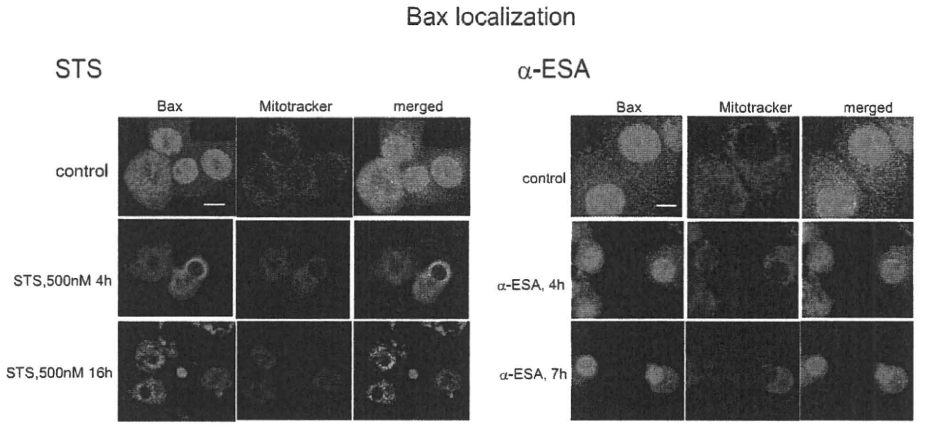

FIGURE 8. **Bax localization of α-ESA-treated cells.** The differentiated PC12 cells were treated with staurosporine (1 μM) or α-ESA (2 μg/ml), and stained with anti-Bax and MitoTracker Red CM-H2Ros. Bax migrated into the mitochondria in the STS-treated cells, whereas Bax did not in the α-ESA-treated cells.

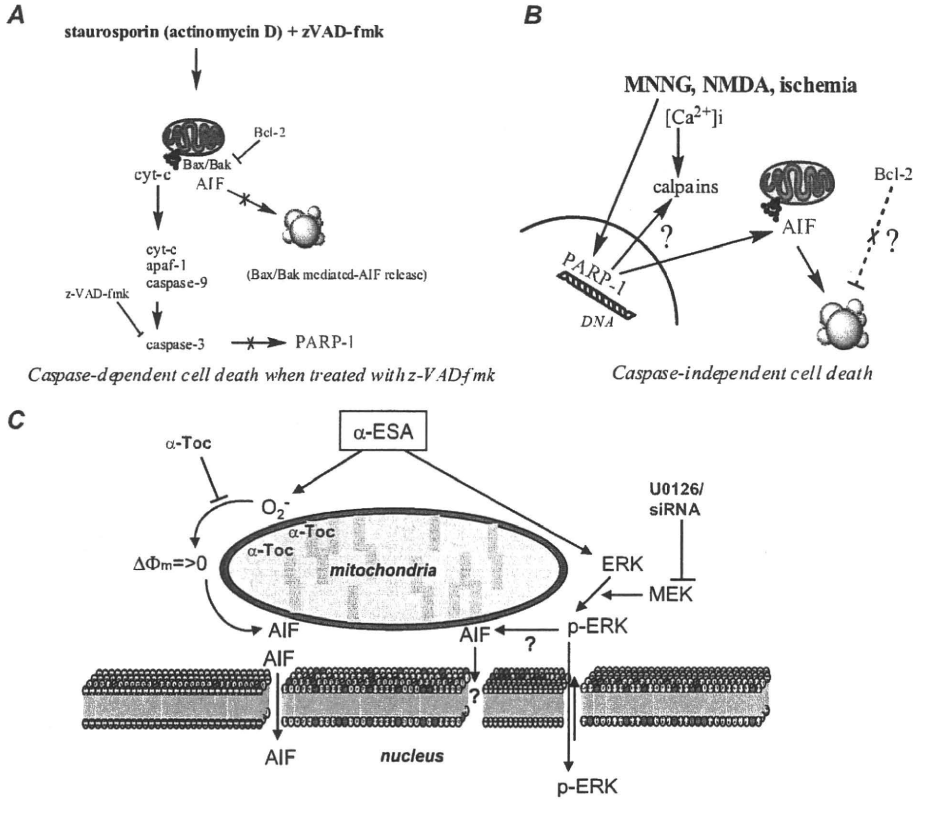

FIGURE 9. **A speculative mechanism of α-ESA-mediated cell death.** *A*, staurosporine + Z-VAD-fmk treatment induces Bax/Bak-mediated Cyt-c, AIF, and other apoptotic mitochondrial proteins such Smac/Diablo, although caspase is blocked by Z-VAD-fmk. This type of apoptosis is blocked by pro-survival Bcl-2 protein. *B*, MNNG, NMDA, or hypoxia ischemia induces the increase in an intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), intracellular ROS production, and DNA alkylation followed by PARP-1 activation, which results in PARP-1-mediated AIF release, leading to the caspase-independent cell death. *C*, α-ESA induces PARP-1-independent AIF-release, resulting in a novel caspase-independent apoptosis. U0126 and MEK1/2 knockdown block the cell death by unknown mechanisms. ERK1/2 is certainly involved in the cell death. α-Toc blocks ROS production in the mitochondria and cell death without the influence on ERK1/2. PARP-1 is not involved in α-ESA-mediated cell death. α-ESA appears to act separately on MEK1/2-ERK1/2 and superoxide production leading to the reduction of the membrane potential.

Diablo, Omi/HtrA2 (Fig. 9*A*). The excitotoxic NMDA or DNA-alkylating agent MNNG induces DNA damage leading to PARP-1 activation, which results in PARP-1-dependent AIF release and cell death (Fig. 9*B*). In caspase-independent apoptosis, PARP-1 is believed to be a death signal. On the other hand, α-ESA induces PARP-1-independent AIF release through the production of superoxide and ERK1/2 phosphorylation, resulting in caspase-independent and PARP-1-independent cell death (Fig. 9*C*). α-Toc protects the α-ESA-mediated cell death because mitochondrially localized α-Toc can scavenge superoxides. This is a novel and alternative pathway for caspase-independent apoptosis without the involvement of other apoptotic proteins.

