HETEROCYCLES, Vol. 65, No. 10, 2005, pp. 2431 - 2439
Received, 4th July, 2005, Accepted, 10th August, 2005, Published online, 12th August, 2005

ISOLATION, SYNTHESIS AND BIOLOGICAL ACTIVITY OF GRIFOLIC ACID DERIVATIVES FROM THE INEDIBLE MUSHROOM *ALBATRELLUS DISPANSUS*

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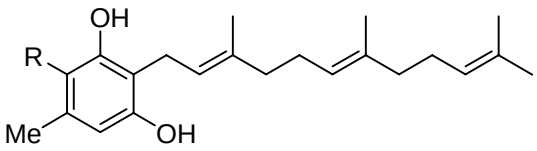
Abstract-Grifolin, grifolic acid, grifolic acid methyl ester and a new compound grifolinol were isolated from methanolic extract of the inedible mushroom *Albatrellus dispansus* (Scutigeraceae). Their structures were established by a combination of 2D NMR, MS spectrum and chemical reaction. In addition, (+)- and (−)-daurichromenic acids were obtained by cyclization of grifolic acid. The inhibitory activity of TNF-α, anti HIV and antimicrobial activity of grifolic acid derivatives were examined.

1. INTRODUCTION

Since grifolin and neogrifolin were isolated from the inedible mushroom *Albatrellus ovinus*,[1] not only the chemical constitutions of other *Albatrellus* sp. but also their useful biological activities have been reported. The previous publications showed that grifolin derivatives possess broad-spectrum of biological activity, such as antimicrobial activity,[2,3] plant growth inhibitory,[4] tyrosinase inhibitory,[5] anticholesteremic activities level in blood and liver,[6] promotion melanin synthesis by B16 melanoma cells,[7] activity on human and rat vanilloid receptor 1 (VR1).[8] In the course of our investigation on the biological active substances from inedible mushrooms, we also reported the antioxidant activity of neogrifolin derivatives from *A. ovinus*.[9] In continuation, we could obtain a large amount of the fruit bodies of A. *dispansus*, which allowed us to study its

chemical constitutions, biological activity and also chemical conversions. The outcome of these efforts is the subject of the current paper.



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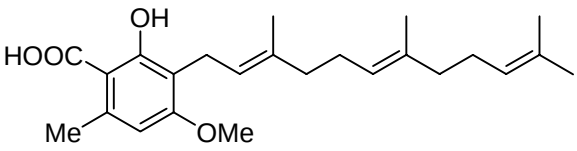

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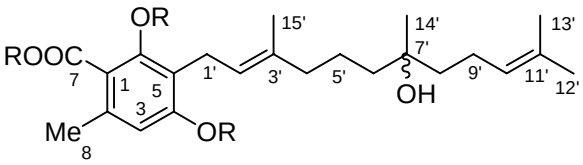

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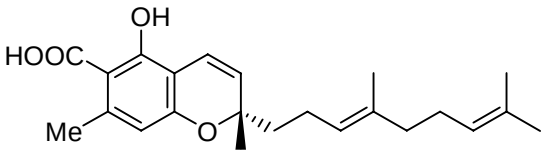
7. R = Me



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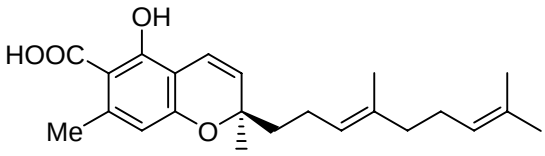

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Figure 1. Structures of **1**-**7**.

2. RESULTS AND DISCUSSION

2.1. Isolation and characterization of grifolin derivatives:

The methanolic extract of the fruit bodies of *A. dispansus* was partitioned between EtOAc and water, then the EtOAc layer was concentrated and subjected repeatedly to silica gel, reverse-phase (C-18) column to give grifolic acid (**1**),[10] grifolin (**2**),[10] grifolic acid methyl ester (**3**)[11] and a new compound, grifolinol (**4**) as described in **EXPERIMENTAL**.

