Bioorganic & Medicinal Chemistry 16 (2008) 9467–9470

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

An endoplasmic reticulum (ER) stress-suppressive compound and its analogues from the mushroom *Hericium erinaceum*

Keiko Ueda [a], Megumi Tsujimori [b], Shinya Kodani [a,b], Akiko Chiba [b], Masakazu Kubo [d], Kazuhiko Masuno [e], Atsushi Sekiya [f], Kaoru Nagai [c,*], Hirokazu Kawagishi [a,b,*]

[a] *Graduate School of Science and Technology, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan*
[b] *Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan*
[c] *Depertment of Epigenetic Medcine, Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi, Yamanashi 409-3898, Japan*
[d] *Kubo Industry Co., Ltd, 580 Ikigaya, Oaza, Chikuma, Nagano 387-0003, Japan*
[e] *Nagano Prefecture General Forest Research Center, 5739 Kataoka, Shiojiri, Nagano 399-0711, Japan*
[f] *Forestry and Forest Products Research Institute, 1 Matsunosato, Tsukuba-shi, Ibaraki 305-8687, Japan*

ARTICLE INFO

Article history:
Received 26 August 2008
Revised 14 September 2008
Accepted 16 September 2008
Available online 19 September 2008

Keywords:
Mushroom
Hericium erinaceum
Endoplasmic reticulum stress suppression
Hericenone
Neurodegenerative disease

ABSTRACT

Three new compounds, 3-hydroxyhericenone F (**1**), hericenone I (**2**), and hericenone J (**3**), were isolated from the mushroom *Hericium erinaceum*. The structures of **1**–**3** were determined by the interpretation of spectral data. Compound **1** showed the protective activity against endoplasmic reticulum (ER) stress-dependent Neuro2a cell death, however, compounds **2** and **3** did not.

1. Introduction

The importance of the endoplasmic reticulum (ER) in triggering a specific program of cell death has been recently reported.[1,2] The ER is the major organelle for Ca^{2+} acquisition and signaling, and its main role controlled by Ca^{2+}-dependent proteins is the regulation of synthesis, folding, and export of proteins.[3] Sustained Ca^{2+} depletion in the ER impaired their functions, which is so-called ER stress.[4] ER stress induces apoptotic pathways with a signaling between ER and mitochondria.[5] By triggering apoptosis on neural cells, the stress is a major cause of degenerative diseases such as Alzheimer disease. The protective compounds against ER stress-dependent cell death are required not only to cure or prevent the diseases but also to investigate the complex system of apoptosis. The demand for new protective substances to the ER-stress-dependent cell death prompted us to screen the protective activity of mushroom extracts. Recently, we found dilinoleoyl-phosphatidylethanolamine as one of protective principles from the mushroom *Hericium erinaceum*.[6] In the course of the further extensive screening, we found a new active compound. Here, we describe the isolation of the new protective compound and two related ones from the mushroom *H. erinaceum*.

2. Results and discussion

2.1. Extraction and isolation of 1–3

Fresh fruiting bodies of *H. erinaceum* were extracted with EtOH and acetone, successively. The combined extract was subjected to two-layer-partitioning using H_2O and $CHCl_3$. The $CHCl_3$ extract was separated using silica gel column chromatography, followed by reversed-phase MPLC and HPLC to afford compound **1** (3-hydroxyhericenone F). On the other hand, dried fruiting bodies were extracted with hexane, EtOAc, and EtOH, successively. The hexane extract was subjected to silica gel column chromatography twice, followed by HPLC to give compounds **2** (hericenone I) and **3** (hericenone J).

2.2. Structure determination of 1–3

3-Hydroxyhericenone F (**1**) was isolated as brown oil. Its molecular formula was determined as $C_{35}H_{54}O_7$ by HRESIMS *m/z*

* Corresponding authors. Tel.: +81 55 273 9554; fax: +81 55 273 9561 (K.N.), tel./fax: +81 54 238 4885 (H.K).
E-mail addresses: kaoru@yamanashi.ac.jp (K. Nagai), achkawa@agr.shizuoka.ac.jp (H. Kawagishi).

