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Neogrifolin derivatives possessing anti-oxidative activity from the mushroom *Albatrellus ovinus*

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Abstract

Three neogrifolin derivatives, 3-hydroxyneogrifolin, 1-formylneogrifolin and 1-formyl-3-hydroxyneogrifolin along with grifolin and neogrifolin were isolated from the Japanese mushroom *Albatrellus ovinus* belonging to Scutigeraceae. Their structures were established by a combination of two-dimensional NMR spectroscopic analyses and by chemical synthesis. 3-Hydroxyneogrifolin and 1-formyl-3-hydroxy-neogrifolin showed more potent antioxidative activity properties than either α-tocopherol or BHA. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Mushroom; Scutigeraceae; *Albatrellus ovinus*; Neogrifolin derivatives; 3-Hydroxyneogrifolin; 1-Formylneogrifolin; 1-Formyl-3-hydroxyneogrifolin; Two-dimensional NMR; Antioxidative activity

1. Introduction

About 1500 species of mushrooms are now known in Japan of which 300 are edible and 1200 are inedible, with 50 being toxic (Kazuno and Miura, 1984). A lot of inedible mushrooms have bitter and pungent tastes, especially those belonging to the Polyporaceae which have been used as medicinal drugs (anti-cancer etc.) in China since ancient times (Ding, 1982). Little attention has been paid to the chemical constituents of inedible mushrooms in Japan, except for the poisonous species. We are interested in non-toxic biologically active substances present in inedible mushrooms and have studied their chemistry and pharmacology as well as their application as a source of medicinal or agricultural drugs. Recently, we found that an inedible mushroom *Cryptoporus volvatus* (Polyporaceae) contained a large amount of novel bitter drimane sesquiterpenoids, cryptoporic acids (CPAs) A–G (Asakawa et al., 1992; Hashimoto et al., 1987, 1988, 1989), which showed the inhibitiory activities of superoxide anion radical release (Asakawa, 1993; Hashimoto and Asakawa, 1998), colon and skin cancer development on mouse (Matsunaga et al., 1991; Narisawa et al., 1992) and anti-HIV-I (Human Immunodeficiency virus-I) (Hashimoto and Asakawa, 1998). We also reported that chemical investigation of the ethyl acetate extract of *Daldinia vernicosa*, belonging to the Xylariaceae, led to the isolation of 16 novel 10-phenyl-[11]-cytochalasans (Buchanan et al., 1995, 1996a,b) possessing a strong cytotoxicity on the KB cell line (Hashimoto and Asakawa, 1998), apotosis induction in HCT116 Cells (Nagasawa et al., 2000) and plant growth inhibitory activity (Hashimoto and Asakawa, 1998), respectively. Some farnesylphenols, grifolin (**1**), neogrifolin (**2**), grifolic acid (**3**) and their derivatives (Fig. 1) possessing antimicrobial and 5-lipoxygenase inhibitory activities were also isolated from the inedible mushrooms *Albatrellus confluens*, *A. dispansus* and *A. cristatus* belonging to the Scutigeraceae (Ishii et al., 1988; Zechlin et al., 1981).

In the course of our investigation of antioxidative substances from mushrooms (Nukata et al., 1996), we isolated three new neogrifolin derivatives, 3-hydroxyneogrifolin (**4**), 1-formylneogrifolin (**5**) and 1-formyl-3-hydroxyneogrifolin (**6**) with grifolin (**1**) and neogrifolin (**2**) from the Japanese *A. ovinus* belonging to Scutigeraceae (Fig. 1). In this article, we report the

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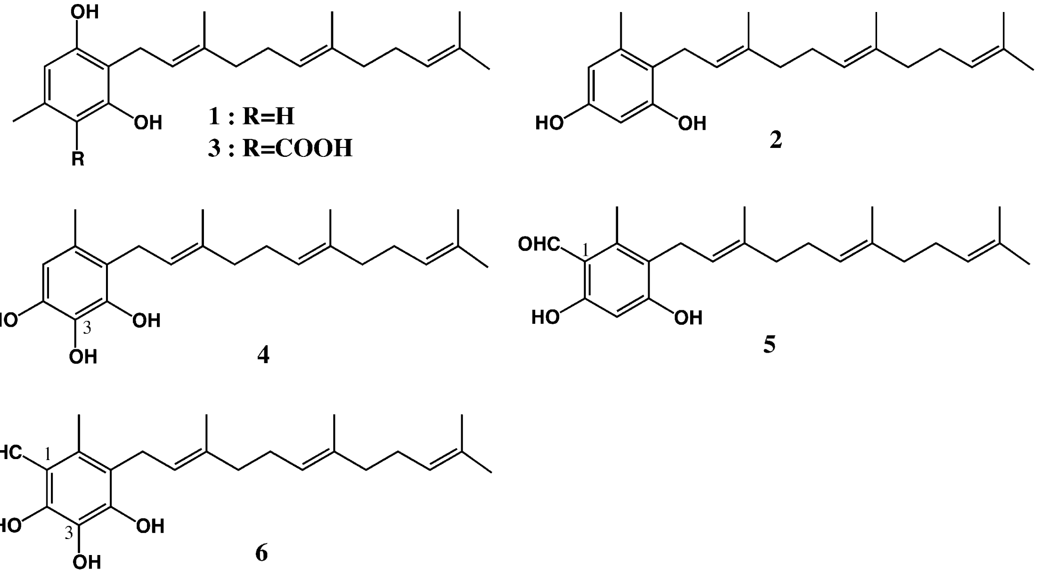

Fig. 1. Grifolin and neogrifolin derivatives isolated from *Albatrellus* species.

relative structures of compounds **4**–**6**, their antioxidative activity and their chemical synthesis.

