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10-Phenyl-[11]-cytochalasans from Indonesian mushroom *Microporellus subsessilis*

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Abstract

10-Phenyl-[11]-cytochalasans (**4**–**6**), together with three known derivatives (**1**–**3**), were isolated from the MeOH extract of the Indonesian mushroom *Microporellus subsessilis* by a bioassay-guided fractionation. The compounds **6** and **1**–**3** induced immotility in *Artemia salina*.

Keywords: Mushroom; *Microporellus subsessilis*; *Artemia salina*; Immotility; Cytochalasin; Conjugated fatty acid

1. Introduction

In tropical forests, the fungi are a major component of biodiversity; for example, they are essential to the survival of other organisms and are a source of bioactive compounds. Many mushroom species have thus been evaluated with respect to the quality and quantity of their bioactive compounds for potential application as antibiotics, anticancer drugs, growth promoters, and immunosuppressants to treat human diseases. Their products are found in some of the most important commercial compounds (Hawksworth, 2002; Bentley and Bennett, 1988). In the course of our investigations for new bioactive compounds from Indonesian mushrooms, the MeOH extract of *Microporellus subsessilis* was found to induce immotility in *Artemia salina*. A bioactivity-guided fractionation of this extract revealed six members of the cytochalasin family, including three new 10-phenyl-[11]-cytochalasans (**4**–**6**) together with three known derivatives (**1**–**3**) (Fig. 1). In this paper, we report the isolation, structural elucidation and biological activities of the new compounds **4**–**6**.

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2. Results and discussion

2.1. Isolation and structural elucidation of compounds 4–6

The freshly collected *Microporellus subsessilis* was extracted with methanol, and metabolite isolation was guided by motile activity using a brine shrimp (*Artemia salina*) assay. Compounds **1**–**6** were purified by silica gel chromatography of the crude extract using a gradient elution with *n*-hexane–EtOAc–MeOH, followed by further column chromatography and HPLC on both normal- and reversed-phase silica gel. Compounds **1**–**3** were identified as 10-phenyl-[11]-cytochalasan derivatives isolated from *Daldinia* fungal species by comparing their physicochemical and spectroscopic data with those previously reported (Buchanan et al., 1995, 1996).

Compound **4** was isolated as needles from methanol. The molecular formula of compound **4** was established to be $C_{29}H_{39}NO_5$, by FAB-HRMS data (*m*/*z* 482.2904 $[M+H]^+$) together with analysis of the ^1H and ^{13}C NMR spectroscopic data. This is the same molecular formula for compound **3**, thus requiring 11 degrees of unsaturation. The occurrence of two major fragments at *m*/*z* 464 $[M-18]^+$ and 414 $[M-36]^+$ in the MS indicated the loss

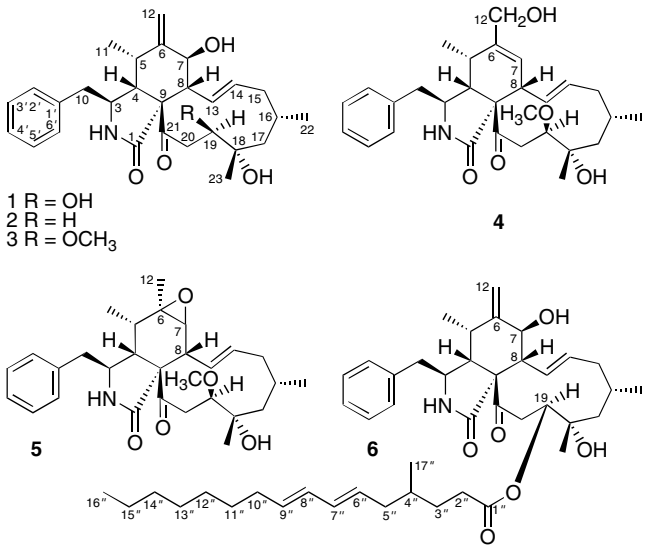

Fig. 1. Structures of compounds **1**–**6**.

