ORIGINAL ARTICLE

Production of a COX-2 inhibitor, 2,4,5-trimethoxybenzaldehyde, with submerged cultured *Antrodia camphorata*

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Abstract

Aims: To investigate the active ingredient in fruiting bodies and to produce it with cultured mycelium in *Antrodia camphorata* (BCRC 35398).
Methods and Results: The volatile components from the fruiting bodies, the liquid cultured broth of *A. camphorata* and *Cinnamomum kanehirae* wood were separately isolated by steam distillation–solvent extraction and identified by gas chromatography–mass spectrometry. In the fruiting bodies, a COX-2 inhibitor 2,4,5-trimethoxybenzaldehyde (TMBA) was found to be the most abundant constituent, but was totally absent in its cultured broth and its natural host, *C. kanehirae* wood. On feeding with the acid-digested sawdust of *C. kanehirae* wood or vanillin to the broth for culture, TMBA was produced in both cultured broths.
Conclusion: The TMBA identified in fruiting bodies was an active ingredient whose functions consisted with the reported experiences of this mushroom. Feeding vanillin to culture broth could produce TMBA containing mycelium product like its fruiting bodies did.
Significance and Impact of the Study: This study found an active ingredient in fruiting bodies of *A. camphorata* and elucidated this compound derived from digested sawdust of *C. kanehirae* wood. A feasible method was also developed to produce TMBA containing mycelium by feeding vanillin.

Introduction

Antrodia camphorata, a unique mushroom known in Taiwan as 'Niu-chang-chih' or 'Chang-chih', has been traditionally used for the treatment of food- or drug intoxication, diarrhoea, abdominal pain, hypertension, skin itching and cancers (Wu *et al.* 1997). Being quite different from the common *Genoderma* species, it bears a stronger bitterness and camphor-like aroma and plate-like or bell-like shape. *Antrodia camphorata* is a well-known fungus that specifically grows on the Taiwan endemic tree *Cinnamomum kanehirae* Hay. *Cinnamomum kanehirae* tree, preferentially growing in forests within altitude 200–2000 m, recently has become an extinguishing species due to illegal cutting to harvest the mushroom grown on it. Literally, the word 'Niu-chang-chih' means exactly in Chinese 'The unique mushroom that grows on the rotten wood of Niu-chang tree'.

Ecologically, its fruiting bodies grow extremely slowly on *C. kanehirae*, hence the production quantity is limited. Consequently the fruiting bodies of *A. camphorata* become very expensive (US$2500 kg^{-1} wet weight at least).

Pharmacological studies evidenced that the extracts from its fruiting bodies or mycelia possess many functions such as anti-inflammatory (Shen *et al.* 2004a,b), anti-viral hepatitis B (Lee *et al.* 2002; Shen *et al.* 2005), anti-cholinergic and anti-serotonergic activities (Chen and Yang 1995). Because of the difficulty to cultivate its fruiting bodies, some submerged culture methods have been developed to produce its mycelia by fermentors (Song and Yen 2002; Yang *et al.* 2003). However, a great

aroma difference does exist between the fruiting bodies and the cultured mycelia, and moreover, the constituents in these two products have never been documented. This study is considered to be the pioneer regarding to such investigations.

Materials and methods

Plant material

Antrodia camphorata wild fruiting bodies, purchased from a local herb store in Hsinchu, were identified by Dr T.-T. Chang (Division of Forest Protection, Taiwan Forestry Research Institute). The sawdust of *C. kanehirae* wood was obtained from a local wood plant in Chungli, Taiwan. Both were ground into powder and sieved through a #60 mesh.

Strain and cultivation

Antrodia camphorata (BCRC 35398), purchased from Bioresources Collection and Research Center in Food Industry Research and Development Institute (Hsinchu, Taiwan), was grown and stored as reported previously (Shen *et al.* 2005). Briefly, a piece of 5 mm × 5 mm *A. camphorata* agar culture was inoculated into 1 l of the broth consisting of the following ingredients (g l^{-1}): glucose, 1·0; soya bean powder, 0·5; peptone, 0·5; $MgSO_4$, 0·01. The pH was adjusted to 4·0 with 1 mol l^{-1} HCl. The whole medium was placed in a 2 l Hinton flask and cultivated at 28°C on a 100 rev min^{-1} rotary shaker for 28 days. For feeding experiment, 1 g vanillin dissolved in 10 ml of propylene glycol passed through 0·45-μm filter was added aseptically to the cultured broth at the fifth day after inoculation. The sampling was conducted at the 7th, 14th, 21st and 28th days after vanillin feeding. In sawdust feeding cultivation, 10 g sawdust of *C. kanehirae* wood in 30 ml of 6 mol l^{-1} HCl was digested at 121°C for 10 h. A volume of 5 ml of this digested solution was added to 1-l broth described as above and pH was adjusted to 4·0 with NaOH granules. After sterilization and inoculation, the broth was cultivated as described above and sampled once every week until the fourth week.

