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Meroterpenoids produced by *Pseudocosmospora* sp. Bm-1-1 isolated from *Acanthus ebracteatus* Vahl

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ABSTRACT

Fractionation of ethyl acetate extract obtained by culturing the endophytic fungus *Pseudocosmospora* sp. Bm-1-1 resulted in the isolation of four new meroterpenoids, cosmosporin A (**1**), 6-carboxy-cosmosporin A (**2**), *rel*-(6a*S*,10a*R*)-Δ^9-tetrahydrocannabiorcolic acid B (**3**) and 8′-hydroxy-cannabiorcichromenic acid (**4**), in addition to four known compounds **5–8**. Structures were elucidated by spectral analysis, as well as by directly comparing the spectral data of new compounds with those of known compounds. Cannabiorcichromenic acid (**5**), decarboxy-cannabiorcichromenic acid (6), and *rel*-(6a*S*,10a*R*)-decarboxy-Δ^9-tetrahydrocannabiorcolic acid B (**8**) restored growth of a *Saccharomyces cerevisiae* mutant strain involving Ca^{2+} signal transduction. Furthermore, compounds **3** and **8** had cytotoxic activity against HL60 cells (**3**: IC_{50} = 24.1 μM and **8**: IC_{50} 1.6 μM).

1. Introduction

Endophyte fungi are known to produce many different types of secondary metabolites with a wide variety of biological activities such as antimicrobial, antimalarial, cytotoxic and antioxidant activities. Our previous work on the mangrove endophytic fungus *Cosmospora vilior* IM2-155 resulted in the isolation of the dichroloresorcinol derivatives cosmochlorins A, B, and C. Cosmochlorins A and B inhibit glycogen synthase kinase (GSK)-3β activity (Shiono et al., 2016). These unique halogenated compounds, cosmochlorins, accumulated only in culture media containing 3% NaCl. This indicated that in *C. vilior* IM2-155, metabolite production was significantly influenced by the presence of NaCl in the culture media (Shiono et al., 2016). In this regard, it is not surprising that the metabolite profiles of chemically prolific endophytes from marine origins are modulated by environmental NaCl, and also that these species tolerate high concentrations of salt (Gogoi et al., 2008; Orfali et al., 2015). Because fungal endophytes are most often isolated from terrestrial plants, rather than from those of marine origin, we have chosen to focus on marine-derived fungi in order to yield a wide variety of novel metabolites that may possess differential biological effects than those produced by terrestrial species.

In continuing study of bioactive compounds from microorganisms isolated from mangrove plants, the fungal strain *Pseudocosmospora* sp. Bm-1-1 was isolated from *Acanthus ebracteatus* Vahl collected in Pontianak, Indonesia. This mangrove plant belongs to the *Acanthaceae* family (Tomlinson, 1986). This plant is a shrub with ranging from 50 to 120 cm tall and naturally reproduces vegetatively and also by seeds, thus generation length is difficult to determine for this plant (Robertson and Alongi, 1992). They are often sympatric with *Acanthus ilicifolius* L. and inhabit in all soil type, especially muddy areas along the mid to high intertidal regions of South and Southeast Asia (India, Brunei Darussalam, China, South Taiwan, India, Malaysia, Philippines, Singapore, Thailand, Viet Nam, Cambodia, and Indonesia) and Australasia (northeast and northwest Australia, Papua New Guinea, and the

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Solomon Island) (Ellison et al., 2010).

Chemical investigation undertaken on the fermented extract of this fungus led to the isolation of four new compounds, cosmosporin A (**1**), 6-carboxy-cosmosporin A (**2**), an analogue of tetrahydrocannabiorcolic acid B (**3**), and 8′-hydroxy-cannabiorcichromenic acid (**4**). The extract also contained four known compounds, cannabiorcichromenic acid (**5**), decarboxy-cannabiorcichromenic acid (**6**), 6-carboxyl-4-dechloro-ascochlorin (**7**), and *rel*-(6a*S*,10a*R*)-decarboxy-Δ^9-tetrahydro cannabiorcolic acid B (**8**). The present report concerns the isolation and structural determination of the new compounds, including spectral data for **7**, which has not been previously reported in the primary literature, and the compounds' activities against Ca^{2+} signal transduction in mutant yeast and HL60 cells.

2. Results and discussion

A large fermentation of *Pseudocosmospora* sp. Bm-1-1 was conducted in unpolished rice medium. A crude MeOH extract was prepared from the fermented media, and was separated by column chromatography, followed by flash column chromatography or HPLC purification to yield compounds **1**-**8**. Based on the NMR and HRESITOFMS data and comparison to the reported data, three known compounds were identified as cannabiorcichromenic acid (**5**), decarboxy-cannabiorcichromenic acid (**6**) (Quaghebeur et al., 1994; Liu et al., 2013), *rel*-(6a*S*,10a*R*)-decarboxy-Δ^9-tetrahydrocannabiorcolic acid B (**8**) (Zhou et al., 2007) (Fig. 1). The detailed structural elucidations of the new compounds and **7** are described below.