0968-0896/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2008.09.044

609.3782 $[M+Na]^+$ (calcd for $C_{35}H_{54}NaO_7$, 609.3767). The complete assignment of all the protons and carbons was accomplished by DEPT, HMQC, COSY, and HMBC experiments as shown in Table 1. The HMBC correlations are illustrated in Figure 1. The chroman skeleton was elucidated by the HMBC correlations (H6/C8, C7-CH_2, C5, C4a; H4/C8a, C4a, C3, C2; C7-*CH_2*/C8, C7, C6; H1′/C3, C2, C2-Me; C2-*Me*/C3, C2, C1′). The position of the formyl group at the chroman was elucidated by the HMBC correlations from the formyl proton to C8 and C7. The position of the methoxy group was also assigned by the HMBC correlation from the methoxy to C5. The presence of the ketone (C2′) was indicated by the characteristic chemical shift at δ_C 201.8. The side chain moiety attached to C2 was elucidated by the HMBC correlations (H1′/C2′; H3′/C5′, C4′-Me, C2′; H5′/C4′, C4′-Me, C3′; C4′-*Me*/C5′, C′4, C3′). Since the alkyl protons, H2″ (δ_H 2.36), H3″ (δ_H 1.64), H4″ to H15″ (overlapped from δ_H 1.20 to1.30 and estimated as 24 protons) and the terminal methyl (δ_H 0.86) were observed, the presence of palmitate was suggested. The ester bond between the chroman and the palmitate was determined by the HMBC correlations from C7-*CH_2*, H3″ and H2″ to the carboxy carbon (δ_C 173.2). The relative stereochemistry was determined by 1D NOE difference and NOESY experiments; NOEs were observed between H3 and C2-*Me*. As a result, the structure of **1** was elucidated as (2*S**,3*S**)-8-formyl-3-hydroxy-5-methoxy-2-methyl-2-(4-methyl-2-oxopent-3-enyl)chroman-7-ylmethyl palmitate. Its dehydroxy analogue, hericenone F, has been already reported as an NGF stimulator.[7] Compound **1** was suggested to be racemic by the CD spectrum data.

Hericenone I (**2**) was purified as colorless crystals. Its molecular formula was determined as $C_{19}H_{22}O_5$ by HRESIMS *m/z* 353.1328 $[M+Na]^+$ (calcd for $C_{19}H_{22}NaO_5$, 353.1365). The structure elucidation using NMR was accomplished in the same manner as **1** (Fig. 1 and Table 1). The chroman skeleton was determined by the COSY correlations between H3/H4 and the HMBC correlations as shown in Figure 2 (H6/C9a, C5, C4a; H4/C10, C5, C4a, C3; H3/C4, C2; C2-*Me*/C3, C2, C1′; C5-*OMe*/C5). The structure of the side chain (position 1′–5′, 4′-Me) was elucidated as identical with that of **1** in the similar manner. The γ-lactone moiety was determined by the HMBC correlations (H9/C10, C9a, C7, C6a; H6/C7). As a result, the plane structure of **2** was determined as 5-methoxy-2-methyl-2-(4-methyl-2-oxopent-3-enyl)-3,4-dihydro-2*H*-furo[3,4-*h*]chromen-7(9*H*)-one. The CD spectrum of **2** also indicated that this compound was racemic (Scheme 1).

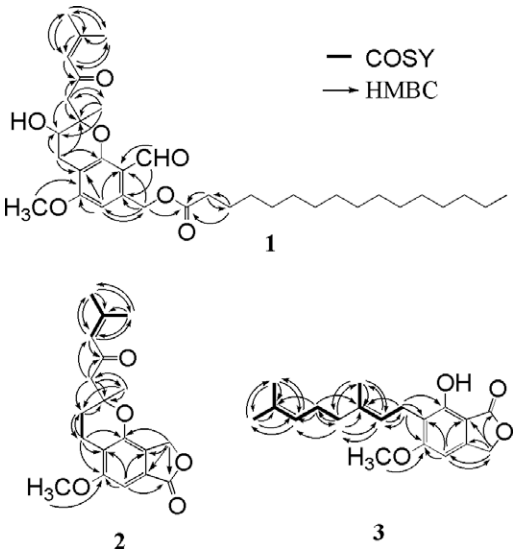


Figure 1. COSY and HMBC correlations of **1**–**3**.