The HRFABMS spectrum of grifolinol (**4**) showed the molecular ion peak at *m/z* 413.2318 corresponding to the molecular formula $C_{23}H_{34}O_5Na$ (calcd for $C_{23}H_{34}O_5Na$, m/z 413.2304). The IR spectrum revealed the absorption band of a carboxyl group attached to aromatic ring (2660-3400 and 1615 cm^{-1}), confirmed in the UV spectrum with maxima at 260 and 300 nm and the chemical shift at δ_C 175.9. Interpretation of 2D NMR spectrum of **4** allowed to the assignment of three vinylic methyls, two olefinic protons, one aromatic methyl, and two phenolic carbons. In comparison of its NMR spectral data (Table 1) with those of grifolic acid[10] suggested that **4** is formed

from grifolic acid (**1**) by addition of one water molecule to the double bond C6'-7' and the hydroxyl group linked to a tertiary carbon C-7' (δ_C 73.7). This is proved by the HMBC correlations between H-14' and C-6', C-7' and C-8'. Methylation of **4** (MeI/K_2CO_3/reflux) afforded trimethylgrifolinol (**7)** with the molecular formula of $C_{26}H_{40}O_5$ established by HRFABMS. Investigation of its NMR spectrum revealed the presence of three methoxyl groups, which were located at C-4, C-6 and C-7 due to the HMBC correlations between the protons of methoxyl groups and C-4, C-6 and C-7. Consequently, **4** was determined to be 6',7'-dihydro-7'-hydroxygrifolic acid as shown in figure 1 and named grifolinol.

2.2. Preparation of (±)-daurichromenic acids:

Since (+)-daurichromenic acid (**5**) was reported to show highly potent anti-HIV activity with an EC_{50} value of 0.00567 μg/mL,[12] it has been totally synthesized in five steps with 49% overall yield[13] and even better in four steps.[14] However, none of them mentioned about (-)-daurichromenic acid (**6**) and how to purified two isomers so far. Thus, (±)-daurichromenic acids (**5**,**6**) were successfully prepared from grifolic acid (**1**), a major component from this fungus with only one step in the presence of dichlorodicyanobenzoquinone (DDQ) in benzene and stirred for 30 min at 90°C, in good yield (73 %). Then, (+) and (-)-daurichromenic acids were separated by HPLC equipped with chiral column (Figure 2). Nevertheless, their spectral data are identical, except for their optical rotations. In comparison their spectral data with those of previous publication,[12] compounds (**5**) and (**6**) were determined to be (+) and (-)-daurichromenic acids with the optical rotation values of +30.9° and –29.9°, respectively. This is the first report of (-)-daurichromenic acid (**6**).

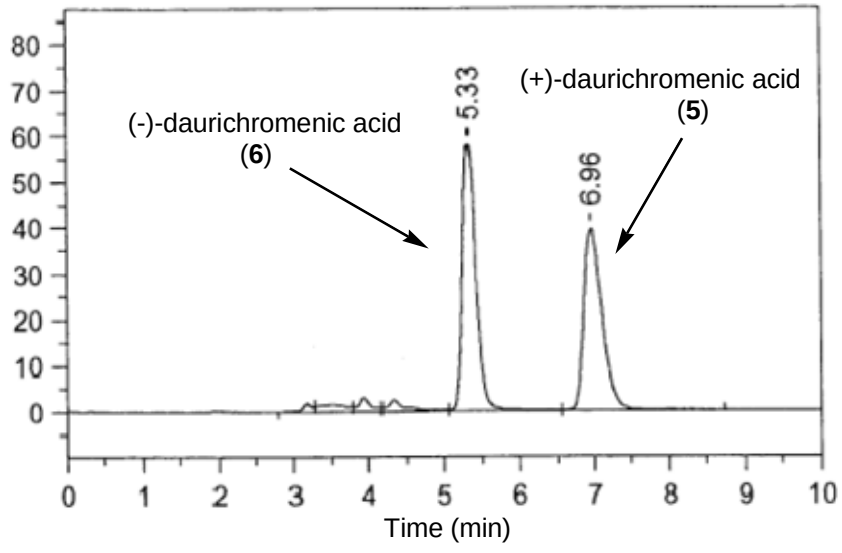

Figure 2. Isolation of (+) and (–)–daurichromenic acids by HPLC with chiral column (flow rate 1mL/min, UV detection at 254 nm).

