# Activity *in vitro* and *in vivo* against Plant Pathogenic Fungi of Grifolin Isolated from the Basidiomycete *Albatrellus dispansus*

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Z. Naturforsch. **60 c,** 50–56 (2005); received April 14/September 27, 2004

In the course of screening for novel naturally occurring fungicides from mushrooms in Yunnan province, China, the ethanol extract of the fruiting bodies of *Albatrellus dispansus* was found to show antifungal activity against plant pathogenic fungi. The active compound was isolated from the fruiting bodies of *A. dispansus* by bioassay-guided fractionation of the extract and identified as grifolin by IR, $^{1}$H and $^{13}$C NMR and mass spectral analysis. Its antifungal activities were evaluated *in vitro* against 9 plant pathogenic fungi and *in vivo* against the plant disease of *Erysiphe graminis*. *In vitro*, *Sclerotinina sclerotiorum* and *Fusarium graminearum* were the most sensitive fungi to grifolin, and their mycelial growth inhibition were 86.4 and 80.9% at 304.9 $\mu$M, respectively. Spore germination of *F. graminearum*, *Gloeosporium fructigenum* and *Pyricularia oryzae* was almost completely inhibited by 38.1 $\mu$M grifolin. *In vivo*, the curative effect of grifolin against *E. graminis* was 65.5% at 304.9 $\mu$M after 8 days.

*Key words*: Grifolin, *Albatrellus dispansus*, Antifungal Activity, Plant Pathogenic Fungi

## Introduction

Research during the last decade has convincingly shown that natural products play an important role, not only in pharmacology, but also in agriculture as a rich source of bioactive components that can be used in crop protection (Wink, 1993). Some successful examples could be given in the past investigations, such as, pyrrolnitrin, produced by *Pseudomonas pyrrocinia*, and strobilurin A, produced by *Strobilurus tenacellus*. From pyrrolnitrin, two synthetic phenylpyrroles, fenpiclonil (Nevill *et al*., 1988) and fludioxonil (Gehmann *et al*., 1990), have been developed as seeddressing agents and fumigants against numerous fungal pathogens. Strobilurin A and oudemansin A are fungicidal natural products found in the basidiomycete fungus *S. tenacellus* (Pers. ex Fr.) Singer (Anke *et al*., 1977) and *Oudemansiella mucida* Hoehn (Musilek *et al*., 1969), respectively. Several fungicides, such as azoxystrobin (Godwin *et al*., 1992), kresoxim-methyl (Ammerman *et al*., 1992), metominostrobin (Mizutani *et al*., 1995) have been synthesized by using the mentioned two fungal metabolites as indicator and were recently commercialized. The mushrooms, as one of the main biological resources, are still an unexplored source of new agricultural chemicals.

Yunnan province, in the southwest of China, is one of the areas with the richest and diverse bioresources in the world based on its unique geoenvironment, diverse geomorphology and three differentiations of climate. About 1500 species of mushrooms are now known from Yunnan. Edible and inedible are about 600 and 900 species, including 10 poisonous species. Mushrooms in bio-resources belong to the very productive biological sources which produce a large and diverse variety of secondary metabolites. We have been interested in the biologically active substances present in the untapped and diverse source of mushrooms from Yunnan province. In our previous paper, we reviewed the new results of investigations on some basidiomycetes and ascomycetes of Yunnan including the structures of novel terpenoids, phenolics and nitrogen-containing compounds and their biological activities (Liu, 2002). Several prenylphenols from basidiocarps of European and Chinese *Albatrellus* spp., namely grifolin, neogrifolin, confluentin, scutigeral, and albaconol, were investigated concerning their activities in test models for vanilloid receptor modulation (Hellwig *et al*.,

0939–5075/2005/0100–0050 $ 06.00 © 2005 Verlag der Zeitschrift für Naturforschung, Tübingen · http://www.znaturforsch.com · D

2003). As part of a search for antifungal compounds from mushrooms in Yunnan province, in the southwest of China, grifolin was isolated from the fruit bodies of *A. dispansus* by bioassay-guided fractionation. Its activity against plant pathogenic fungi has been described in this report.

## Results and Discussion

The antifungal activities of crude ethanol extracts of 20 mushroom species of Yunnan were tested. The antifungal activities were assayed against 9 plant pathogenic fungi. The growth inhibitory activities of the ethanol extracts of the different mushrooms against plant pathogenic fungi are summarized in Table I. The data indicated that ethanol extract from fruit bodies of *A. dispansus* has considerable activities *in vitro* against all plant pathogenic fungi. It was found that the extract from *A. dispansus* almost completely inhibited the mycelial growth of *Sclerotinina sclerotiorum* (Lib.) de Bary and *Fusarium graminearum* Schw.