Hericenone J (**3**) was purified as colorless crystals. Its molecular formula was determined as $C_{19}H_{24}O_4$ by HRESIMS *m/z* 339.1549 $[M+Na]^+$ (calcd for $C_{19}H_{24}NaO_4$, 339.1572). The structure elucidation using NMR was accomplished in the same manner as **1** and **2** (Fig. 1 and Table 1). The structure of the side chain (position 1′–8′) was determined by the COSY cross-peaks (C7′-*Me*/H6′, H6′/H5′, H5′/H4′, C3′-*Me*/H2′, C3′-*Me*/H1′, H2′/H1′) and the HMBC correlations (H1′/C3′, C2′; H2′/C4′, C3′-Me, C1′; H4′/C6′, C5′, C3′, C3′-Me, C2′; H5′/C7′, C6′, C4′, C3′; H6′/C8′, C7′-Me; H8′/C7′, C7′-Me,

Table 1
Chemical shifts values of **1**–**3** in $CDCl_3$

3-Hydroxyhericenone F (**1**)			Hericenone I (**2**)			Hericenone J (**3**)		
Position	^1H δ (multiplicity, *J* in Hz)	^{13}C δ	Position	^1H δ (multiplicity, *J* in Hz)	^{13}C δ	Position	^1H δ (multiplicity, *J* in Hz)	^{13}C δ
2		73.3	2		76.5	1		172.8
3	4.91 (dd, 7.6, 9.8)	89.2	3	1.94 (m)	30.3	3	5.21 (s)	70.3
4	3.10 (dd, 16.4, 9.8)	27.0		2.04 (m)		3a		145.9
	3.16 (dd, 16.4, 7.6)		4	2.70 (m)	17.6	4	6.46 (s)	96.0
4a		114.0	4a		116.8	5		164.8
5		160.3	5		159.3	6		117.0
6	6.49 (s)	103.6	6	6.88 (s)	97.0	7		154.5
7		140.3	6a		125.1	7a		104.3
8		111.4	7		171.8	5-OCH_3	3.88 (s)	56.1
8a		165.9	9	5.11 (d, 15.1)	68.0	1′	3.34 (d, 7.0)	21.6
2-CH_3	1.15 (s)	21.5		5.15 (d, 15.1)		2′	5.16 (t, 7.0)	121.3
5-OCH_3	3.87 (s)	55.6	9a		128.0	3′		135.8
7-CH_2	5.45 (s)	64.2	10		148.5	4′	1.94 (t, 7.6)	39.7
8-CHO	10.22 (s)	187.6	2-CH_3	1.41 (s)	24.7	5′	2.03 (m)	26.7
1′	2.63 (d, 16.8)	49.2	5-OCH_3	3.87 (s)	56.0	6′	5.04 (t, 6.7)	124.3
	2.85 (d, 16.8)		1′	2.65 (d, 14.4)	52.3	7′		131.2
2′		201.8		2.76 (d, 14.4)		8′	1.62 (s)	25.6
3′	6.04 (d, 1.2)	124.4	2′		197.7	3′-CH_3	1.75 (s)	16.1
4′		158.9	3′	6.05 (s)	125.0	7′-CH_3	1.55 (s)	17.6
5′	1.89 (s)	27.9	4′		156.2			
4′-CH_3	2.16 (s)	21.2	5′	1.87 (s)	27.9			
1″		173.2	4′ -CH_3	2.14 (s)	20.9			
2″	2.36 (t, 7.5)	34.4						
3″	1.64 (m)	25.1						
4″–15″	1.20–1.30 (m)	22.7–32.0						
16″	0.86 (t, 6.9)	14.1						

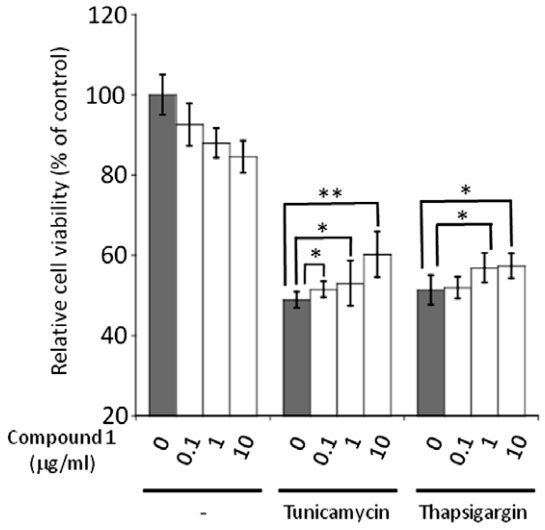
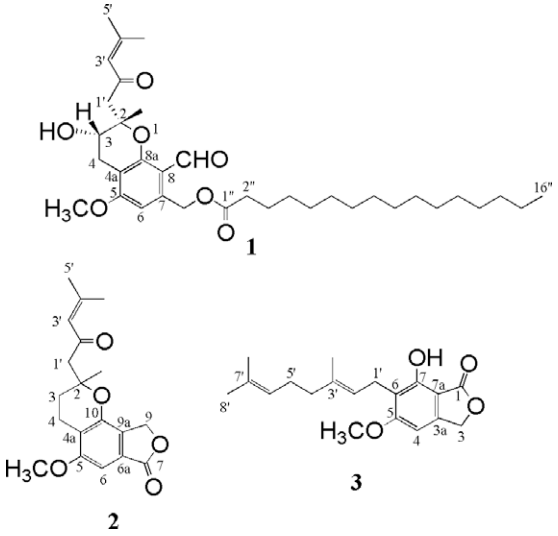


