A NOVEL QUINONE ANTIBIOTIC FROM *Malbranchea cinnamomea* TAIM 13T54

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A novel quinone antibiotic named malbranicin was isolated from the culture filtrate and mycelium of *Malbranchea cinnamomea* TAIM 13T54, a thermophilic fungus. The antibiotic was elucidated to be 6-(1-acetylethyl)-2-methoxy-2,5-cyclohexadiene-1,4-dione by spectral analysis. Malbranicin exhibited antimicrobial and cytotoxic activities against Gram-positive bacteria and mammalian cell lines, respectively.

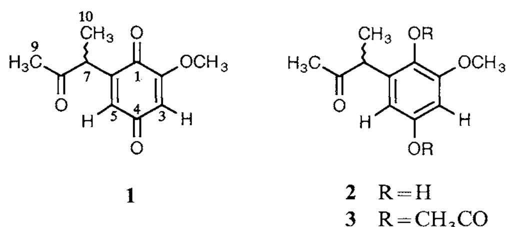
In the screening course of new metabolites from microorganisms, thermophilic fungi showed a very high ratio in producing biological active substances. The authors screened these rare fungi for production of antibiotics. Among strains isolated, TAIM 13T54 identified as *Malbranchea cinnamomea* was a new record in Taiwan. A novel quinone antibiotic named malbranicin was isolated from the culture filtrate and mycelium of the isolate. In this paper we describe the isolation and identification of the strain, and we also describe the production, isolation, structural determination and biological activities of malbranicin (Fig. 1).

Results and Discussion

Discovery of the Producing Strain

Various strains were isolated from field soil in Taiwan. Soil samples were heated at 60°C in water bath for 15 minutes, then incubated at 50°C on PDA containing chloramphenicol and Rose bengal. During the screen described as Experimental, we succeeded in isolating a thermophilic fungal strain TAIM 13T54 exhibiting strong activities against *Staphylococcus aureus* and *Bacillus subtilis* by disc agar diffusion assay method. Microorganisms were expected to produce special physiological active compounds after heat shock treatment as reported in finding novel polyketide antibiotic in *Streptomyces venezuelae*[1], but in our case, heat treatment was only used as a procedure for isolation of thermophilic fungi.

Fig. 1. The structure of malbranicin and its derivatives.



Taxonomy

Cultural and morphological characteristics of the strain TAIM 13T54 are as follows. On potato dextrose agar the strain showed good growth at 40°C, reaching 45 mm long in 10 days; slow growth at 50°C and no growth at 30°C. The central area of

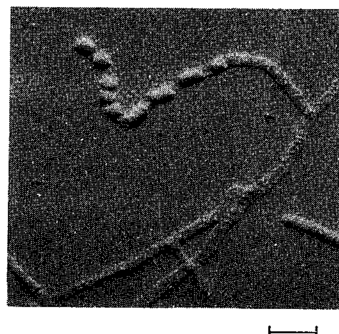
a colony was white initially and changed to a mixture of pale Rhodonite pink, white and yellow; the reverse was Kaiser brown or dark red brown, with a strong rotten fish meal odor when matured. In description above, the capitalized color names noted was from Ridgeway[2].

Vegetative hyphae were 2～8 μm in diameter and hyaline, with conspicuous swells near septa (5～9 μm in diameter). No conidiophore differentiated. Fertile tips usually bent into small loops or coils, and finally articulated into arthroconidia becoming single or chained. Conidia were yellow green, cubic and broadly elliptical or slightly curved, differing in size between 3.2～4.5 × 3.9～7.7 μm, with conspicuous frills at both ends (Fig. 2).

From the mycological characteristics described above, strain TAIM 13T54 was identified as the genus *Malbranchea* following to the classification of thermophilic fungi[3]. Because this strain showed good agreement with the description of *Malbranchea cinnamomea* (Lib.) VAN OOSCHOT and DE HOOG[4], we have designated this strain as *Malbranchea cinnamomea* TAIM 13T54.

