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# Grifolin derivatives from *Albatrellus caeruleoporus*, new inhibitors of nitric oxide production in RAW 264.7 cells

Dang Ngoc Quang,[a,d] Toshihiro Hashimoto,[a] Yuuki Arakawa,[a] Chie Kohchi,[b,c] Takashi Nishizawa,[b] Gen-Ichiro Soma[b,c,*] and Yoshinori Asakawa[a,*]

[a]*Faculty of Pharmaceutical Sciences, Tokushima Bunri University, Yamashiro-cho, Tokushima 770-8514, Japan*
[b]*Institute for Health and Sciences, Tokushima Bunri University, Yamashiro-cho, Tokushima 770-8514, Japan*
[c]*Institute of Drug Delivery Systems, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba 278-8510, Japan*
[d]*Faculty of Chemistry, Hanoi University of Education, 136 Xuan Thuy Road, Cau Giay, Hanoi, Vietnam*

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**Abstract**—Two new farnesyl phenols named grifolinones A and B, together with known grifolin and neogrifolin, were isolated from methanolic extract of the inedible mushroom *Albatrellus caeruleoporus*. Their structures were characterized by a combination of 2D NMR, MS, IR, and UV spectra. Grifolinone B was composed of two grifolin molecules, which were connected by a C–C bond. Grifolinones A and B, grifolin, and neogrifolin exhibited inhibitory activity against nitric oxide (NO) production stimulated by lipopolysaccharide (LPS) in RAW 264.7 cells with $IC_{50}$ values of 23.4, 22.9, 29.0, and 23.3 μM, respectively.


## 1. Introduction

On inflammatory stimulation, macrophages produce NO, prostanoids, and proinflammatory cytokines such as interleukin-1β and TNF-α.[1] NO is generated by NO synthase (NOS) and induces tissue injury at the inflammatory site.[2] There are three distinct isoforms of NOS, neural, endothelial, and inducible (iNOS).[3] Among them, iNOS is induced to express and generate a large amount of NO.[4] Overproduction of NO by iNOS has been implicated in various pathological processes including septic shock, tissue damage following inflammation, and rheumatoid arthritis.[5–9] Therefore, suppression of NO production using drugs might be useful for the treatment of inflammatory diseases.

A previous chemical investigation of *Albatrellus* sp. belonging to the Scutigeraceae family has revealed that they are abundant sources of grifolin (**3**) and its isomer neogrifolin (**4**).[10] These compounds and their derivatives possess many interesting biological activities, such as anti-oxidative,[10] antimicrobial,[11–13] plant growth inhibitory,[14] tyrosinase inhibitory,[15] controlling the anticholesteremic activity level in blood and liver,[16] promoting melanin synthesis by B16 melanoma cells[17] and activity on human and rat vanilloid receptor 1 (VR1).[18]

In the course of our investigation of the biologically active substances, we have reported the antioxidant, antimicrobial, TNF-α inhibitory, and anti-HIV activities of grifolic acid derivatives.[10,13] In continuation, we studied the chemical constituents of the inedible mushroom *Albatrellus caeruleoporus* and isolated two new compounds, one of which is a dimer of grifolin derivatives. We report here their isolation, structural characterization, and inhibition of NO production stimulated by LPS in RAW 264.7 cells.

## 2. Results and discussion

Methanolic extract of *A. caeruleoporus* was condensed under reduced pressure and low temperature. Sephadex LH-20, silicagel column chromatography, followed by further purification using MPLC, led to the isolation of four compounds (**1**–**4**) (Fig. 1). Their identification was conducted by HR-MS, 2D NMR, UV, and IR

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* Corresponding authors. Tel.: +81 88 62 29 611; fax:+81 88 65 53 051 (Y. A.); tel.: +81 88 62 29 611; fax: +81 88 62 23 2 17 (G. S.); e-mail addresses: sma5628@tokushima.bunri-u.ac.jp, asakawa@ph.bunri-u.ac.jp