The EtOH extract of A. *dispansus* was decanted and evaporated and the residue was extracted with $CH_2Cl_2$/MeOH 1:1 v/v. Antifungal activity was detected in the extract of $CH_2Cl_2$/MeOH 1:1. The antifungal active extract was further chromatographed on a silica gel column. Fractions 1–4 eluted with $CHCl_3$/AcOEt (8:2, v/v) showed a high level of antifungal activity against 9 plant pathogenic fungi. The antifungal active fractions were rechromatographed on a silica gel column using petroleum ether/ethyl acetate (19:1, 9:1, v/v). The active compound was obtained as colorless needles after recrystallization from hexane. Comparison of the physicochemical properties with the reported data allowed to identifying this active

Table I. Radial growth inhibition of the ethanol extracts of 20 different mushrooms against 9 plant pathogenic fungi[a].

| Ethanol extract[c] | Plant pathogenic fungi[b] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| A | – | – | – | ± | – | ± | + | ± | – |
| B | – | – | ± | ± | – | ± | – | – | – |
| C | – | – | – | + | ± | + | ± | – | – |
| D | – | – | – | + | – | ± | + | ± | – |
| E | ++ | +++ | ++ | ++ | + | +++ | + | ++ | ++ |
| F | – | – | – | + | + | + | – | – | – |
| G | – | – | – | ± | – | – | – | – | – |
| H | – | – | – | ± | – | ± | ± | + | – |
| I | – | – | – | ± | ± | – | + | – | – |
| J | – | – | – | – | – | ± | ± | – | – |
| K | – | – | – | ± | + | + | ++ | ± | ± |
| L | – | + | ± | ± | + | + | ± | – | ± |
| M | – | – | – | – | ± | – | + | – | – |
| N | – | – | – | – | ± | – | + | – | – |
| O | – | – | ± | ± | – | – | ± | – | – |
| P | – | – | ± | – | ± | ± | ± | – | – |
| Q | – | – | – | ± | – | – | – | – | – |
| R | – | ++ | – | + | ± | ++ | ± | – | + |
| S | – | – | – | – | – | – | – | – | – |
| T | – | – | – | + | – | ± | – | – | – |

[a] The extract concentration (dry matter) in the culture medium was 1.0 mg/ml in all cases. The inhibition was reported as (–) <10%, (±) between 10 and 20%, (+) between 20 and 40%, (++) between 40 and 80%, (+ + +) 80% growth inhibition.

[b] 1, *Rhizoctonia solani* Khum; 2, *Sclerotinina sclerotiorum* (Lib.) de Bary; 3, *Botrytis cinerea* Per. ex Tris.; 4, *Gloeosporium fructigenum* Berk.; 5, *Pyricularia oryzae* Br. et Cav.; 6, *Fusarium graminearum* Schw.; 7, *Gaeumannomyces graminis* (Sacc.) Arx Oliver; 8, *Alternaria alternata* (Fries) Keissler; 9, *Fulria fulva* (Cooke) Ciferri.

[c] A, *Lactarius deliciosus* (Fr.) S. F. Gray; B, *Suillus granulatus* (Fr.) Kuntze; C, *Suillus placidus* (Bon.) Sing.; D, *Lactarius volemus* (Fr.) Fr.; E, *Albatrellus dispansus* (Lloyd) Canf. et Gilbn.; F, *Oligoporus floriformis*; G, *Auricularia polytricha* (Mont.) Sacc.; H, *Boletus rubellus* Krombh.; I *Cantharellus tubaeformis* (Bull.) Fr.; J, *Agaricus campestris* L.: Fr.; K, *Albatrellus ellisii* (Berk.) Pouz.; L, *Lactarius subzonarius* Hongo; M, *Auricularia auricula* (L.: Hook.) Underw.; N, *Heimiella retispora* (Pat. et Bak.); O, *Cantharellus cinnabarinus* Schw.; P, *Ganoderma lucidum* (Leyss.: Fr.) Karst.; Q, *Scleroderma citrinum* Pers.; R, *Tricholoma imbricatum* (Fr.: Fr.) Kummer; S, *Boletus griseus* Frost.; T, *Boletus* sp.

compound as grifolin (Fig. 1) (Goto *et al*., 1963; Mahiiou *et al*., 1995).