2. Results and discussion

2.1. Isolation of neogrifolin derivatives

The MeOH extract of dry material of *A. ovinus* was subjected to column chromatography using Sephadex LH-20 ($CHCl_3$:MeOH = 1:1), silica gel (*n*-hexane-EtOAc gradient) and reversed phase HPLC (Nucleosil $5C_{18}$; CH_3CN) to afford three new neogrifolin derivatives, 3-hydroxyneogrifolin (**4**), 1-formylneogrifolin (**5**) and 1-formyl-3-hydroxyneogrifolin (**6**) along with grifolin (**1**) and neogrifolin (**2**) (Fig. 1).

2.2. 3-Hydroxyneogrifolin

3-Hydroxyneogrifolin (**4**) has a molecular formula of $C_{22}H_{32}O_3$ (M^+ m/z 344.2359) possessing one more oxygen in comparison with that of **1** and **2** as indicated by its high resolution EI mass (HR–EIMS) spectrum. The IR and UV spectra of **4** indicated the presence of a phenolic hydroxyl group (ν 3466 and 3295 cm^{-1}) and a benzene ring (ν 1610 cm^{-1}, λ_{max} 210 and 275 nm). The ^1H NMR spectrum (Table 1) of **4** in $CDCl_3$ showed signals for four vinyl methyls [δ 1.58 (*d*, $J = 0.5$ Hz), 1.59 (*d*, $J = 0.5$ Hz), 1.67 (*d*, $J = 1.1$ Hz), 1.79 (*d*, $J = 0.8$ Hz)], a phenyl methyl [δ2.17(*s*)], three substituted olefinic protons, [δ 5.07 (*m*), 5.08 (*m*), 5.17 (*dq*, $J = 0.8$, 7.1 Hz)] and an aromatic proton [δ 6.33 (*s*)]. The ^1H and ^{13}C spectra of the farnesyl part of **4** were similar to those of **1** and **2** as shown in Tables 1 and 2. The methylation (MeI/K_2CO_3/reflux) of **4** afforded a trimethyl ether (**7**) [$C_{25}H_{38}O_3$ (HRMS: M^+ m/z 386.2831); δ_H 3.83, 3.83, 3.85 (each 3H, *s*)] indicating the presence of three phenolic hydroxyl groups. The structure of **4** was deduced from the analysis of 2D NMR spectra including HMBC and NOESY of **4** and **7**. In the HMBC correlations (Fig. 2) of **4**, H-1 were correlated with C-2, C-3, C-5, C-6 and C-8. In the NOESY correlations of **7** (Fig. 3), the NOEs between (i) H-1 and 2-OMe (ii) H-1 and H-8, (iii) H-1′ and H-8, and (iv) H-1′ and 4-OMe were observed. Thus, the structure of **4** was determined as the C-3 hydroxylated derivative of neogrifolin (**2**). As *Albatrellus ovinus* is a very rare mushroom in Japan, the synthesis of **4** from a commercial available 5-methylpyrogallol (**8**) with Na and farnesyl bromide was carried out to afford compound **4** in 22% yield with the starting material (**8**; 68%), and its biological activity was tested.

2.3. 1-Formylneogrifolin

The spectral data (IR, ^1H and ^{13}C NMR) of 1-formylneogrifolin (**5**) ($C_{23}H_{32}O_3$) (HRMS: $[M]^+$ m/z 356.2376) were similar to those of **2** and **4**. The IR and UV spectra of **5** indicated the presence of phenolic hydroxyl (ν 3235 cm^{-1}) and aromatic aldehyde (ν 1631 cm^{-1}, λ_{max} 239, 293 and 338 nm) groups whose UV absorption band shifted to lower wavelength because of intramolecular hydrogen bonding between a phenolic hyroxy and a formyl group. The ^1H and ^{13}C NMR spectra (Tables 1 and 2) of **5** in $CDCl_3$ showed a formyl group [δ_H 10.16 (s), δ_C 193.6 (*d*)], an aromatic proton [δ_H 6.24 (s); δ_C 101.3 (*d*)], and a hydroxyl proton [δ_H 12.50 (1H, *br. s*)] possessing a hydrogen bond with a formyl group and a phenyl methyl [δ_H 2.48(s)] which was deshielded by ca. 0.3 ppm relative to that of **2** and **4** because of the anisotropic effect of the formyl group.

The methylation (MeI/K_2CO_3/reflux) of **5** afforded the dimethyl ether **9** [δ_H 3.89, 3.90 (each 3H, *s*)] indicating the presence of two phenolic hydroxyl groups. The structure of **5** was deduced from the analysis of the 2D NMR (HMBC and NOESY) spectra of **5** and **9**. In the NOESY correlations of **9** (Fig. 4), NOEs between (i) H-7 and 2-OMe (ii) H-7 and H-8, (iii) H-1′ and H-8, and (iv) H-1′ and 4-OMe were observed. Thus, the structure of **5** was determined as the C-1 formylated derivative of neogrifolin (**2**).