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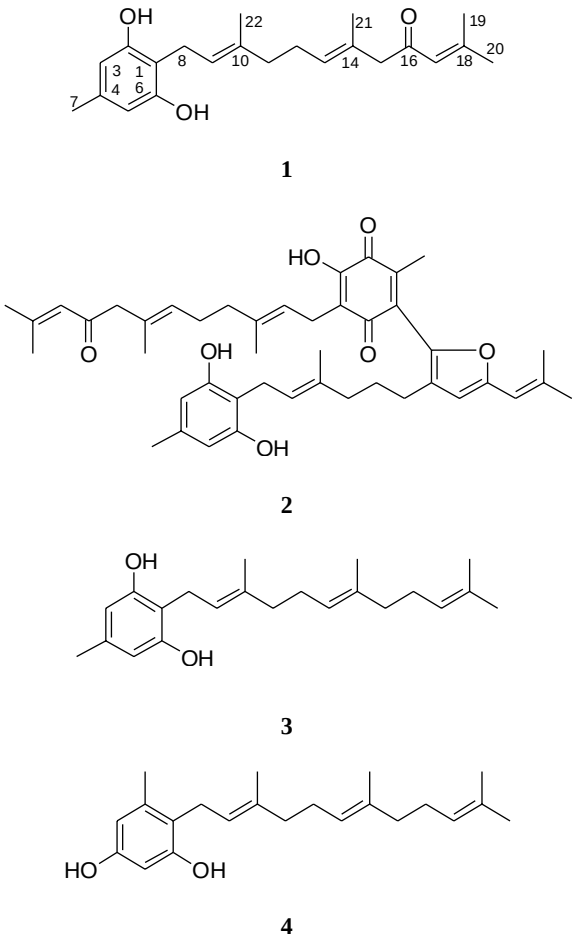


**Figure 1.** Structures of **1**–**4**.

spectra, and compared with those of previous publications. Accordingly, **1** and **2** are new compounds, while **3** and **4** are known and have been reported as grifolin (**3**)[19] and neogrifolin (**4**).[20]

Grifolinone A (**1**) was isolated as an oil and was assigned the molecular formula as $C_{22}H_{30}O_3$ on the basis of HREIMS, possessing one more oxygen and two less hydrogen atoms as compared with those of **3** and **4**. Its IR and UV spectra displayed absorption at 1674, 1615, 1516 $cm^{-1}$, and 234, 302 nm, suggesting that **1** contained a conjugated ketone and aromatic groups. The $^1H$ NMR spectrum (Table 1) showed the presence of three olefinic protons, two meta-coupling aromatic protons, four vinyl methyls, and one phenyl methyl. Further investigation of its $^{13}C$ NMR spectrum (Table 2) revealed that **1** contained a conjugated ketone ($\delta_C$ 200.2) and two phenolic carbons ($\delta_C$ 154.9). A comparison of its spectral data with those of previous publication[19] shows that compound **1** is a grifolin derivative, with a notable difference in the ketone group. The location of this ketone was determined to be at C-16, partly corroborated by HMBC correlations between C-16 and H-15, H-17, and partly by a low-field shift of H-17 ($\delta_H$ 6.12) due to a conjugation between the double bond $C_{17–18}$ and this ketone. Two geometries at $C_{9–10}$, $C_{13–14}$