DISCUSSION

α-ESA is a conjugated trienoic fatty acid and an isomer of nonconjugated linolenic acid. It has been reported to suppress both tumor growth *in vivo* and angiogenesis *in vitro* by inducing caspase-3 via lipid peroxidation at a concentration of 20 μM (23, 24). α-ESA has been shown to be cytotoxic in human tumor cell lines such as DLD-1, HepG2, and A549, although MCF-7 cells lacking caspase-3 were relatively resistant to the α-ESA-mediated apoptosis (22). These reports suggest that a higher concentration of α-ESA can activate caspase-dependent pathways in non-neuronal cells.

First of all, in this study apoptosis induced by α-ESA was not blocked by both the broad spectrum caspase inhibitor Z-VAD-fmk and caspase-3 inhibitor, but it was inhibited by the MEK inhibitor U0126 and the lipophilic antioxidant α-Toc. The α-ESA-mediated apoptosis was abrogated by as little as 23 nM (0.01 μg/ml) of α-Toc. If the peroxidation of α-ESA is involved in the α-ESA-mediated neuronal death, a similar molar concentration of antioxidants should be needed to protect the cells. In addition, intracellular ROS production caused by the peroxidation of α-ESA can deactivate phosphatases by the oxidation in the active center, leading to the increase in ERK1/2 phosphorylation. This phosphorylation can be inhibited by antioxidants. However, α-Toc did not block the increase in ERK1/2 phosphorylation. Even when α-Toc was

added to the cells 1 h after treatment with α-ESA, it blocked the α-ESA-mediated cell death. Moreover, the cell death was not mediated by α-ESA-Me (methyl ester of α-ESA). Together with MitoSOX Red analysis, these results suggest that α-Toc blocks the α-ESA-mediated superoxide production in a small quantity at least in the mitochondria and the resulting cell death.

α-Toc reportedly up-regulated pro-survival Bcl-2 and protected against hydrogen peroxide (H_2O_2)-induced cell death in cortical neurons 24 h after pretreatment with 10 nM α-Toc through ERK1/2 activation (37). The amount of Bcl-2 protein was not changed in this study.[3] Thus, the effect of α-Toc on the up-regulation of Bcl-2 is not important in this apoptotic cell death. This is supported by Bcl-2 overexpression study.

Second, U0126 completely prevented the α-ESA-mediated cell death. U0126 blocked the migration of AIF. The knockdown experiments of MEK1 and MEK2 revealed that MEK1/2 is an important pathway in the α-ESA-mediated cell death.

Caspase-3 and PARP-1 were not activated by α-ESA during the apoptotic cell death process. PARP-1 activation followed by AIF migration is considered to be important for caspase-independent apoptotic cell death. In this study, AIF was released from the mitochondria and migrated to the nucleus by α-ESA. By 7 h, the nucleus was condensed, and AIF was localized throughout the nucleus. However, the cell death was not inhibited by the PARP-1 inhibitor DPQ. PAR proteins were not observed in α-ESA-treated cells at various time points. In addition, PARP-1 knockdown had no protective effect on the α-ESA-mediated cell death. The combination of PARP-1 knockdown and DPQ pretreatment showed no effect. The PAR polymer is considered to be a death signal. AIF is thought to be mediated by PARP-1 activation in caspase-independent apoptotic cells (21, 29, 38, 39). To our knowledge, these are the novel findings of AIF-mediated and caspase-independent apoptosis without PARP-1 activation.

Third, STS or actinomycin D in the presence of Z-VAD-fmk may induce apoptosis without activation of caspase and PARP-1. However, Bax already migrated to the mitochondria to form Bax/Bak channels to release apoptotic proteins such as Cyt-c, AIF, and Smac/Diablo. Bax was not translocated to the mitochondria in the α-ESA-treated cells, whereas Bax did migrate to the mitochondria in the STS-treated cells. Moreover, Cyt-c was not released into the cytosol before AIF. These results suggest that Bax-mediated apoptosis is not involved in the α-ESA-mediated apoptosis. Thus, this type of pathway is excluded. In addition, manganese superoxide dismutase was released into the cytosol, suggesting mitochondrial remodeling or membrane permeabilization associated with the reduction of mitochondrial membrane potential during the apoptotic process, independently of PARP-1 and other signaling molecules. Scattered and condensed mitochondria were observed (Fig. 7*E*).

Fourth, ERK1/2 is reportedly activated by CPT, etoposide (40), and MNNG (31). The apoptotic cell death induced by etoposide and MNNG can be blocked by MEK1/2 inhibitors PD98059 and U0126, the latter of which protects against brain ischemia (41). These reports suggest that ERK1/2 is a key regulator of apoptosis (42). Kauppinen *et al.* (31) reported on the regulation of PARP-1 by ERK1/2 and the inhibition of neuronal cell death by DPQ. However, DPQ at concentrations of up to 100 μM was unable to prevent the cells from the α-ESA-mediated apoptosis. MNNG or NMDA induces PARP-1-dependent AIF translocation to the nucleus. Thus, MNNG or NMDA-treated and the α-ESA-treated cells appear to exert different signaling pathways. Calpain I can mediate AIF release from the mitochondria in ischemia neuronal injury (43). Moubarak *et al.* (21) reported that PARP-1 was activated upstream of calpains in MNNG-induced murine embryo fibroblasts. Recently, however, calpain was reported to be unnecessary for AIF translocation in PARP-1-dependent cell death (44). In this study, the α-ESA-mediated cell death was not abrogated by the calpain inhibitors *N*-Acetyl-Leu-Leu-Nle-CHO ALLN and calpeptin and by the Ca^{2+}-dependent- and independent phospholipase A_2 inhibitors, bromoenol lactone, methylarachidonyl fluorophosphonate (MAFP), and palmitoyl trifluoromethyl ketone.