of one and two H_2O molecules, respectively. The fragment at *m*/*z* 432 $[M-31]^+$ indicated the loss of a methoxyl from the molecular ion, and the fragment at *m*/*z* 91 suggested the presence of a benzyl group. The ^1H NMR spectrum (Table 1) of **4** was very similar to that of 10-phenyl-[11]-cytochalasin **3**. The difference was the appearance of an olefinic proton H-7 [δ_H 5.76 (1H, *m*); δ_C 128.5], and an hydroxymethyl group at C-12 [δ_H 4.09 (2H, *s*); δ_C 63.3] in compound **4**, instead of the presence of a C-12 exomethylene and C-7 secondary hydroxyl functionalities, respectively. This difference strongly suggested that a double bond was newly introduced between C-6 and C-7, and that a hydroxymethyl moiety was located at C-6. These were confirmed by the correlations between H-7 and H-8 in the ^1H–^1H COSY spectrum of **4**, and between the hydroxymethyl protons H_2-12 (δ_H 4.09) and the methine carbon C-5 (δc 34.6), as well as between the hydroxymethyl protons and olefinic carbons C-6 (δc 142.6) and C-7 (δc 128.5) in the HMBC spectrum. The relative stereochemistry of **4** was established by analysis of the NOESY spectrum. Significant NOEs were observed as follows: H-3/H-11; H-4/H-5 and H-11; H-5/H-8; H-7/H-13; H-8/H-14; H-13/H-15; H-14/H-16; and H-22/H-23. Consequently, the structure of **4** was determined to be (16*S**,18*S**,19*R**)-12,18-dihydroxy-19-methoxy-16,18-dimethyl-10-phenyl-[11]-cytochalasa-6(7),13(*E*)-diene-1,21-dione.

The molecular formula of compound **5** was established to be $C_{29}H_{39}NO_5$ by FAB-HRMS data (*m*/*z* 482.2886 $[M+H]^+$) together with analysis of the ^1H and ^{13}C NMR spectroscopic data, indicating that the molecular formula of **5** was the same as those of **3** and **4**. Detailed assignments of the ^1H and ^{13}C NMR spectroscopic data of **5** showed the correct assignment for the cytochalasin skeleton of **4** with different points, which were explained by the appearance of new signals of an oxygenated quaternary carbon C-6 [δ_C 57.3], a tertiary methyl group (H_3-12)[δ_C 19.4; δ_H 1.21, 3H, *s*] and an oxygenated methine carbon C-7 [δ_C 61.5; δ_H 2.96, 1H, *d*, 6.0] in **5**. These data indicated that the six-membered ring of **4** was modified by formation of an epoxide group at C-6 and C-7. This linkage was supported by the fact that the methyl protons H_3-12 (δ_H 1.21) showed correlations with C-5 (δc 36.5) and C-7 (δc 61.5) in the HMBC spectrum of **5**. The relative stereochemistry of **5** was established by analysis of the NOESY spectrum. Significant NOEs were observed as follows: H-3/H-11 and H-12; H-4/H-5 and H-11; H-5/H-11; H-7/H_3-12 and H-13; and H-8/H-14. Thus compound **5** was determined to be a new member of the cytochalasin family, (6*R**,7*S**,16*S**,18*S**,19*R**)-6,7-epoxy-18-hydroxy-19-methoxy-16,18-dimethyl-10-phenyl-[11]-cytochalasa-13(*E*)-ene-1,21- dione.

Compound **6** was isolated as an amorphous solid. Its molecular formula was established to be $C_{45}H_{65}NO_6$ by FAB-HRMS data (at *m*/*z* 716.4900 $[M+H]^+$) together with analysis of the ^1H and ^{13}C NMR spectroscopic data, thus requiring 14 degrees of unsaturation. The NMR spectroscopic data of **6** were very similar to those of **1**, with the

Table 1
^1H and ^{13}C NMR spectroscopic data for compound **4**[a]

