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Aegyptolidines A and B: New pyrrolidine alkaloids from the fungus *Aspergillus aegyptiacus*

Sabrin R.M. Ibrahim [a,*], Gamal A. Mohamed [b], Ahmad M. Moharram [c], Diaa T.A. Youssef [d]

[a] Department of Pharmacognosy, Faculty of Pharmacy, Assiut University, Assiut 71526, Egypt
[b] Department of Pharmacognosy, Faculty of Pharmacy, Al-Azhar University, Assiut Branch, Assiut 71524, Egypt
[c] Department of Botany & Microbiology, Faculty of Science, Assiut University, Assiut 71526, Egypt
[d] Department of Natural Products and Alternative Medicine, Faculty of Pharmacy, King Abdulaziz University, Jeddah 21589, Saudi Arabia

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ABSTRACT

Chemical examination of the fungus *Aspergillus aegyptiacus* isolated from cotton textile yielded two new pyrrolidine alkaloids: aegyptolidine A (**3**) and aegyptolidine B (**4**), together with two known ophiobolin sesterterpenoids: (±)-5-deoxovariecolin (**1**) and variculanol (**2**). Compound **1** is reported for the first time from a natural source. The structures of the isolated compounds were unambiguously elucidated on the basis of extensive spectroscopic data analysis (1D (^{1}H and ^{13}C) and 2D (^{1}H–^{1}H COSY, HMQC, HMBC, and NOESY) NMR, MS, IR, and UV) and comparison with literature data. Compounds **1** and **2** showed moderate cytotoxic activity against murine lymphoma cancer cell line L5178Y with ED_{50} 7.6 and 8.1 μg/mL, respectively.

1. Introduction

Recently, numerous metabolites possessing uncommon structures and potent bioactivity have been isolated from strains of bacteria and fungi collected from diverse environments such as soils, animals, plants, and sediments (Kerr and Kerr, 1999; Fenical, 1993). This clearly indicates that the microorganisms are indeed a rich source of chemically unique and biologically potent substances (Kerr and Kerr, 1999; Fenical, 1993). The fungal microorganisms became a hunting ground for novel drug leads (Nagia et al., 2012; Larsen et al., 2005; Calvo et al., 2002; BúLock, 1961). Thus, many of these compounds are of medical, industrial, and/or agricultural importance. Some natural products are deleterious (e.g. mycotoxins), while others are beneficial (e.g. antibiotics) to humankind (Calvo et al., 2002; Demain and Fang, 2000). *Aspergilli* represent a large diverse genus, containing about 180 filamentous fungal species of substantial pharmaceutical and commercial values (Lubertozzi and Keasling, 2009). *Aspergillus* is one of the most incredible chemical factories known today. In our continued search for new biologically active metabolites from natural sources, we have identified two new pyrrolidine alkaloids: aegyptolidine A (**3**) and aegyptolidine B (**4**), together with two known ophiobolin sesterterpenoids: (±)-5-deoxovariecolin (**1**) and variculanol (**2**) (Fig. 1) from *Aspergillus aegyptiacus* isolated from cotton textile. The chemical structures of the new compounds were identified by one and two dimensional NMR and HRMS analyses. The fungus was cultured then the mycelium and broth were separated by filtration. The concentrated EtOAc extract of broth was subjected to Sephadex LH-20, silica gel, RP-18, and HPLC column chromatography to yield two new (**3** and **4**) and two known compounds (**1** and **2**).