MNNG, NMDA, or hypoxia ischemia can all induce oxidative stress, thereby leading to DNA damage. These stimuli can initiate caspase-independent and PARP-1-dependent signaling cascades. In contrast, α-ESA did not activate PARP-1, although the α-ESA-treated cells became TUNEL-positive. H2AX was not phosphorylated by α-ESA, indicating that DNA strand breaks did not occur. The overexpression of Bcl-2 did not block the α-ESA-mediated apoptotic cell death. Bcl-2 appears not to be enough to protect against α-ESA-mediated apoptosis.

In conclusion, our results show that α-ESA causes a novel PARP-1-independent AIF release and the cell death through the superoxide production and the prolonged ERK1/2 phosphorylation. The cell death was not associated with Bax, Cyt-c, caspase-3, and PARP-1.

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ORIGINAL PAPER

Constituents of cultivated *Agaricus blazei*

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Abstract Two phenylhexane derivatives (**1**, **2**), benzoylergostane (**3**), *N*-benzoyl-L-leucine methyl ester (**4**), two known ergostanes, and highly degraded incisterol were isolated from fruit bodies of *Agaricus blazei*. Compound **3** exhibited strong cytotoxicity toward HepG2 cells (IC_{50} = 6.0 ± 0.33 μM).

Keywords *Agaricus blazei* · Basidomycota · Agaricaceae · Phenylhexane · Ergostane · Cytotoxicity

Introduction

Agaricus (*Agaricus blazei* Murrill) is widely used as a functional food that has potential as a cancer remedy [1–4]. But, some controversial effects have also been reported [5]. *Agaricus blazei* Murrill (local name Cogumelo Piedade) is an endemic mushroom in Piedade, Province of São Paolo, Brazil [6], and is widely cultivated in Japan for medicinal use. Several cytotoxic sterols have been isolated from its fruiting bodies [7], and degraded ergostane derivatives have been isolated from cultured mycelia [8]. Investigation of fruiting bodies, cultivated in Japan, yielded three new compounds (**1–3**) and one new in nature (**4**), along with three known compounds (**5–7**). The structures of the known compounds were identified as demethylincisterol A_3 (**5**) [9], (22*E*,24*R*)-5α,8α-epidioxyergosta-6,22-dien-3-β-ol (ergosterol peroxide) (**6**) [10], and (22*E*,24*R*)-3β,5α,6α-trihydroxyergosta-7,22-diene (**7**) [11, 12]. This paper deals with the structural elucidation of the new compounds and their cytotoxicity.

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Results and discussion

Dried fruiting bodies of *A. blazei* were extracted with MeOH and the concentrated MeOH extract was partitioned with *n*-hexane. The MeOH layer was concentrated and suspended in H_2O, and then successively extracted with EtOAc and 1-BuOH. The EtOAc-soluble fraction was separated by a combination of column chromatographies (CC) to afford three new compounds (**1–3**), one new in nature (**4**), and three known compounds (**5–7**).

Compound **1** was isolated as an amorphous powder and its elemental composition was determined to be $C_{13}H_{20}O_2$ by HR-ESI–MS. The IR spectrum exhibited the absorptions for hydroxyl groups (3367 and 1012 cm^{-1}) and an aromatic ring (1603 and 1512 cm^{-1}). The presence of the aromatic ring was also supported by the UV absorption at 254 nm. In the ^{1}H-NMR spectrum, two aromatic signals coupled in an AA′BB′ system, isolated oxymethylene [δ_H 4.68 (2H, s)] and an oxymethine [δ_H 4.66 (1H, dd, J = 8, 6 Hz)] signals, and a terminal methyl signal [δ_H 0.87 (3H, t, J = 7 Hz)] were observed. The ^{13}C-NMR spectrum of **1** displayed 11 signals, which comprised those of one *para*-substituted aromatic ring, one methyl, four and one methylenes without and with an oxygen functional group, respectively, and one oxymethine carbon (Table 1). The heteronuclear multiple bond correlation (HMBC) spectrum showed key correlations, e.g., the oxymethylene protons (δ_H 4.68) showed a

Table 1 ^{13}C-NMR data for compounds **1** and **2** ($CDCl_3$, 100 MHz)

C	1	2
1	144.4	136.5
2	126.2	128.4
3	127.1	126.7
4	140.1	145.9
5	127.1	126.7
6	126.2	128.4
7	74.5	200.3
8	39.1	38.7
9	25.5	24.2
10	31.7	31.6
11	22.6	22.5
12	14.0	13.9
13	55.1	64.7

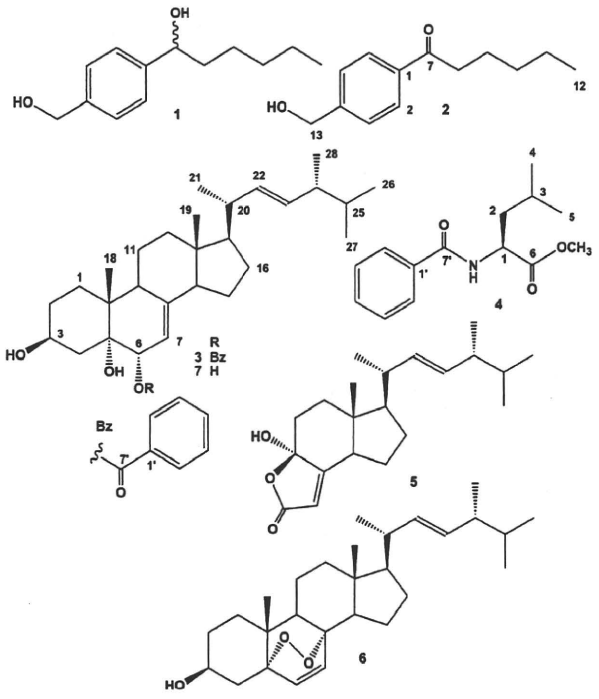

Fig. 1 Structures of isolated compounds

correlation cross peak with one (δ_C 127.1) of the aromatic carbons with protons, and the oxymethine proton (δ_H 4.66) with another aromatic carbon (δ_C 126.2) with protons. Thus, the structure of **1** was established to be as shown in Fig. 1. Since the optical rotation value of **1** was nearly zero, when compared with that of a similar compound, (*S*)-1-phenyl-*n*-hexanol, $[\alpha]_D^{25}$ −36.8 [12], **1** was expected to be an almost racemic mixture. NMR spectra of Mosher's esters, (*R*)- (**1a**) and (*S*)-MTPA (**1b**) esters, were essentially the same, and they were found to be a mixture of diastereomeric compounds (data not shown). Therefore, compound **1** was concluded to be a racemic mixture.