Figure 2. Protective activity of **1** against ER stress-dependent cell death. The cell viabilities were analyzed by MTT assay, and the values were represented as means ± SD of the relative percentage of surviving cells compared with the untreated cells ($n = 8$). $^{*}p < 0.05$, $^{**}p < 0.01$, non-paired t-test.



Scheme 1.

C6′; C7′-*Me*/C8′, C7′, C6′). The benzolactone was constructed by the HMBC correlations (H3/C7a, C4, C3a, C1; H4/C7a, C6, C5, C3). The position of the methoxy was elucidated by the HMBC correlation from H5-OMe to C5. The junction between the side chain and the benzolactone ring was determined by the HMBC correlations (H1′ to C7, C6, C5). All the dates allowed us to conclude that **3** was (*E*)-6-(3,7-dimethylocta-2,6-dienyl)-7-hydroxy-5-methoxyisobenzofuran-1(3*H*)-one.

2.3. Protective activity of 1–3 against ER stress-dependent cell death

Compounds **1–3** were subjected to the protection assay against ER stress-dependent cell death.[6] The ER stress was induced by addition of tunicamycin or thapsigargin into the culture medium of Neuro2a cells in the presence or in the absence of these compounds. Tunicamycin is an inhibitor of N-glycosylation to glycoproteins in the ER and causes protein-misfolding in the ER. Thapsigargin is an ER Ca^{2+}-ATPase inhibitor that causes Ca^{2+} depletion in the ER. Compound **1** showed significant protective activity against both of tunicamycin- and thapsigargin-toxicity dose-dependently (Fig. 2). On the other hand, **2** and **3** did not have any activity even at the concentrations up to 10 μg/ml (data not shown). These results indicated that **1** could protect the neuronal cells by attenuating the ER stress. However, the detailed mechanism of the effects of the compound remains unsolved.

3. Experimental

3.1. General

^{1}H NMR spectra (one- and two-dimensional) were recorded on a JEOL lambda-500 spectrometer at 500 MHz, while ^{13}C NMR spectra were recorded on the same instrument at 125 MHz. The HRESIMS spectra were measured on a JMS-T100LC mass spectrometer. A JASCO grating infrared spectrophotometer was used to record the IR spectra. The CD spectra were measured on a J-820 spectrometer. MPLC was done with a Yamazen MPLC system and UltraPak ODS-S50D column (50 × 300 mm, Yamazen, Japan). HPLC separations were performed with a JASCO Gulliver system using a reverse-phase column (Wakopak Navi C30-5, Wako, Japan, CAPCELL PAK C18 AQ, Shiseido, Japan, Develosil C30-UG-5, Nomura chemistry, Japan). Silica gel plate (Merck F_{254}) and silica gel 60 N (Merck 100–200 mesh) were used for analytical TLC and for flash column chromatography, respectively.

3.2. Fungus materials and cultivation

The strain of *H. erinaceum* was isolated from the fruiting body collected in Nagano Prefecture. Voucher material has been deposited in the research laboratory of Kubo Industry, Nagano prefecture, Japan.

The culture medium (520 g) was prepared by mixing soybean peel (35 g), corn powder (36 g), dried tofu refuse (10 g), activated carbon (2 g), and Japanese beech sawdust and adjusted to 62% moisture by using a moisture meter FD600 (Kett electric laboratory Co., Ltd, Japan). The medium was packed in each polypropylene bottle (520 g/bottle) and autoclaved. The pre-cultured mycelium was inoculated to the bottle. After cultivation under the condition (21 °C, 65% humidity, in the dark) for 2 weeks, fruiting body induction and further cultivation were done under the condition (15 °C, 68% humidity, in the light) for 4 days in an incubator (USC3004, Mikuni Co., Ltd, Japan). The humidity was controlled by a humidity adjuster (H3CR, Omron Co., Ltd, Japan).