2. Results and discussion

Compound **1** was obtained as colorless oil. Its HRESIMS gave a molecular ion peak at 355.2931 $[M+H]^+$, consistent with the molecular formula $C_{25}H_{38}O$. The IR spectrum showed absorption band at 1695 cm^{-1} for α,β-unsaturated aldehyde carbonyl. The ^{1}H NMR spectrum showed an exomethylene group at δ_H 4.72 (d, J = 2.5 Hz, H-24A) and 4.63 (d, J = 2.5 Hz, H-24B), aldehydic proton δ_H 9.18 (s, H-20), olefinic proton at δ_H 6.92 (dd, J = 5.1, 1.1 Hz, H-8), and four methyls at δ_H 0.79 (d, J = 7.3 Hz, H-19), 0.93 (s, H-21), 0.83 (s, H-22), and 1.70 (s, H-25). The ^{13}C NMR spectrum displayed 25 carbon resonances including, four methyls, nine methylenes, eight methines, and four quaternary carbons. The NMR data of **1** were comparable to those of (±)-5-deoxovariecolin, which was previously obtained from synthetic origin (Walker, 2002). This is the first report for its isolation from a natural source. The full assignment of its NMR spectral data was reported here for the first time. The NOESY

* Corresponding author. Tel.: +20 881411330; fax: +20 88 2332776.
E-mail address: sabrinshaur@gmail.com (Sabrin R.M. Ibrahim).

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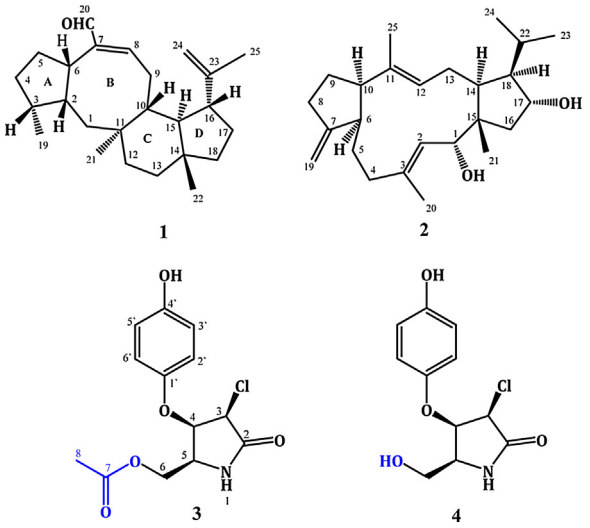

Fig. 1. Structures of the isolated compounds**1**–**4**.

spectrum showed correlations between H-2/H-6, H-10, and H-16 and from H-3 to H-2 and H-22 indicating that they were on the same side of the molecule (Fig. 2). The NOESY correlations of H-19 to H-15 and H-21 suggested that these protons were on the other side of the molecule. On the basis of these evidences, the configuration of **1** was assigned to be same as (±)-5-deoxovariecolin and other related ophiobolin class of sesterterpenes (Walker, 2002; Fujimoto et al., 2000).

Compound **4** was isolated as white amorphous powder. It gave positive ninhydrin and Dragendorff's tests for alkaloids (Harborne, 1984). The HRESIMS spectrum displayed two pseudo-molecular ion peaks at *m/z* 258.0538 ($C_{11}H_{13}{}^{35}ClNO_4$, $[M+H]^+$) and 260.0509 ($C_{11}H_{13}{}^{37}ClNO_4$, $[M+H]^+$) in a ratio of 3:1, indicating the presence of chlorine atom in **4** (Silverstein and Webster, 1998). The molecular formula $C_{11}H_{12}ClNO_4$ suggested six degrees of unsaturation. The ^1H and ^{13}C NMR data of **4** revealed that five of the six units of unsaturation were attributed to phenyl and carbonyl moieties. Thus, the remaining unit of unsaturation indicated the presence of an additional ring in **4**. The UV absorption maxima at 238, 282, and 305 nm indicated the presence of a conjugated chromophore. The ^{13}C NMR spectrum showed resonances for eleven carbons