Compound **2** was isolated as an amorphous powder and its elemental composition was determined to $C_{13}H_{18}O_2$ by HR-ESI–MS. The spectroscopic data for **2** were similar to those for **1**, except for the absence of oxymethine proton and carbon signals, and the presence of a carbonyl carbon signal (δ_C 200.3). Oxymethylene protons also showed correlation cross peaks with the aromatic carbon signal at δ_C 126.7 (C-3′ and 5′), and the aromatic protons (δ_H 7.95) at C-2′ and 6′ with the carbonyl carbon in the HMBC spectrum. Therefore, the structure of **2** was elucidated to be 4′-hydroxymethyl-1-phenyl-*n*-hexan-1-one, as shown in Fig. 1.

Compound **3**, $[\alpha]_D$ +29.1, was isolated as an amorphous powder and its elemental composition was determined to be $C_{35}H_{50}O_4$ by HR-ESI–MS. The IR spectrum exhibited absorptions for aliphatic hydrocarbons (2957, 2931, and 2871 cm^{-1}) and an ester carbonyl (1716 cm^{-1}). The ^{1}H-NMR spectrum showed resonances for five aromatic protons, four doublet methyls and two singlet methyls, and two olefinic protons for one *trans* double bond. In the ^{13}C-NMR spectrum, four signals, two of which were of double strength, and one carbonyl carbon were assignable to a benzoate moiety, the remaining 28 carbons comprising six methyls, seven methylenes, six methines, two oxymethines, two quaternary carbons, one oxygenated quaternary carbon, and di- and trisubstituted double bonds (Table 2). The above evidence implied that compound **3** was a benzoyl derivative of ergostane, which co-occurs in *A. blazei* as (22*E*,24*R*)-3β,5α,6α-trihydroxyergosta-7,22-diene (**7**) [11]. The ^{13}C-NMR spectral chemical shifts of **3** were essentially the same as those of **7**, except for C-5, 6, 7, and 8 (Table 2). As a result of acylation, the C-6 signal was shifted downfield, and the adjacent C-5 and C-7 signals were shifted upfield. The highly deshielded proton resonating at δ_H 5.54 was assigned to an oxymethine proton at the 6-position, which showed a cross peak with the benzoyl carbonyl carbon (δ_C 166.2) in the HMBC spectrum (Fig. 2). Other HMBC correlations shown in Fig. 2 supported the structure to be **3**, as shown in Fig. 1.

Compound **4**, $[\alpha]_D$ −10.3, was isolated as colorless crystals and its elemental composition was determined to be $C_{14}H_{19}O_3N$ by HR-ESI–MS. The most distinct IR absorption was due to a carbonyl functional group at 1744 cm^{-1}. The NMR spectra revealed the presence of a benzoyl group and a methyl ester. The remaining six carbons and one nitrogen atom formed a core skeleton. Two terminal methyls, one methylene, one methine, one relatively deshielded methine at δ_C 51.2 with a proton at δ_H 4.87, and one carbonyl carbon at δ_C 173.7 indicated that the core moiety is an amino acid, such as leucine. One amide proton appeared at δ_H 6.49 as a doublet. Thus, the structure of **4** was expected to be *N*-benzoyl leucine methyl

Table 2 ^{13}C-NMR spectroscopic data for compounds **3** and **7** ($CDCl_3$, 100 MHz)

C	3	7
1	31.5	31.7
2	30.8	30.7
3	67.4	67.5
4	39.6	39.3
5	75.6	76.1
6	74.5	70.4
7	115.2	119.6
8	144.5	142.2
9	43.7	43.8
10	39.2	39.3
11	21.4	21.5
12	39.3	39.3
13	43.9	43.8
14	54.9	54.8
15	22.7	22.8
16	28.0	28.1
17	56.0	56.0
18	12.3	12.3
19	18.1	17.8
20	40.4	40.4
21	21.2	21.2
22	135.5	135.5
23	132.2	132.2
24	42.9	42.9
25	33.1	33.2
26	20.0	20.0
27	19.7	19.7
28	17.6	17.6
1′	130.2	
2′, 6′	129.8	
3′, 5′	130.2	
4′	133.2	
7′	166.2	

ester, which is known as a synthetic material [13, 14], this being the first time it had been isolated from nature (Fig. 1). The negative optical rotation sign of **4** assigned the absolute configuration of the leucine to be *S* (L).

Compound **5** was identified as demethylincisterol A_3. Incisterol A_3, a highly degraded sterol, was first isolated from a marine sponge, *Dictyonella incisa* [15], and demethylincisterol A_3 (**5**) was prepared from ergosterol by photodegradation [16]. Mansoor et al. [17] isolated demethylincisterol A_3 (**5**) as a known compound from marine sponge *Homaxinella* sp. Although they isolated **5** for the first time as a natural product, our isolation of **5** from *A. blazei* is for the first from a terrestrial basidiomycetes.