Grifolin isolated from *A. dispansus* inhibited the mycelial growth of some of the plant pathogenic fungi tested (Fig. 2). However, the fungal sensitivity varied with the species. *S. sclerotiorum* and *F. graminearum* were the most sensitive fungi to grifolin, and their mycelial growth inhibition was 86.4 and 80.9% at 304.9 $\mu$M. The growth of the other fungi, including *Botrytis cinerea* Per. ex Tris., *Alternaria alternata* (Fries) Keissler, *Gloeosporium fructigenum* Berk., and *Venturia nashicola* Tanaka et Yamamoto, were also obviously inhibited at the same dose. To determine the fungicidal sensitivity of grifolin, this compound and the commercially available fungicide carbendazim were compared under the same assay conditions at 304.9 $\mu$M. We found that carbendazim was more effective to inhibit mycelia1 growth of test fungi except *A. alternata* and *V. nashicola*. The growth of *A. alternata* and *V. nashicola* was not affected by carbendazim, in contrast, while 304.9 $\mu$M grifolin inhibited the growth by 70.2 and 74.3%, respectively.

The dose response curves of antifungal activity for grifolin against spore germination of *Pyricularia oryzae* Br. et Cav., *F. graminearum*, *G. cingulata* and *B. cinerea* are shown in Fig. 3. Grifolin showed inhibitory activities to various phytopathogenic fungi. Spore germination of *F. graminearum*, *G. fructigenum* and *P. oryzae* was almost completely inhibited by 38.1 $\mu$M grifolin. At this dose, *B. cinerea* was only slightly inhibited. The spore germination of *B. cinerea* was completely inhibited at 76.2 $\mu$M grifolin. Its inhibition of spore germination to *P. oryzae* was 81.9% at 19.1 $\mu$M, the inhibition of spore germination to the other fungi was less than 50% at the same dose.

Based on the above results, the activity of grifolin was tested for its control effects on wheat powdery mildew in greenhouse. The fungicidal activities of isolated grifolin from *A. dispansus* against *Erysiphe graminis* when treated with 609.8, 304.9 and 152.4 $\mu$M were determined *in vivo*,

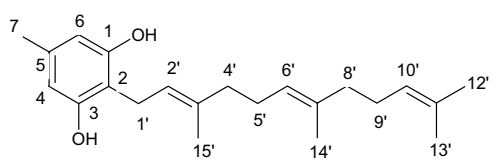


Fig. 1. The structure of grifolin isolated from *A. dispansus*.

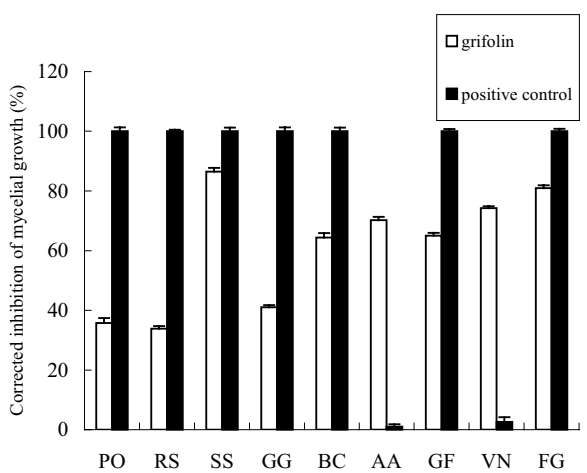


Fig. 2. Antifungal activity spectrum of grifolin and carbendazim (positive control) at 304.9 $\mu$M against nine pathogenic fungi: PO, *Pyricularia oryzae*; RS, *Rhizoctonia solani*; SS, *Sclerotinina sclerotiorum*; GG, *Gaeumannomyces graminis*; BC, *Botrytis cinerea*; AA, *Alternaria alternata*; GF, *Gloeosporium fructigenum*; VN, *Venturia nashicola*; FG, *Fusarium graminearum*. Each value is the mean of three replicates and error bars represent the standard deviation of the mean.