Position	δ_C	δ_H (integral, mult., *J* = Hz)
1	174.6	–
2	–	5.69 (1H, *br s*)
3	54.3	3.48 (1H, *m*)
4	49.3	2.84 (1H, *dd*, 2.4, 3.6)
5	34.6	2.57(1H, *m*)
6	142.6	–
7	128.5	5.76 (1H, *m*)
8	50.1	2.80 (1H, *m*)
9	68.5	–
10	44.6	2.73 (1H, *dd*, 13.6, 5.2) 2.46 (1H, *dd*, 13.6, 5.2)
11	12.3	1.04 (3H, *d*, 5.6)
12	63.3	4.09 (2H, *s*)
13	129.4	6.12 (1H, *dd*, 15.2, 10.0)
14	133.1	5.15 (1H, *ddd*, 15.2, 11.0, 4.4)
15	42.4	1.72 (1H, *dd*, 12.6, 4.4) 2.05 (1H, *m*)
16	29.5	1.28 (1H, *m*)
17	43.9	1.28 (1H, *m*) 1.82 (1H, *m*)
18	75.4	–
19	79.8	3.30 (1H, *d*, 5.6)
20	43.4	4.27 (1H, *d*, 19.6) 1.73 (1H, *m*)
21	211.8	–
22	25.1	1.24 (3H, *d*, 7.2)
23	23.8	1.28 (3H, *s*)
1′	136.4	–
2′,6′	129.4	7.08 (1H, *d*, 6.8)
3′,5′	128.7	7.27 (1H, *dd*, 7.2, 6.8)
4′	127.0	7.25 (1H, *t*, 7.2)
–OCH_3	58.8	3.50 (3H, *s*)

[a] Taken in $CDCl_3$ at 400 MHz for ^1H and at 100 MHz for ^{13}C.

difference point being a down-field shift of H-19 and newly observed signals comprising two methyls, two double bonds, nine methylenes, one methine and one ester carbonyl, respectively. These data strongly suggested that the hydroxyl at C-19 was acylated with a C_{17} fatty acid containing two double bonds and a branched methyl. To determine the structure of the fatty acid moiety of **6**, methanolysis of **6** was carried out and the resulting materials were analyzed on TLC and purified using prep-HPLC. A spot identical to **1** was observed on silica gel TLC, strongly indicating that **6** was an ester consisting of **1** and a fatty acid. GC–MS analysis of the methanolysis product revealed a single peak of the methyl ester at R_t 11.28 min, with a molecular ion peak at m/z 280 $[M]^+$. In the FAB-MS data of **6**, the occurrence of the fragments at m/z 466 $[M-250]^+$ and 450 $[M-266]^+$ indicated the loss of a fatty acid moiety from the molecular ion. Because the amount of the fatty acid moiety resulting from hydrolysis of **6** was limited, we tried to isolate the identical fatty acid from an *n*-hexane extract. The fatty acid isolated from the *n*-hexane layer was converted to its fatty acid methyl ester (FAME) derivative, and GC–MS and NMR spectroscopic analyses showed that the resulting FAME was identical to that generated from **6**.

The FAME was isolated as colorless oil. The molecular formula was established as $C_{18}H_{32}O_2$ by HR-EIMS data (m/z 280.2409 $[M]^+$) together with analysis of the ^1H and ^{13}C NMR spectroscopic data (Table 2). The position of the branched methyl was first determined as follows. Significant fragment peaks at m/z 115 $[M-169]^+$ and m/z 87 $[M-193]^+$, arising from a cleavage between C-4″ and C-5″ and a cleavage between C-3″ and C-4″, respectively, indicated that the position of the branched methyl was C-4″. This position was also confirmed by analysis of the MS of the saturated FAME (Appelqvist, 1972). Catalytic hydrogenation of FAME yielded the saturated FAME, which showed a molecular ion peak at m/z 284 $[M]^+$ and the same significant fragments at m/z 115 $[M-169]^+$ and m/z 87 $[M-193]^+$ corresponding to the signals for the branched methyl. The ^1H–^1H COSY spectrum of FAME suggested that the two double bonds were located at C-6″–C-7″ and C-8″–C-9″. The positions of these conjugated double bonds were confirmed by GC–MS analyses of the Diels–Alder adduct with 4-methyl-1,2,4-triazoline-3,5-dion (MTAD) (Dobson, 1998). The fragment peaks at m/z 294 $[M-99]^+$ and m/z 244 $[M-129]^+$ were derived from cleavage of two sides of the six-membered ring. The geometric configurations around the two double bonds of FAME were determined to be *E*/*E* by comparing the chemical shifts of H-6″, H-7″, H-8″ and H-9″ in the ^1H NMR spectrum with those of the four geometric isomers of conjugated linoleic acids. The chemical shifts of H-6″ (δ_H 5.59, δ_C 129.7), H-7″ (δ_H 5.99, δ_C 120.0), H-8″ (δ_H 5.99, δ_C 131.8) and H-9″ (δ_H 5.59, δ_C 132.7) agreed more closely with those of the *E*/*E* isomer than the other three isomers (Lie Ken Jie et al., 1997; Ogawa et al., 2001). All the spectroscopic data of the FAME indicated that the fatty acid moiety in **6** was a novel fatty acid, 4-methyl-6(*E*),8(*E*)-hexadecadienoic acid. The absolute configuration of methyl at C-4″ remains unsolved. Consequently, the structure of **6** was determined to be (7*S**,16*S**,18*S**,19*R**)-7,18-dihydroxy-19-*O*-(4-methyl-6(*E*),8(*E*)-hexadecadienoyl)-16,18-dimethyl-10-phenyl-[11]-cytochalasa-6(12),13(*E*)-diene-1,2-dione. To our knowledge, **6** is the first member of the cytochalasin family found to contain a long-chain fatty acid moiety.

