Germination Inhibitor from the Japanese Cedar *Cryptomeria japonica*

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(1*S*,6*R*)-2,7(14),10-Bisabolatrien-1-ol-4-one was identified as a germination inhibitor from the methanol extract of Japanese cedar wood, *Cryptomeria japonica*. The occurrence of this compound in 1 g fresh wood was 2.0 mg, and showed a maximum of 60% germination inhibition at the dose of 20 mg/filter paper (157 μg/cm^2) against both of lettuce and rice seeds for 4 d. A selective activity between Dicotyledoneae and Monocotyledoneae seeds was not observed.

Key words: (1*S*,6*R*)-2,7(14),10-Bisabolatrien-1-ol-4-one, *Cryptomeria japonica,* Germination Inhibitor

Introduction

Japanese cedar, *Cryptomeria japonica* D. Don, is one of the most popular indigenous trees in Japan and has been afforested in the mountain region throughout the country. Many studies on chemical components of the wood of this tree have been carried out in the long term by many groups. To date, many compounds were identified from woods of *C. japonica*, but only a few compounds were reported to have biological activities including termiticides (Sogabe *et al.*, 2000), acaricides (Yatagai *et al.*, 1991; Morita *et al.*, 1991; Morita and Yatagi, 1994) and a growth inhibitor against fungi (Nakajima *et al.*, 1980). Recently, we identified four compounds, secuirin-C, agatharesinol, cubebol and 2,7(14),10-bisabolatrien-1-ol-4-one, as antifeedants to a snail from a wood of *C. japonica* (Chen *et al.,* 2001a, b). Yatagai *et al.* (1991) reported that the extracts of cedar woods showed growth inhibition activities against plant seeds, but its structure(s) have not yet been elucidated.

Here we report the isolation of (1*S*,6*R*)-2,7(14),10-bisabolatrien-1-ol-4-one from the methanol extract of *C. japonica* wood which was identified as a germination inhibitor.

Abbreviations: Fr., fraction; FW, fresh wood equivalent; I, inhibition; Rt, retention time.

Materials and Methods

Plant material

Fresh *C. japonica* wood used in this study was collected from the Reihoku area in Kochi Prefecture, Japan. Lettuce (*Lactuca sativa* L. cv. Cisco) and rice (*Oryza sativa* L. cv. Toyonishiki) seeds used were purchased from Maekawasyubyo Nursery (Kochi, Japan) and the Agricultural Cooperative Society of Kochi (Kochi, Japan), respectively.

Instruments

Gas liquid chromatography (GLC) analyses were carried out using a fused silica capillary column (HR1710; 0.25 μm film thickness, 25 m $\times$ 0.2 mm i.d.) with a Shimazu GC-14A instrument. Samples were introduced via a split injector at 250 °C and a flame ionization detector was set at 250 °C. The column temperature was kept at 100 °C for 2 min and then programmed to 250 °C at a rate of 10 °C/min. Gas chromatography-mass spectrometry (GC-MS) analyses were conducted on a JEOL MS600 mass spectrometer using a fused silica capillary column (HP-5; 0.25 μm film thickness, 30 m $\times$ 0.32 mm i.d.) at 70 eV programmed from 100 °C (2-min hold) to 250 °C at a rate of 10 °C/min. High performance liquid chromatography (HPLC) was carried out using a silica gel column (YMC-Pack SIL06-A; 300 $\times$ 10 mm

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i.d.) with a Hitachi L-6000 pump system equipped with a Shodx RI-71 refractive index detector. Infra-red spectra were measured on a Shimadzu FT-IR-4300 instrument, using the liquid film method. NMR spectra were recorded on a JEOL Lambda 400 spectrometer in $CDCl_3$ using tetramethylsilane as an internal standard. Optical rotation was measured on a HORIBA SEPA-200 using a microcell in methanol solution at 23 °C.

Bioassay

The filter paper (9 cm i.d., #2; Toyo Advantec) treated with 1 ml of test solution at various concentrations, after air drying, was placed on a glass Petri dish (9 cm i.d.). The control was treated in the same manner with solvent only. Then the filter paper was moistened with 2 ml of water and 25 lettuce (or rice) seeds were sown on it. The Petri dish was covered with a lid and sealed with a parafilm in order to prevent dryness. The bioassay apparatus was maintained at 24 ± 3 °C with a 14-h period of illumination, and seeds were daily observed for 4 d. Inhibition [I (%)] was calculated from the equation $I = 100 \times [(C - S)/C]$, where S and C was the numbers of the germinated seeds on the treated and control filter paper, respectively. After the data were statistically analyzed using a Fisher's exact test ($P < 0.01$), the strong inhibition activities (++) were distinguished from the weak activities (+) by whether I was over or below 50%.

Extraction and purification procedure

Fresh *C. japonica* wood (300 g), which was cut into small pieces (5 cm long, 1 cm thickness), was extracted with methanol (2 × 2 l) at room temperature for 3 d. After evaporating the solvent *in vacuo*, the residue of the combined extracts (6.4 g) was dissolved in 200 ml of water, and then the solution partitioned between water and the following solvents successively: *n*-hexane, diethyl ether, ethyl acetate and *n*-butanol (250 ml, 4 times each). The ethyl acetate layer was chromatographed on a silica gel column (350 × 20 mm i.d.; Wakogel C-300, Wako Pure Chemical Industry) by eluting successively with the following solvents: diethyl ether, 10% diethyl ether in ethyl acetate, 30% diethyl ether in ethyl acetate, 70% diethyl ether in ethyl acetate and ethyl acetate (500 ml each). The diethyl ether fraction was further fractionated over a silica gel column (250 × 18 mm i.d.) by stepwise elution with *n*-hexane, 10% ethyl acetate in *n*-hexane, 30% ethyl acetate in *n*-hexane, 70% ethyl acetate in *n*-hexane and finally diethyl ether (300 ml each). Subsequently the 10% ethyl acetate in *n*-hexane fraction was separated into three fractions, Fr. A (Rt = 0.0–32.4 min), Fr. B (Rt = 32.4–35.6 min) and Fr. C (Rt = 35.6–55.0 min), by using preparative HPLC eluting with the solvent system *n*-hexane/ethyl acetate/ethanol (90:10:1 v/v/v) at a flow rate of 2 ml/min. Fr. B consisted only of compound **1** at Rt = 34.2 min and the yield of compound **1** was 2.0 mg/g fresh wood equivalent (g FW).