Steam distillation with solvent extraction

Total of 200 ml cultured broth or 5 g ground powder of fruiting bodies or sawdust of *C. kanehirae* wood in 200 ml of H_2O was placed in a modified Likens–Nickerson apparatus (Filek *et al.* 1995). The solvent mixture (50 ml) of *n*-pentane and diethyl ether (column distilled, 1 : 1, v/v; Merck, Darmstadt, Germany) was used as an extractant. After 200 μl of the internal standard [11 mg of *cis*-3-hexenyl lactate (Aldrich, Milwaukee, WI, USA) dissolving in a 10 ml of 1 : 1 mixture of diethyl ether/*n*-pentane (v/v)] was added, the volatile compounds of cultured broth were immediately subjected to the isolation process. The simultaneous steam-distillation and solvent extraction was allowed to proceed for 2 h, and the extract thus obtained was dried over anhydrous Na_2SO_4 (Merck) and filtered through Whatman no. 4 filter paper. The filtered extract was then concentrated at 40°C to dryness using a Vigreux column (i.d. 1·5 × 100 cm, Tung Kawn Glass Co., Hsinchu, Taiwan) and the resulting extract was stored at −20°C prior to chemical analyses. More details with respect to the design and function of the steam distillation with solvent extraction (SDSE) apparatus can be found in the previous report (Filek *et al.* 1995).

GC/MS analysis and identification

The gas chromatography/mass spectrometry (GC/MS) analysis was performed using a Hewlett Packard 6890 Gas Chromatography linked to a Hewlett Packard 5973A Mass Spectrometer system equipped with a 60-m long, 0·25 mm i.d., 0·25-μm thickness CP Wax 52CB capillary column (Chrompack Inc., Middelburg, the Netherlands). The ionization energy was 70 eV. The temperature of the injection

Table 1 The volatile compounds in the fruiting bodies of *Antrodia camphorata*

Compounds	Kovats index (CP wax 52CB)*	Peak area (%)†
Ethyl acetate	877	1·06
Acetic acid‡	1436	1·43
1-Terpineol	1586	1·48
4-Terpineol‡	1601	1·26
α-Terpineol‡	1694	0·66
β-Nerolidol	1994	12·79
8-epi-β-Bisabolol	2128	2·78
α-Cadinol	2142	0·59
T-cadinol	2162	0·19
T-muurolol	2207	1·52
Ethyl hexadecanoate‡	2233	0·67
2,4,5-Trimethoxybenzaldehyde	2279	21·07
1,2,3,4-Tetramethoxy benzene	2383	0·22
Ethyl oleate‡	2455	1·08
Ethyl linoleate‡	>2500	0·91
Methyl 3,4,5-trimethoxy benzoate	>2500	0·22
4,4′-Biguaiacol	>2500	2·13
2,6-Dihydroxy-4-methoxy-acetophenone	>2500	0·25
Hexadecanoic acid‡	>2500	2·54
Total		52·85

*Retention indices determined on a CP wax 52CB column using *n*-alkanes (C_6–C_{25}) as references.
†Area per cent was determined using the total ion chromatogram of GC-MSD area method and from the averaged triplicates.
‡Compounds were identical by referring to those of authentics.

block was 250°C. The GC oven temperature was programmed as follows: initial 40°C followed by increasing 3°C min^{-1} up to 220°C. Carrier gas was helium with a flow rate set at 1·0 ml min^{-1}. Components were identified on the basis of gas chromatographic retention indices, mass spectra from Wiley MS Chemstation Libraries (6th edn, G1034, Rev. C.00.00; Hewlett-Packard, Palo Alto, CA, USA) and other published mass spectra (Adams 1995).