Table 2
^1H and ^{13}C NMR spectroscopic data for FAME of compound **6**[a]

Positions	δ_C	δ_H (integral, mult., J = Hz)
1″	174.2	–
2″	31.9	1.33 (1H, *m*)[b] 2.33 (1H, *m*)[c]
3″	31.8	1.34 (1H, *m*)[b] 2.34 (1H, *m*)[c]
4″	32.6	1.52 (1H, *m*)
5″	39.8	1.97 (1H, *dt*, 7.2, 6.8) 2.15 (1H, *dt*, 7.2, 6.8)
6″	129.7	5.59 (1H, *m*)
7″	129.9	5.99 (1H, *m*)
8″	131.8	5.99 (1H, *m*)
9″	132.7	5.59 (1H, *m*)
10″	32.9	2.02 (1H, *m*) 1.33 (1H, *m*)[b]
11″	29.2	1.44 (2H, *m*)
12″	31.4	1.52 (1H, *m*) 1.76 (1H, *m*)
13″	29.5	1.33 (2H, *m*)[b]
14″	29.1	1.33 (2H, *m*)[b]
15″	22.6	1.33 (2H, *m*)[b]
16″	14.1	0.82 (3H, *t*, 6.8)
17″	19.1	0.95 (3H, *d*, 6.4)
–OCH_3	51.4	3.73 (3H, *s*)

[a] Taken in $CDCl_3$ at 400 MHz for ^1H and at 100 MHz for ^{13}C.
[b,c] Assignments may be interchanged.

2.2. Immotile activity of 1–6

The immotile activity of compounds **1**–**6** was evaluated in brine shrimp by counting the shrimp that showed immotility 24 h after administration. Compounds **1**, **2**, **3** and **6** showed immotility rates of 72%, 78%, 64% and 63% in brine shrimp at 10 ppm, respectively, while **4** and **5** showed no immotility. This indicated that the hydroxyl group at C-7 and the exomethylene at C-6 in the active compounds **1**–**3** and **6** were essential components to exhibiting immotility. The effect of fatty acid moiety as well as the substituents at C-19 were not significant because the activities of compound **1**–**3** and **6** were almost equal. These results were in good agreement with structure activity relationship studies of cytochalasins which showed the important role of the hydroxyl group at C-7 in exhibiting biological activity (Evidente et al., 2002; Burres et al., 1991; Capasso et al., 1991; Hirose et al., 1990).

2.3. Conclusions

Three new 10-phenyl-[11]-cytochalasans (**4–6**), together with three known derivatives (**1–3**), were isolated from the mushroom of *Microporellus subsessilis*, and their structures were determined by spectroscopic analysis. Cytochalasins exhibit a broad spectrum of activity, including antibiotic and antitumor activity (Beno et al., 1977; Thilly and Wogan, 1978; Natori and Yahara, 1991; Carter, 1967), inhibition of HIV-1 protease (Betina, 1989), and phytotoxic activity (Katagiri and Matsuura, 1971), but their most important activities include various effects on mammalian cells (Lingham et al., 1992; Well et al., 1976). The toxic effects of cytochalasins against brine shrimp (*Artemia salina*) has already been reported (Dagne et al., 1994; Evidente et al., 2003), but the present report is the first to find immotile activity in brine shrimp.