Table 1. ^{1}H and ^{13}C NMR data of compounds (**4**) and (**7**) ($CDCl_3$).

	Compound (**4**)		Compound (**7**)	
position	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C
1		103.9		120.7
2		142.3		135.3
3	6.23 s	111.6	6.48 s	108.2
4		160.0		159.3
5		111.9		121.1
6		163.6		156.6
7		175.9		169.0
8	2.50 s	24.2	2.30 brs	19.9
1’	3.39 d (6.9)	21.9	3.31 d (6.6)	22.6
2’	5.25 t (6.9)	121.9	5.15 tq (6.9, 1.1)	123.0
3’		137.6		134.8
4’	2.01 m	39.9	1.95 t (7.4)	40.1
5’	1.46 m	21.9	1.38 m	22.2
6’	1.46 m	41.4	1.38 m	41.5
7’		73.7		72.8
8’	1.46 m	41.0	1.45 m	41.5
9’	2.01 m	22.6	2.00 q (7.4)	22.6
10’	5.10 t (7.1)	124.3	5.11 tq (7.1, 1.1)	124.5
11’		131.8		131.7
12’	1.67 s	25.7	1.61 brs	25.7
13’	1.60 s	17.6	1.68 d (1.1)	17.6
14’	1.17 s	26.6	1.14 s	26.8
15’	1.79 s	16.1	1.74 d (1.1)	16.0
6-OH	11.93 s			
4-OMe			3.82 s	55.7
6-OMe			3.75 s	62.6
7-OMe			3.90 s	52.1

2.3. Biological activity of grifolic acid derivatives:

Biological testing against human immuno-deficiency virus type 1 (HIV-1) were carried out with MT-4 cells ($1x10^4$ cells/ml). The MT-4 cells were infected with HIV-1 and cultured in the presence of various concentrations of **1**-**3**. After four days incubation at 37^oC, the number of viable cells was determined.[15] Accordingly, compounds (**1**-**3**) were devoid of anti-HIV activity (Table 2).

Table 2: Anti-HIV effects of grifolic acid derivatives.

Samples	EC_{50} (μM)	CC_{50} (μM)
Grifolic acid (**1**)	> 40	40
Grifolin (**2**)	> 38	38
Grifolic acid methyl ester (**3**)	> 53	53
(+)-Daurichromenic (**5**)*	0.00567 μg/ml (0.15 μM)	

* Kashiwada et al., 2001.

Tumor necrosis factor-alpha (TNF-α) is one of the most important proinflammatory cytokines and is produced mainly by activated monocytes and marcrophages.[16] It induced various biological responses including tissue injury, shock and apoptosis.[16,17] TNF-α also induces the secretion of cytokines such as interleukin-1 (IL-1), IL-6, and IL-10 and activates T-cells and other inflammatory cells.[18] Thus, inhibition of production of TNF-α by using drugs might be useful for treatment of inflammatory diseases. The suppression of the production of TNF-α of grifolic acid derivatives (**1**-**3, 5** and **6**) was tested and their results were illustrated in table 3. Grifolin (**2**) strongly suppressed TNF-α production with IC_{50} values of 3.98 μM (Table 3), while others compounds exhibited weak and similar inhibitory activity. The finding suggested that the presence of the carboxylic group at C-1 in **1**, **3** and **5**, **6** decreased the inhibitory activity.

Table 3: Inhibitory activity of TNF-α of grifolic acid derivatives.