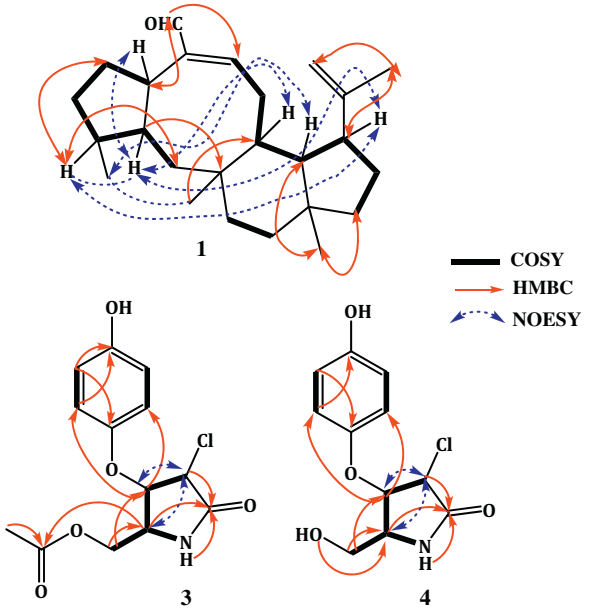

Fig. 2. Key COSY, HMBC, and NOESY correlations of **1**, **3**, and **4**.

including, one amide carbonyl, three aliphatic methines including one oxymethine, an oxymethylene, and six aromatic C-atoms for one benzene moiety. The ^1H and ^{13}C NMR data of **4** in association with its ^1H–^1H COSY and HMQC spectra indicated the presence of 1,4-*di*substituted phenyl moiety at δ_H 7.59 (2H, d, J = 8.7 Hz, H-2′, 6′) and 8.14 (2H, d, J = 8.7 Hz, H-3′, 5′) (Table 1). The HMBC correlations (Fig. 2) from H-2′ and H-6′ to C-4′ (δ_C 146.7) and H-3′ and H-5′ to C-1′ (δ_C 151.1) and C-4′ established the 1,4-dihydroxy phenyl moiety. ^1H and ^{13}C NMR spectra of **4** displayed signals for N*H*-bonded methine at δ_H 3.95 (m, H-5)/δ_C 56.9 (C-5), an oxymethine at δ_H 5.08 (H-4)/δ_C 69.4 (C-4), methine at δ_H 6.44 (s, H-3)/δ_C 66.6 (C-3), and N*H* group at δ_H 8.33 (d, J = 9.2 Hz, H-1), indicating the presence of 4-hydroxy pyrrolidine-2-one moiety. This was established by the observed ^1H–^1H COSY cross peaks and further confirmed by the HMBC correlations of H-1, H-3, H-4, and H-5 to the amide carbonyl at δ_C 163.6 (C-2) (Fig. 2). Moreover, resonances for two exchangeable protons at δ_H 5.12 (4′-OH) and 5.95 (6-OH) were observed in the ^1H NMR spectrum. In addition, a multiplet for an oxymethylene group at δ_H 3.38 (H-6), correlating with the carbon at δ_C 60.5 (C-6) in HMQC spectrum was observed. Its HMBC correlations to C-4 (δ_C 69.4) and C-5 (δ_C 56.9) indicated its attachment at C-5 of 4-hydroxy pyrrolidine-2-one moiety. The connectivity of 1,4-dihydroxy-phenyl moiety at C-4 of 4-hydroxy pyrrolidine-2-one nucleus was established based on the ^4J HMBC correlations of H-4 to C-2′ and C-6′, H-2′ and H-6′ to C-4, and ^3J HMBC cross peak of H-4 to C-1′. The relative stereochemistry of **4** was deduced from the observed coupling constants in the ^1H NMR spectrum. The small J values of H-3, H-4, and H-5 indicated α-orientation of these protons. Furthermore, it was confirmed by the observed correlations of H-3 to H-5 and H-4 to H-3 and H-5 in NOESY spectrum (Fig. 2). On the basis of the previous mentioned data, the structure of **4** was assigned as 3-chloro-5-(hydroxymethyl)-4-(4-hydroxyphenoxy)pyrrolidin-2-one and named aegyptolidine B. Structurally similar pyrrolidine alkaloids had been reported previously from *Aspergilli* (Liu et al., 2011; Schwartz et al., 1988).