Kovats indices

Values of *Kovats indices* were determined relative to the retention times of a series of *n*-paraffin hydrocarbons (C_6–C_{25}) on a Hewlett Packard 6890 Gas Chromatograph and 5973A MSD using a fused-silica capillary column like the one used above (Schomberg and Dielmann 1973). Analytical conditions were similar.

Results

Isolation of the volatile constituents by SDSE from *A. camphorata* fruiting bodies, whole cultured broth and *C. kanehirae* wood were conducted respectively. Identification of the various compounds was performed by comparing the mass spectra, retention indices of the obtained data with those cited as well as those of commercially available authentic compounds (Adams 1995). Among 19 compounds identified (Table 1), 2,4,5-trimethoxybenzaldehyde (TMBA) is the major constituent in fruiting bodies. In contrast, we failed to detect TMBA in *C. kanehirae* wood and cultured broth of *A. camphorata* (Tables 2 and 3). β-Nerolidol, was existed in all the three samples, but other alcohols such as 8-epi-β-bisabolol, 1-terpineol, 4-terpineol, α-terpineol and T-muurolol cannot be found in cultured broth. 4-Terpineol and two other major compounds, 1-terpineol and α-terpineol, were major components in *C. kanehirae*. Two groups of characteristic compounds were noted to exist in the volatiles from cultured broth. One was the lactones including γ-valerolactone, γ-octalactone, γ-decalactone and γ-dodecalactone. γ-Lactones are usually associated with the fruity, coconut-like, buttery sweet and nut-like odour. The other

Table 2 The volatile compounds of *Cinnamomum kaehirae* wood

Compounds	Kovats index (CP wax 52CB)*	Peak area (%)†
α-Terpinene	1180	4·59
γ-Terpinene‡	1245	0·98
ρ-Cymenyl	1426	0·45
Linalool oxide‡	1453	0·05
Camphor‡	1496	0·23
Linalool‡	1542	0·09
1-Terpineol	1586	18·19
4-Terpineol‡	1601	40·36
β-Terpineol‡	1624	0·48
Sabinaketone	1632	0·16
α-Terpineol‡	1694	13·10
Piperitone	1716	0·58
1(7)-ρ-Menthen-9-ol	1741	1·24
Cuminal	1763	0·11
ρ-Cymen-8-ol	1830	2·12
Carvenol	1888	0·19
ρ-Menth-1-en-9-ol	1908	0·14
β-Nerolidol	1994	0·09
cis-α-Bisabolene + cuminol	2078	1·92
γ-Curcumene	2109	0·79
8-epi-β-Bisabolol	2128	1·94
T-Cadinol + thymol‡	2162	1·49
T-muurolol	2207	2·78
Total		92·07

*Retention indices determined on a CP wax 52CB column using *n*-alkanes (C_6–C_{25}) as references.
†Area per cent was determined using the total ion chromatogram of GC-MSD area method and from the averaged triplicates.
‡Compounds were identical with the authentics.

Table 3 Composition of volatile compounds in the liquid culture of *Antrodia camphorata*

Compounds	Kovats index (CP wax 52CB)*	Peak area (%)†
2-Butanol	1077	2·22
Isoamyl propionate‡	1181	0·28
Isoamyl alcohol‡	1195	10·35
3-Octanone‡	1235	0·33
1-Octen-3-ol	1434	18·76
E-2-octenyl acetate	1474	0·27
Benzaldehyde‡	1484	1·62
Linalool‡	1495	0·44
Methyl furoate	1557	0·53
1-Octanol‡	1594	1·50
γ-Valerolactone	1605	1·75
Methyl benzoate‡	1616	0·26
2-Methyl-3-furancarboxylic acid	1655	0·81
2-Methyl cyclo-octanone	1685	11·57
Methyl phenylacetate‡	1725	1·06
Phenethyl alcohol‡	1886	7·82
γ-Octalactone‡	1894	3·36
2-Ethyl hexanoic acid‡	1910	0·91
5-*n*-Butyl-2(5H)-furanone	1937	25·12
β-Nerolidol	1994	1·61
Methyl (2-methoxyphenyl) acetate	2024	1·02
γ-Decalactone‡	2057	0·70
γ-Dodecalactone‡	2343	3·70
Dihydro-5-(2-octenyl)-2(3H)-furanone	2365	2·84
Total		98·81

*Retention indices determined on a CP wax 52CB column using *n*-alkanes (C_6–C_{25}) as references.
†Area per cent was determined using the total ion chromatogram of GC-MSD area method and from the averaged triplicates.
‡Compounds were identical with the authentics.

was C8 alcohols or ketones such as 3-octanone, 1-octen-3-ol and *E*-2-octen-1-ol that contribute general mushroom-like aroma.