Samples	IC_{50} (μM)
Grifolic acid (**1**)	20.3
Grifolin (**2**)	3.98
Grifolic acid methyl ester (**3**)	28.0
(+)-Daurichromenic (**5**)	27.8
(–)-Daurichromenic (**6**)	28.4

Previous studies repeatedly pointed out that grifolic acid derivatives possessed an interesting antimicrobial activity.[2,3] However, only a few microorganisms have been tested. In this time, grifolic acid derivatives were tested against seven microorganisms, together with gentamicin (standard for anti-bacteria) and nystatin (standard for anti-fungi). Their antimicrobial activities were shown in Table 4. These samples have exceedingly good activity, in some cases three times stronger than that of standards. Especially, *K. pneumoniae*, an important medicinal pathogen was the most susceptible to the samples.

Table 4: Antimicrobial activity of grifolic acid derivatives (diameter of the zone of growth inhibition, bactericidal or fungicidal zone in mm, including the diameter of disc, 6 mm).

Microorganism/Sample	**1**	**2**	**3**	**5**	**6**	Gentamicin	Nystatin
E. coli	30	27	20	32	32	16	nt
K. pneumoniae	44	40	42	43	44	14	nt
P. aeruginosa	27	20	16	24	22	16	nt
S. aureus	27	19	17	24	25	15	nt
S. enteritidis	28	18	26	26	38	18	nt
A. niger	29	18	23	28	29	nt	18
C. albicans	32	20	26	30	28	nt	17

nt = not tested.

In conclusion, grifolic acid derivatives from *Albatrellus dispansus* possess potent inhibitory activity of TNF-α and antimicrobial activity. (+)-Daurichromentic acids (**5**) demonstrated potent anti-HIV activity could be synthesized from grifolic acid (**1**) with only one step. Further studies on these biological activities of these compounds should be done, since this fungus produces a large amount of grifolic acid derivatives.

3. EXPERIMENTAL

3.1. General

TLC was performed on silica gel plates (Kieselgel 60 F254, Merck) and reverse-phase C_{18} silica gel plates (Merck). Column chromatography was carried out on silica gel 60 (0.2-0.5 mm, 0.04-0.063 mm, Merck) and reverse-phase C-18 (Cosmosil 75C_{18}-OPN, Nacalai Tesque). HPLC was performed on a Shimadzu liquid chromatograph LC-10AS with RID-10A and SPD-10A

detectors. IR spectra were measured on a Perkin Elmer Spectrum One FT-IR spectrometer. UV spectra were obtained on a Shimadzu UV-1650PC in MeOH solution. Optical rotations were measured on a JASCO DIP-1000 polarimeter with $CHCl_3$ as a solvent. NMR spectra were recorded on a Varian Unity 600 (600 MHz for ^1H NMR and 150 MHz for ^{13}C NMR) using $CDCl_3$ as solvent. Mass spectra including high-resolution FAB mass spectra were recorded on a JEOL JMS AX-500 spectrometer.

3.2. Material

Fruit bodies of *Albatrellus dispansus* were collected in October 2002 in Okutama, Tokyo, Japan, and identified by Mr. Yasuhiko Gotoh. A voucher specimen (No.GY020101) has been deposited at the Faculty of Pharmaceutical Sciences, Tokushima Bunri University, Japan.

3.3. Extraction and isolation

Fresh fruit bodies of *Albatrellus dispansus* (715.4 g) were extracted with MeOH (2 x 2L) at room temperature for two weeks and the methanolic extract was partitioned between EtOAc and water. The EtOAc layer was concentrated to give a residue (23.17 g), which was chromatographed on SiO_2 column, using a linear gradient of 5% EtOAc-hexane to 100% EtOAc to afford fifteen fractions (Fr. 1-15). Fr-2 (5.5 g) was purified by reverse-phase column, eluting by H_2O-CH_3CN (1:4) to afford grifolic acid (**1**) (2.35 g) and grifolic acid methyl ester (**3**) (0.65 g). Fr. 3-4 (10.4 g) was recrystallized in n-hexane-Et_2O to give grifolic acid (**1**) (7.92 g) as a white crystal (mp 93-94^oC) and a mother liquid. The mother liquid (3.3 g) was further subjected to reverse-phase column, H_2O-CH_3CN (1:4) as eluent, to obtain grifolic acid methyl ester (**3**) (0.17 g) and a mixture (1.47 g) which was purified by silica gel column using hexane-EtOAc gradient to yield grifolic acid (**1**) (0.61 g) and grifolin (**2**) (0.63 g). Fr-8 (0.31 g) was purified by reverse-phase column, H_2O-CH_3CN (1:9) to give grifolinol (**4**) (40.1 mg).