Table 1
^{1}H NMR spectral data of grifolin (**1**), neogrifolin (**2**), 3-hydroxyneogrifolin (**4**), 1-formylneogrifolin (**5**), 1-formyl-3-hydroxyneogrifolin (**6**), and methyl ethers **7**, **9** and **10**[a]

	(**1**)[b]	(**2**)[b]	(**4**)	(**5**)		(**6**)	(**7**)	(**9**)	(**10**)
H-1	6.20 (*s*)	6.23 (*d*, 3.0)	6.33 (*s*)		H-1		6.50 (*s*)		
H-3	6.20 (*s*)	6.18 (*d*, 3.0)		6.24 (*s*)	H-3			6.33 (*s*)	
H-7				10.16 (*s*)	H-7	10.16 (*s*)		10.52 (*s*)	10.46 (*s*)
H-8	2.18 (*s*)	2.21 (*s*)	2.17 (*s*)	2.48 (*s*)	H-8	2.43 (*s*)	2.23 (*s*)	2.53 (*s*)	2.46 (*s*)
H-1′	3.37 (*d*, 7.0)	3.27 (*d*, 7.0)	3.30 (*d*, 7.1)	3.34 (*d*, 6.6)	H-1′	3.35 (*d*, 6.3)	3.29 (*d*, 6.9)	3.33 (*d*, 6.9)	3.37 (*d*, 6.6)
H-2′	5.26 (*t*, 7.0)	5.11 (*t*, 7.0)	5.17 (*tq*, 7.1, 0.8)	5.06 (*tq*, 6.6, 1.1)	H-2′	5.04 (*tq*, 6.3, 0.8)	5.05 (*tq*, 6.3, 0.8)	4.97 (*tq*, 6.9, 1.1)	4.99 (*tq*, 6.6, 1.1)
H-4′	1.90–2.10 (*m*)	1.84–2.04 (*m*)	2.04 (*m*)	2.02 (*m*)	H-4′	2.00 (*m*)	1.99 (*m*)	1.97 (*m*)	2.00 (*m*)
H-5′	1.90–2.10 (*m*)	1.84–2.04 (*m*)	2.10 (*m*)	2.10 (*m*)	H-5′	2.07 (*m*)	2.07 (*m*)	2.05 (*m*)	2.07 (*m*)
H-6′	5.06 (*m*)	5.04 (*m*)	5.07 (*m*)	5.07 (*m*)	H-6′	5.07 (*m*)	5.08 (*m*)	5.06 (*m*)	5.06 (*m*)
H-8′	1.90–2.10 (*m*)	1.84–2.04 (*m*)	1.96 (*m*)	1.95 (*m*)	H-8′	1.94 (*m*)	1.93 (*m*)	1.93 (*m*)	1.93 (*m*)
H-9′	1.90–2.10 (*m*)	1.84–2.04 (*m*)	2.05 (*m*)	2.03 (*m*)	H-9′	2.01 (*m*)	2.03 (*m*)	2.01 (*m*)	2.02 (*m*)
H-10′	5.06 (*m*)	5.04 (*m*)	5.08 (*m*)	5.07 (*m*)	H-10′	5.07 (*m*)	5.08 (*m*)	5.06 (*m*)	5.06 (*m*)
H-12′	1.66 (*s*)	1.66 (*s*)	1.67 (*d*, 1.1)	1.68 (*d*, 1.1)	H-12′	1.67 (*d*, 1.1)	1.68 (*d*, 1.1)	1.66 (*d*, 1.1)	1.66 (*d*, 0.8)
H-13′	1.57 (*s*)	1.58 (*s*)	1.59 (*d*, 0.5)	1.58 (*d*, 0.5)	H-13′	1.58 (*d*, 0.5)	1.59 (*br. s*)	1.58 (*br.* s)	1.58 (*br. s*)
H-14′	1.57 (*s*)	1.58 (*s*)	1.58 (*d*, 0.5)	1.58 (*d*, 0.5)	H-14′	1.57 (*d*, 0.5)	1.57 (*br. s*)	1.58 (*br.* s)	1.57 (*br. s*)
15′	1.80 (*s*)	1.80 (*s*)	1.79 (*d*, 0.8)	1.79 (*d*, 1.1)	H-15′	1.77 (*d*, 0.8)	1.77 (*d*, 1.1)	1.75 (*d*, 1.1)	1.77 (*d*, 1.1)
OH			6.20 (*s*)	6.24, 12.50 (each *s*)	OH	5.74, 6.32, 12.52 (each *s*)			
2-OMe					2-OMe		3.83 (*s*)	3.90 (*s*)	3.95 (*s*)
3-OMe					3-OMe		3.85 (*s*)		3.88 (*s*)
4-OMe					4-OMe		3.83 (*s*)	3.89 (*s*)	3.91 (*s*)

[a] Chemical shifts from TMS (multiplicity, *J* in Hz) in $CDCl_3$ and assignments from ^{1}H–^{1}H–2D COSY spectra.

[b] ^{1}H NMR spectral data of grifolin (**1**) and neogrifolin (**2**) (Ishii et al., 1988).