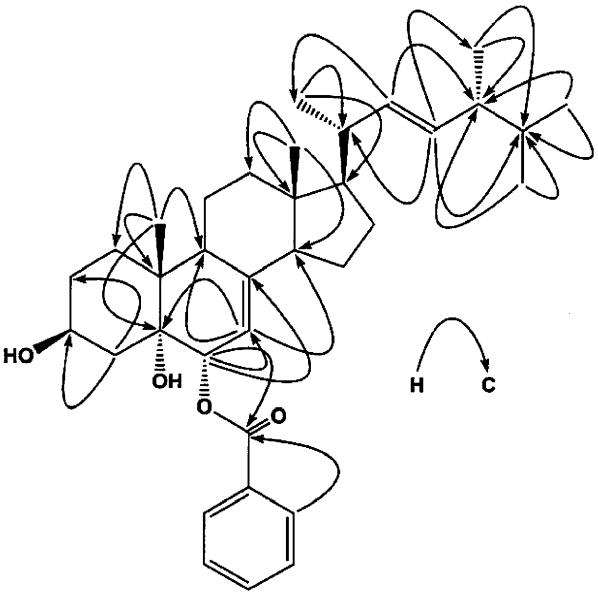

Fig. 2 Selected HMBC correlations for compound **3**

The cytotoxicity of the new compounds toward HepG2 cells was assayed using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) method. Compound **4** was inactive ($IC_{50} > 100$ μM). Compounds **1** and **2** showed moderate activities, $IC_{50} = 39.2 \pm 5.68$ and 28.9 ± 2.06 μM, respectively. Compound **3** exhibited strong activity, $IC_{50} = 6.0 \pm 0.33$ μM, whereas that of a positive control, etoposide, was 0.63 ± 0.11 μM.

Experimental

General experimental procedures

Melting points were measured on a Yanagimoto micro-melting point apparatus and are uncorrected. Optical rotations were measured on a JASCO P-1030 polarimeter. IR and UV spectra were measured on Horiba FT-710 and JASCO V-520 UV/Vis spectrophotometers, respectively. ^{1}H- and ^{13}C-NMR spectra were taken on a JEOL JNM α-400 spectrometer at 400 and 100 MHz, respectively, with tetramethylsilane as an internal standard. Positive-ion HR-ESI–MS was performed with an Applied Biosystem QSTAR XL system ESI (Nano Spray)-TOF-MS.

(*R*)- and (*S*)-MTPA were the products of Wako Pure Chemical Industry Co., Ltd (Tokyo, Japan).

Plant material

Fruiting bodies of *A. blazei* were cultivated and donated by Marukura Co., Ltd (Onojo City, Fukuoka, Japan).

Extraction and isolation

Air-dried fruit bodies of *A. blazei* (11.0 kg) were extracted three times with MeOH (4.5 l) at 25°C for 1 week and then concentrated to 3 l in vacuo. The extract was washed with *n*-hexane (3 l, 177 g) and then the MeOH layer was concentrated to a gummy mass. The latter was suspended in water (3 l) and then extracted with EtOAc (3 l) to give 70.9 g of an EtOAc-soluble fraction. The aqueous layer was extracted with 1-BuOH (3 l) to give a 1-BuOH-soluble fraction (190 g), and the remaining water layer was concentrated to furnish 1.31 kg of a water-soluble fraction.

The EtOAc-soluble fraction (70.0 g) was subjected to silica gel (1.00 kg) ($\Phi = 8$ cm, $L = 45$ cm) CC, with *n*-hexane–EtOAc [(50:1, 6 l), (20:1, 6 l), (10:1, 6 l), (5:1, 6 l), (2:1, 6 l), and (1:1, 3 l)], 6-l fractions being collected. The fourth fraction (8.4 g) was separated by silica gel (200 g) ($\Phi = 8$ cm, $L = 45$ cm) CC again with $CHCl_3$ (1.2 l) and $CHCl_3$–MeOH [(100:1, 1.2 l), (50:1, 1.2 l), (40:1, 1.2 l), (30:1, 1.2 l), (10:1, 1.2 l), (2:1, 1.2 l), and (1:1, 1.2 l)], 300-ml fractions being collected. The residue (1.18 g) in fractions 18–20 was separated by Sephadex LH-20 ($\Phi = 3$ cm, $L = 90$ cm) CC with $CHCl_3$–MeOH (1:1), 10-g fractions being collected, and the residue (20.3 mg) in fractions 21–26 was finally purified by HPLC [GL Science Inertsil Diol, $\Phi = 6$ mm, $L = 250$ mm, *n*-hexane–EtOH (19:1), 1 ml/min] to give 4.9 mg of **7** from the peak at 33 min.

The fifth fraction (10.2 g) was separated by ODS {Cosmosil 75C_{18}-OPN (Nacalai Tesque, Kyoto, Japan) [$\Phi = 50$ mm, $L = 25$ cm, linear gradient of MeOH–H_2O (3:7, 1 l) → (1:0, 1 l), fractions of 13 g being collected]} CC to give two fractions. The first fraction was purified by HPLC [Nacalai Tesque Cosmosil 5 C18-Ar-II, $\Phi = 10$ mm, $L = 250$ mm, MeOH–H_2O (1:1), 2 ml/min] to give crude **4** (29.4 mg) and 3.6 mg of **2** from the peaks at 50–66 and 69 min, respectively. The crude **4** was repeatedly purified under the same conditions to afford 2.1 mg of pure **4** from the peak at 64 min. The latter was purified by HPLC [Merck Hibar, $\Phi = 20$ mm, $L = 250$ mm, MeOH–H_2O (19:1), 0.5 ml/min] to give impure **5** (65.9 mg), **6** (26.7 mg), and **3** (44.3 mg) from the peaks at 17, 34, and 37 min, respectively. These impure compounds were separately purified by repeated HPLC under the following conditions [GL Science Inertsil Diol, $\Phi = 10$ mm, $L = 250$ mm, *n*-hexane–EtOH (199:1), 2.8 ml/min), GL Science Inertsil Diol, $\Phi = 6$ mm, $L = 250$ mm, *n*-hexane–EtOH (199:1), 1.6 ml/min and GL Science Inertsil Diol, $\Phi = 6$ mm, $L = 250$ mm, *n*-hexane–EtOH (19:1), 1.6 ml/min, respectively] to give 10.7 mg of **5**, 6.0 mg of **6**, and 5.0 mg of **3** from the peaks at 48, 20, and 39 min, respectively.