Fig. 2. The morphology of *Malbranchea cinnamomea* TAIM 13T54.

Bar represents 10 μm.

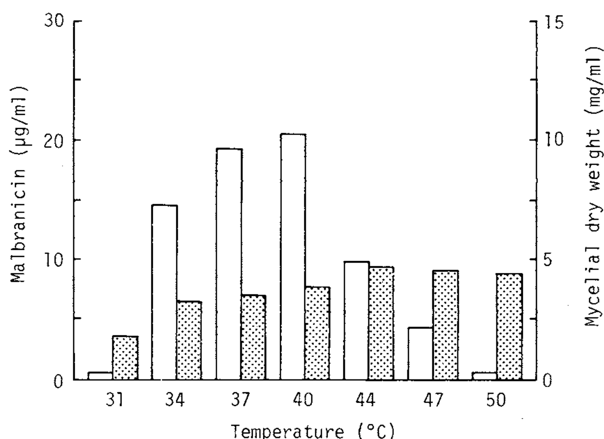


Fermentation

To improve the fermentation conditions, we investigated the effects of temperature, nitrogen sources and carbon sources. The strain produced malbranicin only between 34～44°C without much variation in growth. The highest yield appeared at 40°C (Fig. 3). The results of the study about nitrogen sources showed that yeast extract was more effective than others (Table 1), but Polypepton supplied a longer plateau. So mixtures of them in

Fig. 3. Temperature effect on the yields of malbranicin.

Open: malbranicin, closed: mycelial dry weight.



Fermentation medium: soluble starch 1.5%, yeast extract 0.4%, K_2HPO_4 0.1% and $MgSO_4 \cdot 7H_2O$ 0.05%, pH 7.0.

All of the batches were incubated in L-tubes on a temperature gradient shaker for 48 hours after inoculated with 1% seed culture which was shaked with 110 rpm at 40°C for 30 hours.

Table 1. Effect of nitrogen sources on malbranicin production.

Nitrogen sources	%	Mycelial dry weight (mg/ml)	Malbranicin (μg/ml)
Polypepton	0.2	5.00	15.1
	0.4	6.15	9.2
	0.6	5.25	<1.0
C.S.L.	0.2	2.94	20.5
	0.4	5.59	14.2
	0.6	3.17	8.1
Yeast extract	0.2	4.89	<1.0
	0.4	5.76	27.5
	0.6	5.18	5.7
Soybean meal	0.2	6.8	2.2
	0.4	5.7	3.6
	0.6	8.1	2.1
Meat extract	0.2	7.9	4.6
	0.4	8.1	16.7
	0.6	8.3	18.5
Yeast extract/Polypepton			
	0.3/0.1	5.3	34.5
	0.2/0.2	5.4	22.3
	0.1/0.3	6.7	2.0

Basal medium: soluble starch 1.5%, K_2HPO_4 0.1% and $MgSO_4 \cdot 7H_2O$ 0.05%, pH 7.0.

All of the batches were fermented for 48 hours at 40°C with 110 rpm on a reciprocal shaker after inoculated with 1% seed culture.

Table 2. Effect of carbon sources on malbranicin production.

Carbon sources (3%)	Mycelial dry weight (mg/ml)	Malbranicin (μg/ml)
Soluble starch 2%	5.4	59.0
3%	5.7	66.2
4%	5.9	50.2
Dextrin	6.3	43.0
Soybean oil	15.1	9.4
Sucrose	5.1	3.3
Lactose	2.2	<1.0
Fructose	5.1	17.7
Glucose	7.4	3.9
Ribose	8.3	13.8
Glycerin	7.2	37.4
Mannitol	3.7	10.0
Erythritol	6.3	12.5
Malonic acid	1.9	<1.0
Succinic acid	2.4	9.0