Cosmosporin A (**1**) was obtained as a white amorphous powder. Its molecular formula was determined by HRESITOFMS to be $C_{22}H_{34}O_4$, with six degrees of unsaturation. The UV spectrum of **1** showed absorption maxima bands at 274 and 282 nm, indicating the presence of an aromatic moiety. The IR spectrum showed absorption at 3310 cm^{-1}, indicating the presence of hydroxyl group. The ^{13}C NMR spectral data (Table 1) of **1** displayed 22 carbons, including five methyls, five methylenes, four methines, and eight quaternary carbons identified using a DEPT experiment. The ^1H NMR spectral data (Table 2) of **1** displayed characteristic signals for a singlet aromatic methine [δ_H 6.21 (2H, s, H-4 and H-6)], suggesting the presence of a symmetrical 1,2,3,5-tetra-substituted benzene ring, as well as resonances for three tertiary olefinic methyls at δ_H 1.57 (s, H-14′), 2.16 (s, Me-7) and 1.75 (s, H-15′); two tertiary methyls at δ_H 1.14 (s, H-12′) and 1.19 (s, H-13′); two olefinic methine protons at δ_H 5.18 (t, J = 6.0 Hz, H-2′) and 5.10 (t, J = 6.0 Hz, H-6′); an oxymethine proton at δ_H 3.37 (dd, J = 10.2, 1.2 Hz, H-10′) and five methylenes at δ_H 1.37–1.41 (*m*, H-9′), 2.04–2.07 (m, H-4′ and H-8′), 2.09–2.14 (m H-5′), 2.17–2.20 (m, H-8′), 3.35 (d, J = 6.6 Hz, H-1′) and 1.37–1.41 (*m*, H-9′). The HMBC correlations (Fig. 2) from Me-7 to C-4, C-5 and C-6, and from H-4 to C-2 and C-6, and from H-1′ to C-1 and C-3 indicated the presence of a 1,3-dihydroxy-5-methyl-benzene ring moiety. The COSY spectrum indicated three partial structures including H-1′/H-2′, H-4′/H-5′/H-6′, and H-8′/H-9′/H-10′ units. The HMBC correlations from H-15′ to C-2′, C-3′ and C-4′, from H-14′ to C-6′, C-7′ and C-8′, and from H-12′ and H-13′ to C-10′ and C-11′, revealed a sesquiterpene moiety from C-1′ to C-15′ (Fig. 2). The *E* stereochemistry of trisubstituted Δ2′ and Δ6′ double bonds was determined based on the relative upfield shift of the vinylic methyl groups (C-14′ and C-15′) (Blunt et al., 1985). Finally, the absolute configuration at C-10′ in **1** was determined using the modified Mosher's method (Ohtani et al., 1991). The ^1H chemical-shift differences between the (*S*)-2-methoxy-2-phenyl-2-(trifluoromethyl) acetic acid (MTPA) esters (**1a**) of **1** and (*R*)-(**1b**) of **1** are shown in Fig. 3, and suggested the *R* configuration for C-10′, establishing the absolute structure of **1**.

6-Carboxy-cosmosporin A (**2**) was obtained as a white amorphous powder. Its molecular formula was deduced as $C_{23}H_{34}O_6$ from HRESITOFMS and ^{13}C NMR (Table 1) data. The IR spectrum indicated the presence of OH (3310 cm^{-1}) and carboxyl (1620 cm^{-1}) groups. Comparison of the NMR spectra of **2** with those of **1** showed that they were very similar expect for an additional carboxylic group (δ_C 174.3), the absence of signals assigned to symmetrical 1,2,3,5-tetrasubstituted benzene ring (δ_H 6.21, H-4 and H-6) in **1**, and the presence of characteristic signals of penta-substituted benzene rings at δ_H 6.17 (1H, s, H-4), δ_C 163.4 (C-1), 112.6 (C-2), 159.8 (C-3), 110.2 (C-4), 103.7 (C-5), and 140.6 (C-6). These observations indicated that the carboxylic group was at C-6, and this conclusion was supported by the HMBC spectrum (Fig. 2). Therefore, compound **2** was established as 6-carboxy-cosmosporin A. The absolute configuration of C-10′ in **2** was determined to be *R* by comparing the optical rotation value of **2** [$[\alpha]_D$ + 6.3°, (*c* 0.33, MeOH)] to those of **1** [$[\alpha]_D$ + 10.0°, (*c* 0.32, MeOH)]. Further, the similar metabolite, (*S*)-10′,11′-dihydroxyneogrifolic acid ($[\alpha]_D$ − 4.0°, (*c* 0.2, MeOH) differing from **2** in the aromatic substitution pattern and the *β*-hydroxyl group at C-10′ has been found in endophyte *Penicillium* sp. T2-8 (Duan et al., 2016).

Compound **3** was obtained as a white amorphous solid. Its molecular formula was established by HRESITOFMS to be $C_{18}H_{22}O_4$,

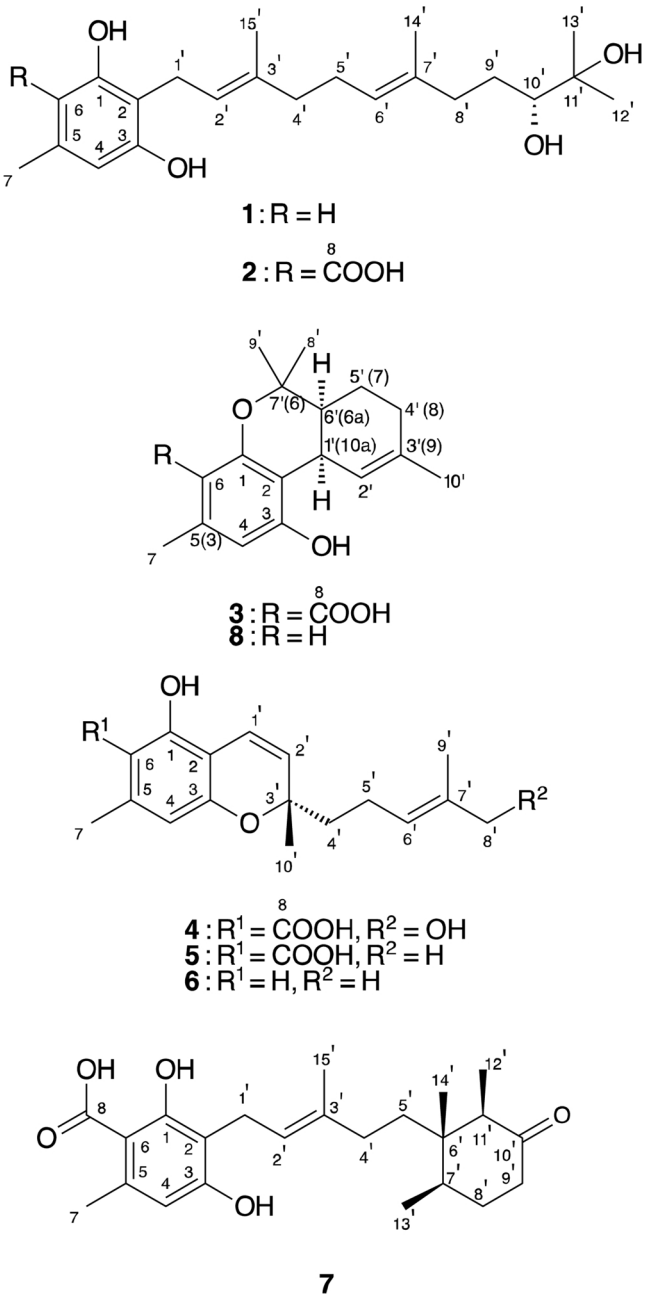

Fig. 1. Structures of compounds **1**-**8**.

