This article contains supplementary materials.

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