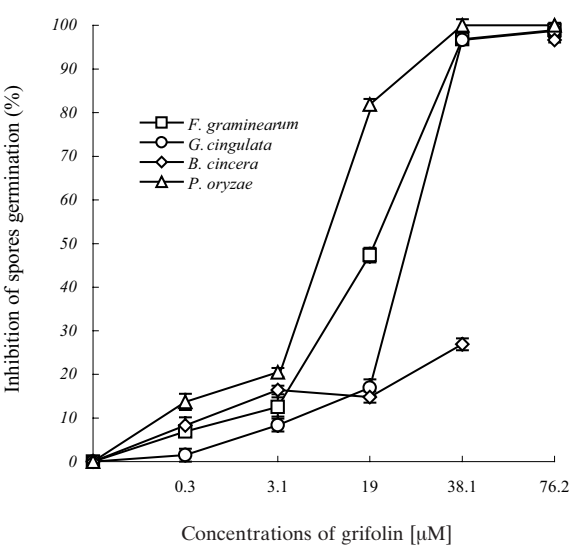


Fig. 3. Inhibition of spore germination of *Pyricularia oryzae*, *Fusarium graminearum*, *Glomerella cingulata* and *Botrytis cinerea* by grifolin isolated from *A. dispansus*. Error bars represent the standard error of the mean of three replicates. The activity of grifolin was determined by measurement of the percentage of spore germination (considered inhibited when length was less than 1.5 times the length of the spore) after 5, 12, 12 and 6 h, respectively.

respectively (Table II). The data indicated that the preventive effect of grifolin was obviously inferior to triadimefon. The curative effect of grifolin was 65.5% at 304.1 $\mu$M after 8 days.

The present work showed that grifolin isolated from *A. dispansus* exhibited significant inhibitory effects to mycelial growth of *S. sclerotiorum*, *F. graminearum* and spore germination of *P. oryzae in vitro*. In particular, it had a high level of curative activity against plant disease of *E. graminis in vivo*. Modern fungicides are generally selective, systemic and curative, achieving control with limited numbers of applications and at low rates. However, the development of fungicide resistance is often a problem leading to a requirement for new fungicides with different modes of action. In this respect, the active compounds isolated from *A. dispansus* may play an important role as a potential candidate for development of a new fungicide. At the same time grifolin was firstly reported that it shows antifungal activity against plant phytogenic fungi.

## Experimental

### *General*

$^1$H NMR (400 MHz, $CDCl_3$) and $^{13}$C NMR (100 MHz, $CDCl_3$) were recorded on a Bruker AM-400 spectrometer with TMS as internal standard. MS were measured with a VG Autospec 3000 mass spectrometer. Silica gel (200–300 mesh) was used for column chromatography (CC). Thin layer chromatography was done on pre-coated silica gel plates ($GF_{254}$). Spots were detected on TLC under UV or by heating after spraying with 5% $H_2SO_4$ in $C_2H_5OH$.

### *Materials*

The mushrooms were collected at Wuding in Yunnan province of China, in July 2001. The voucher specimen was deposited at the herbarium of Kunming Institute of Botany, the Chinese Academy of Sciences. Carbendazim and triadimefon were used as positive controls and purchased from Hebei Shuangji Chemical Co. Ltd., China.

### *Preparation of ethanol extracts of mushrooms*

10 g air-dried fruit bodies of 20 mushroom species were shaken in 100 ml EtOH at 40 °C for 48 h (40 cycles/min). The insoluble material was filtered and the filtrate evaporated to dryness under reduced pressure at 40 °C. The extracts were weighed and dissolved in DMSO to 1 mg/ml final concentration for antifungal test against mycelia growth.

### *Isolation of active principle*

The entire freshly collected fruiting bodies of *A. dispansus* (dry weight 0.7 kg) were immersed in 95% EtOH and left at room temperature for several days. Then the EtOH extract was decanted and evaporated and the residue was extracted with $CH_2Cl_2$/MeOH 1:1 (6 times). The extract (285.0 g) was fractionated by column chromatography (silica gel, $CHCl_3$/AcOEt 8:2). The combined fractions 1–4 were purified by column chromatography (silica gel, petroleum ether/AcOEt 19:1, 9:1, v/v) to give the active compound (4.8 g) after recrystallization from hexane.

Table II. *In vivo* control of *Erysiphe graminis* with preventive and curative spray applications of grifolin[a].

| Compound | Dose [$\mu$M] | 1 d prior to inoculation | 1 d after inoculation |
|---|---|---|---|
| | | Preventive effect (%) (±SD)[b] | Curative effect (%) (±SD)[b] |
| Grifolin | 609.8 | 23.0 (±4.4) a | 70.3 (±4.8) A |
| | 304.9 | 22.6 (±3.5) a | 62.5 (±4.6) A |
| | 152.4 | 22.0 (±3.3) a | 51.0 (±2.4) B |
| Triadimefon | 340.7 | 87.5 (±2.6) b | 64.1 (±4.6) A |

[a] Disease incidence observed in untreated control was about 100%. Disease incidence was determined 8 d after inoculation.