Table 1
^{13}C NMR (150 MHz) spectroscopic data for compounds **1**–**4**, and **7**.

No.	**1**[1] δ_C	**2**[2] δ_C	**3**[2] δ_C	**4**[2] δ_C	**7**[2] δ_C
1	155.0 s	163.4 s	151.6 s	160.7 s	163.4 s
2	111.0 s	112.6 s	114.6 s	106.6 s	112.4 s
3	155.0 s	159.8 s	157.9 s	157.6 s	159.8 s
4	110.2 d	110.2 d	108.9 d	110.8 d	110.1 d
5	108.9 s	103.7 s	109.9 s	104.9 s	103.8 s
6	110.2 d	140.6 s	135.2 s	143.4 s	140.6 s
7	21.3 q	23.1 q	18.7 q	23.1 q	23.1 q
8		174.3 s	171.3 s	174.0 s	174.3 s
1’	22.2 t	21.4 t	31.4 d	116.6 d	21.5 t
2’	122.9 d	122.9 d	122.2 d	125.9 d	122.9 d
3’	135.2 s	133.8 s	135.2 s	79.3 s	134.2 s
4’	39.4 t	39.6 t	29.1 t	41.0 t	32.6 t
5’	25.6 t	26.3 t	20.7 t	22.0 t	35.5 t
6’	124.8 d	124.3 d	39.7 d	124.9 d	43.4 s
7’	137.4 s	134.5 s	77.2 s	134.9 s	35.7 d
8’	36.6 t	36.4 t	24.0 q	67.5 t	30.8 t
9’	29.3 t	29.4 t	24.7 q	26.0 q	41.0 t
10’	78.2 d	77.7 d	22.5 q	12.3 q	215.8 s
11’	73.6 s	72.6 s			50.1 d
12’	23.3 q	24.1 q			6.63 q
13’	26.4 q	23.8 q			14.0 q
14’	16.0 q	14.9 q			14.4 q
15’	16.0 q	15.0 q			15.1 q

[1] Recorded in $CDCl_3$.
[2] Recorded in CD_3OD.

suggesting eight degrees of unsaturation. The UV spectra, with absorption maxima at 220 and 261 nm, were indicative of a conjugated chromophore. The IR spectrum, with absorption bands at 3178 and 1700 cm^{-1}, suggested the presence of OH and carbonyl groups. The ^{1}H and ^{13}C NMR spectral data (Tables 1 and 2) revealed similarities to compounds **1**, **4** and **5**. The NMR spectral data of **3** indicated the presence of a penta-substituted benzene ring [δ_H 6.18 (s, H-4), δ_C 151.6 (C-1), 114.6 (C-2), 157.9 (C-3), 108.9 (C-4), 109.9 (C-5), and 135.2 (C-6)] (Fig. 4A). The presence of the 3′-methyl-2′-cyclohexene was supported by COSY and HMBC correlations (Fig. 4B). Substructures A and B were connected using key HMBC correlations, including H-1′ to C-2 and C-3, indicating a bond between C-2 and C-1′. The downfield chemical shift value for C-7′ supported oxygenation at this position. Therefore, the planar structure of **3** was assigned as shown in Fig. **1**. The relative configurations of C-1′ and C-6′ were assigned by analyzing ^{1}H-^{1}H coupling constants in an ^{1}H-^{1}H homonuclear decoupling experiment. Coupling constant (J = 5.0 Hz) was exhibited by H-1′ with H-6′ suggesting a *cis*–juncture of the pyran and cyclohexene rings. In addition, literature searches revealed that **3** is a new *cis* (rel-6a*S*,10a*R*) isomer of (6a*R*,10a*R*)-Δ^9-tetrahydrocannabiorcolic acid B described previously in the patent literature (Cooper and Levy, 2017). However, to our knowledge, the spectral data of (6a*R*,10a*R*)-Δ^9-tetrahydrocannabiorcolic acid B have not been reported in the primary literature.

8′-hydroxy-cannabiorcichromenic acid (**4**) was obtained as a white amorphous powder. Its molecular formula was determined by HRESITOFMS to be $C_{18}H_{22}O_5$. Comprehensive analysis of the ^{1}H and ^{13}C NMR spectroscopic data (Tables 1 and 2), as well as HSQC correlations, indicated the presence of three methyls, three methylenes, nine methines (four olefinic), and eight other carbons with no hydrogen attached (including one conjugated ketone group at δ_C 174.0). Two partial structures, H-1′/H-2′ and H-4′/H-5′/H-6′ were established by the ^{1}H–^{1}H COSY spectrum as shown in Fig. 2. A literature search showed that the NMR data of **4** was very similar to those of **5**, which was previously isolated from *Cylindrocarpon olidum* (Quaghebeur et al., 1994). The only difference between **4** and **5** was the presence of an oxymethylene signal [δ_H 3.85, δ_C 67.5] in **4** instead of a methyl group in **5**. The molecular formula of **4**, which has one more oxygen atom than that of **5**, and the HMBC correlations among the oxymethylene and C-6′and C-9′, suggesting that the hydroxyl group was attached at C-8′. The *E* stereochemistry of the trisubstitute Δ6′ double bond was determined based on the NOE correlations from H-9′ to H-5′ and from H-8′ to H-6′ (Fig. S28 and 29).

Compound **7** was obtained as a white amorphous powder. Its molecular formula was determined to be $C_{23}H_{32}O_5$ by HRESITOFMS. Compound **7** showed IR and UV spectra similar to those of **1**, indicating

Table 2
^{1}H NMR (600 MHz) spectroscopic data for compounds **1**–**4**, and **7**.