The sixth fraction (4.2 g) was separated by ODS [Nacalai Tesque Cosmosil 75C_{18}-OPN, $\Phi = 50$ mm, $L = 25$ cm, linear gradient of MeOH–H_2O (1:9, 1 l) → (1:0, 1 l), fractions of 13 g being collected]. The residue (209 mg) in fraction 91–124 was purified by HPLC [Merck Hibar, $\Phi = 20$ mm, $L = 250$ mm, MeOH–H_2O (3:2), 3 ml/min] to yield 17.7 mg of **1** from the peak at 22 min.

1-[4-(Hydroxymethyl)phenyl]hexan-1-ol (**1**)

Amorphous powder, $[\alpha]_D^{26}$ ~0.0 (*c* 1.18, $CHCl_3$). IR ν_{max} (film) cm^{-1}: 3367, 2930, 2858, 1603, 1512, 1456, 1402, 1208, 1012, 821. UV λ_{max} (MeOH) nm (log ε): 254 (3.39), 220 (3.64). ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 7.33 (4H, m, H-2, 3, 5, and 6), 4.68 (2H, s, H_2-13), 4.66 (1H, dd, $J = 8$, 6 Hz, H-7), 1.79 (1H, m, H-8a), 1.67 (1H, m, H-8b), 1.29 (6H, m, H_2-9, 10 and 11), 0.87 (3H, t, $J = 7$ Hz, H_3-12). ^{13}C-NMR ($CDCl_3$, 100 MHz): Table 1. HR-ESI–MS (positive-ion mode) *m/z*: 231.1360 $[M+Na]^+$ (Calcd for $C_{13}H_{20}O_2Na$: 231.1355).

1-[4-(Hydroxymethyl)phenyl]hexan-1-one (**2**)

Amorphous powder, IR (film) ν_{max} cm^{-1}: 3138, 2954, 2928, 2863, 1679, 1609, 1508, 1413, 1043. UV (MeOH) λ_{max} nm (log ε): 250 (4.00), 212 (3.73). ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 7.95 (2H, d, $J = 8$ Hz, H-2′ and 6′), 7.45 (2H, d, $J = 8$ Hz, H-3′ and 5′), 4.77 (2H, s, H_2-13), 2.95 (2H, t, $J = 7$ Hz, H_2-8), 1.74 (2H, m, H_2-9), 1.37 (4H, m, H_2-10 and 11), 0.91 (3H, t, $J = 7$ Hz, H_3-12). HR-ESI–MS (positive-ion mode) *m/z*: 231.1194 $[M+Na]^+$ (Calcd for $C_{13}H_{18}O_2Na$: 229.1199).

3β,5α,6α-Trihydroxyergosta-7,22-diene 6-*O*-benzoate (**3**)

Amorphous powder, $[\alpha]_D^{28}$ +29.1 (*c* 0.37, $CHCl_3$). IR ν_{max} cm^{-1}: 3,429, 2,956, 2,935, 2870, 1716, 1455, 1274, 1112, 1069, 1025, 757, 713. UV (MeOH) λ_{max} nm (log ε): 275 (3.25), 228 (3.96). ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 8.07 (2H, d, $J = 7$ Hz, H-2′ and 6′), 7.57 (1H, t, $J = 7$ Hz, H-4′), 7.45 (2H, t, $J = 7$ Hz, H-3′ and 5′), 5.54 (1H, br d, $J = 2$ Hz, H-6), 5.23 (1H, dd, $J = 15$, 7 Hz, H-23), 5.16 (1H, dd, $J = 15$, 8 Hz, H-22), 5.02 (1H, br d, $J = 2$ Hz, H-7), 4.02 (1H, dddd, $J = 11$, 11, 5, 5 Hz, H-3), 2.13 (1H, m, H-9), 2.10 (2H, m, H_2-12), 2.01 (2H, H-4a and 20), 1.88 (1H, m, H-24), 1.84 (1H, m, H-2a), 1.75 (1H, m, H-16a), 1.72 (2H, m, H-11a and 16b), 1.59 (1H, m, H-4b), 1.57 (2H, m, H-1a and 11b), 1.54 (1H, m, H-15a), 1.53 (1H, m, H-1b), 1.46 (2H, m, H-2b and 25), 1.40 (1H, m, H-15b), 1.31 (1H, m, H-14), 1.30 (1H, m, H-17), 1.08 (3H, s, H_3-19), 1.03 (3H, d, $J = 7$ Hz, H_3-21), 0.91 (3H, d, $J = 6$ Hz, H_3-28), 0.84 (3H, d, $J = 6$ Hz, H_3-27), 0.82 (3H, d, $J = 6$ Hz, H_3-26), 0.59 (3H, s, H_3-18). ^{13}C-NMR ($CDCl_3$, 100 MHz): Table 1. HR-ESI–MS (positive-ion mode) *m/z*: 557.3602 $[M+Na]^+$ (Calcd for $C_{35}H_{50}O_4Na$: 557.3601).