3. Experimental

3.1. General experimental procedures

Melting points were measured on a Yamato micro-melting-point apparatus and were uncorrected. Optical rotations were measured on a Horiba model SEPA-300 polarimeter. UV spectra were recorded on a Hitachi model U-3210 spectrophotometer, whereas IR spectra were obtained using a JASCO FT/IR-460plus spectrometer. Mass spectra were recorded on a JMS-700 mass spectrometer with glycerol as a matrix, along with a Kratos PC-Kompact MALDI instrument, whereas ^{1}H and ^{13}C NMR spectra were obtained with a JEOL JNM A-400 spectrometer. Chemical shifts are given in a δ (ppm) scale with TMS as an internal standard. Column chromatography (CC) separations were carried out on Wakogel C-200 (Wako Pure Chemical Industries), silica gel G-60 (Merck; 70–200 and 230–400 mesh) and Chromatorex ODS (Fuji Silysia, Japan). Analytical and preparative HPLC were carried out on Inertsil ODS-3 HPLC columns ($\varnothing$ 4.6 × 250 mm and $\varnothing$ 10 × 250 mm, GL Sciences Inc. Japan). GC was carried out on an FFAP column ($\varnothing$ 0.25 mm × 50 m, GL Sciences Inc. Japan). TLC analysis was performed on precoated silica gel 60 F_{254} plates and RP-18 F_{254S} (Merck; 0.2 mm), and spots were visualized by UV absorption at 254 nm and fluorescence at 365 nm, or by spraying with 10% H_2SO_4 in ethanol followed by heating.

3.2. Mushroom material

Microporellus subsessilis mushrooms were collected on Tangkuban Parahu mountain in the Regency of Bandung District and on Sunda mountain in the Regency of Subang District, West Java, Indonesia, in June 2002. The mushroom species was identified by staff members of the laboratory of Plant Taxonomy at Herbarium Bogorienesess, Bogor, Indonesia. A voucher of a specimen was deposited at the Herbarium of the Bandung Institute of Technology, Bandung, Indonesia.

3.3. Isolation of compounds 1–6

The fresh body of mushroom *Microporellus subsessilis* (15 kg) was extracted with MeOH (30 L). The MeOH extract (26.8 g) was partitioned between EtOAc and H_2O to afford an active EtOAc extract (619 mg), and this extract was subjected to Wakogel C-200 (*n*-hexane–EtOAc–MeOH by 10% stepwise) chromatography to obtain seven active fractions **8–15** (80–100% EtOAc eluates and 10–40% MeOH eluates). The active fractions were combined and re-applied to the Wakogel C-200 (clusted with *n*-hexane–Me_2CO, 10% stepwise) to yield two active fractions **I** (40–60% Me_2CO eluates) and **II** (70–90% Me_2CO eluates). The active fraction **I** was then applied to an ODS eluted with MeOH–H_2O with increasing ratios of MeOH in 5% steps, and active fractions were sequentially subjected to silica gel G-60 CC, eluted with *n*-hexane–Me_2CO with 5% stepwise increasing ratios of Me_2CO followed by ODS chromatography eluted with MeOH–H_2O (65:35, v/v) to yield an active compound **1** (15 mg). Active fraction **II** was applied to an ODS column, eluted with MeOH–H_2O with increasing ratios of MeOH in 5% steps to yield three active fractions, **II.a** (MeOH–H_2O (7:3), eluate), **II.b** (75:25–80:20 MeOH–H_2O eluates) and **II.e** (MeOH eluate) together with two inactive fractions, **II.c** (MeOH–H_2O (85:15, v/v) eluate) and **II.d** MeOH–H_2O (90:10–95:5, v/v) eluates. Further purification of active fraction **II.a** by chromatography on silica gel G-60 eluted with $CHCl_3$–Me_2CO (85:15), and on ODS eluted with MeOH–H_2O (60% MeOH), yielded an active compound **2** (5 mg). The active fraction **II.b** was applied to an ODS column, eluted with MeOH–H_2O increasing ratios of MeOH in 5% steps, followed by chromatography on ODS eluted with MeOH–H_2O (MeOH–H_2O (7:3, v/v)) to yield active compound **3** (14 mg). Inactive fraction **II.c** was further purified by chromatography on silica gel G-60 eluted with *n*-hexane–Me_2CO in 5% steps as well as on ODS eluted with MeOH–H_2O in 5% steps, and then finally by prep-HPLC using Inertsil ODS-3 ($\varnothing$ 10 × 250 mm; solvent: MeOH–H_2O (4:1); 3.2 ml/min; A_{254} nm), to yield an inactive compound **4** (4 mg) at R_t 17.8 min. Repeated purification of inactive fraction **II.d** by sequential chromatography was next carried out as follows: silica gel G-60 eluted with *n*-hexane–Me_2CO (80:20); Chromatorex ODS eluted with MeOH–H_2O in 5% steps; silica gel G-60 eluted with *n*-hexane–Me_2CO (80:20) and then finally by prep-HPLC using Inertsil ODS-3 ($\varnothing$ 10 × 250 mm; solvent: MeOH–H_2O (95:5); 3.2 ml/min; A_{254} nm), to yield an inactive compound **5** (4 mg) at R_t 21.12 min. Further purification of active fraction **II.e** by chromatography on silica gel G-60 eluted with *n*-hexane–Me_2CO in 10% steps, on ODS eluted with MeOH–H_2O in 5% steps, on silica gel G-60 eluted with *n*-hexane–Me_2CO in 10% steps and finally by prep-HPLC using Inertsil ODS-3 ($\varnothing$ 10 × 250 mm; solvent MeOH–