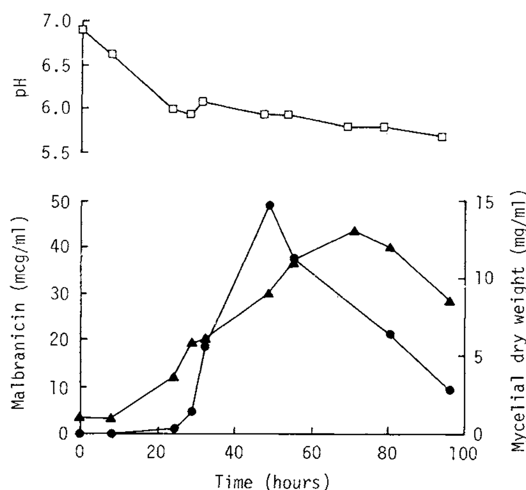
Basal medium: yeast extract 0.3%, Polypepton 0.1%, K_2HPO_4 0.1% and $MgSO_4 \cdot 7H_2O$ 0.05%, pH 7.0.

All of the batches were fermented at 40°C for 48 hours after inoculated with 1% seed culture.

different ratios were tested and a mixture of 0.3% yeast extract and 0.1% Polypepton was found to be the better choice. The nitrogen basal medium thus obtained was used to examine the carbon source effect. The higher yields were appeared in batches using soluble starch as carbon source (Table 2). The best concentration was found to be 3%. The time course of fermentation for malbranicin in the medium thus found is shown in Fig. 4.

Fig. 4. Time-course of malbranicin production by *Malbranchea cinnamomea* TAIM 13T54.

Malbranicin (●); determined by the HPLC method. Mycelial dry weight (▲). pH (□).



Fermentation medium: soluble starch 3%, yeast extract 0.3%, Polypepton 0.1%, K_2HSO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, pH 7.0.

The batches were shaked with 110 rpm at 40°C after inoculated with 1% seed which was cultured in seed medium under the same condition.

Isolation

The culture filtrate (10 liters) of the strain was extracted with equal volume of ethyl acetate at pH 7.0. The mycelial mat was extracted twice with 70% acetone. After removal of acetone, the resulting syrup was extracted three times with 3 liters of ethyl acetate at pH 7.0. The combined ethyl acetate extracts were dried over anhydrous sodium sulfate and concentrated to dryness *in vacuo*. The residue (*ca.* 1.3 g) was subjected to silica gel column

Table 3. Physico-chemical properties of malbranicin.

Appearance	Yellow needles
MP (°C)	112~114
$[\alpha]_D^{25}$	−18° (*c* 0.01, MeOH)
HREI-MS *m/z* $(M)^+$	208.0735
EI-MS *m/z*	208 $(M)^+$, 166, 151, 138
Molecular formula	$C_{11}H_{12}O_4$
Elemental analysis	Found: C 63.36, H 5.72%
	Calcd: C 63.45, H 5.81%
Color test	Positive: 2,4-dinitrophenylhydrazine, vanillin-H_2SO_4
	Negative: I_2, Dragendorff
Solubility	Soluble: chloroform, benzene, EtOAc, MeOH
	Insoluble: petroleum ether
UV λ_{max}^{MeOH} nm (ε)	258 (12,400), 360 (970)
IR ν_{max} (Neat) cm^{-1}	2980 (w), 2950 (w), 1700 (s), 1680 (s), 1645 (s), 1240 (s)
TLC[a] (Rf)	0.4
HPLC[b] (Rt)	3.69 minutes
GC[c] (Rt)	7.16 minutes

[a] Merck silica gel F_{254}; benzene - EtOAc, 5:1 (v/v).
[b] NOVA PAK C_{18} (5×250 mm); mobile phase, MeOH - H_2O, 60:40 (v/v); flow rate, 1 ml/minute; detection, UV 254 nm.
[c] Capillary Column OV-1 (TC-1, 0.25×30 m), 150~250°C (5°C/minute).

chromatography (18×400 mm) using chloroform - ethyl acetate as an eluant. The 33% ethyl acetate eluate showed antibiotic activity against *Bacillus subtilis*. The active fraction was concentrated *in vacuo* (*ca.* 450 mg), and rechromatographed on a silica gel column (14×250 mm) using a 5:1 mixture of chloroform - ethyl acetate. The elution was monitored by TLC under a UV lamp. The active fractions showing one spot on TLC were combined and then concentrated to dryness *in vacuo*. After washed with hexane and benzene, a faint yellow powder was first crystallized in a chloroform - methanol mixture, and then recrystallized in methanol. Malbranicin thus obtained was proved to be homogeneous by silica gel TLC, reverse phase HPLC and GC.