Compound **3** was isolated as white amorphous powder. Similar to **4**, compound **3** showed two pseudo-molecular ion peaks in the HRESIMS at *m/z* 300.0641 and 302.0611 in the ratio of 3:1 suggesting the presence of chlorine atom in the molecule. These two pseudomolecular ion peaks are corresponding to the two isotopes of the chlorinated **3** with the molecular formula $C_{13}H_{15}{}^{35}ClNO_5$ (*m/z* 300.0639, $[M+H]^+$) and $C_{13}H_{15}{}^{37}ClNO_5$ (*m/z* 302.0611, $[M+H]^+$). Compound **3** is 42 mass units and one degree of unsaturation more than **4**. The IR spectrum showed absorption bands at 3496 (hydroxyl), 1740 (ester carbonyl), and 1658 (amide carbonyl) cm^{-1}. The ^1H and ^{13}C NMR data for **3** and **4** were quite similar, but new signals at δ_H 1.98 (3H, s, H-8)/δ_C 21.0 (C-8) and 171.0 (C-7), suggesting the presence of an acetoxy group were observed (Table 1). This was supported by the IR absorption band at 1740 cm^{-1} and further confirmed by the fragment ion peak at *m/z* 257.0457 $[M+H\text{-acetyl}]^+$. The HMBC cross peaks of H-5 and H-6 to the carbonyl of acetyl group confirmed the placement the acetyl moiety at C-6 (Fig. 2). The relative configuration of **4** was established based on the observed NOESY correlations (Fig. 2). Therefore, the structure of **3** was determined as (4-chloro-3-(4-hydroxyphenoxy)-5-oxopyrrolidin-yl)methyl acetate and the name aegyptolidine A was given to it.

Compound **2** was identified as variculanol by analysis of the spectroscopic data (1D, 2D NMR, and MS) and comparison of its data with those in literature (Singh et al., 1991). Variculanol was previously isolated from *Aspergillus variecolor* (Singh et al., 1991). This is the first report of its isolation from *A. aegyptiacus*.

All isolated compounds were evaluated for their cytotoxic activity against murine lymphoma L5178Y cell line using MTT assay. Compounds **1** and **2** showed moderate activity with ED_{50} 7.6 and

Table 1
NMR data of compounds **3** and **4** (DMSO-d_6, 400 and 100 MHz).

No.	**3**			**4**		
	δ_H [mult., J (Hz)]	δ_C (mult.)	HMBC	δ_H [mult., J (Hz)]	δ_C (mult.)	HMBC
1	8.56 d (8.7)	–	2	8.33 d (9.2)	–	2
2	–	164.3 (C)	–	–	163.6 (C)	–
3	6.42 s	66.7 (CH)	2	6.44 s	66.6 (CH)	2
4	5.02 brs	70.2 (CH)	2′, 6′	5.08 brs	69.4 (CH)	2, 6, 1′, 2′, 6′
5	4.23 m	54.1 (CH)	7	3.95 m	56.9 (CH)	2, 6
6	4.11 t (10.3)	63.7 (CH_2)	5, 7	3.38 m	60.5 (CH_2)	4, 5
7	–	171.0 (C)	–	–	–	–
8	1.98 s	21.0 (CH_3)	7	–	–	–
1′	–	150.5 (C)	–	–	151.1 (C)	–
2′, 6′	7.63 d (8.4)	128.1 (CH)	4, 4′, 3′, 5′	7.59 d (8.7)	127.4 (CH)	4, 4′
4′	–	147.1 (C)	–	–	146.7 (C)	–
3′, 5′	8.18 d (8.4)	123.8 (CH)	1′, 4′, 2′, 6′	8.14 d (8.7)	122.8 (CH)	1′, 4′
6-OH	–	–	–	5.95 brd (3.9)	–	5, 6
4′-OH	6.28 brs	–	–	5.12 s	–	4′

8.1 μg/mL, respectively compared to kahalalide F (6.7 μg/mL, positive control). However, **3** and **4** exhibited weak activity (Table 2).