H_2O (95:5); 3.2 ml/min; A_{254} nm), gave an active compound **6** (4.9 mg) at R_t 14.58 min.

3.4. Isolation and esterification of fatty acid from n-hexane extract

The *n*-hexane extract (221.9 mg) was subjected to Wako-gel C-200 (*n*-hexane–EtOAc by 1% stepwise) chromatography to obtain seven fractions containing the fatty acid. Fractions (5%, 6% EtOAc eluates) were combined and applied to a silica gel G-60 column eluted with *n*-hexane–EtOAc (100 ml:20 μl). Fractions **126–166** were combined and subjected to esterification by heating at 90 °C for 1 h in a mixture solution (*n*-hexane–H_2O–methanolic-HCl (0.6N); 0.5:1:1) to yield a mixture of fatty acid methyl esters (FAMEs). For further purification, FAMEs were subjected to ODS chromatography eluted with MeOH–H_2O (93% MeOH) and finally on prep-HPLC using Inertsil ODS-3 (∅ 10 × 250 mm; solvent MeOH–H_2O (95:5); 3.2 ml/min; A_{254} nm) to yield a FAME identical to that derived from compound **6** (3.8 mg) at R_t 22.35 min. The FAME product was purified by prep-HPLC using Inertsil ODS-3 (∅ 10 × 250 mm; solvent: MeOH–H_2O (95:5); 3.2 ml/min; A_{254} nm) and then was analyzed on gas chromatography fitted with a FFAP capillary column (∅ 0.32 mm × 50 m). The chromatograph was programmed for an injection temperature of 200 °C, a column temperature of 250 °C and a FID detector temperature of 250 °C. The final temperature was maintained for 10 min.

3.5. Hydrogenation of fatty acid methyl ester (FAME)

FAME (1–2 mg) in a test tube was dissolved in 1 ml of MeOH, to which 1 mg of Adam's catalyst (platinum oxide) was added. The tube was connected via a two-way tap, with both ways leading to a reservoir of hydrogen at just above atmospheric pressure. The tube was alternately evacuated and flushed with hydrogen several times to remove any air, and then it was shaken vigorously while an atmosphere of hydrogen at a slight positive pressure was maintained. After 2 h, the hydrogen supply was disconnected, the tube was flushed with nitrogen gas and the solution was filtered to remove the catalyst. The solvent was evaporated under reduced pressure. The resulting saturated ester was taken up in *n*-hexane and was then analyzed using gas chromatography.

3.6. Diels–Alder adduct for conjugated double bonds

The fatty acid methyl ester (FAME) (220 μg; 1.15 mM) and MTAD (425 μg; 5.8 mM) in CH_2Cl_2 (650 μl) were mixed in a test tube at 0 °C by agitation for less than 10 s. The reaction was immediately stopped by the addition of 1,3-hexadiene, followed by agitation for a few seconds. Excess reagents were removed with a stream of nitrogen at 30 °C, and the sample was redissolved in CH_2Cl_2 for analysis by GC–MS.

3.7. (16S*,18S*,19R*)-12,18-dihydroxy-19-methoxy-16,18-dimethyl-10-phenyl-[11]-cytochalasa-6(7),13(E)-diene-1,21-dione (**4**)

Colorless needles (MeOH); mp 136–138 °C; $[\alpha]_D^{28}$ − 102 ($CHCl_3$, *c* 0.00031); UV λ_{max}^{MeOH} nm (log ε) end absorption; IR ν_{max}^{KBr} cm^{-1}: 3392, 2924, 2853, 2686; ^1H NMR and ^{13}C NMR spectra (Table 1); FAB-HRMS (found *m/z* 482.2904 $[M+H]^+$) (calcd. for $C_{28}H_{35}O_3N$ 482.2906). FAB-MS *m/z* (rel. int.): 482 (26), 464 (28), 432 (7), 414 (5), 91(49).