Acid-digested sawdust of *C. kanehirae* wood was added to the culture broth and sampling was conducted once every week. Dry cell weights were 1·4 ± 0·1, 4·2 ± 0·1, 8·7 ± 0·3 and 9·8 ± 0·2 g l^{-1} in the 7th, 14th, 21st, 28th days respectively. TMBA could not be detected until the fourth week (Fig. 1 top and Table 4). In contrast, TMBA was totally absent in the acid-digested wood liquor (data not shown). Alternatively, vanillin, a common food flavour ingredient with benzaldehyde nucleus, was further tested to afford the precursor for TMBA synthesis. Dissolved vanillin sterilized with filter was applied into the cultured broth on the fifth day 1·2 g l^{-1} dry cell weight after inoculation to avoid evaporation loss in autoclave. Feeding of vanillin too early would result in longer lag phase of mycelium growth. A volume of 3 mgl^{-1} TMBA could be detected in vanillin-feeding cultured broth (Fig. 1 bottom and Table 4), but it still required 4 weeks after vanillin feeding for conversion. Dry cell weight of *A. camphorata* mycelium for four samples are 1·5 ± 0·2, 4·9 ± 0·2, 8·5 ± 0·4 and 9·3 ± 0·2 g l^{-1} respectively.

Discussion

Because the fruiting bodies only grow on the rotten trunk of *C. kanehirae*, it implicated that the compound TMBA may be derived from some precursors pre-existing in *C. kanehirae* wood. Among them, the lignin-degraded products in wood were strongly suspected, but it remains to be examined. TMBA has been found to exhibit selectively inhibitory effect on the enzyme activity of COX-2 when compared with aspirin, ibuprofen, naproxen and celebrex (Momin *et al.* 2003).

COX-2 inhibitors have been reported to possess many functions such as anti-inflammation, pain reduction and chemoprotective effects against colon and breast cancers (Koki *et al.* 1999). The identification of TMBA in wild fresh fruiting bodies of *A. camphorata* actually indicated a good consistency with most of the experienced and reported therapeutic functions of this folk medicinal mushroom. The fermented broth of *A. camphorata* has been reported to inhibit COX-2 expression in MDA-MB-231 cells (Tsai *et al.* 2005); however, investigations of the direct inhibition on COX-2 enzyme activity still have never been exploited.