**Table 2.** $^{13}C$ NMR data of compounds **1** and **2** ($CDCl_3$, 150 MHz)

| Compound **1** | | Compound **2** | | | |
|---|---|---|---|---|---|
| Position | $\delta_C$ | Position | $\delta_C$ | position | $\delta_C$ |
| 1 | 110.6 | 1 | 110.5 | 1′ | 136.9 |
| 2 | 154.9 | 2 | 154.9 | 2′ | 150.6 |
| 3 | 108.9 | 3 | 108.8 | 3′ | 184.1 |
| 4 | 137.6 | 4 | 137.4 | 4′ | 137.3 |
| 5 | 108.9 | 5 | 108.8 | 5′ | 135.5 |
| 6 | 154.9 | 6 | 154.9 | 6′ | 185.2 |
| 7 | 21.1 | 7 | 21.1 | 7′ | 13.4 |
| 8 | 22.1 | 8 | 22.1 | 8′ | 22.2 |
| 9 | 122.4 | 9 | 122.4 | 9′ | 119.9 |
| 10 | 137.3 | 10 | 137.5 | 10′ | 137.3 |
| 11 | 39.2 | 11 | 39.3 | 11′ | 39.2 |
| 12 | 26.3 | 12 | 27.8 | 12′ | 26.7 |
| 13 | 128.7 | 13 | 25.4 | 13′ | 129.3 |
| 14 | 129.9 | 14 | 131.3 | 14′ | 129.3 |
| 15 | 55.2 | 15 | 111.1 | 15′ | 55.1 |
| 16 | 200.2 | 16 | 154.8 | 16′ | 200.3 |
| 17 | 123 | 17 | 114.1 | 17′ | 123 |
| 18 | 156.2 | 18 | 137.3 | 18′ | 156.4 |
| 19 | 20.8 | 19 | 20.3 | 19′ | 20.8 |
| 20 | 27.7 | 20 | 27.2 | 20′ | 27.8 |
| 21 | 16.4 | 21 | 140.4 | 21′ | 16.4 |
| 22 | 16.1 | 22 | 16 | 22′ | 16.2 |

**Table 1.** $^1H$ NMR data of compounds **1** and **2** ($CDCl_3$, 600 MHz)

| Compound **1** | | Compound **2** | | | |
|---|---|---|---|---|---|
| Position | $\delta_H$ (*J* in Hz) | Position | $\delta_H$ (*J* in Hz) | Position | $\delta_H$ (*J* in Hz) |
| 3,5 | 6.24 s | 3,5 | 6.22 s | 3′,5′ | |
| 7 | 2.20 s | 7 | 2.18 s | 7′ | 2.06 s |
| 8 | 3.38 d (6.6) | 8 | 3.34 d (7.1) | 8′ | 3.18 d (7.4) |
| 9 | 5.24 dt (1.1, 6.6) | 9 | 5.20 m | 9′ | 5.15 dt (1.1, 7.4) |
| 11 | 2.08 t (7.4) | 11 | 1.97 m | 11′ | 1.97 m |
| 12 | 2.16 m | 12 | 1.65 m | 12′ | 2.08 m |
| 13 | 5.19 t (6.6) | 13 | 2.20 m | 13′ | 5.19 m |
| 15 | 3.04 s | 15 | 6.20 s | 15′ | 3.03 s |
| 17 | 6.12 s | 17 | 6.02 s | 17′ | 6.11 t (1.1) |
| 19 | 2.15 d (1.1) | 19 | 1.97 s | 19′ | 2.15 d (1.1) |
| 20 | 1.87 d (1.1) | 20 | 1.91 s | 20′ | 1.88 d (1.1) |
| 21 | 1.60 s | 21 | | 21′ | 1.58 s |
| 22 | 1.80 s | 22 | 1.76 s | 22′ | 1.72 s |
| | | HO-2,6 | 5.49 brs | HO-2′ | 7.08 s |

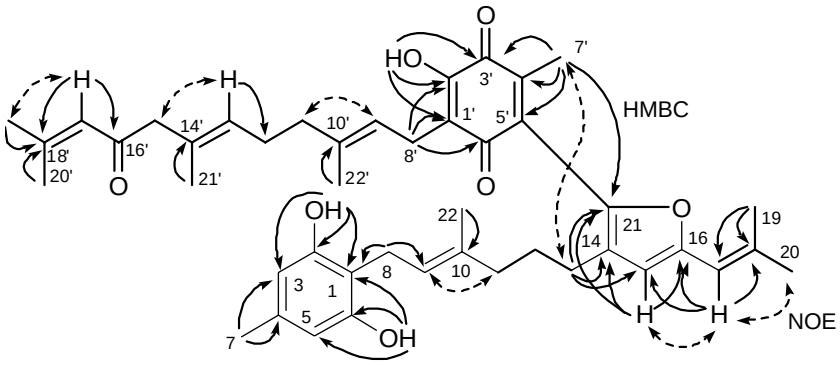


**Figure 2.** Important HMBC and NOESY correlations of **2**.

were *E-form*, as indicated by the NOESY spectrum. From the above discussion, grifolinone A (**1**) was characterized as 2,6-dihydroxy-4-methylphenyl-1-(3,7,11-trimethyldodeca-2*E*,6*E*,10-trien-4-one-1-yl).

