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# Antioxidant Activity of Abietane-Type Diterpenes from Heartwood of *Taiwania cryptomerioides* Hayata

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*Keywords*

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**Summary**

Nine abietane-type diterpenes were isolated from the heartwood of *Taiwania cryptomerioides* and their structures identified by spectral analyses. Among these nine compounds, six diterpenes were isolated for the first time from *T. cryptomerioides*, including 6,7-dehydroferruginol, 6α-hydroxysugiol, 5,6-dehydro-6-hydroxysugiol, 11-hydroxyferruginol, secoabietane dialdehyde and isohinokiol. We suggest that abietane-type diterpenes are the dominant diterpenes in the heartwood of *T. cryptomerioides*. A possible biosynthesis pathway is proposed. In addition, a 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay was performed to evaluate the antioxidant activity of these diterpenes. This study demonstrates that ferruginol exhibits the strongest antioxidant activity among the diterpenes isolated from *T. cryptomerioides* heartwood.

## Introduction

The secondary, derived metabolites of trees (wood extractives) contribute to specific properties and unique features of the wood, such as color, odour, durability, etc. *Taiwania cryptomerioides* Hayata is a native tree that grows at elevations from 1800–2600 m in Taiwan's central mountains. Recently, we characterized the putative bioactivity of some specific chemical constituents isolated from the heartwood of *T. cryptomerioides*, including photodiscoloration (Chang *et al.* 1999a), antifungal activity (Chang *et al.* 1998, 1999b, 2000a) and antitermitic activity (Chang *et al.* 2001a). Furthermore, we have studied antibacterial and antimite activities of the heartwood extractives, essential oils and their derived compounds (Chang *et al.* 2000b, 2001b). Potential usages of the phytochemicals isolated from *T. cryptomerioides* in pharmacological applications have been evaluated by our research group. For instance, taiwanin A, a dominant lignan isolated from the heartwood of *T. cryptomerioides*, confers significant cytotoxicity against three specific human tumor cells, *i.e.* A-549 lung carcinoma, MCF-7 human breast adenocarcinoma and HT-29 colon adenocarcinoma (Chang *et al.* 2000c). The observed anti-tumor cell activity of taiwanin A functions *via* an apoptotic pathway as demonstrated by morphology-based evaluation, flow cytometric analysis and DNA fragmentation assays (Chang *et al.* 2000c).

Although many interesting and useful compounds have been isolated from and characterized for *T. cryptomerioides* by Chang and coworkers, in this study we identified several diterpenes from the heartwood of the plant, which have not been reported. Terpenoids, the most widespread and chemically diverse group of natural products, are considered to play different roles in the ecology and physiology of plants (Brielmann 1999). During the past several years, a broad range of various natural products from plants have been characterized as useful as pharmaceuticals or nutraceuticals, and some of them have drastically increased in market availability and public usage worldwide, *e.g.*, *Echinacea* spp. and *Ginkgo biloba* plant extracts. Phytomedicines, as an alternative to synthetic drugs, have played an important role at the level of basic, public health care in various countries, especially in Asia.

Reactive oxygen species (ROS) encompass a spectrum of diverse chemical species including superoxide anions, hydrogen peroxide, hydroxyl radicals, nitric oxide and peroxynitrite. ROS have been shown to be involved in cellular signaling, cell growth regulation and energy production under *in vivo* conditions in animal and plant systems. (Halliwell 1997). The potential of antioxidant activities to reduce oxidative stress *in vivo* have inspired various investigators to search for effective antioxidants from plant sources. Thus, to evaluate the antioxidant activity of diterpenes isolated from *T. cryptomerioides,* this study uses the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity assay.

## Materials and Methods

High Performance Liquid Chromatography (HPLC) was performed with a Jasco model PU-980 pump equipped with an RI-930 RI detector and a Hibar Lichrosorb Si 60 (25 × 1 cm i.d.) column. The infrared (IR) spectra were recorded on a Bio-Rad

model FTS-40 spectrophotometer. The mass spectra (MS) were obtained on a Finnigan MAT-95S mass spectrometer. The nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance-200, 300 and 500 MHz FT-NMR.