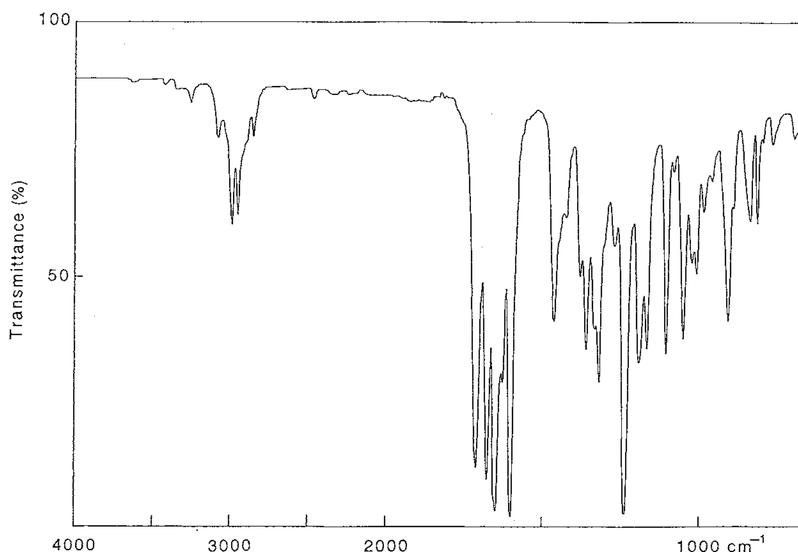
Physico-chemical Properties

Malbranicin (**1**) is soluble in chloroform, ethyl acetate, acetone, methanol and ether, sparingly soluble in hexane and benzene, and almost insoluble in petroleum ether. It gave a positive color reaction with 2,4-dinitrophenylhydrazine, suggesting that it has a ketone group. The crystals of **1** showed a melting point of 112~114°C with $[\alpha]_D^{25}$ −18° (*c* 0.01, MeOH). The molecular formula of **1** was determined to be $C_{11}H_{12}O_4$ by EI-MS: *m/z* 208 (M^+), CI-MS: *m/z* 209 $(M+1)^+$, FAB-MS: *m/z* 209 $(M+1)^+$, high resolution mass spectrometry [*m/z* 208.0735 (M^+), calcd for $C_{11}H_{12}O_4$: 208.0736] and elemental analysis. The physico-chemical properties of **1** are summarized in Table 3.

Structure

The molecular formula of **1** was determined to be $C_{11}H_{12}O_4$ by HRMS and elemental analysis, so the unsaturation number of **1** was counted to be six. The existence of two double bonds and three carbonyl carbons was indicated by ^1H and ^{13}C NMR spectra (Table 4), so **1** was deduced to contain a ring. A ketone at 1700 cm^{-1} and two conjugated ketones at 1680 and 1645 cm^{-1} were also suggested by IR spectrum (Fig. 5), and the UV spectrum of **1** also revealed it belonging to a benzoquinone. A methoxyl group (δ_H 3.84, δ_C 56.2) was suggested to locate on benzoquinone because of a benzenoid signal at

Fig. 5. IR spectrum of malbranicin.



δ_C 158.6. The absorption in UV spectrum as well as the marked difference in the ε value between 258 nm ($\varepsilon = 12{,}400$) and 360 nm ($\varepsilon = 970$) resulted in the conclusion that **1** belongs to a alkylated 2-methoxy-1,4-benzoquinone[5].

