

## Isolation and Identification of an Antifungal Sesquiterpene Alcohol from Amboyna Wood

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**Abstract:** An antifungal compound was isolated from the n-hexane solubles in methanolic extracts of amboyna wood by bioassay-guided fractionation using *Pleurotus pulmonarius*, a wood-rotting fungus. The compound was identified as  $\beta$ -eudesmol, a sesquiterpene alcohol with an eudesmane skeleton, based on instrumental analyses (Ultraviolet absorption, Mass spectrometry and <sup>1</sup>H and <sup>13</sup>C-Nuclear Magnetic Resonance spectroscopy) and a melting point test. The compound showed 21% of the antifungal activity of the methanolic extract as a control. Antifungal assays against *P. pulmonarius* showed that addition of  $\beta$ -eudesmol at different concentrations affected the inhibition of fungal growth. Furthermore, the activity to inhibit the growth of *P. pulmonarius* seemed to be dependent on the concentration of  $\beta$ -eudesmol.

**Key words:** Amboyna wood, antifungal compound,  $\beta$ -eudesmol, bioassay-guided fractionation, sesquiterpene alcohol, wood-rotting fungi

### INTRODUCTION

As part of our on-going research into biologically active compounds from trees and their production by tissue culture<sup>[1-6]</sup>, previous study conducted the isolation and identification of antifungal compounds from amboyna wood.

Amboyna, *Pterocarpus indicus* Willd. (leguminosae), a large deciduous tree, is an important timber native to tropical countries. The quality of its wood makes this species an excellent source of timber and material for furniture in southern Asia. Amboyna grows throughout the Philippines and other tropical countries such as India, Borneo, Celebes, New Guinea and the Caroline Islands<sup>[7]</sup>. Traditionally, the kinos, young leaves and roots of amboyna have been used to remedy sores of the mouth, diarrhea, syphilitic sores, prickly heat and ulcers by Malaysians and Javanese. Parts of this tree are also used as an antibacterial, antimalarial, antidysenteric, antidiarrheal, astringent, purgative and mouthwash to treat thrush. The red latex of amboyna is used as a remedy for tumors, especially of the mouth, while the leaves were reported to significantly inhibit the growth of Ehrlich ascites carcinoma cells in mice<sup>[8-13]</sup>.

Previous study on this plant has made known the occurrence of a sesquiterpene alcohol, pterocarpol, and two flavonoids, liquiritigenin and isoliquiritigenin. To the best of our knowledge, little is known about the antifungal

activity of amboyna wood. Therefore, this study deals with the isolation and structural determination of an antifungal compound from the wood.

### MATERIALS AND METHODS

**Wood samples:** Amboyna wood samples were collected in Indonesia in 2001. The wood was sawn to convert it into lumber. The lumber was converted into woodmeal with a Wiley Mill. The woodmeal was passed through # 40 mesh prior to extraction. A voucher specimen was deposited in the laboratory.

**Test fungi:** Cultures of *Pleurotus pulmonarius* (isolated from Kalimantan, Indonesia) were maintained in onion extract agar (1 L of medium contains 125 g of onion, 50 g of glucose, 0.3 g of K<sub>2</sub>HPO<sub>4</sub>, 0.3 g of KH<sub>2</sub>PO<sub>4</sub>, 0.2 g of MgSO<sub>4</sub>·7H<sub>2</sub>O, 5 g of peptone and 30 g of agar powder at pH 6.0) and seven-day old cultures were used for the antifungal assays. Subculture of the fungi was conducted every 30 days during the experiments.

**Chemicals:** Authentic  $\beta$ -eudesmol was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The silica gel used for column chromatography was a Wakogel C-200, 75-150  $\mu$ m (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Thin Layer Chromatography (TLC)

aluminum sheets (Silica gel 60 F<sub>254</sub>, 20x20 cm) and glass plates (RP-8 F<sub>254</sub>, 5x10 cm) were obtained from Merck (Darmstadt, Germany). Spots were detected under a UV lamp or by heating after spraying with conc. sulfuric acid. All other materials or solvents were of the highest purity or High-performance Liquid Chromatography (HPLC) grade.