No.	**1**[1] δ_H (J in Hz)	**2**[2] δ_H (J in Hz)	**3**[2] δ_H (J in Hz)	**4**[2] δ_H (J in Hz)	**7**[2] δ_H (J in Hz)
4	6.21 s	6.17 s	6.18 s	6.14 s	6.17 s
6	6.21 s				
7	2.16 s	2.43 s	2.19 s	2.47 s	2.48 s
1’	3.35 d (6.6)	3.23 d (7.2)	3.51 m	6.66 d (10.2)	3.25 d (6.6)
2’	5.18 t (6.0)	5.18 t (6.0)	6.23 m	5.50 d (10.2)	5.25 t (6.0)
4’	2.04–2.07 m	1.91–1.94 m	1.85–1.94 m	1.64–1.73 m	1.84 td (12.6, 4.8) 1.97 td (12.6, 4.8)
5’	2.09–2.14 m	2.01–2.06 m	1.42–1.47 m 1.95–1.99 m	2.11 q (7.8)	1.33 ddd (16.2, 12.0, 4.8) 1.40 ddd (16.2, 12.0, 4.8)
6’	5.10 t (6.0)	5.12 t (6.0)	1.74 ddd (9.0, 5.0, 3.0)	5.35 t (7.2)	
7’					1.99–2.04 m
8’	2.04–2.07 m 2.17–2.20 m	1.91–1.94 m 2.16 ddd (14.4, 10.2, 4.8)	1.24 s	3.85 s	1.49–1.57 m 1.79–1.81 m
9’	1.37–1.41 m 1.56–1.61 m	1.28 m 1.63 m	1.35 s	1.58 s	2.16 ddd (13.8, 5.0, 1.2) 2.35 td (13.8, 5.0)
10’	3.37 dd (10.2, 1.2)	3.20 dd (10.2, 1.2)	1.63 s	1.35 s	
11’					2.55 q (6.0)
12’	1.14 s	1.09 s			0.85 d (6.0)
13’	1.19 s	1.16 s			0.83 d (6.0)
14’	1.57 s	1.55 s			0.50 s
15’	1.75 s	1.73 s			1.77 s

[1] Recorded in $CDCl_3$.
[2] Recorded in CD_3OD.

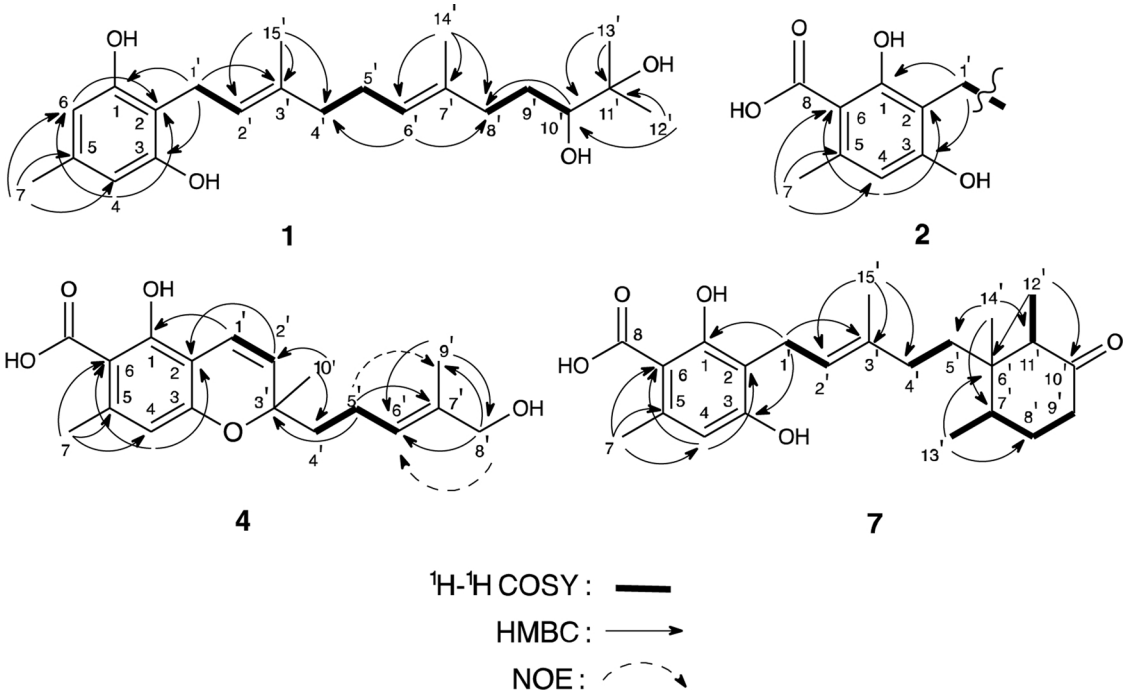

Fig. 2. Selected ^{1}H-^{1}H COSY and HMBC correlations for compounds **1**, **2**, **4** and **7** and NOEs correlations for compound **4**.

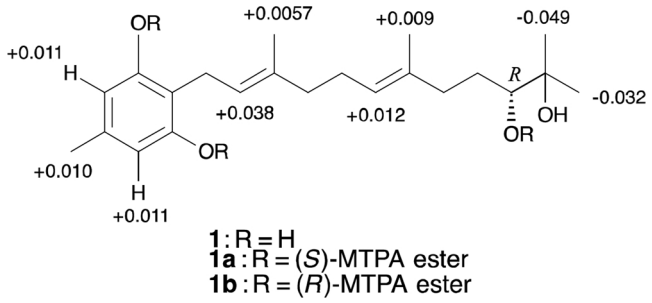

Fig. 3. Chemical shift differences for the (*S*)-(-)-MTPA ester (**1a**) and (*R*)-(+)-MTPA ester (**1b**) in ppm.

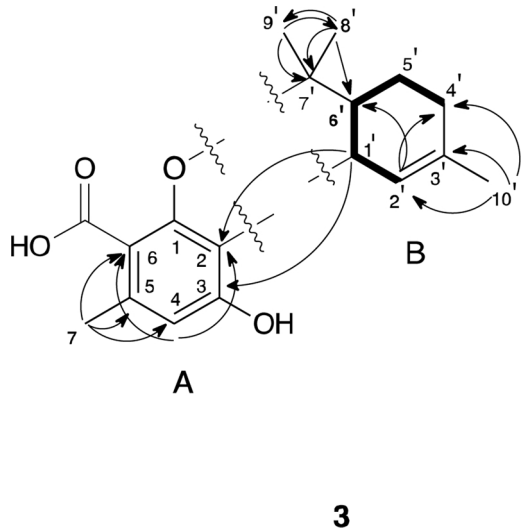

Fig. 4. Selected ^{1}H-^{1}H COSY and HMBC correlations for compound **3**.

that **7** was also a related compound of **1** and **2**. In the ^{1}H NMR spectrum (Table 1), signals of a sesquiterpene group that was present in **1** and **2** was not observed, but signals assigned to 3-methyl-2-pentene (C-1′-C-5′/C-15′) and 2,3,4-trimethyl-cyclohexan-1-one (C-6′-C-11′/C-12′/C-13′/C-14′) moieties were found. The pentene moiety was connected at C-6′ using the HMBC correlations from Me-14′ to C-5′ (Fig. 2). The relative stereochemistry of **7** was the same of that of cylindrol A (Singh et al., 1995) and nectchlorin A (Isaka et al., 2014) at all chiral centers of cyclohexanone, as determined by NOE correlations H-7′/H-11′ and, ^{13}C NMR chemical shifts. Although **7** was reported to be synthesized in a Japanese patent (Tamura et al., 2002), no spectroscopic data was available, and **7** was tentatively named 6-carboxyl-4-dechloro-ascochlorin.