A signal at δ_C 145.9 was assigned to a carbon to be linked with another substituent. Considering the coupling constant ($J = 2.4$ Hz) between the signals at δ_H 5.95 (δ_C 107.3) and 6.53 (δ_C 133.5), these two methine protons were speculated to be meta-positioned, so alkyl substitute would be at C-6. The remaining signals composed of C_4H_7O was equivalent to this aliphatic side chain which was assigned to acetyl (δ_C 206.7 and δ_H 2.29, δ_C 28.7) and ethylidene (δ_H 1.35d, δ_C 14.3; δ_H 3.95dq, δ_C 45.2) residues. The only way of combination came to a acetyl-ethyl chain. This elucidated substitute is just the same as acetoin, and was further confirmed by direct comparison of ^{1}H and ^{13}C NMR spectra. The fragment ions at m/z 166, 151 and 138 in the EI-MS spectrum (Table 3) also supported the presence of this side chain. Finally the structure of **1** was concluded as 6-(1-acetylethyl)-2-methoxy-2,5-cyclohexadiene-1,4-dione.

Table 4. ^{1}H and ^{13}C NMR data of malbranicin in $CDCl_3$.

Carbon	δ_C	Proton	δ_H
1	186.7*		—
2	158.6		—
3	107.3	3-H	5.95 (d, 2.4)
4	181.3*		—
5	133.5	5-H	6.53 (dd, 2.4, 1.2)
6	145.9		—
7	45.2	7-H	3.95 (dq, 7.3, 1.2)
8	206.7		—
9	28.7	$-COCH_3$	2.29 (s)
10	14.3	$-CH_3$	1.35 (d, 7.3)
11	56.2	$-OCH_3$	3.84 (s)

* May be exchanged.

Based on the long range coupling constant ($J_{ax} = 1.2$ Hz) between the quartet methine and the quinone proton at δ_H 6.53, this quinone proton was determined at C-5. Finally from the results deduced from ^{1}H-^{1}H and ^{1}H-^{13}C COSY NMR spectra, the assignment of all signals of ^{1}H and ^{13}C was completed except two ketone signals of δ_C 186.7 and 181.3. These two signals were temporarily assigned to C-1 and C-4, because in the ketone carbons of benzoquinone, one on the side of stronger electron-negativity always produces a signal in a lower field than the other one[6]. The total assignment is shown in Table 4.

Fig. 6. A perspective view of diacetate (3) of dihydromalbranicin.

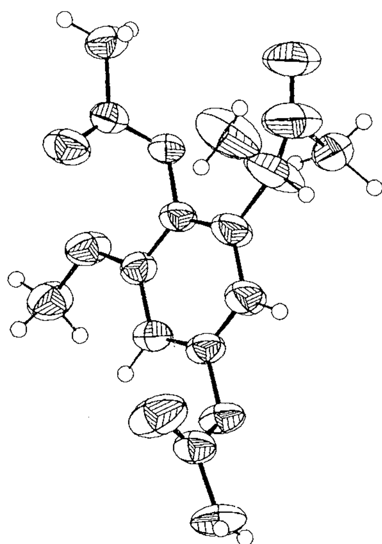


Table 5. Antimicrobial spectrum of malbranicin.

Microorganisms	MIC (μg/ml)
Staphylococcus aureus IFO3060	25
S. epidermidis IFO209D	100
S. xylosus ATCC29971	100
Bacillus subtilis ATCC6633	25
Micrococcus luteus ATCC9341	100
M. roseus IFO3768	6.25
Enterococcus faecalis IFO12968	>100
Escherichia coli IFO3301	>100
Serratia marcecens IFO12648	>100
Pseudomonas aeruginosa IFO13275	>100
Candida albicans IFO589	>200
Saccharomyces cerevisiae	>200
Aspergillus niger IFO4416	>200
Fusarium oxysporum IFO5880	200
Penicillium chrysogenum IFO4897	>200

Validity of the proposed conclusion about the structure of **1** was chemically checked as follows: hydrogenation of **1** yielded dihydromalbranicin (**2**), which had two phenolic hydroxyl groups, then **2** was converted to the corresponding diacetate (**3**) by the method described in Experimental. The UV and IR spectra of the two derivatives clearly showed the transformation from quinoid to benzenoid. Only the saturated ketone signal was observed to be unchanged at $1700\,cm^{-1}$ in **3**. The computer-generated perspective view of **3**, which was deduced from X-ray crystallographic analysis, is shown in Fig. 6. We will report the details of this study elsewhere in future[7].