**Apparatus:** Melting points were measured on a Yanaco micro melting point apparatus (Yanaco Co., Ltd., Kyoto, Japan) and were uncorrected. Ultraviolet (UV) spectra,  $\lambda_{\text{max}}$  (nm), were measured on a Shimadzu UV-VIS 1200 spectrophotometer (Shimadzu Corp., Kyoto, Japan); HPLC analysis was performed with a Shimadzu system (Shimadzu Corp.) consisting of a LC-10 ADvp pump equipped with a CTO 10Avp column oven, SPD 10 Avp UV-visible spectrophotometric detector and a FRC 10A fraction collector; peak analysis and assignment was done with Class-LC 10 software; Mass spectra were recorded on a Shimadzu GC-MS QP 5050A (Shimadzu Corp.) at an electron energy of 70 eV (direct inlet); <sup>1</sup>H-NMR and <sup>13</sup>C-NMR spectra were recorded on a JEOL JNM-EX400 (JEOL, Ltd., Tokyo, Japan) at 400 MHz and 100 MHz, respectively. Chemical shifts are expressed as  $\delta$  in ppm and TMS was used as an internal standard. Coupling constants (J) were recorded in Hz.

**Antifungal assay:** The agar dilution method was used for the antifungal assays. Sterile onion extract agar (20 mL) and 2 g of woodmeal or the respective amount of wood extract dissolved in acetone were mixed on a 90 mm petri dish and sterilized by autoclave (121°C, 20 min). Control plates contained culture medium and acetone only. Mycelial plugs were punched out with a sterile 4 mm diameter cork borer from the test fungi grown on each agar medium and placed at the center of each petri dish. Inoculated agar plates were incubated in the dark at 25°C for 7 days. Antifungal activity was determined based on inhibition using the formula:

$$\text{Percent inhibition} = (1 - T/C) \times 100$$

where, T is hyphal extension of the treated sample and C is hyphal extension of the control. IC<sub>50</sub> was defined as the lowest concentration of extract, which inhibited 50% of fungal growth.

**Extraction:** Woodmeal of amboyna (1.2 kg) was twice treated with methanol at its boiling point to obtain the methanolic extract. The extract solution was filtrated under suction and concentrated with a rotary evaporator under reduced pressure to give the methanolic extract (420.16 g).

**Isolation of the active compound:** The methanolic extract was suspended in water and then partitioned with n-hexane. The organic fraction, where the antifungal activity was found, was dried over anhydrous sodium sulfate and concentrated to give the n-hexane solubles (26.5 g). A portion of the n-hexane solubles (12.8 g) was separated by chromatography with a silica gel column (gradient of 2-50% methanol in chloroform). The fractions collected were first subjected to TLC and visualized by UV, and the TLC plates were sprayed with concentrated sulfuric acid. The fractions containing the same spots were combined and gave 5 fractions (C.1-C.5). The antifungal assay against *P. pulmonarius* showed that fraction C.4 (6.4 g) was most active. Therefore, part of fraction C.4 (4.4 g) was further chromatographed on a silica gel and eluted with a gradient of chloroform and methanol (0.4-10% methanol). After TLC analysis, the combined eluates were concentrated into 7 fractions (C.4.1-C.4.7). Fraction C.4.3 (2.8 g) was most active in the antifungal assay. Further column chromatography over silica gel of fraction C.4.3 (1.4 g) with a gradient of chloroform and methanol (0.5-10% methanol) gave 5 fractions (C.4.3.1-C.4.3.5). Part of fraction C.4.3.2 (370 mg), where the most antifungal activity in the five fraction was found, was subjected to column chromatography on silica gel eluted with a gradient of chloroform and methanol (1-50% methanol). Based on the TLC profiles, the fractions collected were combined and concentrated into 7 fractions (C.4.3.2.1-C.4.3.2.7). The most active fraction (C.4.3.2.2) was further separated by preparative HPLC to isolate three more fractions (Fr. 1-3). The preparative HPLC-based separation of fraction C.4.3.2.2 was conducted on a 5  $\mu$ m Capcell Pak C18 MG (20 mm i.d.x250 mm) column (Shiseido Co., Ltd., Tokyo, Japan) with an isocratic elution using methanol-water (80% methanol) at a flow rate of 7 mL min<sup>-1</sup>. Peaks were detected at a UV wavelength of 205 nm and peak fractions were collected automatically by the fraction collector. The antifungal activities of the three fractions were checked by the method mentioned above. An active compound (compound A) was isolated from Fr. 2. as an oily product. Compound A was obtained as a colorless crystalline product, mp. 80-81°C after the crystallization of Fr. 2 from ethyl acetate and n-hexane.