Compound **2** was obtained as a purple oil and the FAB-MS spectrum indicated its molecular weight to be 694, and the high-resolution MS measurement pointed to the elemental composition as $C_{44}H_{54}O_7$. This was confirmed by the $^1H$ and $^{13}C$ NMR spectra, in which the signals of all hydrogens and carbons were visible. The structure could then be elucidated by analysis of the data obtained in 2D NMR experiments, whose HMBC and NOESY correlations are shown in Figure 2. The presence of two grifolin molecules was suggested by comparing their spectral data with those of **1**, **3**, and **4**. One of them has identical NMR spectral data with those of **1** from C-8′ to C-22′. A careful analysis of 2D NMR of this part from C-1′ to C-7′ revealed that the phenolic proton (2′-OH) coupled to C-1′, C-2′, and C-3′ ($\delta_C$ 184.1) and the phenyl methyl (H-7′) coupled to C-3′, C4′, and C-5′ in the HMBC spectrum, indicating the presence of *para*-quinone partial structure of **2**. This was confirmed by measuring the absorption band at 532 nm and 1646 $cm^{-1}$ in the UV and IR spectra. The absorption band of *para*-quinone shifted toward to a longer wavelength in its IR spectrum due to the hydroxyl (2′-OH) at *peri*-position.[21] This quinone is linked to C-8′ based on HMBC correlations between H-8′/C-1′, C-2′, and C-6′ ($\delta_C$ 185.2). The second part also possessed very similar NMR spectral data with those of grifolin (**3**) from C-1 to C-13, except for C-14 to C-21. In addition, C-16 and C-21 were rather low-field shifts, indicating that they must be connected to a strongly electron-withdrawing group. HMBC correlations from H-13 to C-14, C-15, and C-21, and from H-15 to C-14, C-16, and C-21 were shown, suggesting the presence of a furane ring in **2**. This furane ring was attached to C-17 due to HMBC correlations between H-17/C-15, C16, and C-18. Finally, the linkage between two monomers was clearly detected from C-5′ to C-21 by the HMBC correlation from the phenyl methyl (H-7′) and C-21 of the furane ring. All geometries were *E-form* based on the correlations in the NOESY spectrum (Fig. 2). From the above discussion, grifolinone B (**2**) was determined to be a dimer of grifolin derivatives. This is the first report on the isolation of a dimeric grifolin, which is connected via carbon–carbon bond, and the second example of grifolin having furane ring.[12] Interestingly, the finding of this dimeric compound possessing a *para*-quinone supports a slightly purple color of the fruit bodies of this fungus.[22]

### 2.1. Effects of 1–4 on LPS-induced production of NO

RAW 264.7 cells were preincubated for 1 h at 37 °C in a medium containing five different dilution of 40, 30, 20, 10 and 0 μM of each of compound **1**, **2**, **3**, or **4**. Then, LPS (1 μg/mL) was added to the cells and incubated further for 24 h. NO concentration in the conditioned medium was determined. All four compounds (**1**–**4**) were also examined as to whether they could activate RAW 264.7 or not, but, no result was obtained. In addition, when cells were preincubated with **1**–**4**, and then stimulated by LPS, there was a significant reduction in NO production as compared with the cells treated with LPS only. Moreover, the lower the concentration of the sample added, the higher amount of NO production detected (Fig. 3). This phenomenon indicated that **1**–**4** strongly inhibited the LPS-induced production of NO at 24 h with the $IC_{50}$ values of 23.4, 22.9, 29.0, and 23.3 μM, respectively. Especially, all four samples suppressed almost their NO production at a concentration of 40 μM and were lacked significant activity at 10 μM. A higher concentration (more than 50 μM) was also tested; however, cell viability was reduced due to their cytotoxicity.