Compounds **1**–**8** were tested for cytotoxicity against human promyelocytic leukemia HL60 cells. Among the compounds tested, **3** and **8** had substantial cytotoxic effects, with IC_{50} of 24.1 μM and 1.6 μM, respectively.

We next examined the biological activities of the isolates using a mutant yeast screening system to search for inhibitors of Ca^{2+} signaling (Shiono et al., 2009). Ca^{2+} signaling affects the progress of the G2/M cell cycle multilaterally in the yeast *Saccharomyces cerevisiae*. The mutant *S. cerevisiae* strain (*zds1Δ erg3Δ pdr1Δ pdr3Δ*: YNS17 strain) used in this study cannot grow at high $CaCl_2$ concentrations, as growth is arrested during the G2 phase by hyperactivation of cellular Ca^{2+} signaling. Inhibition of Ca^{2+} signal transduction can therefore detect the compounds' ability to restore cell growth, as quantified by a growth zone around a paper disc and/or spot containing the active compound (Shitamukai et al., 2000; Ogasawara et al., 2008).

The Ca^{2+} signaling pathways for growth regulation (cell cycle) are comprised of several signaling molecules, including the Ca^{2+} channel (target of anti-hypertension drugs), calcineurin (target of immunosuppressant drugs), Pkc1 protein kinase C (target of anti-cancer drugs), Mpk1 MAPK (target of anti-cancer drugs), and Mck1 GSK-3 (target of anti-diabetes and Alzheimer's disease drugs). In fact, the calcineurin inhibitor FK506 (2.5 ng/spot) had a growth zone of yeast cells in this screening system (Ogasawara et al., 2008). This screening system revealed that **5**, **6**, and **8** had dose-dependent growth-restoring activity in the mutant yeast (from 0.63 μg/spot to 0.01 μg/spot) (Fig. 5),

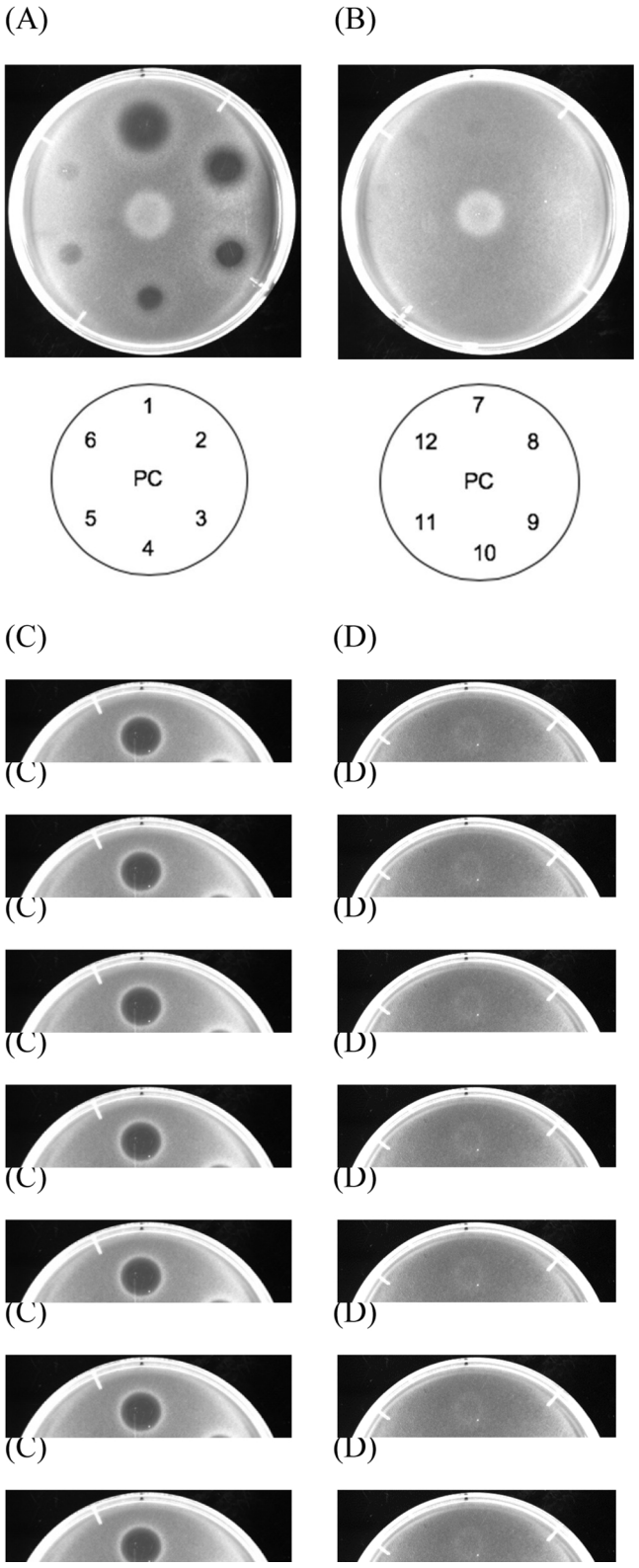

Fig. 5. Growth restored activities of **5** (A and B), **6** (C and D), and **8** (E) against *S. cerevisiae* YNS17 strain (*zds1Δ erg3Δ pdr1Δ pdr3Δ*) in the presence of 0.3 M $CaCl_2$. 1: 5.0 μg/spot, 2: 2.5, 3: 1.25, 4: 0.63, 5: 0.31, 6: 0.16, 7: 0.078, 8: 0.039, 9: 0.020, 10: 0.0098, 11: 0.0048, 12: 0.0024 (μg/spot), PC (FK506): 2.5 ng/spot.

suggesting inhibition of Ca^{2+} signal transduction. However, **1**, **2**, **3**, **4**, and **7** exhibited no activity and/or faint activity even at a dose of 5.0 μg/spot (data not shown).