**The isolated active compound A:** Colorless needles, m.p. 80-81°C (lit. 80-81°C)<sup>[14]</sup>. UV MeOH  $\lambda_{\text{max}}$  nm (log  $\epsilon$ ): 205 (2.55). EIMS m/z (rel.int.) 222 [M<sup>+</sup>](3), 204 (4), 189 (6), 164 (20), 149 (44), 108 (25), 59 (100). <sup>1</sup>H-NMR (CDCl<sub>3</sub>, 400 MHz):  $\delta$  4.72 (1H, bs, H<sub>A</sub> or H<sub>B</sub>), 4.55 (1H, bs, H<sub>B</sub> or H<sub>A</sub>), 2.29~2.33 (1H, bd, J=13.2 Hz, H-3 <sub>$\beta$</sub> ), 1.99~2.01 (1H, m, H-3 <sub>$\alpha$</sub> ), 1.76~1.78 (1H, bd, J=12.2 Hz, H-5), 1.65-1.12 (12H, eleven

cyclic protons and OH), 1.21 (6H, s, two methyl groups at C-12 and C-13), 0.70 (3H, s, one methyl group at C-14). <sup>13</sup>C-NMR (CDCl<sub>3</sub>, 100 MHz): δ 16.3 (CH<sub>3</sub>, C-14), 22.4 (CH<sub>2</sub>, C-8), 23.5 (CH<sub>2</sub>, C-2), 25.0 (CH<sub>3</sub>, C-12), 27.1 (CH<sub>2</sub>, C-13), 27.2 (CH<sub>2</sub>, C-9), 35.9 (C, C-10), 36.9 (CH<sub>2</sub>, C-3), 41.1 (CH<sub>2</sub>, C-1), 41.8 (CH<sub>2</sub>, C-6), 49.5 (CH, C-7), 49.8 (CH, C-5), 72.9 (COH, C-11), 105.3 (=CH<sub>2</sub>, C-15), 151.1 (=C, C-4). EIMS, UV, <sup>1</sup>H-NMR and <sup>13</sup>C-NMR spectra of compound A were in good agreement with those of the authentic β-eudesmol. The mixed-melting point test of compound A and authentic β-eudesmol was undepressed.

**Benzoate of compound A:** Compound A (7 mg) and 3, 5-dinitrobenzoyl chloride (20 mg) in dry pyridine (0.5 mL) were heated in a hot waterbath for 10 min. without contact with moisture and stood overnight at room temperature. The mixture was diluted with ice-cold water and extracted with chloroform (2 times). The chloroform solution was washed with aq. NaHCO<sub>3</sub> followed by a 1 N HCl solution, and dried over anhydrous sodium sulfate. After evaporation of the solution, solid reaction products were obtained. The products were recrystallized from the mixture of chloroform and ethanol to furnish the pure 3, 5-dinitrobenzoate of compound A, mp. 135-137°C. The melting point and mass spectrum [EIMS m/z (rel. int.) 416 (M<sup>+</sup>) (1), 212 (5), 204 (75), 189 (52), 175 (9), 161 (100), 149 (42), 122 (31), 105 (42)] of 3, 5-dinitrobenzoate obtained from compound A were identical with those of the 3, 5-dinitrobenzoate from authentic β-eudesmol (Fig. 1).

## RESULTS

Isolation and identification of compound A: An antifungal assay against *P. pulmonarius* showed that n-hexane solubles of the methanolic extract of amboyna wood exhibited significant antifungal activity with 35% inhibition. Bioassay-guided fractionation of the n-hexane solubles demonstrated that several fractions were active against *P. pulmonarius* (Fig. 2). Silica gel column chromatography of n-hexane solubles gave fraction C.4 with 75% activity. Repeated column chromatography of the C.4 fraction over silica gel resulted in the isolation of the active fractions, C.4.3, C.4.3.2 and C.4.3.2.2 with 83, 85 and 60% activity, respectively. Finally, a preparative HPLC-based separation furnished an active compound (compound A) from fraction C.4.3.2.2. Compound A, C<sub>15</sub>H<sub>26</sub>O, (M<sup>+</sup>) [(molecular ion peak)=222], mp. 80-81°C, was composed of colorless crystals. The EI mass spectrum of compound A was characteristic of sesquiterpenoids with a base peak at m/z 59 matched to an isopropyl alcohol moiety [(CH<sub>3</sub>)<sub>2</sub>COH<sup>+</sup>]. Several important