Table 2
^{13}C NMR spectral data of grifolin (**1**), neogrifolin (**2**), 3-hydroxyneogrifolin (**4**), 1-formylneogrifolin (**5**), 1-formyl-3-hydroxyneogrifolin (**6**), and methyl ethers **7**, **9** and **10**[a]

	(**1**)[b]	(**2**)[b]	(**4**)	(**5**)	(**6**)		(**7**)	(**9**)	(**10**)
C-1	109.3 (*d*)	109.9 (*d*)	109.3 (*d*)	113.8 (*s*)	112.9 (*s*)	C-1	109.3 (*d*)	117.4 (*s*)	124.5 (*s*)
C-2	137.2 (*s*)	154.1 (*s*)	141.6 (*s*)	164.4 (*s*)	149.7 (*s*)	C-2	151.0 (*s*)	163.7 (*s*)	156.8 (*s*)
C-3	109.3 (*d*)	101.2 (*d*)	129.9 (*s*)	101.3 (*d*)	128.4 (*s*)	C-3	140.3 (*s*)	92.1 (*d*)	143.6 (*s*)
C-4	154.6 (*s*)	155.2 (*s*)	142.7 (*s*)	162.4 (*s*)	149.5 (*s*)	C-4	151.8 (*s*)	162.3 (*s*)	156.7 (*s*)
C-5	111.0 (*s*)	118.4 (*s*)	118.1 (*s*)	119.4 (*s*)	119.9 (*s*)	C-5	126.3 (*s*)	122.5 (*s*)	131.2 (*s*)
C-6	154.6 (*s*)	138.7 (*s*)	127.8 (*s*)	142.0 (*s*)	133.1 (*s*)	C-6	132.0 (*s*)	141.7 (*s*)	135.4 (*s*)
C-7				193.6 (*d*)	194.2 (*d*)	C-7		191.3 (*d*)	192.3 (*d*)
C-8	21.0 (*q*)	20.2 (*q*)	19.4 (*q*)	13.8 (*q*)	12.9 (*q*)	C-8	19.7 (*q*)	20.2 (*q*)	15.7 (*q*)
C-1′	22.3 (*t*)	25.2 (*t*)	25.7 (*t*)	24.6 (*t*)	24.4 (*t*)	C-1′	25.5 (*t*)	24.2 (*t*)	25.2 (*t*)
C-2′	122.0 (*d*)	122.3 (*d*)	122.1 (*d*)	121.5 (*d*)	121.8 (*d*)	C-2′	123.1 (*d*)	122.3 (*d*)	122.2 (*d*)
C-3′	138.4 (*s*)	137.3 (*s*)	137.7 (*s*)	137.5 (*s*)	135.7 (*s*)	C-3′	134.8 (*s*)	135.1 (*s*)	135.6 (*s*)
C-4′	39.7 (*t*)	39.8 (*t*)	39.7 (*t*)	39.7 (*t*)	39.7 (*t*)	C-4′	39.7 (*t*)	39.7 (*t*)	39.7 (*t*)
C-5′	26.7 (*t*)	26.6 (*t*)	26.3 (*t*)	26.3 (*t*)	26.5 (*t*)	C-5′	26.6 (*t*)	26.6 (*t*)	26.5 (*t*)
C-6′	123.7 (*d*)	123.9 (*d*)	123.7 (*d*)	123.6 (*d*)	124.0 (*d*)	C-6′	124.1 (*d*)	124.1 (*d*)	124.0 (*d*)
C-7′	135.4 (*s*)	135.4 (*s*)	135.5 (*s*)	135.4 (*s*)	135.0 (*s*)	C-7′	135.0 (*s*)	135.0 (*s*)	135.0 (*s*)
C-8′	39.7 (*t*)	39.8 (*t*)	39.7 (*t*)	39.7 (*t*)	39.7 (*t*)	C-8′	39.7 (*t*)	39.7 (*t*)	39.7 (*t*)
C-9′	26.7 (*t*)	26.8 (*t*)	26.7 (*t*)	26.7 (*t*)	26.7 (*t*)	C-9′	26.7 (*t*)	26.7 (*t*)	26.7 (*t*)
C-10′	124.5 (*d*)	124.5 (*d*)	124.3 (*d*)	124.3 (*d*)	124.3 (*d*)	C-10′	124.4 (*d*)	124.4 (*d*)	124.3 (*d*)
C-11′	131.0 (*s*)	131.4 (*s*)	131.4 (*s*)	131.3 (*s*)	131.3 (*s*)	C-11′	131.3 (*s*)	131.2 (*s*)	131.2 (*s*)
C-12′	25.7 (*q*)	25.8 (*q*)	25.6 (*q*)	25.7 (*q*)	25.7 (*q*)	C-12′	25.7 (*q*)	25.7 (*q*)	25.7 (*q*)
C-13′	17.7 (*q*)	17.8 (*q*)	17.7 (*q*)	17.7 (*q*)	17.6 (*q*)	C-13′	17.7 (*q*)	17.7 (*q*)	17.6 (*q*)
C-14′	16.1 (*q*)	16.4 (*q*)	16.0 (*q*)	16.0 (*q*)	16.0 (*q*)	C-14′	16.0 (*q*)	16.0 (*q*)	16.0 (*q*)
C-15′	16.0 (*q*)	16.2 (*q*)	16.2 (*q*)	16.3 (*q*)	16.2 (*q*)	C-15′	16.2 (*q*)	16.2 (*q*)	16.2 (*q*)
2-OMe						2-OMe	61.0 (*q*)	55.8 (*q*)	62.3 (*q*)
3-OMe						3-OMe	55.9 (*q*)		60.8 (*q*)
4-OMe						4-OMe	60.8 (*q*)	55.6 (*q*)	61.0 (*q*)

[a] Chemical shifts from TMS in $CDCl_3$ and assignments from HMQC and HMBC spectra.