3.3. Extraction and isolation

The fresh fruiting bodies of *H. erinaceum* (4.0 kg) were extracted with EtOH (15 L, four times) and then acetone (15 L). The combined solution was concentrated under reduced pressure and partitioned between $CHCl_3$ and H_2O and then the aqueous layer was further extracted with EtOAc. The $CHCl_3$-soluble part (186.3 g) was fractionated by silica gel flash column chromatography (100%, 90%, 70% $CHCl_3$/acetone, 90%, 70%, 50%, 30% $CHCl_3$/MeOH, MeOH) to obtain fourteen fractions, and the fourth fraction (2.5 g) was separated by reversed-phase MPLC (99% MeOH) to obtain 215 fractions. The 13th fraction (82.3 mg) was further separated by reversed-phase HPLC (95% MeOH) to afford compound **1** (1.5 mg).

Powder of the dried fruiting bodies of *H. erinaceum* (1.9 kg) was successively extracted with hexane (1.75 L, four times) and then EtOAc (2.4 L, three times). The hexane-soluble part (30.3 g) was fractionated by silica gel flash column chromatography (95%, 90%, 80%, 50% *n*-hexane/acetone, acetone, EtOH, 80% EtOH, 70%,

2–5 L each) to obtain seventeen fractions. The ninth fraction (977.6 mg) was further separated by silica gel flash column chromatography (95%, 90%, 70%, 50% *n*-hexane/acetone, acetone, EtOH, 1–2 L each) and nine fractions were obtained. The seventh fraction (30.4 mg) was separated by reversed-phase HPLC (99% MeOH) to afford compounds **2** (0.8 mg) and **3** (1.5 mg).

3.3.1. Compound 1

HRESIMS *m/z* 609.3782 $[M+Na]^+$ (calcd for $C_{35}H_{54}NaO_7$, 609.3767). IR ν_{max} (NaCl) cm^{-1}: 3446, 2916, 2833, 1750.

3.3.2. Compound 2

HRESIMS *m/z* 353.1328 $[M+Na]^+$ (calcd for $C_{19}H_{22}NaO_5$, 353.1365). Mp 90–92 °C. IR ν_{max} (NaCl) cm^{-1}: 1768, 1682.

3.3.3. Compound 3

HRESIMS *m/z* 339.1549 $[M+Na]^+$ (calcd for $C_{19}H_{24}NaO_4$, 339.1572). Mp 85–90 °C. IR ν_{max} (NaCl) cm^{-1}: 3426, 1732.

3.4. Cell viability

Cell viability analysis was performed by 3-(4,5-dimethyl-2-thiazolyl) 2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay.

Cell protection assay on ER stress by MTT assay was performed as follows. Neuro2a cells were cultured in 96-well plates at cell density 5000 cells/well. After one-day cultivation, the cells were cultured in D-MEM without FBS, and 0.5 μg/ml of tunicamycin (or thapsigargin) and varying concentrations of compounds **1**–**3** were applied to the medium. The cells were incubated for 24 h, and then the viability was measured by MTT assay, as described previously.[8] Briefly, 0.25 mg/ml of MTT in D-MEM without FBS were added onto the cells and incubated for 2 h. The incubation was terminated by addition of 20% SDS (v/w) and 50% dimethylformamide (v/v) in water. The absorbance at 570 nm of the reaction mixture was measured by a microplate reader (Molecular Devices, USA).

Acknowledgments

This work was partially supported by Grant-in-Aid for scientific research on priority areas 'Creation of Biologically Functional Molecules' (No. 17035037) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References and notes

1. Nakagawa, T.; Zhu, H.; Morishima, N.; Li, E.; Xu, J.; Yankner, B. A.; Yuan, J. *Nature* **2000**, *403*, 98.
2. Nakagawa, T.; Yuan, J. *J. Cell Biol.* **2000**, *150*, 887.
3. Meldolesi, J.; Pozzan, T. *J. Cell Biol.* **1998**, *142*, 1395.
4. Pahl, H. L. *Physiol. Rev.* **1999**, *79*, 683.
5. Hacki, J.; Egger, L.; Monney, L.; Conus, S.; Rosse, T.; Fellay, I.; Borner, C. *Oncogene* **2000**, *19*, 2286.
6. Nagai, K.; Chiba, A.; Nishino, T.; Kubota, K.; Kawagishi, H. *J. Nutr. Biochem.* **2006**, *17*, 525.
7. Kawagishi, H.; Ando, M.; Shinba, K.; Skamoto, H.; Yoshida, S.; Ojima, F.; Ishiguro, Y.; Ukai, N.; Fukuwara, S. *Phytochemistry* **1993**, *32*, 175.
8. Liu, Y.; Peterson, D. A.; Kimura, H.; Schubert, D. *J. Neurochem.* **1997**, *69*, 581.