N-Benzoyl-L-leucine methyl ester (**4**)

Colorless crystals (MeOH), mp. 104–106°C, $[\alpha]_D^{28}$ −10.3 (*c* 0.21, EtOH). IR ν_{max} (film) cm^{-1}: 3317, 2955, 1744, 1637, 1543, 1207, 1163, 1024. UV (MeOH) λ_{max} nm (log ε): 226 (4.08). ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 7.80 (2H, dt, J = 7, 2 Hz, H-2′ and 6′), 7.51 (1H, tt, J = 7, 2 Hz, H-4′), 7.44 (1H, tt, J = 7, 2 Hz, H-3′ and 5′), 6.49 (1H, br d, J = 8 Hz, –NH–), 4.87 (1H, ddd, J = 8, 8, 5 Hz, H-1), 3.77 (3H, s $-OCH_3$), 1.79–1.65 (3H, m, H_2-2 and H-3), 1.00 (3H, d, J = 6 Hz, H_3-4), 0.98 (3H, d, J = 6 Hz, H_3-5). ^{13}C-NMR ($CDCl_3$, 100 MHz) δ: 173.7 (C-6), 167.1 (C-7′), 134.1 (C-1′), 131.7 (C-4′), 128.6 (C-3′ and 5′), 127.1 (C-2′ and 6′), 52.4 ($-OCH_3$), 51.2 (C-1), 42.1 (C-2), 25.1 (C-3), 22.8 (C-4), 22.2 (C-5). HR-ESI–MS (positive-ion mode) *m/z*: 272.1256 $[M+Na]^+$ (Calcd for $C_{14}H_{19}O_3NNa$: 272.1257).

(*R*)- (**1a**) and (*S*)-MTPA (**1b**) esters of **1**

A solution of **1** (0.9 mg) in 1 ml of dehydrated CH_2Cl_2 was reacted with (*R*)-MTPA (46 mg) in the presence of 1-ethyl-3-(3-dimethylaminopropyl)cardodiimide hydrochloride (EDC) (43 mg) and *N*,*N*-dimethyl-4-aminopyridine (4-DMAP) (22 mg), and then the mixture was occasionally stirred at 25°C for 30 min and then at 40°C for 5 min. After the addition of 1 ml of CH_2Cl_2, the solution was washed with H_2O (1 ml), 4 N HCl (1 ml), saturated aqueous $NaHCO_3$, and then brine (1 ml), successively. The organic layer was dried over Na_2SO_4 and then evaporated under reduced pressure. The residue was purified by preparative TLC [silica gel (0.25 mm thickness), being applied for 18 cm, developed with $CHCl_3$–$(CH_3)_2CO$ (20:1) for 9 cm, and then eluted with $CHCl_3$–MeOH (9:1)] to furnish ester **1a** (1.3 mg). Through a similar procedure, ester **1b** (1.2 mg) was prepared from **1** (0.9 mg) using (*S*)-MTPA (51 mg), EDC (44 mg), and 4-DMAP (24 mg). The ^{1}H-NMR spectra of **1b** and **1c** were ones of essentially the same diastereomeric mixture.

Assaying of cytotoxicity toward HepG2 cells

The cytotoxicities of the isolated compounds toward HepG2 cells were determined by means of the MTT assay. HepG2 cells were inoculated at a density of 3×10^3 cells/well in 90 μl of DMEM containing 10% FCS, supplemented with amphotericin B (Sigma Co., Ltd) (0.5 μg/ml) and kanamycin sulfate (Meiji Confectionery Co., Ltd) (100 μg/ml), on 96-well plates and then incubated at 37°C under 5% CO_2. After 24 h, 10 μl of a sample solution and etoposide in 10% DMSO was added, followed by further incubation for 72 h. The medium was removed and 100 μl of MTT (0.5 mg/ml) in DMEM was added. After 1.5 h, the medium was removed, 100 μl of DMSO was added to lyse the cells, and then the absorbance (*A*) of MTT formazan in each well was measured with a microplate reader at L_1 = 540 nm and L_2 = 620 nm. Etoposide was used as a positive control [18–20].

Activity was calculated as follows:

$$\% \text{ Inhibition} = [1 - \text{sample}(A_{L_1} - A_{L_2})/\text{control}(A_{L_1} - A_{L_2})] \times 100.$$

The results are expressed as the means with standard deviations (SD) for triplicate experiments.

Known compounds, isolated

Demethylincisterol A_3 (**5**): colorless oil, $[\alpha]_D^{26}$ +174.3 (*c* 0.81, $CHCl_3$) [9]. (22*E*,24*R*)-5α,8α-Epidioxyergosta-6,22-dien-3-β-ol (ergosterol peroxide) (**6**): colorless crystals (MeOH), mp. 180–181°C. $[\alpha]_D^{26}$ −18.3 (*c* 0.60, MeOH) [21]. (22*E*,24*R*)-3β,5α,6α-Trihydroxyergosta-7,22-diene (**7**): colorless needles (MeOH), mp. 230–232°C. $[\alpha]_D^{24}$ +11.6 (*c* 0.37, $CHCl_3$) [22].

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アジサイによる中毒と原因毒

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Hydrangea Poisoning and Its Responsible Substance

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昨年2008年6月，相次いでアジサイ *Hydrangea macrophylla* による食中毒が発生して話題となった．まず6月13日，茨城県つくば市の飲食店で，料理に添えられていたアジサイの葉を食べた10人のうち8人が，食後30分から吐き気・めまいなどの症状を訴えた．つづいて6月26日，大阪市の居酒屋で，男性1名が，だし巻き卵の下に敷かれていたアジサイの葉を食べ，40分後に嘔吐や顔面紅潮などの中毒症状を起こした．いずれも重篤には至らず，軽症で済んだのは不幸中の幸いだった．茨城県では，この症状が青酸中毒に似ていることから，原因はアジサイに含まれる青酸配糖体と特定した．これを受けて厚労省は，7月1日「アジサイの喫食による青酸食中毒について」という通知を出し，アジサイの販売・喫食をしないよう，全国の飲食店・消費者に注意を促した[1]．ところがその後詳細に調べても，原因物質とされた青酸配糖体は検出されず，8月18日再度通知を出し，現時点では，青酸配糖体が含有されているとの知見は十分ではないと修正した[2]．