*Extraction and isolation*

A 27 year-old *T. cryptomerioides* tree collected from the Experimental Forest of National Taiwan University provided the experimental material in this study. *T. cryptomerioides* heartwood chips were prepared from the freshly cut tree and the air-dried chips (5.7 kg) were completely extracted with methanol (MeOH). The MeOH extracts were condensed to *ca.* 286.4 g by a rotary evaporator and extracted successively with *n*-hexane (*n*-$C_6H_{14}$), chloroform ($CHCl_3$), ethyl acetate (EtOAc) and methanol (MeOH) to yield the *n*-$C_6H_{14}$, $CHCl_3$, EtOAc, MeOH soluble fractions and MeOH insoluble fraction. The *n*-$C_6H_{14}$ soluble fraction (20 g) was divided into 41 subfractions (H1-H41) using a silica-gel column eluted with EtOAc/*n*-$C_6H_{14}$ by 0/100 to 100/0 gradient elution. Compounds **1**, **2**, **3**, **4** and **5**, as shown in Figure 1, were isolated and purified from the H4 subfraction by semi-preparative HPLC with a Si-60 column (Mobile phase: EtOAc/*n*-$C_6H_{14}$ = 30/70; Flow rate: 1 ml/min; Retention time (R.t.) = 12.8, 19.6, 20.4, 21.0 and 28.6 min, respectively). Compound **6** was obtained from the H10 subfraction by recrystallization with EtOAc and acetone. Compound **7** (R.t. = 27.6 min) was also isolated from the H18 subfraction by the same HPLC system (Mobile phase: EtOAc/ *n*-$C_6H_{14}$ = 30/70; Flow rate: 1 ml/min).

In addition, the EtOAc soluble fraction (5 g) was divided into 13 subfractions (E1-E13) by chromatography with a silica-gel column eluted with EtOAc/*n*-$C_6H_{14}$ (gradient elution was performed by changing from 0/100 to 100/0). Compound **8** (Mobile phase: EtOAc/*n*-$C_6H_{14}$ = 35/65; Flow rate: 6 ml/min; R.t. = 8.7 min) and compound **9** (Mobile phase: EtOAc/*n*-$C_6H_{14}$ = 40/60; Flow rate: 2 ml/min; R.t. = 16.2 min) were isolated and purified from E5 and E6 subfractions by an HPLC.

*Ferruginol (**1**) (65 mg)*

Yellow oil; EIMS for $C_{20}H_{30}O$ found 286; IR $\upsilon_{max}$ : 3370, 1612, 1579, 1501 $cm^{-1}$; $^1$H-NMR (in $CDCl_3$) : δ (ppm) 0.89 (3H, s, H-18), 0.93 (3H, s, H-19), 1.15 (3H, s, H-20), 1.22 (3H, d, *J*=7.0 Hz, H-16), 1.29 (3H, d, *J*=7.0 Hz, H-17), 2.77 (1H, ddd, *J*=7.0, 10.4, 16.8 Hz, H-7α), 2.81(1H, ddd, *J*=2.1, 6.5, 16.8 Hz,, H-7β), 3.11 (1H, sept, *J*=7.0 Hz, H-15), 4.81 (s, OH), 6.62 (1H, s, H-11), 6.81 (1H, s, H-14).

*6,7-Dehydroferruginol (**2**) (15 mg)*

Colorless oil; EIMS for $C_{20}H_{28}O$ found 284; IR $\upsilon_{max}$ : 3365, 1612 $cm^{-1}$; $^1$H-NMR (in $CDCl_3$) : δ (ppm) 0.90 (3H, s, H-18), 0.96 (3H, s, H-19), 1.03 (3H, s, H-20), 1.22 (3H, d, *J*=7.0 Hz, H-16), 1.24 (3H, d, *J*=7.0 Hz, H-17), 3.12 (1H, sept, *J*=7.0 Hz, H-15), 5.84 (1H, dd, *J*=9.2, 3.0 Hz, H-6), 6.48 (1H, dd, *J*=9.2, 3.0 Hz, H-7), 6.54 (1H, s, H-11), 6.88 (1H, s, H-14).

*Sugiol (**3**) (28 mg)*

Colorless crystal; mp: 291-292 °C (292-293 °C, Su *et al.* 1994); EIMS for $C_{20}H_{28}O_2$ found 300; IR $\upsilon_{max}$ : 3110, 1637, 1583, 1500, 1372 $cm^{-1}$; $^1$H-NMR (in $CDCl_3$): δ (ppm) 0.99 (3H, s, H-18), 1.00 (3H, s, H-19), 1.19 (3H, s, H-20), 1.20 (3H, d, *J*=7.0 Hz, H-16), 1.24 (3H, d, *J*=7.0 Hz, H-17), 1.83 (dd, *J*=4.5, 13.0 Hz, H-5), 2.18 (dd, *J*=13.0, 18.0 Hz, H-6β), 2.62 (1H, dd, *J*=4.5, 1.8 Hz, H-6α), 3.13 (1H, sept, *J*=7.0 Hz, H-15), 6.68 (1H, s, H-11), 7.89 (1H, s, H-14).