In the literature, **5** and **6** were originally isolated from the culture of *Cylindrocarpon olidum,* and characterized as fungal antagonists (Quaghebeur et al., 1994; Iwata and Kitanaka, 2011). Although **6** has been reported to inhibit histamine release from mast cells (Iwata and Kitanaka, 2011), to date there has been little research regarding the biological activity of **5**, with exception to preliminary antimicrobial activity and nematodes with mixture of a chloro derivative, 8-chloro-cannabiorcichromenic acid (Quaghebeur et al., 1994). In the present assay system, clear zones were found in **5** and **6,** indicating antifungal activity against the mutant YNS17 strain as well. In addition, **3,** which has a carboxylic acid group, had weaker activity than that of **8**. It is likely that the hydrophobicity is also important for antifungal activity. Furthermore, the cytotoxicity of **3** and **8** against HL60 cells had IC_{50} values at 24.1 μM and 1.6 μM, respectively. Compound **8** was isolated for the first time from a natural origin, and to date very few reports have examined its biological activity. Although further studies of these compounds' mechanisms of action are needed, **5**, **6**, and **8** are newly identified potential Ca^{2+} signal transduction inhibitors.

3. Experimental

3.1. General methods

Column chromatography (CC): SiO_2 (200–300 mesh; *Kanto Chemical Co*., Inc. Tokyo, Japan), ODS (100 μm; *Fuji Silysia*, Aichi, Japan) and flash (25μm, SNAP Ultra, Biotage Japan, Tokyo, Japan). Semi-preparative HPLC was carried out with Shimadzu pump and UV LC-10A detector (210 nm) on Mightysil ODS column (6.0 mm i.d. x 250 mm) at the flow rate of 1.5 ml min^{-1}. Thin-layer chromatography (TLC): SiO_2 GF_{254} plates (20 × 20 cm, *Merck*, Darmstadt, Germany), and spots were detected by spraying with 10% vanillin in H_2SO_4 followed by heating, or by UV irradiation. Optical rotations: *Horiba SEPA-300* polarimeter (*HORIBA*, Kyoto, Japan). UV spectrum: *Shimadzu UV mini-1240* spectrophotometer (*SHIMADZU,* Kyoto, Japan); λ_{max} (log ε) in nm. IR spectra: *Jasco J-20A* spectrophotometer (*JASCO Co.,* Tokyo, Japan); KBr pellets; ν in cm^{-1}. Mass spectra were obtained with a Synapt G2 (*Waters Co*., Meliford, MA, USA). ^{1}H-, ^{13}C-, and 2D-NMR spectra: *Jeol ECZ-600* spectrometer at 600 MHz for ^{1}H and 125 MHz for ^{13}C (*JEOL*, Tokyo, Japan). Chemical shifts are given on a δ (ppm) scale with TMS as an internal standard.

3.2. Biological material

The fungal strains of Bm-1-1 was isolated from a surface sterilized branch of *Acanthus ebracteatus* Vahl, collected at Kapuas River in Pontianak, West Kalimantan, Indonesia (northern latitude: 0°14′22″; east longitude: 109° 9′ 54″). A voucher specimen (No. AM2017091) is deposited at Department of Food, Life, and Environmental Science, Faculty of Agriculture, Yamagata University and Department of Geography, Faculty of Mathematics and Natural Sciences, Universitas Negeri Makassar, Makassar, where Dr. Abdul Malik identified the plant material. Fungal strains Bm-1-1 was identified as *Pseudocosmospora* sp. by using a DNA analysis of the 18S rDNA region and was submitted in GenBank: LC440319 (the sequence data are available on and after 14 June 2019). This strain has been deposited at our laboratory in the Faculty of Agriculture of Yamagata.

3.3. Fermentation, extraction, and isolation

The strain was grown under static conditions at 25 °C for 30 days in 1 L Erlenmeyers containing 100 g of the steamed unpolished rice and 150 ml of water. The moldy unpolished rice (1000 g) was extracted with methanol, and the methanol extract was concentrated. The resulting aqueous concentrate was partitioned into *n*-hexane (300 ml) and EtOAc (300 ml × 3) layers. The purification from the EtOAc layer was guided by the intense blue characteristic coloration with vanillin-

sulfuric acid solution on TLC plates. The EtOAc layer (3.6 g) was chromatographed on a silica gel CC using first a stepwise of *n*-hexane/EtOAc (100:0 − 0:100, *v/v*; each 500 ml) and then a mixture of EtOAc/MeOH (50:50) as eluting solvents to give 13 fractions (Frs. 1-1 - 1-13). Frs. 1–3 and 1–4 (300 mg) were subjected to ODS CC by eluting stepwise with H_2O and an increasing ratio of MeOH to afford **5** (380 mg) and crude **6** and **8**, which were finally purified by semi-preparative HPLC eluted with H_2O/MeOH (20:80) to afford **6** (6.0 mg, t_R = 10.1 min) and **8** (10.3 mg, t_R = 9.0 min). Frs. 1–4 and 1–5 (420 mg) were chromatographed on a silica gel CC using a stepwise gradient of $CHCl_3$/EtOAc (100:0 − 0:100, *v/v*; each 300 ml) to give 12 fractions (Frs. 2-1 - 2–12). Fr. 2–4 (300 mg) was subjected to ODS CC by eluting stepwise with H_2O and an increasing ratio of MeOH (100:0 − 0:100, *v/v*; each 100 ml) to afford 12 fractions (Frs. 2–4-1 - 2–4-12). Fr. 2–4-5 (150 mg) was subject to flash silica gel CC (*n*-hexane/EtOAc, 5:1) to afford **1** (4.0 mg), **3** (25 mg) and Fr. 2–4-5-1. Fr. 2–4-5-1 was purified by semi-preparative HPLC eluted with H_2O/MeOH (40:80) to yield **4** (3.7 mg, t_R = 10.5 min). Fr. 1–7 (335 mg) was chromatographed on silica gel CC using a stepwise gradient of $CHCl_3$–EtOAc to give fractions 1–12 (Frs. 3-1 - 3–12). Frs. 3–6 and 3–7 (90 mg) were subjected to ODS CC by eluting stepwise with H_2O and an increasing ratio of MeOH to afford crude compounds **2** and **7**, which were finally purified by flash silica gel CC ($CHCl_3$/MeOH, 50:1) to afford **2** (3.0 mg) and **7** (63.0 mg).