The inhibitory activities of the NO synthesis of these compounds are strong as compared to $IC_{50}$ 88.4 μM of NG-methyl-L-arginine (L-NMMA), a known inhibitor of nitric oxide synthase.[23] Obviously, small structural differences in these compounds caused significance changes in activity. The presence of the conjugated ketone group at C-16 in **1** and **2** increased their activities, as compared to **3**. In addition, the location of phenolic hydroxyls also clearly affected the activity, that of neogrifolin (**4**) was stronger than that of grifolin (**3**). However, the presence of the furane ring and *para*-quinone in **2** did not support the inhibitory properties. Preliminary investigation of iNOS mRNA expression of compound

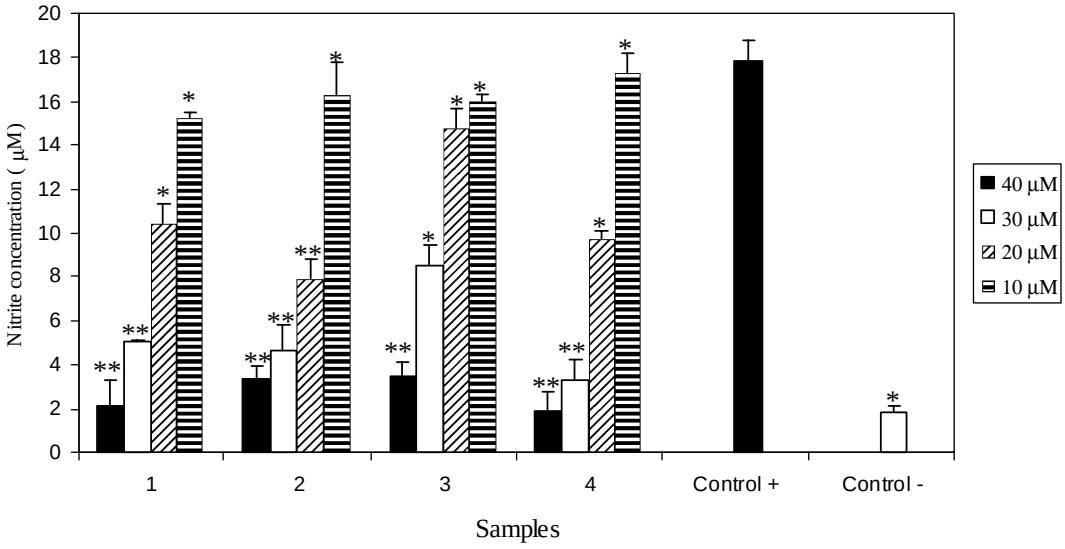


**Figure 3.** Inhibition of NO production of **1**–**4**. RAW cells were incubated in the presence of the indicated concentrations from 40 μM to 10 μM of **1**–**4** for 24 h in the presence (+) or absence (−) of LPS. From the supernatant, NO was detected and analyzed using Griess reagents. Data were derived from the three independent experiments. *$p < 0.05$ and **$p < 0.01$ indicate statistically significant differences from the LPS-treated group.

**1** by total RNA extraction, followed by RT-PCR, suggested that the inhibition of NO production was stimulated by LPS in RAW cells due to the suppression of iNOS gene expression. This finding suggests that these grifolin derivatives may have anti-inflammatory activity.

## 3. Experimental

### 3.1. General experimental procedures

IR spectra were measured on a PerkinElmer Spectrum One FT-IR spectrometer. UV spectra were obtained on a Shimadzu UV-1650PC instrument in MeOH. Column chromatography was carried out on silica gel 60 (0.2–0.5 mm, 0.04–0.063 mm, Merck) and Sephadex LH-20 (Amersham Pharmacia Biotech). Preparative medium-pressure liquid chromatography (MPLC) was performed with Work-21 pump (Lab-Quatec Co., Ltd, Japan) and a Lobar column (Merck). HPLC was performed on a Shimadzu Liquid chromatograph LC-10AS with RID-6A and SPD-10A detectors using a Waters 5C 18-AR-II or 5 SL-II column. The $^{1}H$ and $^{13}C$ NMR spectra were recorded on a Varian Unity 600 NMR spectrometer (600 MHz for $^{1}H$ and 150 MHz for $^{13}C$), using $CDCl_3$ as solvent. Chemical shifts are given relative to TMS ($\delta$ 0.00) as internal standard ($^{1}H$) and $\delta$ 77.0 (ppm) from $CDCl_3$ as standard ($^{13}C$). Mass spectra including high-resolution mass spectra were recorded on a JEOL JMS AX-500 spectrometer.