*6α-Hydroxysugiol (**4**) (10 mg)*

Colorless crystal; mp: 208 °C (207-208 °C, Su *et al.* 1994); EIMS for $C_{20}H_{28}O_3$ found 316; IR $\upsilon_{max}$ : 3330, 3150, 1660, 1600, 1495, 1380 $cm^{-1}$; $^1$H-NMR (in $CDCl_3$): δ (ppm) 1.18 (3H, s, H-18), 1.20 (3H, s, H-19), 1.22 (3H, s, H-20), 1.23 (3H, d, *J*=7.0 Hz, H-16), 1.24 (3H, d, *J*=7.0 Hz, H-17), 1.33 (1H, s, H-20), 1.81 (1H, d, *J*=12 Hz, H-5), 3.12 (1H, sept, *J*=7.0 Hz, H-15), 4.60 (1H, d, *J*=12.0 Hz, H-6), 6.68 (1H, s, H-11), 7.91 (1H, s, H-14).

*5,6-Dehydro-6-hydroxysugiol (**5**) (8 mg)*

Colorless crystal; mp: 189-190 °C (189-190 °C, Su *et al.* 1994); EIMS for $C_{20}H_{26}O_3$ found 314; IR $\upsilon_{max}$ : 3326, 1675, 1601, 1502, 1380 $cm^{-1}$; $^1$H-NMR (in $CDCl_3$): δ (ppm) 1.25 (3H, d, *J*=7.0 Hz, H-16), 1.28 (3H, d, *J*=7.0 Hz, H-17), 1.41 (6H, s, H-18, -19), 1.46 (3H, s, H-20), 2.25 (1H, ddd, *J*=3.0, 4.9, 13.0 Hz, H-1β), 3.16 (1H, sept, *J*=7.0 Hz, H-15), 6.82 (1H, s, H-11), 7.13 (s, OH), 7.99 (1H, s, H-14).

*11-Hydroxyferruginol (**6**) (12 mg)*

Colorless oil; EIMS for $C_{20}H_{30}O_2$ found 302; $^1$H-NMR (in $CDCl_3$): δ (ppm) 1.02 (3H, s, H-18), 1.13 (3H, s, H-19), 1.20 (3H, s, H-20), 1.22 (3H, d, *J*=7.0 Hz, H-16), 1.26 (3H, d, *J*=7.0 Hz, H-17), 1.33 (s, H-20), 1.70 (d, *J*=12 Hz, H-5), 3.12 (1H, sept, *J*=7.0 Hz, H-15), 4.67 (-OH), 4.76 (-OH), 6.59 (1H, s, H-14).

*Secoabietane dialdehyde (**7**) (4 mg)*

White solid; mp: 191-192 °C (192 °C, Fang *et al.* 1986); EIMS for $C_{20}H_{28}O_3$ found 316; IR $\upsilon_{max}$ : 3327, 2720, 1705, 1658, 1570, 1500 $cm^{-1}$; $^1$H-NMR (in $CDCl_3$): δ (ppm) 0.68 (3H, s, H-18), 0.99 (3H, s, H-19), 1.23 (3H, d, *J*=7.0 Hz, H-17), 1.27 (3H, d, *J*=7.0 Hz, H-16), 1.24 (3H, s, H-20), 1.49 (1H, s, H-20), 3.07 (1H, d, *J*=4.0 Hz, H-5), 3.10 (1H, sept, *J*=7.0 Hz, H-15), 4.50 (1H, s, -OH), 6.88 (1H, s, H-11), 7.82 (1H, s, H-14), 9.83 (1H, d, *J*=4.0 Hz, H-6), 10.48 (1H, s, H-7).

*Hinokiol (**8**) (30 mg)*

Colorless crystal; mp: 234-235 °C (233-234 °C, Chang *et al.* 1998); EIMS for $C_{20}H_{30}O_2$ found 302; IR $\upsilon_{max}$ : 3530, 3270, 1608, 1506, 1460 $cm^{-1}$; $^1$H-NMR (in $CDCl_3$): δ (ppm) 0.86 (3H, s, H-18), 1.04 (3H, s, H-19), 1.16 (3H, s, H-20), 1.23, 1.25 (each 3H, d, *J*=7.0 Hz, H-16, -17), 2.17 (1H, brd, *J*=12.0 Hz, H-1β), 2.74 (1H, ddd, *J*=18.0, 11.0, 7.0 Hz, H-7α), 2.86(1H, ddd, *J*=18.0, 7.0, 2.0 Hz, H-7β), 3.08 (1H, sept, *J*=7.0 Hz, H-15), 3.27 (1H, dd, *J*=10.0, 6.0 Hz, H-3), 6.59 (1H, s, H-11), 6.82 (1H, s, H-14).