[b] ^{13}C NMR spectral data of grifolin (**1**) and neogrifolin (**2**) (Ishii et al., 1988).

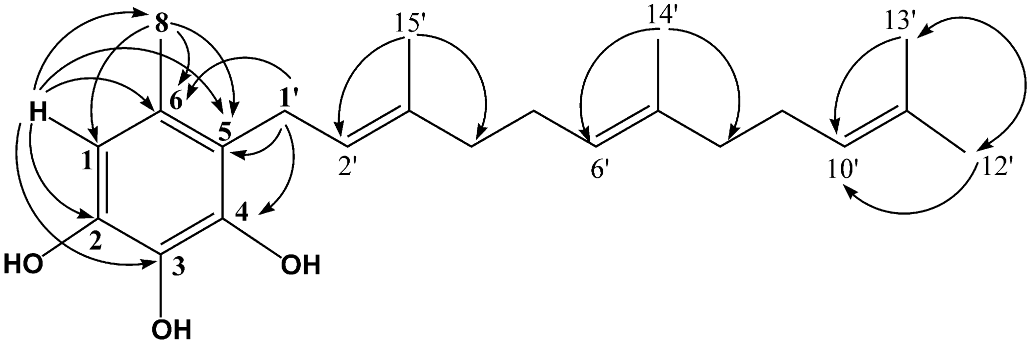

Fig. 2. HMBC correlations of 3-hydroxyneogrifolin (4).

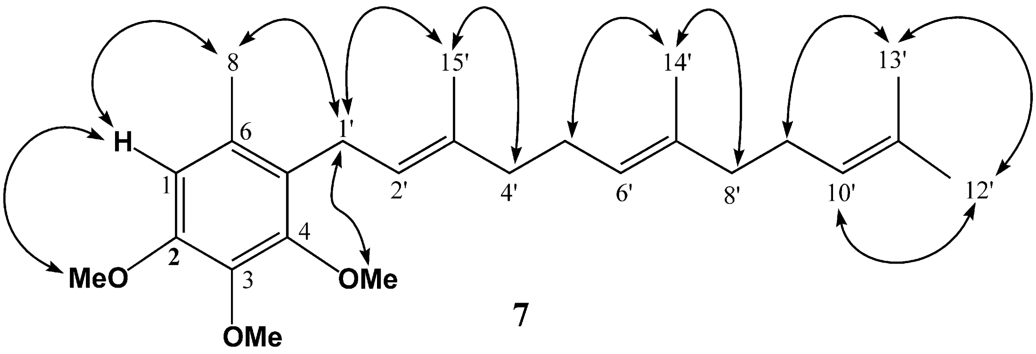

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Fig. 3. NOESY correlations of compound 7.

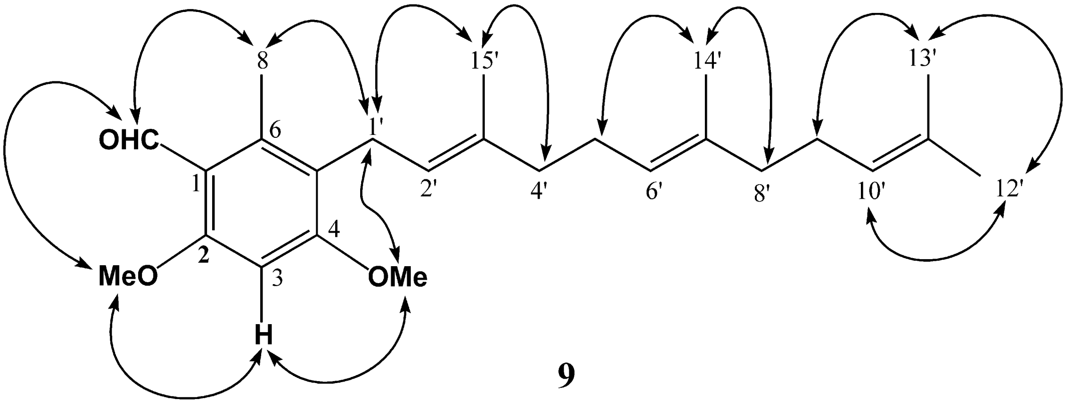

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Fig. 4. NOESY correlations of compound 9.

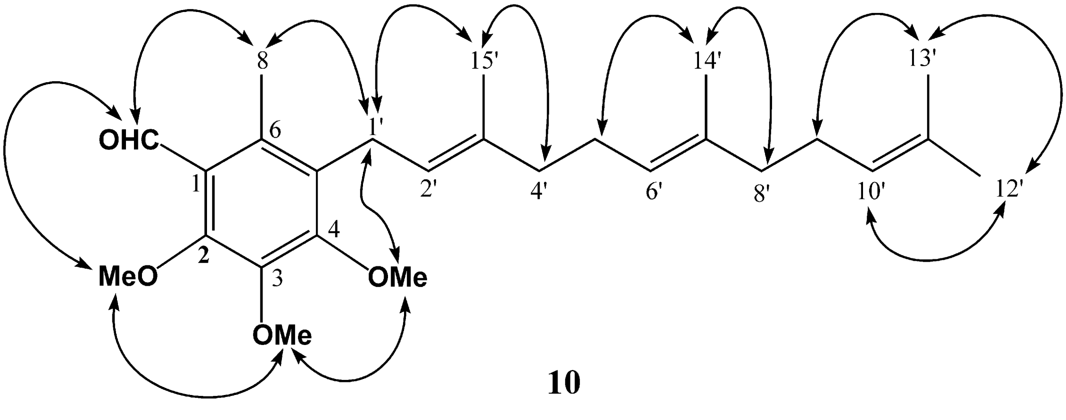

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Fig. 5. NOESY correlations of compound 10.