Cosmosporin A (**1**). White amorphous powder; $[\alpha]_D^{25}$ + 10.0 (*c* 0.32, MeOH); UV (MeOH) λ_{max} (log ε) 228 (4.1), 274 (3.1), 282 (3.1); IR (KBr)ν_{max} 3300 (OH), 2930, 1600, 1234, 567 cm^{-1}; ^{13}C NMR (150 MHz, $CDCl_3$) and ^{1}H-NMR (600 MHz, $CDCl_3$) data, see Tables 1 and 2; HRESITOFMS *m/z* 385.2355 ($[M + Na]^+$, calcd for $C_{22}H_{34}NaO_4$, 385.2354)

6-Carboxyl-cosmosporin A (**2**). White amorphous powder; $[\alpha]_D^{25}$ + 6.3 (*c* 0.33, MeOH); UV (MeOH) λ_{max} (log ε) 221 (4.3), 267 (4.0), 302 (3.5); IR (KBr)ν_{max} 3310 (OH), 2929, 1620(CO), 1379, 1423, 1265, 1076, 667 cm^{-1}; see Tables 1 and 2 for ^{1}H ($CDCl_3$, 600 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz); ^{13}C NMR (150 MHz, CD_3OD) and ^{1}H-NMR (600 MHz, CD_3OD) data, see Tables 1 and 2; HRESITOFMS *m/z* 429.2251 ($[M + Na]^+$, calcd for $C_{23}H_{34}NaO_6$, 429.2252).

rel-(6a*S*,10a*R*)-Δ^9-Tetrahydrocannabiorcolic acid B (**3**). White amorphous powder; $[\alpha]_D^{25}$ −18.0 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 220 (4.1), 261 (3.5); IR (KBr)ν_{max} 3178 (OH), 2927, 1700 (CO), 1600, 1450, 1253, 1079, 848 cm^{-1}; ^{13}C NMR (150 MHz, CD_3OD) and ^{1}H NMR (600 MHz, CD_3OD) data, see Tables 1 and 2; HRESITOFMS *m/z* 303.1598 ($[M+H]^+$calcd for $C_{18}H_{23}O_4$, 303.1596).

8′-Hydroxy-cannabiorcichromenic acid (**4**). White amorphous powder; $[\alpha]_D^{25}$ + 10.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 252 (4.6), 260 (4.6); IR (KBr)ν_{max} 3317 (OH), 1650 (CO), 1519, 137, 1264, 1176, 671 cm^{-1}; ^{13}C NMR (150 MHz, CD_3OD) and ^{1}H-NMR (600 MHz, CD_3OD) data, see Tables 1 and 2; HRESITOFMS *m/z* 341.1359 ($[M + Na]^+$, calcd for $C_{18}H_{22}NaO_5$, 341.1364).

6-Carboxyl-4-dechloro-ascochlorin (**7**). White amorphous powder; $[\alpha]_D^{25}$ + 5.3 (*c* 0.32, MeOH); UV (MeOH) λ_{max} (log ε) 221 (4.4), 268 (4.0), 308 (3.5); IR (KBr)ν_{max} 3320 (OH), 2969, 1700 (CO), 1620 (CO), 1519, 1434, 1168, 671 cm^{-1}; ^{13}C NMR (150 MHz, CD_3OD) and ^{1}H-NMR (600 MHz, CD_3OD) data, see Tables 1 and 2; HRESITOFMS *m/z* 411.2146 ($[M + Na]^+$, calcd for $C_{23}H_{32}NaO_5$, 411.2147).

Cannabiorcichromenic acid (**5**). Brown amorphous powder; $[\alpha]_D^{25}$ + 10.2 (*c* 0.48, MeOH); UV (MeOH) λ_{max} (log ε): 252 (4.6), 259 (4.6); IR (KBr)ν_{max} cm^{-1}: 3300 (OH), 2970, 1650, 1457, 713; ^{13}C NMR (150 MHz, $CDCl_3$): δ 176.7 (C-8), 160.7 (C-1), 159.3 (C-3), 144.7 (C-6), 132.0 (C-7′), 126.4 (C-2′), 123.9 (C-6′), 116.7 (C-1′), 110.2 (C-4), 107.1 (C-2), 103.7 (C-5), 80.7 s (C-3′), 41.8 (C-4′), 27.3 (C-10′), 25.8 (C-8′), 24.6 (C-7), 22.7 (C-5′), 17.7 (C-9′); ^{1}H-NMR (600 MHz, $CDCl_3$): δ 6.72 (1H, d, *J* = 10.2 Hz, H-1′), 6.23 (1H, s, H-4), 5.46 (1H, d, *J* = 10.2 Hz, H-2′), 5.08 (1H, t, *J* = 6.0 Hz, H-6′), 2.53 (3H, s, H-7), 2.08 (m, H-5′), 1.65 (3H, s, H-8′), 1.64–1.76 (2H, m, H-4′), 1.57 (3H, s, H-9′), 1.40 (3H, s, H-10′); HRESITOFMS *m/z* 325.1412 ($[M + Na]^+$, calcd for $C_{18}H_{22}NaO_4$, 325.1416)