3.8. (6R*,7S*,16S*,18S*,19R*)-6,7-epoxy-18-hydroxy-19-methoxy-16,18-dimethyl-10-phenyl-[11]-cytochalasa-13(E)-ene-1,21-dion (**5**)

An amorphous solid (MeOH); $[\alpha]_D^{20}$ − 115 ($CHCl_3$, *c* 0.0007,); UV λ_{max}^{MeOH} nm (ε) end absorption; IR ν_{max}^{KBr} cm^{-1}: 3381, 2927, 2852, 1684, 1105, 1086; ^1H NMR ($CDCl_3$): δ 5.69 (1H, *br s*, NH-2), 7.08 (2H, *d*, *J* = 6.8 Hz, H-2′ and H-6′), 7.27 (2H, *dd*, *J* = 7.6, 6.8 Hz, 3H′ and H-5′), 7.25 (1H, *t*, *J* = 7.6 Hz, H-4′), 6.12 (1H, *dd*, *J* = 15.6, 9.8 Hz, H-13), 5.15 (1H, *ddd*, *J* = 15.6, 11.4, 4.8 Hz, H-14), 1.04, (3H, *d*, *J* = 7.2 Hz, H-11), 1.10 (3H, *d*, *J* = 6.4 Hz, H-22), 1.28 (3H, *s*, H-23), 3.62 (1H, *t*, *J* = 6.4 Hz, H-3), 2.84 (1H, *d*, *J* = 6.4, Hz, H-4), 1.85 (1H, *m*, H-5), 2.96 (1H, *d*, *J* = 6.0 Hz, H-7), 2.56 (1H, *dd*, *J* = 9.8, 6.0 Hz, H-8), 1.18 (1H, *m*, H-16), 3.30 (1H, *d*, *J* = 4.4 Hz, H-19), 2.62 (2H, *dd*, *J* = 13.6, 7.6 Hz, H-10), 1.21 (3H, *s*, H-12), 1.75 (1H, *dd*, *J* = 13.2, 4.8 Hz, H_a-15) and 1.85 (1H, *m*, H_b-15), 1.19 (1H, *m*, H_a-17) and 1.81 (1H, *m*, H_b-17), 1.73 (1H, *m*, H_a-20) and 4.27, (1H, *d*, *J* = 18.8 Hz, H_b-20), 3.50 (3H, *s*, –OCH_3). ^{13}C NMR ($CDCl_3$): δ 174.5 (C-1), 53.3 (C-3), 48.8 (C-4), 36.5 (C-5), 57.3 (C-6), 61.5 (C-7), 49.7 (C-8), 65.3 (C-9), 44.8 (C-10), 12.5 (C-11), 19.4 (C-12), 128.3 (C-13), 133.9 (C-14), 42.4 (C-15), 29.3 (C-16), 43.9 (C-17), 76.2 (C-18), 79.6 (C-19), 43.7 (C-20), 210.6 (C-21), 24.9 (C-22), 23.9 (C-23), 136.2 (C-1′), 129.2 (C-2′ and C-6′), 128.7 (C-3′ and C-5′), 127.1 (C-4′), 58.8 (–OCH_3); FAB-HRMS found *m/z* 482.2886 $[M+H]^+$ (calcd. for $C_{28}H_{35}O_3N$ 482.2906). FAB-MS *m/z* (rel. int.): 482 (43), 464 (100), 432 (82), 414 (10), 91(85).