2.4. 1-Formyl-3-hydroxyneogrifolin

HR–EIMS showed that 1-formyl-3-hydroxyneogrifolin (**6**) has a molecular formula, $C_{23}H_{32}O_4$ (M^+ m/z 372.2296) possessing more one oxygen in comparison with that of **5**. The spectral data (IR, UV, ^1H and ^{13}C NMR) of **6** were very similar to those of **5** except for the disappearance of the C_3-aromatic proton. The methylation (MeI/K_2CO_3/reflux) of **5** afforded the trimethyl ether **10** [δ_H 3.88, 3.91, 3.95 (each 3H, *s*)] indicating the presence of three phenolic hydroxyl groups. The structure of **5** was based on the analysis of 2D NMR (HMBC and NOESY) spectra. In the NOESY correlations of **10** (Fig. 5), the NOEs between (i) H-7 and 2-OMe (ii) H-7 and H-8, (iii) H-1′ and H-8, and (iv) H-1′ and 4-OMe were observed. Thus, the structure of **6** was established as the C-3 hyroxylated derivative of 1-formylneogrifolin (**5**).

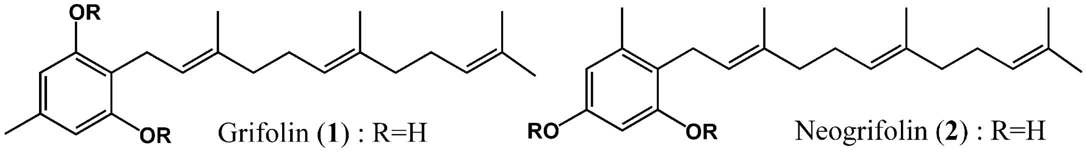
Table 3
Distribution of grifolin (**1**), neogrifolin (**2**), grifolic acid (**3**), compounds **4–6**, and compounds **11–14** in *Albatrellus* species

Species	**1**	**2**	**3**	**4–6**	**11–14**
Japanese *A. ovinus*	+[a]	+ +		+ + + +	
European *A. ovinus*	+ + + +	+ + + +			+ + + +
A. confluens	+ + + +	+ + + +			
A. dispansus	+ + +	+	+ + + +		
A. caeruleoporus	+ + + +	+ + + +			
A. yasudai	+	+	+ + + +		

[a] The symbol (+ ∼ + + + +) was shown on the base of a concentration of products.

2.5. Distribution of grifolin, neogrifolin and their derivatives in Albatrellus species

Table 3 shows the distribution of grifolin (**1**), neogrifolin (**2**) and related compounds that are the most important chemical markers in *Albatrellus* species. Grifolin (**1**) and neogrifolin (**2**) were isolated from *A. confluens* and *A. caeruleoporus* as the main products. Grifolic acid (**3**) was isolated from *A. dispandus* and *A. yasudai* as the main product. Vrkoc (1977) reported the isolation and structural characterization of grifolin (**1**), neogrifolin (**2**), their dimethyl ethers (**11** and **12**) and their diacetates (**13** and **14**) from a European *A. ovinus* (Fig. 6). It is noteworthy that the Japanese *A. ovinus* elaborates the



Grifolin (**1**) : R=H
11 : R=Ac
12 : R=Me

Neogrifolin (**2**) : R=H
13 : R=Ac
14 : R=Me

Fig. 6. Chemical constituents isolated from European *Albatrellus ovinus*.