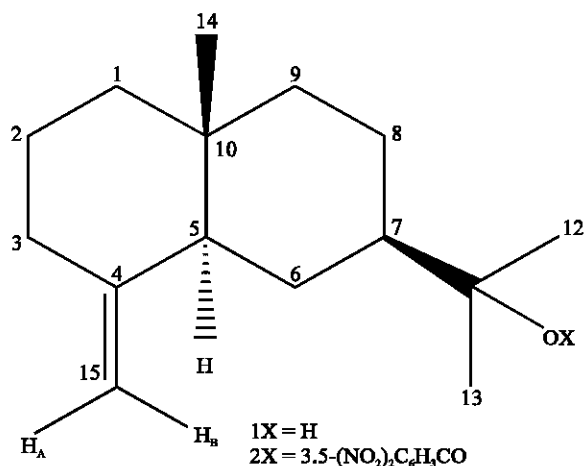


Fig. 1: Chemical structure of β-eudesmol (1) and 3,5-dinitrobenzoate (2)

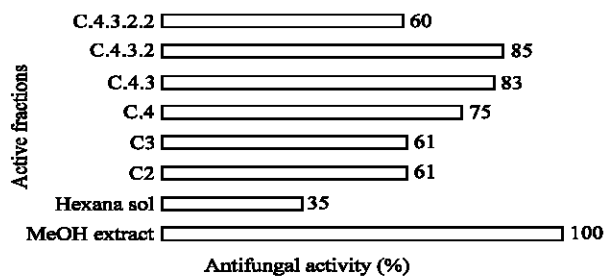


Fig. 2: Antifungal active fraction from Amboyna wood against *P. pulmonarius*

fragment ions, at m/z 204, 189, 164, 149, and 108 were also observed. From the analysis of the mass spectrum, compound A was suggested to be β-eudesmol, a sesquiterpenoid having an eudesmane skeleton. The UV spectrum of compound A showed a maximum absorbance at 205. In the <sup>1</sup>H-NMR spectrum of compound A, signals from two exomethylene protons at 4.71 and 4.48 ppm, three allylic protons at 2.31, 1.99 and 1.77 ppm, a gem dimethyl group at 1.21 ppm, one angular methyl proton at 0.70 ppm were observed. These values agree with those recorded for β-eudesmol<sup>[15,16]</sup>. Furthermore, eleven cyclic protons and one proton of a hydroxygroup at 1.65-1.12 ppm were observed in the NMR spectrum. From the results obtained above, compound A was suggested to be β-eudesmol. In the <sup>13</sup>C-NMR spectrum, the carbon signal of the angular methyl group (C-14) was visible at 16.3 ppm and an exocyclic methylene group (C-4) was observed at 151.1 ppm. The <sup>13</sup>C-NMR spectrum of compound A was identical to that of β-eudesmol<sup>[17,18]</sup>. The mixed-melting point test of compound A and authentic β-eudesmol was undepressed. Furthermore,

characteristics of the 3,5-dinitrobenzoate from compound A were completely identical with those of authentic 3, 5-dinitrobenzoate from  $\beta$ -eudesmol. From the present results compound A was identified as  $\beta$ -eudesmol.

**Antifungal activity of  $\beta$ -eudesmol at different concentrations:**

The effect of the concentration of  $\beta$ -eudesmol on the antifungal activity against *P. pulmonarius* was evaluated.  $\beta$ -eudesmol was added to the medium in several concentrations (0.1, 1, 5, 10, 20 mg/dish) and the growth of fungal mycelia was compared with the control (without addition of  $\beta$ -eudesmol). The result of the antifungal activity of  $\beta$ -eudesmol at several concentrations can be seen in Fig. 3 showed that after the seven-day incubation at 25°C in the dark, significantly different levels of antifungal activity were observed at different concentrations of  $\beta$ -eudesmol (0.1, 1, 5, 10, 20 mg/dish). The antifungal activity at each concentration was 9, 21, 53, 60 and 68%, respectively. Increasing the concentration of  $\beta$ -eudesmol from 0.1 to 1 mg significantly increased the inhibition of fungal growth. Furthermore, a similar trend was observed when the concentration was raised from 1 to 5 mg, which resulted in an increase in the inhibitory effect by more than 2 fold. However, increasing the concentration from 5-10 mg and 10-20 mg seemed to be less effective. The results showed that the activity of  $\beta$ -eudesmol to inhibit the growth of *P. pulmonarius* increased with an increase in the concentration to about 5 mg/dish. The results also demonstrated that 50% inhibition (IC<sub>50</sub>) by  $\beta$ -eudesmol of *P. pulmonarius* can be achieved at a concentration of less than 5 mg/dish.