3.9. (7S*,16S*,18S*,19R*)-7,18-dihydroxy-19-O-(4-methyl-6(E),8(E)-hexadecadienoyl)-16,18-dimethyl-10-phenyl-[11]-cytochalasa-6(12),13(E)-diene-1,21-dione (**6**)

An amorphous solid (MeOH); $[\alpha]_D^{28}$ − 22 ($CHCl_3$, *c* 0.0024); UV λ_{max}^{MeOH} nm (log ε): 232 (55.409); IR ν_{max}^{KBr} cm^{-1}: 3384, 2956, 2925, 2854, 2360, 2342, 1733, 1686; ^1H NMR ($CDCl_3$): δ 5.52 (1H, *m*, NH-2), 7.08 (2H, *d*, *J* = 6.8 Hz, H-2′ and H-6′), 7.27 (2H, *t*, *J* = 6.8 Hz, 3H′ and H-5′), 7.25 (1H, *t*, *J* = 7.6 Hz, H-4′), 6.14 (1H, *dd*, *J* = 15.6, 10.0 Hz, H-13), 5.3 (1H, *ddd*, *J* = 15.6, 11.0, 4.8 Hz, H-14), 13.0, (3H, *d*, *J* = 6.8 Hz, H-11), 1.42 (3H, *d*, *J* = 6.4 Hz, H-22), 1.11 (3H, *s*, H-23), 3.31 (1H, *m*, H-3), 2.94 (1H, *dd*, *J* = 2.4, 3.2 Hz, H-4), 2.85 (1H, *m*, H-5),

4.13 (1H, *d*, $J = 10.0$ Hz, H-7), 2.51 (1H, *t*, $J = 10.0$ Hz, H-8), 1.73 (1H, *m*, H-16), 5.23 (1H, *d*, $J = 8.0$ Hz, H-19), 2.72 (1H, *dd*, $J = 4.8$, 8.8 Hz, H_a-10) and 2.5 (1H, *m*, H_b-10), 5.33 (1H, *s*, H_a-12) and 5.1 (1H, *s*, H_b-12), 1.37 (1H, *m*, H_a-15) and 2.0 (1H, *dd*, J= 4.8, 13.2 Hz, H_b-15), 1.81 (1H, *m*, H_a-17) and 2.16 (1H, *d*, $J = 13.6$ Hz, H_b-17), 1.93 (1H, *m*, H_a-20) and 4.2 (1H, *d*, $J = 18.8$ Hz, H_b-20). ^{13}C NMR ($CDCl_3$): δ 173.3 (C-1), 52.6 (C-3), 4.0 (C-4), 32.2 (C-5), 148.8 (C-6), 71.3 (C-7), 51.6 (C-8), 63.7 (C-9), 43.9 (C-10), 13.0 (C-11), 114.3 (C-12), 128.8 (C-13), 135.7 (C-14), 45.6 (C-15), 29.4 (C-16), 42.8 (C-17), 75.4 (C-18), 72.6 (C-19), 39.2 (C-20), 208.6 (C-21), 25.2 (C-22), 22.6 (C-23), 136.3 (C-1′), 129.3 (C-2′ and C-6′), 128.7 (C-3′ and C-5′), 126.9 (C-4′); FAB-HRMS found *m/z* 716.4900 $[M+H]^+$ (calcd. for $C_{28}H_{35}O_3N$ 716.4890); FAB-MS *m/z* (rel. int.): 716 (8), 698 (8), 446 (5), 450 (15), 432 (38), 414 (10), 91 (24).

3.10. Fatty acid methyl ester (FAME): 4-methyl-6(E),8(E)-hexadecadienoate

A colorless oil (*n*-hexane); $[\alpha]_D^{20} - 5.9$ ($CHCl_3$, *c* 0.00072); UV λ_{max}^{MeOH} nm ($\log\varepsilon$): 219 (16.646); IR ν_{max}^{KBr} cm^{-1}: 2926, 2855, 1742, 988 and 986; ^{1}H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) spectra (Table 2); HREIMS found *m/z* 280.2409 $[M]^+$ (calcd. 280.2402 for $C_{18}H_{32}O_2$); IE-MS *m/z* (rel. int.): 280 (20), 249 (15), 206 (16), 193 (23), 165 (10), 152 (14), 150 (13), 115 (30), 109 (22), 95 (29), 87 (3), 81 (33), 67 (48), 55 (27).

3.11. Bioassay

The eggs of brine shrimp, *Artemia salina*, were hatched in a beaker filled with artificial seawater according to the partially modified Meyer method (Meyer et al., 1982). Into a sample tube, 100 μl of a methanol extract or varying amounts of the sample to be tested were added. After the solvent was removed, 2 ml of seawater containing nauplii was poured into the sample tube. At each sample, 20–30 nauplii were treated at each dosage, and the nauplii that showed immotility were counted every hour for 24 h after the administration began.

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