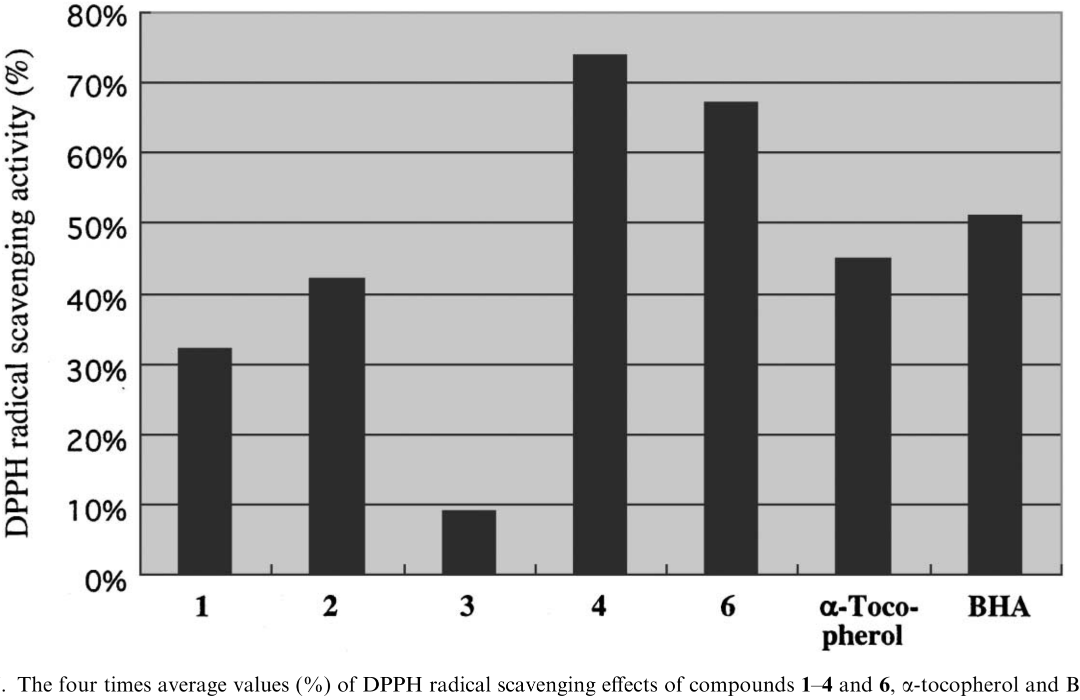


Fig. 7. The four times average values (%) of DPPH radical scavenging effects of compounds **1–4** and **6**, α-tocopherol and BHA.

characteristic 1-formyl and 3-hydroxy derivatives (**4**–**6**) of **2** without compounds **11**–**14**.

2.6. Anti-oxidative activities of grifolin, neogrifolin and their related compounds

The diphenyl-*p*-picrylhydrazyl (DPPH) radical scavenging effects of compounds **4** and **6** were compared with those of compounds **1**–**3**, and known anti-oxidants, α-tocopherol and BHA (*tert*-butylhydroxy-anisole) at a concentration of 20 μM, as the indicator of their anti-oxidative activity. DPPH radical scavenging effects of compounds **4** and **6** were stronger than compounds **1**–**3**, α-tocopherol and BHA as shown in Fig. 7.

3. Experimental

3.1. General

IR spectra were measured on a Jasco FT-IR 500 spectrophotometer. ^1H and ^{13}C NMR were recorded on Varian Untiy 600 (^1H; 600 MHz, ^{13}C; 150 MHz) or Varian Unity 200 (1H; 200 MHz, ^{13}C; 50 MHz) spectrometers. The solvent used for NMR spectra was $CDCl_3$ unless otherwise stated. Mass spectra were measured on either Jeol JMS HX-100 or Jeol AX-500 spectrometer. The specific rotation and the UV spectra were taken on JASCO DIP-140 polarimeter and a Hitachi U-3000 spectrometer, respectively. Silica gel 60 for column chromatography was purchased from Merck.

3.2. Plant material

A. ovinus was collected in Musashimurayama, Tokyo, Japan, in October 2000, and identified by Mr. Y. Goto. A voucher specimen was deposited at the Institute of Food Culture, Kurashiki Sakuyo University.

3.3. Extraction and isolation

Dry material of *A. ovinus* (3.18 g) was extracted with MeOH (200 ml×3) ultrasonically for 2 h at rt. After filtration, the brown solution was evaporated in vacuo to afford a brown residue (1.23 g) which was partitioned between H_2O and EtOAc and the EtAOc layer evaporated in vacuo to afford a residue (723.8 mg). The residue was subjected to Sephadex LH-20 chromatography $CHCl_3$:MeOH (1:1) to give 4 fractions. From fr. 4, 3-hydroxyneogrifolin (**4**) (127.0 mg) was obtained as white amorphous powder. A yellow oil (290.2 mg) from fr. 3 was applied to a silica gel (100 g) column with a gradient solvent system of *n*-hexane–EtOAc increasing the amounts of EtOAc stepwise in 5% increments to give 52 fractions. 1-Formyl-3-hydroxyneogrifolin (**6**) (61.9 mg) and neogrifolin (**2**) (94.6 mg) were obtained from 30% EtOAc–*n*-hexane (Fr. 14–18) and 50% EtOAc-*n*-hexane (Fr. 40–52), respectively. The crude oil (290.2 mg) from the 25% EtOAc-*n*-hexane eluate (Fr. 8–12) was further purified by reversed phase HPLC (Nucleosil $5C_{18}$; CH_3CN) to give grifolin (**1**) (10.6 mg), 1-formylneogrifolin (**5**) (19.5 mg) and 1-formyl-3-hydroxyneogrifolin (**6**) (3.2 mg), respectively. The spectral data (IR, ^1H and ^{13}C NMR) of compounds **1** and **2** were identical with those of grifolin (**1**) and neogrifolin (**2**) (Ishii et al., 1988).

*3.4. 3-Hydroxyneogrifolin (**4**)*

White amorphous powder, HR–MS: *m*/*z* 344.2359, $C_{22}H_{32}O_3$ requires 344.2351; EI–MS: *m*/*z* 344 (M^+, 11%), 207, 191, 153 (100%), 69; FT–IR (KBr) cm^{-1}: 3466, 3295 (OH), 1610, 1514, 1296, 1082, 1049; UV (EtOH) λ_{max}nm (logε): 210 (4.60), 276 (3.17); ^1H and ^{13}C NMR ($CDCl_3$) (Tables 1 and 2).