### 3.2. Fungus materials

Fruit bodies of *A. caeruleoporus* were collected in September 2004 in Okutama, Tokyo, Japan, and were identified by Mr. Yasuhiko Gotoh. A voucher specimen (No. 0401GY) has been deposited at the Faculty of Pharmaceutical Sciences, Tokushima Bunri University, Japan.

### 3.3. Extraction and isolation

Fresh fruit bodies of *A. caeruleoporus* (86.5 g) were extracted with MeOH and then the methanolic extract was concentrated to obtain 3.0 g of crude extract, which was separated by Sephadex LH-20 column chromatography, using MeOH–$CHCl_3$ (1:1) to give six fractions. Fraction 3 (251 mg) was subjected further to silica gel column, solvent system $CHCl_3$–MeOH (30:1), followed by MPLC using the same solvent system to give **2** (16.7 mg). Fraction 4 (190.9 mg) was separated by $SiO_2$ column, using $CHCl_3$–MeOH gradient from 0% MeOH, then MPLC, eluting with $CHCl_3$–MeOH (30:1), and finally by preparative HPLC, mobile-phase $CH_3CN$–$H_2O$ (4:1), flow rate 0.7 ml/min to yield **1** (4.4 mg). Fraction 5 (1115.5 mg) was purified by $SiO_2$ column, using hexane–EtOAc (3:1) to obtain **1** (23.0 mg), **3** (184.4 mg), and **4** (693.1 mg).

**3.3.1. Grifolinone A (1).** Oil; UV (MeOH) $\lambda_{max}$ (log $\varepsilon$) 302 (3.0), 234 (4.2) nm; IR (KBr) $\nu_{max}$ 3391, 1674, 1615, 1516 $cm^{-1}$; $^{1}H$ and $^{13}C$ NMR ($CDCl_3$) (Tables 1 and 2); EIMS *m/z* 342 $[M]^+$ (11), 328 (5), 244 (13), 205 (14), 191 (22), 177 (14), 175 (36), 137 (94), 83 (100), 69 (20), 55 (23), 43 (15), 41 (14); HREIMS *m/z* 342.2194 (calcd for $C_{22}H_{30}O_3$, 342.2195).

**3.3.2. Grifolinone B (1).** Oil; UV (MeOH) $\lambda_{max}$ (log $\varepsilon$) 532 (3.2), 395 (3.4), 272 (4.3), 235 (4.4) nm; IR (KBr) $\nu_{max}$ 3407, 1646, 1614, 1433, 1311 $cm^{-1}$; $^{1}H$ and $^{13}C$ NMR ($CDCl_3$) (Tables 1 and 2); FABMS: *m/z* 694 $[M]^+$; HRFABMS *m/z* 694.3829 (calcd for $C_{44}H_{54}O_7$, 694.3870).

### 3.4. Inhibition of NO production

RAW 264.7 cells, mouse macrophage cell line, were grown in RPMI 1640 supplemented with 10% fetal bovine serum, kanamycin (50 μg/mL), and ampicillin (60 μg/mL) at 37 °C in an atmosphere of 5% $CO_2$ and

95% air. Then, 100 μL of RAW-264.7 cells ($8 \times 10^5$ cells/mL) was pipetted in each well and cultured in a 96-well culture plate. After 24 h incubation, 50 μL of medium containing various concentrations of each grifolin derivative, which was diluted in DMSO, was added to each well. Then, 50 μL of LPS (4 μg/mL) or only the medium was added to each well. The cells were incubated further at 37 °C for 24 h. The supernatant (35 μL) of each well was taken, then mixed with Griess reagents[24] (35 μL), and their absorbance at 550 nm was measured using Bio-Rad model 550 Microplate reader. NO concentrations were determined by measuring the amount of nitrite in the cell culture supernatant using Griess reagents, as described in Figure 3.

### 3.5. Statistical analysis

All experiments were repeated three times and the results are expressed as means ± SEM. The data were analyzed by *t* test using SPSS software (12.0 version). The differences were considered statistically significant at *p* value less than 0.05.

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