3. Experimental

3.1. General experimental procedures

Melting points were carried out using an Electrothermal 9100 Digital Melting Point apparatus (Electrothermal Engineering Ltd., Essex, England). Optical rotations were measured on a Perkin-Elmer Model 341 LC polarimeter (Perkin-Elmer, Waltham, MA, USA). UV spectra were recorded in MeOH on a Shimadzu 1601 UV/VIS spectrophotometer (Shimadzu, Kyoto, Japan). The IR spectra were measured on a Shimadzu Infrared-400 spectrophotometer (Shimadzu, Kyoto, Japan). HRESIMS was recorded on a LTQ Orbitrap (ThermoFinnigan, Bremen, Germany). ESIMS spectra were obtained with a LCQ DECA mass spectrometer (Thermo-Finnigan, Bremen, Germany) coupled to an Agilent 1100 HPLC system equipped with a photodiode array detector. 1D and 2D NMR spectra (chemical shifts in ppm and coupling constants in Hz) were recorded on Bruker BioSpin GmbH 400 MHz Ultrashield spectrometer using $CDCl_3$ and DMSO-d_6 as solvents, with TMS as the internal reference (Bruker, Rheinstetten, Germany). Solvents were distilled prior spectroscopic measurements. HPLC separations were performed on an HPLC system consisting of a Lachrom–Merck Hitachi L-7100 pump and a L-7400 UV detector using a C-18 column (300 mm × 8 mm i.d., prefilled with Eurospher 100, Knauer, Berlin, Germany), a flow rate of 5.0 mL/min. Column chromatographic separations were performed on SiO_2 60 (0.04–0.063 mm, Merck, Darmstadt, Germany), Sephadex LH-20 (0.25–0.1 mm, Merck, Darmstadt, Germany), and RP-18 (0.04–0.063 mm, Merck, Darmstadt, Germany). TLC analysis was performed on pre-coated TLC plates with SiO_2 60 F_{254} (0.2 mm, Merck, Darmstadt, Germany). The solvent systems used for TLC analyses were $CHCl_3$:MeOH (97:3, S_1) and $CHCl_3$:MeOH (90:10, S_2). The compounds were detected by UV absorption at λ_{max} 255 and 366 nm followed by spraying with the following reagents: anisaldehyde:H_2SO_4 and heating at 110 °C for 1–2 min, ninhydrin, and Dragendorff's.

Table 2
Cytotoxic activity results of compounds **1**–**4**.

Sample	L5178Y % inhibition (10 μg/mL)	ED_{50} (μg/mL)
1	65	7.6
2	61	8.1
3	28	>10
4	23.5	>10
Kahalalide F		6.7

3.2. Identification of fungal cultures

The fungal strain *A. aegyptiacus* was kindly provided by Assiut University Mycological Centre (AUMC) and deposited in Assiut University Mycological Center (AUMC No. 6122) (http://www.aun.edu.eg/aumc/Catalog.htm). It was originally isolated from cotton textile and identified on morphological bases as follows: Colonies with greenish white to pale green in color on Czapek agar medium; conidiophores smooth, colorless; conidial heads strictly biseriate, loosely columnar; vesicles globose, small; conidia globose or subglobose, smooth; hull cells globose, subglobose or elliptical (Moubasher, 1993).

3.3. Cultivation

A. aegyptiacus was sub-cultured on potato dextrose agar (PDA) for 10 days at 25 °C to obtain inoculums for subsequent cultivation. PDA medium contained (g/L): potato extract 200 g, dextrose 20 g, and agar 20 g. The medium was autoclaved at 121 °C for 20 min and distributed in 10 cm Petri plates which were then inoculated with the fungal strain. Potato dextrose broth (PDB) was similarly prepared without incorporation of agar. PDB was distributed in 2 L-capacity conical flasks (one liter medium per each flask). Ten flasks were prepared and inoculated with 3 agar discs (1 cm diam.) of *A. egyptiacus* grown on PDA. After incubation for 21 days, the growing cultures were homogenized in sterile electric homogenizer for further studies.