[b] Means followed by the same letter are not significantly different ($P = 0.05$) according to the least significant difference test.

*Mycelial growth inhibition test*

The isolated compound dissolved in acetone was tested for antifungal activity *in vitro* by a Poison Food Technique (Agarwal *et al.*, 2001). Potato dextrose agar (PDA) medium was used as the medium for all test fungi. The media incorporating test compound at 304.9 $\mu$M was inoculated at the centre of agar discs of the test fungi (4 mm diameter). Three replicate plates for each fungus were incubated at 26 (±2) °C for all test fungi. Control plates containing media mixed with acetone (1 ml) were included. After incubation for 2–6 d, the mycelial growth of fungi (mm) in both treated ($T$) and control ($C$) Petri dishes was measured diametrically in three different directions till the fungal growth in the control dishes was almost complete. The percentage of growth inhibition ($I$) was calculated using the formula (Sztejnberg *et al.*, 1983):

$$I\,(\%) = [(C - T)/C] \times 100$$

The corrected inhibition ($IC$) was then calculated as follows (Agarwal *et al.,* 2001):

$$IC = [(I - CF)/(100 - CF)] \times 100,$$

where $CF = [(90 - C_0)/C_0] \times 100$; 90 is the diameter (mm) of the Petri dish, and $C_0$ is the growth (mm) of the fungus in the control.

Analysis of variance was performed on the data with the PROCGLM procedure (SAS Institute, Cary, NC, USA). If $P > F$ was less than 0.01, means were separated with the least significant different (LSD) test at the $P = 0.05$ level.

*Spore germination test*

*B. cinerea* was cultured on potato sucrose agar (PSA; 20% potato extract, 2% sucrose, 2% agar) at 22.5 °C in the darkness. After incubation for 5 d, the aerial hyphae were removed by washing with a brush and sterilized distilled water. The culture surfaces were allowed to dry for 30 min and re-incubated for 3 d at the same temperature under continuous near ultraviolet irradiation of an intensive of 360 Mw/cm$^2$ that promoted synchronous spore formation. Spore suspensions were prepared by adjusting to $2 \times 10^5$ spores ml$^{-1}$ with a haemocytometer.

*P. oryzae* was grown on oatmeal-agar medium at 25 °C in the dark for 7–10 d. Spores were collected by agitating with distilled water from cultures 2 d older fluorescent light after removal of spores from the culture plate. The spore suspension was filtered through tissue paper and adjusted to a density of about $1 \times 10^5$ spores ml$^{-1}$ by addition of distilled water with a haemocytometer.

*Glomerella cingulata* (Stonem.) Spauling et Schrenk were obtained from a culture provided by Prof. Sun Guangyu, the Pathogenic Institute, College of Protection, Northwest Sci-Tech University of Agriculture and Forestry, Yangling, Shaanxi, P. R. China. The isolate was grown on potato dextrose agar for 5 d at 28 °C. The culture was overlaid with 10 ml of sterile distilled water, after that conidia were obtained by filtration of the water extract through four layers of sterile cheesecloth to remove mycelia debris. Conidia were washed three times in sterile distilled water by centrifugation and adjusted to $1 \times 10^6$ conidia ml$^{-1}$ with a haemocytometer.

*F. graminearum* obtained from corn was used for test. Colonies of *F. graminearum* were applied to oatmeal-agar medium at 25 °C in the dark for 5 d. 5 ml of sterile distilled water were added to each slant of 5-day-old cultures, gently scraping the agar surface to give a turbid suspension, corresponding to $1 \times 10^8$ spores ml$^{-1}$ with a haemocytometer.

The tests for inhibition of *B. cinerea*, *P. oryzae*, *F. graminearum* and *G. cingulata* spore germination were done on micro slides (Dhiangra and Sinclair, 1986). 30 $\mu$l of the spores suspension were added to 30 $\mu$l of the test compounds (0.3, 3.1, 19.1, 38.1, 76.2 $\mu$M acetone solutions), well mixed with a toothpick and incubated at 25 °C in the dark. 100 spores from each of five replicates were examined under a light microscope to determine the percentage of germinated spores. The experiment was repeated three times and results presented as mean values with standard deviation of the mean.