我々は，この中毒事件に関心を持ち，新聞報道の直後から調査を始めた．しかし，いくら調べてみても，アジサイの青酸配糖体を証明する学術論文は見当たらなかった．ところが，インターネット検索では，Wikipediaをはじめ，まるで常識のように，アジサイには青酸配糖体が含まれているとする記述が続々と見つかってくる．それは，1920年のアメリカでの家畜の中毒例が元になっているらしいことがわかった．アメリカノリノキ *Hydrangea arborescence* というアジサイの近縁種によって牛や馬が中毒し，その原因はhydrangin と名づけられた"glucoside"と報告されている[3]．しかしながら，1963年になってhydranginの構造が再検討され，この化合物は，青酸配糖体に含まれるはずの窒素を含まず，植物一般に見いだされるクマリン誘導体のウンベリフェロン umbelliferone にほかならないことが，種々の化学分析によって証明された[4]．にもかかわらず，その後も青酸配糖体説が一人歩きしてしまったようである．念のため，我々も，富山県中央植物園で栽培されているアメリカノリノキの葉部抽出物のシアン検出テストを行ってみたが，やはり検出できなかった．

このアジサイ中毒は，我々に限らず，他の研究者の関心も誘い，さっそくアジサイのシアン分析を行ったという情報が各地から寄せられた．4例の結果の私信を得たが，うちシアンが検出できたとするのは1例，ほか3例はすべて検出できなかったそうである．しかし，なぜ相反する結果が出るのだろうか．本当に青酸配糖体なのか，あるいは，何かほかの原因物質があるのだろうか？

年が明けて本年3月，日本薬学会第129年会にて，アジサイの青酸配糖体に関する2つの独立した研究報告があった．やはり，相反する結果である．まず，慶応大薬学部の竹田らは，附属薬用植物園で栽培されているアジサイを用いてシアン分析を行い，わずかながらシアン化合物の存在を示唆する結果を得た．そこで葉部の含有成分を調べたが，青酸配糖体は得られず，仮にシアン化合物が存在したとしても微量なので，中毒原因物質とは考えにくいとしている*1．一方，京都薬大の吉川らは，中国四川省産アジサイの葉部・茎部の成分検索を行い，新規青酸配糖体hydracyanoside A（図1），B, Cを得た*2．後に絶対配置決定も含めて，論文として報告している[5]．さらに，つい最近開かれた第56回日本生薬学会に続報を発表し，新たに3種の新規配糖体hydracyanoside D, E, Fを報告した*3．しかし，これら青酸配糖体は，京都産のアジサイ抽出物には含まれず，品種によって成分・含量にかなりの差があるらしいので，実際中毒を起こした品種を特定し，その成分研究をする必要があるとの見解だった．日本原産の

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*1 成川佑次，青木香奈枝，竹田忠紘，アジサイ *Hydrangea macrophylla* f. *macrophylla* の成分研究，日本薬学会第129年会CD要旨集，28P-am108，2009年3月26-28日，京都．

*2 吉川雅之，中村誠宏，王　知斌，松田久司，アジサイ（*Hydrangea macrophylla*，葉部，茎部）の新規青酸配糖体成分，日本薬学会第129年会CD要旨集，26Z-am13，2009年3月26-28日，京都．

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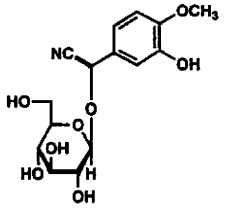

図1. Hydracyanoside A の構造

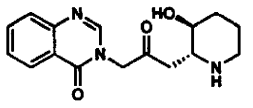

図2. Febrifugine の構造

アジサイは，園芸植物として世界各地に広がり，100 種以上の品種がある．また，その花の色は，産地や同じ株でも赤や青など変化に富むことが知られれていて，俗に「七変化」と言われている．その原因は，色素の構成成分に違いがあることが解明されているが，毒成分にもやはり「七変化」があるのだろうか．

青酸配糖体以外の原因物質は考えられないのだろうか，そう思ってさらに調べていくと，アジサイには，嘔吐性のアルカロイド febrifugine（図2）が含まれていることが分かった[6]．その季節変動も調べられていて，花の咲く夏期のほうが，冬期より含量が高いと報告されている[7]．この化合物は，ジョウザンアジサイ *Dichroa febrifuga* の抗マラリア成分として知られている[8]．本植物は，アジサイの名がついていて同じユキノシタ科 Saxfragacea ではあるが，アジサイ属 *Hydrangea* には属さない．その根が，生薬ジョウザン（常山）として，解熱・催吐・抗マラリア作用を目的とした漢方方剤に用いられる．febrifugine は，もともと第二次世界大戦中のアメリカで，キニーネ代替品として軍事研究をした結果見つかったものであるが，日本でも最近抗マラリア作用が見直され，種々誘導体を合成して構造活性相関が調べられている[9]．中国では，アジサイそのものが八仙花（はちせんか）と呼ばれる生薬で，抗マラリア剤とされるが，やはり嘔吐性が強いので頻用はされないらしい．しかしながら，前述の吉川らによれば，彼らが用いたアジサイ抽出物には，febrifugine は検出できなかったそうである．この成分もやはり「七変化」するのかもしれない．

ところで，アジサイ中毒に関連すると思われるアマチャ（甘茶）による中毒が，今年4月16日岐阜で起こった．新聞によると，岐阜県岐南市の2か所の保育園が，町中の寺院で合同花祭りを開催．甘茶を飲んだ119人の園児のうち28人が嘔吐症状を訴えた．幸いいずれも軽症で，1日以内に全員快方に向かったという．アマチャ *Hydrangea macrophylla* var. *thunbergii* はアジサイの変種なので，同じ成分を含んでいても不思議はない．しかし，古くから薬用として用いられ，日本薬局方にも収載されていて，有毒成分の存在はまったく報告されていない．アジサイだけでなく，アマチャをはじめアジサイ関連植物を徹底的に調べ直す必要がありそうである．

以上のように，アジサイ中毒の原因は，まだまだ混沌としている．アジサイを料理の飾りに使うことは今に始まったことではないようであるが，中毒報告は昨年が初めてのことである．これほど誰でもよく知っている身近な植物で中毒が起こることは，大きな驚きでもあった．それだけに多くの関心を呼び，原因物質解明に向けた研究は精力的に行われているが，いまだ明らかではない．とにかく，アジサイを食べてはいけないことだけは確実である．

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