**DISCUSSION**

Earlier study on the screening of trees having antifungal activity showed that Amboyna wood was the most active against four basidiomycetes, *Pycnoporus coccineus*, *Pleurotus pulmonarius*, *Pleurotus ostreatus* and *Lentinula edodes*. As a continuation of this research, we have isolated a sesquiterpene alcohol, pterocarpol, and two flavonoids, liquiritigenin and isoliquiritigenin, as antifungal compounds from the diethyl ether solubles of Amboyna wood. This study isolated and identified an antifungal sesquiterpenoid from amboyna wood. In the antifungal assay, n-hexane solubles also exhibited promising antifungal activity which inhibited the growth of *P. pulmonarius* by 35%. Repeated silica gel column chromatography of n-hexane solubles combined with the preparative HPLC separation afforded an antifungal compound identified as  $\beta$ -eudesmol by instrumental analyses and by comparison with an authentic sample

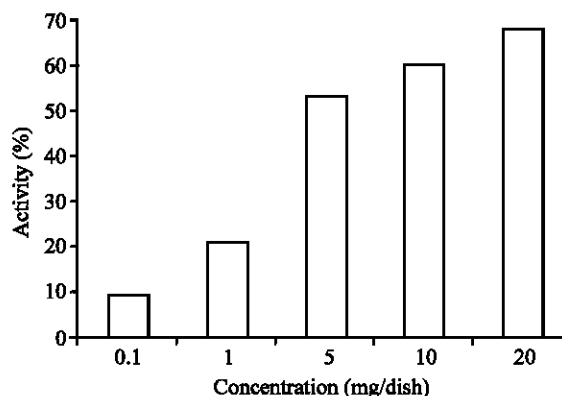


Fig. 3: Activity of  $\beta$ -eudesmol against *P. Pulmonarius* at several concentration

(mmp, UV, MS, <sup>1</sup>H and <sup>13</sup>C-NMR). An antifungal assay to find out the effect of the concentration of  $\beta$ -eudesmol on the inhibition of fungal growth was conducted. Figure 3. shows the activity of  $\beta$ -eudesmol to inhibit the growth of *P. pulmonarius* at several concentrations. An increase in the concentration of  $\beta$ -eudesmol from 0.1-1 and 5 mg significantly affected the activity to inhibit the fungal growth. However, increasing the concentration of  $\beta$ -eudesmol to more than 5 mg seemed to be less effective. In addition, with regard to the trend of antifungal activity (Fig. 3),  $\beta$ -eudesmol seems to have a concentration dependent inhibitory effect.

Plant terpenoids are well-known phytoalexins produced in plant cells after exposure to micro-organisms or treatment with elicitors. Among them, sesquiterpenoids including furanosesquiterpenoids, eudesmane, cadinane, nor-eudesmane eremophilane and so on are the most common class of compounds<sup>[19]</sup>. Eudesmols are sesquiterpene alcohols which serve as precursors for many biologically active derivatives e.g. santonin and panellon<sup>[20, 21]</sup>.  $\beta$ -eudesmol was first isolated by Baker and Smith from the essential oil of *Eucalyptus piperita* towards the end of the nineteenth century<sup>[22]</sup>. Furthermore,  $\beta$ -eudesmol has been isolated from various plants<sup>[23-27]</sup>.  $\beta$ -eudesmol can act as hypotensive, antihepatotoxic or antiepileptic agent<sup>[28-30]</sup> and was also reported to show considerable anti-microbial activity against *Alternaria* fungi<sup>[31]</sup>. However, reports about the activity of natural  $\beta$ -eudesmol against wood-rotting fungi are limited. Fungal attacks cause serious problems in the agricultural and forestry sector. The decline in agricultural yields and damage to wood products caused by fungal attack are a major source of economic losses in the world. Furthermore, many fungi are also reported to be pathogenic to humans and animals causing various diseases. In conclusion, this investigation on the antifungal activity of  $\beta$ -eudesmol will

increase the applicability of the compound not only in the agricultural field, but also in many other areas. Further investigations of the activity of  $\beta$ -eudesmol to inhibit the growth of a broad range of fungi is indispensable.

#### ACKNOWLEDGMENTS

We thank the Integrated Centre for Science, Ehime University for the recording of NMR spectra. We also thank Mr. Kawano, Ehime Rinsan Shoji Company, Ltd. (Matsuyama, Ehime Prefecture, Japan) for providing the Amboyna wood for this study.

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