Among benzoquinones, malbranicin is the first compound with an acetyl-ethyl substitute.

Biological activity

The results of antimicrobial evaluation are summarized in Table 5, showing that **1** had weak activity against Gram-positive bacteria and no activity on Gram-negative bacteria. Slight activity against some fungi was noted. **1** was found to be cytotoxic at 0.7 μg/ml against P388 cells and 2.8 μg/ml to KB cells, indicative of a slight selective toxicity between two cell lines.

Experimental

Isolation of the Strains

Fungal strains were isolated on potato dextrose agar and corn meal agar plates containing chloramphenicol (0.1 mg/ml) and Rose bengal (0.05 mg/ml) after 3 days incubation at 50°C. All of the soil samples were digged out from about 10 cm depth from various districts in Taiwan. Each sample was treated with hot water for 15 minutes at 60°C before spreaded on isolation media.

Screen and Fermentation

The pure cultures were inoculated in 250-ml Erlenmeyer flasks containing 50 ml YpSs medium (soluble starch 1.5%, yeast extract 0.4%, K_2HPO_4 0.1% and $MgSO_4 \cdot 7H_2O$ 0.05%, pH 7.0), and incubated at 40°C for 4 days with shaking at 180 rpm. The detection for production of antibiotics was monitored by disc agar diffusion assay method using *Staphylococcus aureus* as detectors.

Batch cultures for the study on the effects of the temperature, nitrogen sources and carbon sources

were incubated in 500-ml flasks with 120 ml medium for 48 hours on a reciprocal shaker at 110 rpm, after inoculating with 1% of seed cultures incubated at 40°C for 30 hours in seed medium (soluble starch 1.5%, Polypepton 0.4%, yeast extract 0.4%, K_2HPO_4 0.1% and $MgSO_4 \cdot 7H_2O$ 0.05%, pH 7.0). The potency of the production with nitrogen sources was examined at three different concentrations.

The culture fluid for isolation was fermented for 48 hours with a medium composed of soluble starch 3.0%, yeast extract 0.3%, Polypepton 0.1%, K_2HPO_4 0.1% and $MgSO_4 \cdot 7H_2O$ 0.05% (pH 7.0) under the same condition.

Assay of Antibacterial Activities

The test organisms were suspended in YDF medium (glucose 3%, Polypepton 0.2%, yeast extract 0.1%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.1%, NaCl 0.05%, $CaCl_2$ 0.03%, $FeCl_2$ 0.00002%, $ZnSO_4$ 0.00003%, pH 7.0, agar 2%). The samples were first separated into cell mass and supernatant by filtration, then the culture fluids and mycelial mat were extracted with ethyl acetate at pH 7.0 and acetone, respectively. Aliquots of extracts were applied to 8 mm paper discs and bioautographed with silica gel TLC.

Quantitative Analysis

The yields of **1** were determined by HPLC. The standard curve was done by standard solutions prepared from crystals. The aliquots described as above were evaporated and resolved in methanol for quantification.

Biological Activity

The MIC was determined by the serial dilution assay method after a test microorganism was incubated at 38°C for 18~42 hours in Mueller-Hinton agar for bacteria and 27°C for 3 days in Sabouraud Dextrose agar for fungi. IC_{50} values were determined by using 24 hours incubates of 1×10^3 cells/well of KB cell and P388 cell line. After further 3 days incubation with **1** in various concentration, the viability was judged by crystal violet staining.