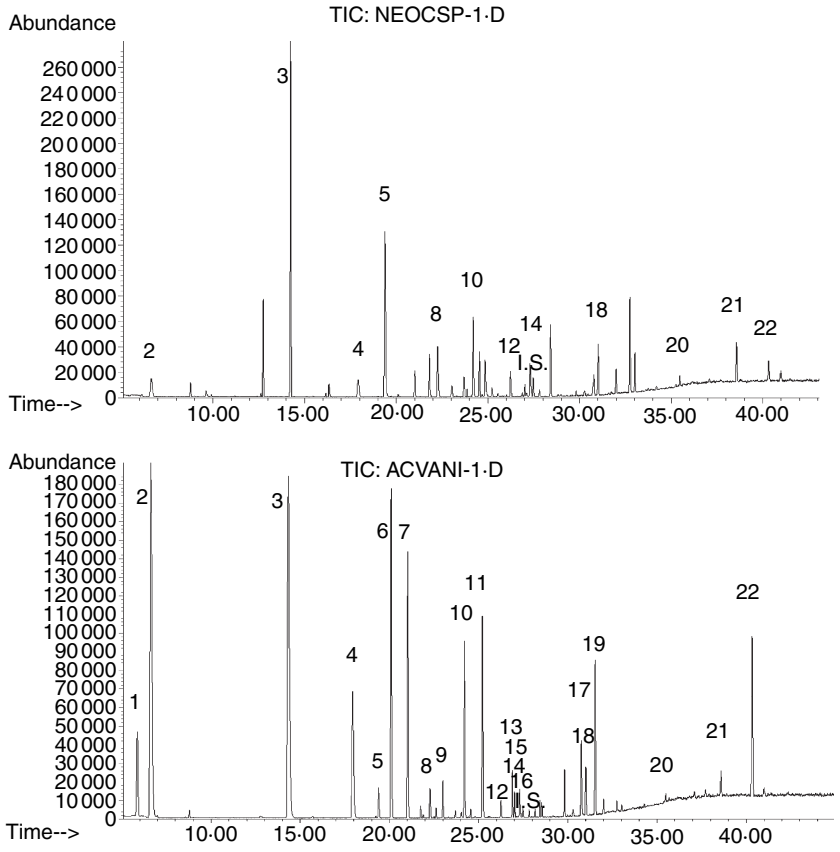

Figure 1 Total ion chromatograms of volatile components from liquid culture of *Antrodia camphorata* after adding *Cinnamomum kanehirai* wood sawdust digests and vanillin. Top: with *Cinnamomum kanehirai* wood sawdust digests; bottom: with vanillin.

Table 4 Quantitative analysis of volatile composition from liquid culture of *Antrodia camphorata* after adding digested *Cinnamomum kanehirae* wood sawdust and vanillin

Peak no.	Compounds	Amount (mg l^{-1})*	
		C. kanehirae	Vanillin
1	Ethyl acetate	ND	17·48 ± 1·0
2	Ethyl alcohol	2·57 ± 0·46	111·45 ± 20·06
3	3-Octanone	20·77 ± 3·68	104·34 ± 12·02
4	3-Octanol	2·06 ± 0·32	31·14 ± 5·67
5	1-Octen-3-ol	15·38 ± 3·48	4·59 ± 1·01
6	*n*-Octyl acetate	ND	43·98 ± 5·13
7	*E*-2-octenyl acetate	ND	36·31 ± 3·05
8	1-Octanol	4·20 ± 0·68	4·75 ± 0·83
9	Methyl 2-furoate	ND	4·76 ± 0·45
10	Methyl benzoate	5·46 ± 1·26	23·56 ± 3·48
11	Ethyl benzoate	0·78 ± 0·22	27·45 ± 4·05
12	1-Cyclohexenyl acetate	1·79 ± 0·62	2·45 ± 0·86
13	Geranyl acetate	ND	4·91 ± 1·24
14	3-Dodecen-1-ol	ND	2·67 ± 1·95
15	Methyl phenylacetate	2·24 ± 0·43	3·68 ± 0·64
IS	*cis*-3-Hexenyl lactate	I.S.	I.S.
16	Ethyl phenylacetate	ND	0·88 ± 0·36
17	Phenethyl alcohol	ND	11·21 ± 2·62
18	γ-Octalactone	3·26 ± 0·24	6·43 ± 1·84
19	2-Methoxy-4-methyl phenol	ND	19·92 ± 3·78
20	β-Nerolidol	5·18 ± 1·65	1·18 ± 0·22
21	2,4,5-Trimethoxybenzaldehyde	2·60 ± 0·46	3·32 ± 1·68
22	γ-Dodecalactone	1·84 ± 0·28	26·39 ± 6·94

*Values given are mean ± SD ($n = 2$).

The absence of TMBA in both the sawdust of *C. kanehirae* wood and the liquid-cultured broth has excluded the possibility that the TMBA nuclear structure could be derived from the aromatic amino acids, the latter otherwise had been abundantly enriched in the ingredients soya bean powder and peptone. Thus the speculation that lignin degradation monomers can afford the precursors for TMBA formation was established. *Antrodia camphorata* has been described as brown-rotton fungus (Chang and Chou 1995), so lignin existed in *C. kanehirae* wood should be degraded first under the help of other micro-organisms.

Vanillin has been used to synthesize 3,4,5-trimethoxybenzaldehyde, a valuable pharmaceutical intermediate, by a three-step process (Rao and Stuber 1983). In view, those two analogues of TMBA, 1,2,3,4-tetramethoxy benzene and methyl 3,4,5-trimethoxy benzoate were simultaneously detected in fruiting bodies. We suggested that there may exist the possibility to produce 3,4,5-trimethoxylbenzaldehyde with *A. camphorata* mycelium by feeding different precursors or varying different parameters in culture.

To sum up, it was suggested that TMBA not only could be derived from the digested sawdust of *C. kanehirae* wood but also from vanillin. However, more potential material was for vanillin but not for the sawdust of *C. kanehirae* if the rarity of *C. kanehirae* wood in nature is to be taken into consideration. In this study, we conclude that a simple process was successfully developed to manufacture functional *A. camphorata* mycelium product with TMBA by feeding vanillin.

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