*3.5. 1-Formylneogrifolin (**5**)*

White plates, mp 98–102 °C, HR–MS: *m*/*z* 356.2376, $C_{23}H_{32}O_3$ requires 356.2351; EI–MS: *m*/*z* 356 (M^+, 10%), 219, 191, 165 (100%), 136; 69; FT–IR (KBr) cm^{-1}: 3235 (OH), 1631 (C=O), 1252, 1179, 1133; UV (EtOH) λ_{max} nm (logε): 202 (4.33), 239 (3.92), 293 (4.03), 338 (3.68); ^1H and ^{13}C NMR ($CDCl_3$) (Tables 1 and 2).

*3.6. 1-Formyl-3-hydroxyneogrifolin (**6**)*

Yellow needles, mp 83–84 °C; HR–MS: *m*/*z* 372.2296, $C_{23}H_{32}O_4$ requires 372.2301; EI–MS: *m*/*z* 372 (M^+, 30%), 235, 219, 181 (100%), 169; FT–IR (KBr)cm^{-1}: 3309 (OH), 1629 (C=O), 1275; UV (EtOH) λ_{max}nm (logε): 203 (4.54), 311 (4.14); ^1H and ^{13}C NMR ($CDCl_3$) (Tables 1 and 2).

*3.7. Methylation of 3-hydroxyneogrifolin (**4**)*

A solution of **4** (15.7 mg) in dry Me_2CO (10 ml) was treated with K_2CO_3 (200 mg) and methyl iodide (0.5 ml). The reaction mixture was refluxed for 24 h and filtered over celite. The filtrate was evapd in vacuo to give a colorless oil (57.5 mg). The crude product was purified by silica gel column chromatography with a hexane–EtOAc gradient to afford trimethyl ether **7** (4.2 mg; 24%) as a colorless oil. HR–MS: *m*/*z* 386.2831, $C_{25}H_{38}O_3$ requires 386.2821; EI–MS: *m*/*z* 386 (M^+, 44%), 249, 218, 195 (100%), 69; FT–IR (KBr) cm^{-1}: 1601, 1580, 1333, 1122; ^1H and ^{13}C NMR ($CDCl_3$) (Tables 1 and 2).

*3.8. Methylation of 1-formylneogrifolin (**5**)*

Compound **5** (4.4 mg) was treated with K_2CO_3 (264 mg) and MeI (1.0 ml) as described above to afford dimethyl ether **9** (3.8 mg; 81%) as a colorless oil, HR–MS: *m/z* 384.2654, $C_{31}H_{28}O_5$ requires 384.2665; EI–MS: *m/z* 384 (M^+, 47%), 247, 233, 193; FT–IR (KBr) cm^{-1}: 1674 (C=O), 1587, 1118, 1088; ^1H and ^{13}C NMR ($CDCl_3$) (Tables 1 and 2).

3.9. Methylation of 1-formyl-3-hydroxyneogrifolin (6)

Compound **6** (21.4 mg) was treated with K_2CO_3 (738 mg) and MeI (1.0 ml) as described above to afford trimethyl ether **10** (17.3 mg; 73%) as a colorless oil. HR–MS: *m/z* 414.2771, $C_{26}H_{38}O_4$ requires 414.2770; EI–MS: *m/z* 414 (M^+, 29%), 277, 247, 223, 191; FT–IR (KBr) cm−1: 1685 (C=O), 1103, 1037; ^1H and ^{13}C NMR ($CDCl_3$) (Tables 1 and 2).

3.10. Synthesis of 3-hydroxyneogrifolin (4)

To a solution of 5-methylpyrogallol (**8**) (40 mg) in benzene (5 ml) was added Na (ca. 20 mg). After the reaction mixture was stirred at room temp for 1 h, farnesyl bromide (97.4 mg) was added dropwise over 10 min. The reaction mixture was further refluxed for 10 h and poured into ice cold 10% HCl solution (50 ml) and extracted with EtOAc (50 ml×2). The organic layer was washed with brine, dried ($MgSO_4$) and evaporated under reduced pressure to give crude product (97.4 mg) which was subjected to preparative TLC (silica gel; *n*-hexane–EtOAc = 1:1) to afford the strating material **8** (27 mg, 68%) and compound **4** (21.4 mg; 22%), the spectral data (MS, IR, ^1H NMR) of which were identical to those of the natural 3-hydroxyneogrifolin (**4**).

3.11. Antioxidative activity

An aliquot of antioxidant solution (20 μM) in ethanol (2 ml) was mixed with the 100 mM sodium acetate buffer (pH 5.5, 2 ml) and then added to 1 ml of 500 μM diphenypicrylhydrazyl (DPPH) in ethanol (final concentration of 100 μM). The mixture was shaken vigorously and allowed to stand for 30 min at room temp in the dark. The absorbance (517 nm) of DPPH was measured with a UV-vis spectrophotometer.

The four times average values (%) of DPPH radical scavenging experiments indicated grifolin (**1**) (31.6 ± 0.4), neogrifolin (**2**) (40.9 ± 0.4), grifolic acid (**3**) (9.6 ± 0.1), 3-hydroxyneogrifolin (**4**) (72.8 ± 0.8), 1-formyl-3-hydroxyneogrifolin (**6**) (65.4 ± 0.4), *dl*-α-tocopherol (45.2 ± 0.3), and BHA (50.8 ± 2.1), respectively.

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