Spectroscopy

Melting points were determined with a Yanagimoto micro melting point apparatus and were uncorrected. UV spectra were recorded on a Shimadzu double beam spectrophotometer UV-180. IR spectra were determined on a Perkin Elmer FT-IR spectrophotometer 1760 or a Hitachi infrared spectrophotometer 260-10. Optical rotations were measured with a JASCO DIP-SL automatic polarimeter. NMR spectra were recorded on a JEOL JNM-GX270 spectrometer. Mass spectra were recorded on a JEOL JMS-AX500 mass spectrometer. Analysis of **1** and its derivatives were fullfilled on a Hitachi liquid chromatograph L-6200 and a Hitachi gas chromatograph G3000.

Dihydromalbranicin (2)

Malbranicin (**1**) (58 mg) was hydrogenated in the presence of 10% Pd-C (1 mg) in MeOH (5 ml) under H_2 gas for 2 hours at room temperature. After catalyst had been filtered off, the filtrate was evaporated *in vacuo*. Dihydromalbranicin (**2**) was obtained as colorless film, Rf 0.05 (benzene - ethyl acetate, 5:1, v/v on a silica gel TLC); Rt 9.26 minutes on GC. EI-MS *m/z* 210 (M^+), 167, 152, 135. ^1H NMR: (270 MHz, CD_3OD) δ 1.35 (3H, d, J=7.0 Hz), 2.12 (3H, s), 3.90 (3H, s), 4.17 (1H, q, J=7.0 Hz), 6.20 (1H, d, J=2.7 Hz), 6.46 (1H, d, J=2.7 Hz), 7.88 (1H, br), 8.75 (1H, br). ^{13}C NMR: (67.5 MHz, CD_3OD) δ 15.9 (q, C-10), 28.3 (q, C-9), 47.9 (d, C-7), 56.4 (q, OCH_3), 99.8 (d, C-3), 106.5 (d, C-5), 128.8 (s, C-6), 137.9 (s, C-2), 149.6 (s, C-4 or 1), 151.4 (s, C-1 or 4), 212 (s, C-8). **2** was immediately subjected to acetylation without purification.

Diacetate (3) of dihydromalbranicin (2)

A mixture of dihydromalbranicin (**2**), acetic anhydride (1.5 ml) and pyridine (1 ml) was kept at room temperature for 24 hours and then poured into H_2O (15 ml). The mixture was extracted with chloroform and washed with 5% HCl solution. After the solvent was evaporated, the residue was purified by silica gel chromatography to give diacetate (**3**) (78 mg). It gave colorless cubic crystals from ether or acetone. Rf 0.53 (benzene - ethyl acetate, 5:1, v/v on a silica gel TLC); Rt 10.54 minutes on GC; mp 101~102°C,

$[\alpha]_D^{25}$ $-81°$ (*c* 0.01, MeOH), UVλ_{max}^{MeOH} nm (ε) 225 (7,640), 273 (5,470), EI-MS *m/z* 294 (M^+), 166, 138, 107. ^{1}H NMR: (270 MHz, $CDCl_3$) δ 1.23 (3H, d, J=7.0 Hz), 1.95 (3H, s), 2.21, 2.24 (each 3H, s, $OCOCH_3$), 3.72 (3H, s), 3.69 (1H, q, J=7.0 Hz), 6.46 (1H, d, J=2.7 Hz), 6.60 (1H, d, J=2.7 Hz). ^{13}C NMR: (67.5 MHz, $CDCl_3$) δ 15.9 (q, C-10), 20.0, 21.0 (each s, $OCOCH_3$), 28.1 (q, C-9), 47.9 (d, C-7), 56.1 (q, OCH_3), 105.3 (d, C-3), 112.6 (d, C-5), 134.5 (s, C-6 or 2), 135.5 (s, C-2 or 6), 149.6 (s, C-4 or 1), 151.9 (s, C-1 or 4), 168.5, 169.0 (each s, $OCOCH_3$), 207.6 (s, C-8).

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