*In vivo assay*

In order to investigate further *in vivo* antifungal activities of the isolated compound such as the duration of preventive and curative activity, the plant disease of wheat powdery mildew (*Erysiphe graminis* DC f sp. *tritici* Marchal graminis) was used in the test. The effects of the test compound on disease development and spread were determined using potted plants in a greenhouse. The potted plants were randomly arranged in two groups and watered twice daily with tap water. The potted plant seedlings were sprayed with the test compounds dissolved in water/acetone (95:5 v:v) con-

taining Tween 20 (250 $\mu$g/ml) as wetter and allowed to stand for 24 h.

For the test of preventive effects, the plants in the first group were inoculated with the pathogen of the plant disease, 1 d after spraying with either the test compound or a standard fungicide at a dose of 609.8, 304.9 and 152.4 $\mu$M. For the test of curative effects, the plants in the second group were firstly inoculated with the pathogen, 1 d before the application of the test compounds and a standard fungicide at a dose of 609.8, 304.9 and 152.4 $\mu$M. Control plants within each group were similarly inoculated with sterile distilled water/acetone containing Tween 20.

For the development of wheat powdery mildew, the treated wheat seedlings at the first stage were inoculated by spraying with a spore suspension (4 mg/ml) of *E. graminis*. The inoculated wheat seedlings were incubated for 8 d at 20 (±1) °C and 60% RH (relative humidity) during the day and 18 (±1) °C and 60% RH during the night with 16 h of daylight per day in artificial climate chambers (RP-300, R. P. China), and then the disease severity was determined. The disease severity was recorded on a 0–5 scale, where 0 = no colonies visible to the unaided eye; 1 = few scattered, small discrete colonies; 2 = larger, but still discrete colonies; 3 = colonies merging to form larger mildew lesions; 4 = mildew covering half the total leaf surface and 5 = mildew covering the total leaf surface (Hickey, 1986).

Pots were arranged as a randomized complete block with three replicates per treatment. The experiment was conducted three times and values are expressed as percentage control (±SD) compared with the control (Song *et al.*, 2004).

The percentage disease incidence was determined using the formulas:

disease incidence (%) = [(Σ scale × number of plants infected)/(highest scale × total number of plants)] × 100 and preventive or curative effects (%) = [(disease incidence of the control – disease incidence of the treatment)/disease incidence of the control] × 100.

Analysis of variance was performed on the data with the PROCGLM procedure (SAS Institute, Cary, NC, USA). If $P > F$ was less than 0.01, means were separated with the least significant different (LSD) test at the $P = 0.05$ level.

*Grifolin*, $C_{22}H_{32}O_2$: M.p. 38–40 °C (lit. 40–41 °C); colorless needles. – IR (KBr): $\nu_{max}$ = 3620 (OH), 2920 (CH), 1635, 1585 $cm^{-1}$ (C=C). – HRMS: *m/z* = 328.2396. – $^1$H NMR ($CDCl_3$), δ in ppm: δ = 1.56 (6H, s, $CH_3$-13′ and $CH_3$-14′), 1.63 (3H, s , $CH_3$-12′), 1.76 (3H, s, $CH_3$-15′), 1.87–2.07 (8H, m, H-4′, 5′, 8′, and 9′), 2.10 (3H, s, $CH_3$-7), 3.33 (2H, d, $J$ = 7 Hz, H-1′), 5.06–5.09 (2H, m, H-6′ and 10′), 5.30 (1H, br t, $J$ = 7 Hz, H-2′), 6.19 (2H, s, H-4 and 6), 7.80 (2H, s, OH-1 and 3). – $^{13}$C NMR (DEPT): δ = 15.6 (C-14′), 15.8 (C-15′), 17.3 (C-13′), 20.8 ($CH_3$-7), 22.1 (C-1′), 25.4 (C-12′), 26.8 (C-5′ and C-9′), 39.8 and 40.0 (C-4′ and 8′), 107.8 (C-4 and 6), 112.0 (C-2), 123.9 (C-2′), 124.5 and 124.6 (C-6′ and C-10′), 130.8 (C-11′), 133.7 (C-7′), 134.6 (C-3′), 136.0 (C-5), 156.0 (C-1 and C-3) (Goto *et al.*, 1963; Mahiiou *et al.*, 1995).

*Acknowledgements*

We wish to acknowledge financial support from the National Natural Science Foundation of China (30225048).

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