Results and Discussion

Fresh *C. japonica* wood (300 g) was extracted twice with methanol and the combined extract completely inhibited germination of lettuce seeds at the dose of 10 g FW/filter paper for 4 d (I = 100%, +). The crude methanol extract was, after evaporating the solvent, dissolved in 200 ml of water and partitioned between water and subsequently the following solvents: *n*-hexane, diethyl ether, ethyl acetate and *n*-butanol. The high inhibition activity was observed in the ethyl acetate layer (I = 62.5%, ++) but weaker activities were also detected in the diethyl ether (I = 45.8%, +) and the butanol (I = 33.3%, +) layers at 10 g FW/filter paper. However, the hexane and the water layer did not show inhibition activities (I = 4.2%, –, and 0.0%, –, respectively) at the same dose. Then the most active ethyl acetate layer was chromatographed on a silica gel column to separate it into five fractions. Judging from the bioassay results, inhibition activity was mainly recovered in the diethyl ether fraction (I = 66.7%, ++) and the other fractions only showed weak or insignificant activities. The diethyl ether fraction was then purified on a silica gel column again and high activity was observed in the 10% ethyl acetate/*n*-hexane fraction (I = 75.0%, ++). Finally the active fraction was separated into three fractions by HPLC (Fr. A: 0.0–32.4 min, Fr. B: 32.4–35.6 min, Fr. C: 35.6–55.0 min), and only Fr. B indicated inhibition activity (I = 62.5%, ++). As Fr. B gave only one peak (Rt = 14.8 min) in the GLC analysis, it was assigned this germination inhibitor as compound **1**. MS, ^1H NMR and ^{13}C NMR spectra of compound **1** were in good agreement with those of our previous data for 2,7(14),10-bisabolatrien-1-ol-4-one (Chen *et al.*, 2001a). The

$[\alpha]_D^{23}$ value of compound **1** of +130° was almost the same as that of our previous data for the (1*S*,6*R*)-isomer isolated from *C. japonica* (Kim *et al*., 2002). So germination inhibitor **1** was identified as (1*S*,6*R*)-2,7(14),10-bisabolatrien-1-ol-4-one (Fig. 1).

It was reported by Yatagai *et al*. (1991) that the germination of radish seeds was inhibited by both the essential oil and the methanol extract of *C. japonica* wood, and they suggested that the active component would be relatively polar. As this was confirmed in our study, it was thought that the identified compound would be an answer for them. This compound was first identified from *C. japonica* by Nagashima and Tazaki (1993), and recently the absolute configuration of this compound was revealed as the (1*S*,6*R*)-isomer by Kim *et al*. (2002). Effects of this compound on some animals were reported as antifeedant against a snail, *Acusta despesta* (Chen *et al*., 2001a), and repellent against a pill-bug, *Armadillidium vulgare* (Morisawa *et al*., 2002). To our knowledge, this is the first report that compound **1** exhibits biological effects on plants.

The inhibition activities at various concentrations of (1*S*,6*R*)-2,7(14),10- bisabolatrien-1-ol-4-one against both lettuce and rice seeds were also examined (Fig. 1). Inhibition against both rice and lettuce seeds were enhanced with the increase in the dose of the compound. The inhibition activities against both rice and lettuce seeds were almost the same at the concentrations of ca. 40 and 80 μM, but the inhibition against rice seeds were 10% weaker than that of lettuce seeds at the concentrations of ca. 4 and 20 μM. However, there were no significant differences between the inhibition against lettuce and rice seeds at all concentrations examined, and it is thought that the compound would not show a selective activity between Dicotyledoneae and Monocotyledoneae. Although inhibition activities of this compound against other plant species have not yet been investigated, this compound may be used as a herbicide inhibiting germination of plant seeds including both Dicotyledoneae and Monocotyledoneae plants.

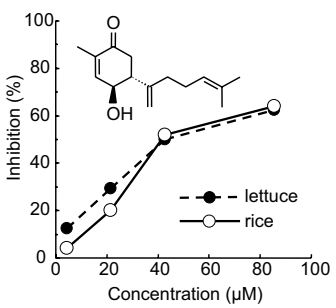

Fig. 1. Structure and germination inhibiting activities against rice and lettuce seeds of (1*S*,6*R*)-2,7(14),10-bisabolatrien-1-ol-4-one.

The inhibition activities of (1*S*,6*R*)-2,7(14),10-bisabolatrien-1-ol-4-one were almost unchanged, despite the increase in concentration from ca. 40 to 80 μM (Fig. 1). It was suggested that this could be the maximum inhibition activities of the isolated compound. However, the original crude extract indicated complete inhibition at 10 g FW/filter paper. So this indicated that other inhibitors may be present in the original methanol extract and disperse to many fractions on the process of purification. Thus a study on minor active constituents is needed.

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