*Grifolinol (**4**)*: $[\alpha]_D^{20}$ – 4.5^o (c 0.62, $CHCl_3$); HRFABMS: *m/z* 413.2318 $[M+Na]^+$, $C_{23}H_{34}O_5Na$, requires 413.2304; IR (KBr) cm^{-1}: 2660-3400, 1615, 1267, 1087; UV λ_{max} (EtOH) nm (logε): 210 (4.3), 221 (4.4), 260 (3.9), 300 (3.5); ^1H and ^{13}C NMR ($CDCl_3$) (Table 1).

3.3.1. Preparation of (+)- and (–)-*daurichromenic acids (**5**,**6**):*

A solution of grifolic acid (600 mg) in dry benzene (60 mL) was added DDQ (550 mg). The

reaction mixture was stirred at 90°C for 30 min, filtered and evaporated to give a residue (994 mg), which was subjected to reverse-phase column, using H_2O-CH_3CN (1:9) to afford a mixture of **5** and **6** (463 mg). The isomer mixture (436 mg) was further separated by HPLC with chiral column (Chiralpak AD-H, 1x25 cm, Daicel Chemical Industries, LTD, Japan), mobile phase MeOH-AcOH (100:0.1), flow rate 1 mL/min, and UV detection at 254 nm to give (-)-daurichromenic acid (**6**) (203 mg), $[\alpha]_D^{20}$ – 29.9° (c 1.03, $CHCl_3$) and (+)-daurichromenic (**5**)(208 mg), $[\alpha]_D^{20}$ + 30.9° (c 1.0, $CHCl_3$), Rt 5.33 and 6.96 min, respectively.

3.3.2. Preparation of trimethylgrifolinol *(**7**):*

A solution of grifolinol (8.4 mg) in dry Me_2CO (10 mL) was added K_2CO_3 (1.363 g) and MeI (1.2 mL). The reaction mixture was refluxed for 10 h and filtered over celite. The filtrate was concentrated *in vacuo* to obtain a residue (30.6 mg), which was purified by silica gel column, hexane-EtOAc gradient to yield **7** (3.4 mg) as oil. HRFABMS: *m/z* 455.2768 $[M+Na]^+$, $C_{26}H_{40}O_5Na$, requires 455.2773; ^1H and ^{13}C NMR ($CDCl_3$) (Table 1).

3.3.3. Biological tests:

Biological testing against human immuno-deficiency virus type 1 (HIV-1) were carried out with MT-4 cells in culture as described previously.[15]

Inhibitory activity of TNF-α of grifolic acid derivatives was tested according to the method reported in Hashimoto et al. (2003).[19]

Antimicrobial activity was assayed in analogy to a conventional plate diffusion assay as described in reference.[20]

ACKNOWLEDGEMENTS

We thank Dr. M. Tanaka and Miss Y. Okamoto (TBU, Japan) for recording NMR and mass spectra, respectively. Thanks are due to Prof. M. Baba (Faculty of Medicine, Kagoshima University, Kagoshima, Japan), Mr. N. Radulovic (University of Niš, Serbia and Montenegro) and Dr. M. Yamada and Dr. T. Kohno (Meiji Milk Products Co. LTD, Japan) for anti-HIV, antimicrobial and inhibition of TNF-α assays, respectively. Our special acknowledgments are also due to Mr. Y. Gotoh and Miss Y. Nomura for their collection and identification of the mushroom and technical assistance.

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