3.4. Extraction and isolation

The mycelium and broth were separated by filtration. The broth was extracted with EtOAc (4× 1 L) and evaporated to dryness under vacuum to afford 1.2 g dark brown residue. The latter was subjected to Sephadex LH-20 using MeOH:$CHCl_3$ (90:10) as an eluent to give four subfractions AE-1 to AE-4. Subfraction AE-1 (370 mg) was chromatographed over silica gel column (70 g × 50 cm × 2 cm) using *n*-hexane:EtOAc gradient elution to give compounds **1** (5.6 mg, colorless oil) and **2** (6.8 mg, colorless needles). RP-18 column chromatography (100 g × 50 cm × 3 cm) of subfraction AE-3 (340 mg) using MeOH:H_2O gradient elution yielded impure **3** and **4**. They were further purified by semi-preparative HPLC to yield **3** (4.3 mg, white amorphous powder) and **4** (5.2 mg, white amorphous powder). The purity of isolated compounds (**1**–**4**) was verified to be >96% based on HLC analysis.

3.5. Spectral data

(±)-5-Deoxovariecolin (**1**). Colorless oil, R_f 0.84, Si 60 F_{254} (S_1); UV λ_{max} (MeOH): 203, 235 nm; IR (KBr) ν_{max} 2945, 1695, 1245 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ_H 1.53 (1H, dd, J = 14.1, 10.9 Hz, H-1α), 1.21 (1H, m, H-1β), 2.64 (1H, m, H-2), 2.40 (1H, m, H-3), 2.55 (1H, m, H-4α), 2.38 (1H, m, H-4β), 1.43 (1H, m, H-5α), 1.25 (1H, m, H-5β), 3.59 (1H, brd, J = 10.1 Hz, H-6), 6.92 (1H, brd, J = 5.1, 1.1 Hz, H-8), 2.86 (1H, brd, J = 14.1 Hz, H-9α), 2.21 (1H, m, H-9β), 2.20 (1H, m, H-10), 1.88 (1H, dt, J = 14.0, 3.9 Hz, H-12α), 1.02 (1H, m, H-12β), 1.48 (2H, m, H-13), 1.40 (1H, m, H-15), 2.40 (1H, m, H-16), 1.98 (1H, m, H-17α), 1.21 (1H, m, H-17β), 1.45 (1H, m, H-18α), 1.23 (1H, m, H-18β), 0.79 (3H, d, J = 7.3 Hz, H-19), 9.18 (1H, s, H-20), 0.93 (3H, s, H-21), 0.83 (3H, s, H-22), 4.72 (1H, d, J = 2.5 Hz, H-24A), 4.63 (1H, d, J = 2.5 Hz, H-24B), 1.70 (3H, s, H-25); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 42.2 (C-1), 39.2 (C-2), 34.7 (C-3), 35.2 (C-4), 29.7 (C-5), 50.3 (C-6), 139.8 (C-7), 160.9 (C-8), 31.5 (C-9), 40.6 (C-10), 39.0 (C-11), 35.4 (C-12), 35.2 (C-13), 43.3 (C-14), 48.6 (C-15), 40.4 (C-16), 29.8 (C-17), 39.8 (C-18), 15.8 (C-19), 193.1 (C-20), 21.8 (C-21), 18.0 (C-22), 150.3 (C-23), 110.7 (C-24), 19.0 (C-25). HRESIMS m/z: 355.2931 (calcd for $C_{25}H_{39}O$, $[M+H]^+$, 355.3001).