2,7-Dimethyl-2-(4-methylpent-3-enyl)-3,4-dihydrochromen-5-ol (**6**). Brown amorphous powder; $[\alpha]_D^{25}$ +28.5 (*c* 0.47, MeOH); UV (MeOH) λ_{max} (log ε): 230 (4.4); IR (KBr)ν_{max} cm^{-1}: 3600 (OH), 2969, 1600, 1542, 671; ^{13}C NMR (150 MHz, $CDCl_3$): δ 154.2 (C-3), 151.1 (C-1), 139.6 (C-1), 131.8 (C-1), 127.3 (C-1), 124.3 (C-1), 116.8 (C-1), 109.9 (C-4), 108.4 (C-1), 106.8 (C-2), 78.3 (C-1), 41.1 (C-1), 26.3 (C-1), 25.8 (C-1), 22.8 (C-1), 21.6 (C-1) 17.7 (C-1); ^{1}H-NMR (600 MHz, $CDCl_3$): δ 6.59 (1H, d, *J* = 10.8 Hz, H-1′), 6.23 (1H, s, H-4), 6.10 (1H, s, H-6), 5.47 (1H, d, *J* = 10.8 Hz, H-2′), 5.08 (1H, t, *J* = 6.6 Hz, H-6′), 2.19 (3H, s, H-7), 2.08 (m, H-5′), 1.64 (3H, s, H-8′), 1.60–1.74 (m, H-4′), 1.57 (3H, s, H-9′), 1.36 (3H, s, H-10′); HRESITOFMS *m/z* 281.1598 ($[M + Na]^+$, calcd for $C_{12}H_{22}NaO_2$, 281.1518). 3,6,6,9-Tetramethyl-6,7,8,10-tetrahydrobenzo[c]chromen-1-ol (**8**). Brown amorphous powder; $[\alpha]_D^{25}$ -4.5 (*c* 0.33, MeOH); UV (MeOH) λ_{max} (log ε): 234 (3.9); IR (KBr)ν_{max} cm^{-1}: 3420 (OH), 3270, 1600, 1519, 775; ^{13}C NMR (150 MHz, $CDCl_3$): δ155.0 (C-3), 154.0 (C-1), 137.4 (C-5), 135.1 (C-3′), 122.0 (C-2′), 110.8 (C-6), 109.4 (C-2), 108.8 (C-4), 76.3 (C-7′), 40.1 (C-6′), 31.5 (C-1′), 29.9 (C-4′), 26.1 (C-8′), 25.3 (C-9′), 23.8 (C-10′), 21.0 (C-7), 20.7 (C-5′); ^{1}H-NMR (600 MHz, $CDCl_3$): δ 6.23 (1H, s, H-6), 6.20 (1H, d, *J* = 1.2 Hz, H-2′), 6.12 (1H, s, H-4), 3.54 (1H, br.s. H-1′), 2.17 (3H, s, H-7), 1.98 (m, H-5′), 1.93 (m, H-4′), 1.70 (1H, t, *J* = 6.0 Hz, H-6′), 1.69 (3H, s, H-10′), 1.38 (3H, s, H-8′), 1.25 (H, s, H-9′); HRESITOFMS *m/z* 281.1558 ($[M + Na]^+$, calcd for $C_{12}H_{22}NaO_2$, 281.1518).

3.4. Modified Mosher's ester for *1*

To **1** (1.0 mg) in dry pyridine were added (*R*)-(-)-α-methoxy-α-trifluoromethyl phenylacetic chloride (MTPACl, 10 μL), the mixture was stirred at room temperature for 24 h. Purification by column chromatography on silica gel (*n*-hexane: EtOAc) afforded the (*S*)-(-)-MTPA ester (**1a**, 0.7 mg). Compound **1** (1.0 mg) was treated with (*S*)-(+)-α-methoxy-α-trifluoromethylphenylacetic chloride (MTPACl, 10 μL) in the same procedure to afford the (*R*)-(+)-MTPA ester (**1b**, 0.6 mg).

1a : ^{1}H (600 MHz, $CDCl_3$) δ_H 1.089 (3H, s, Me-13′), 1.134 (3H, s, Me-12′), 1.30 (3H, s, Me-14′), 1.480 (3H, s, Me-15′), 1.62 (2H, m, H-9′), 1.30 (m, H-4′, H-5′, H-8′), 2.919 (2H, m, H-1′), 3.50 (3H, s, MTPA-O*CH*$_3$), 3.53 (6H, s, MTPA- O*CH*$_3$), 4.792 (1H, t, *J* = 6.0 Hz, H-6′), 4.992 (1H, t, *J* = 6.0 Hz, H-2′), 5.082 (1H, m, H-10′), 6.836 (2H, s, H-4 and H-6), 7.44 (6H, m, MTPA-Ar*H*), 7. 64 (9H, m, MTPA-Ar*H*).

1b : ^{1}H (600 MHz, $CDCl_3$) δ_H 1.183 (3H, s, Me-12′), 1.121 (3H, s, Me-13′), 1.292 (3H, s, Me-14′), 1.423 (3H, s, Me-15′), 1.61 (2H, m, H-9′), 1.87 (m, H-4′, H-5′, H-8′), 2.906 (2H, m, H-1′), 3.50 (3H, s, OMe), 3.53 (6H, s, OMe), 4.780 (1H, t, *J* = 6.0 Hz, H-6′), 4.954 (1H, t, *J* = 6.0 Hz, H-2′), 5.10 (1H, m, H-10′), 6.824 (2H, s, H-4 and H-6), 7.53 (6H, m, MTPA-Ar*H*), 7. 74 (9H, m, MTPA-Ar*H*).

3.5. Cell culture and cytotoxicity

HL60 cells (RCB0041, RIKEN BioResource Center, Tsukuba, Japan) were grown in RPMI 1640 medium supplemented with 10% heat-inactivated FBS and penicillin (50 units/ml)-streptomycin (50 μg/ml) in a humidified atmosphere at 37 °C under 5% CO_2 for 48 h. The cytotoxicity of the compounds was examined by MTT assay, as described previously (Arayama et al., 2016).

3.6. Growth restored activity of samples against YNS17 strain

Screening was performed according to previous described method (Ogasawara et al., 2008). Each sample was dissolved in MeOH and two-fold dilutions of them were used. Difco® yeast-peptone-dextrose (YPD) broth and YPD agar were purchased from Becton Dickinson Biosciences (Franklin Lakes, NJ, USA). The mutant yeast, YNS17 (*MAT*a *zds1::TRP1 erg3::HIS3 pdr1::hisG-URA3-hisG pdr3::hisG*) yeast strains was derivatives of strain W303-1A (Ogasawara et al., 2008). A 5 μl aliquot of samples was spotted on a YPD agar medium containing YNS17 strain

and 0.3 M $CaCl_2$. After 3 days of incubation at 28 °C, the intensity of the growth spot was observed as the result of inhibition of Ca^{2+}-signal transduction. FK506 (2.5 ng/spot) was used as a positive control. FK506 was kindly provided by Fujisawa Pharmaceutical Co., Ltd. (the present Astellas Pharma Inc., Tokyo Japan).

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.phytol.2019.03.014.

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