Variculanol (**2**). Colorless needles, R_f 0.76, Si 60 F_{254} (S_1); mp 187–188 °C; $[\alpha]_D$ +24.5 (c 1.0, $CHCl_3$); IR (KBr) ν_{max} 3345, 2945, 1648, 736 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ_H 4.02 (1H, d, J = 9.8 Hz, H-1), 5.35 (1H, dd, J = 9.8, 1.5 Hz, H-2), 2.05 (1H, m, H-4α), 2.21 (1H, m, H-4β), 2.20 (1H, m, H-5α), 0.75 (1H, m, H-5β), 2.48 (1H, m, H-6), 2.52 (1H, m, H-8α), 2.51 (1H, m, H-8β), 1.95 (1H, m, H-9α), 1.64 (1H, m, H-9β), 3.08 (1H, dd, J = 7.8, 2.3 Hz, H-10), 5.14 (1H, ddd, J = 11.1, 3.5, 1.2 Hz, H-12), 2.18 (1H, m, H-13α), 2.01 (1H, m, H-13β), 2.68 (1H, dd, J = 7.5, 2.1 Hz, H-14), 1.95 (1H, m, H-16α), 1.80 (1H, m, H-16β), 3.98 (1H, d, J = 5.3 Hz, H-17), 1.98 (1H, m, H-18), 4.79 (1H, d, J = 2.1 Hz, H-19A), 4.70 (1H, d, J = 2.1 Hz, H-19B), 1.63 (3H, d, J = 1.5 Hz, H-20), 0.85 (3H, s, H-21), 1.85 (1H, m, H-22), 0.84 (3H, d, J = 6.9 Hz, H-23), 1.34 (3H, d, J = 6.9 Hz, H-24), 1.38 (3H, d, J = 1.2 Hz, H-25); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 75.9 (C-1), 126.7 (C-2), 138.9 (C-3), 38.3 (C-4), 27.9 (C-5), 50.3 (C-6), 156.8 (C-7), 34.0 (C-8), 28.2 (C-9), 51.4 (C-10), 135.2 (C-11), 130.3 (C-12), 26.5 (C-13), 40.3 (C-14), 50.1 (C-15), 49.6 (C-16), 72.7 (C-17), 61.4 (C-18), 102.6 (C-19), 16.4 (C-20), 23.2 (C-21), 25.8 (C-22), 20.2 (C-23), 24.6 (C-24), 13.7 (C-25); ESIMS m/z: 373 $[M+H]^+$.

Aegyptolidine A (**3**). White amorphous powder, R_f 0.77, Si 60 F_{254} (S_2); $[\alpha]_D$ −51.9° (c 0.5, MeOH); UV λ_{max} (MeOH): 240, 286, 309 nm; IR (KBr) ν_{max} 3496, 1740, 1658 cm^{-1}; NMR data: see Table 1; HRESIMS m/z 300.0641 (calcd for $C_{13}H_{15}{}^{35}ClNO_5$, $[M+H]^+$, 300.0639), 302.0611 (calcd for $C_{13}H_{15}{}^{37}ClNO_5$ $[M+H]^+$).

Aegyptolidine A (**4**). White amorphous powder, R_f 0.69, Si 60 F_{254} (S_2); $[\alpha]_D$ −76.4° (c 0.5, MeOH); UV λ_{max} (MeOH): 238, 282, 305 nm; IR (KBr) ν_{max} 3456, 1665, 1058 cm^{-1}; NMR data: see Table 1; HRESIMS m/z 258.0538 (calcd for $C_{11}H_{13}{}^{35}ClNO_4$, $[M+H]^+$, 258.0533), 260.0509 (calcd for $C_{11}H_{13}{}^{37}ClNO_4$ $[M+H]^+$.

3.6. Cell proliferation assay

Antiproliferative activity was examined against the murine lymphoma L5178Y cancer cell line using the micro-culture tetrazolium (MTT) assay and compared to that of untreated controls. All experiments were carried out in triplicates and repeated three times. As negative controls, media with 0.1% (v/v) EtOH were included in all experiments. The depsipeptide kahalalide F was used as a positive control (Ebrahim et al., 2012; Ibrahim et al., 2008).

4. Conclusion

Two new pyrrolidine alkaloids: aegyptolidine A (**3**) and aegyptolidine B (**4**) together with two known ophiobolin sesterterpenoids were isolated from fungus *A. aegyptiacus*. Their structures were elucidated on the basis of extensive spectroscopic data analysis. Compounds **1** and **2** showed moderate cytotoxic activity.

Conflict of interest

There is no conflict of interest associated with the authors of this paper.

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