*Isohinokiol (**9**) (9 mg)*

Colorless crystal; mp: 225-226 °C (224 °C, Akita and Oishi 1981); EIMS for $C_{20}H_{30}O_2$ found 302; IR $\upsilon_{max}$ : 3522, 3305, 1600, 1505, 1459, 1419, 1389, 1237 $cm^{-1}$; $^1$H-NMR (in $CDCl_3$): δ (ppm) 0.92 (3H, s, H-18), 1.00 (3H, s, H-19), 1.20 (6H, d, *J*=7.0 Hz, H-16, -17), 1.55 (3H, s, H-20), 3.09 (1H, sept, *J*=7.0 Hz, H-15), 3.48 (1H, br, H-3), 6.61 (1H, s, H-11), 6.80 (1H, s, H-14).

*Free radical inhibition activity*

Scavenging activity of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals by the diterpenes isolated from *T. cryptomerioides* was measured based on the method of Fejes *et al.* (2000) with slight modifications. Briefly, the reaction mixture contained 1000 µl of 0.1 mM DPPH-ethanol solution, 450 µl of 50 mM Tris-HCl buffer (pH 7.4) and 50 µl of test sample or methanol (as control). Reduction of the DPPH free radical was measured by taking the absorbance at 517 nm after 30 min incubation with the test samples at 25 °C. The inhibition ratio (%) was calculated according to the following equation: % Inhibition = [(Ab-

sorbance of the control – Absorbance of test sample)/ Absorbance of the control] × 100.

## Results and Discussion

Diterpenes are a diverse group of compounds based on four isoprene units, most of which have limited distribution in the living plants. Because of their higher boiling points, they are not considered to be essential oils, and are classically considered to be resins, the material that remains after steam distillation of a plant extract. Diterpenes constitute the second largest class of terpenes, with over 2200 compounds belonging to 130 distinct skeletal types (Dev 1989). According to a review by Wang *et al.* (1997), more than eighteen diterpenes have been isolated from different parts of *T. cryptomerioides*, including root, bark, leaves and heartwood. Lin *et al.* (1995, 1996, 1997) have also identified several diterpenes with novel skeletons (the six-five-six fused-ring) from the leaves of *T. cryptomerioides*. Recently, eleven novel podocarpane-type diterpenes were obtained from the bark of *T. cryptomerioides* (Kuo and Chang 2000; Kuo *et al.* 2000). In this study, we further isolated and characterized several abietane-type diterpenes including six diterpenes reported for the first time from *T. cryptomerioides* heartwood.

### *Isolation and identification of diterpenes from Taiwania heartwood*

With the partition by different polarity ranges of solvents (*n*-$C_6H_{14}$, $CHCl_3$, EtOAc and MeOH), open column chromatogram and HPLC separation, nine abietane type diterpenes (Fig. 1), namely ferruginol (**1**), 6,7-dehydroferruginol (**2**), sugiol (**3**), 6α-hydroxysugiol (**4**), 5,6-dehydro-6-hydroxysugiol (**5**), 11-hydroxyferruginol (**6**), secoabietane dialdehyde (**7**), hinokiol (**8**) and isohinokiol (**9**), were isolated from the heartwood of *T. cryptomerioides*. Spectral analyses and melting points were employed to identify these compounds. The spectral data of these nine abietane-type diterpenes are described in the Material and Methods section and the results have been confirmed with the data in the literature (Akita and Oishi 1981; Fang *et al.* 1986; Su *et al.* 1994; Chang *et al.* 1999b). We summarized the typical spectral characteristics of abietane-type diterpenes as follows based on the results of NMR analyses in this study. Six-five-six fused-ring type, royleanone-type and podocarpane-type diterpenes have been isolated from leaf and bark tissues, respectively, of *T. cryptomerioides* (Wang *et al.* 1997). Abietane-type diterpene was found, in this study, as the dominant diterpene obtained from the heartwood of *T. cryptomerioides.* However, only one isopimarane-type diterpene was identified from the heartwood extract of the plant (Chang *et al.* 2000a). Six diterpenes among the nine characterized compounds, including 6,7-dehydroferruginol (**2**), 6α-hydroxysugiol (**4**), 5,6-dehydro-6-hydroxysugiol (**5**), 11-hydroxyferruginol (**6**), secoabietane dialdehyde (**7**) and isohinokiol (**9**) are identified and reported here for the first time.

### *Possible biosynthesis pathway of diterpenes in Taiwania heartwood*

To understand the importance of wood extractives for the tree, it is necessary to examine the biochemistry of biosynthesis pathways and their integration in the phys-

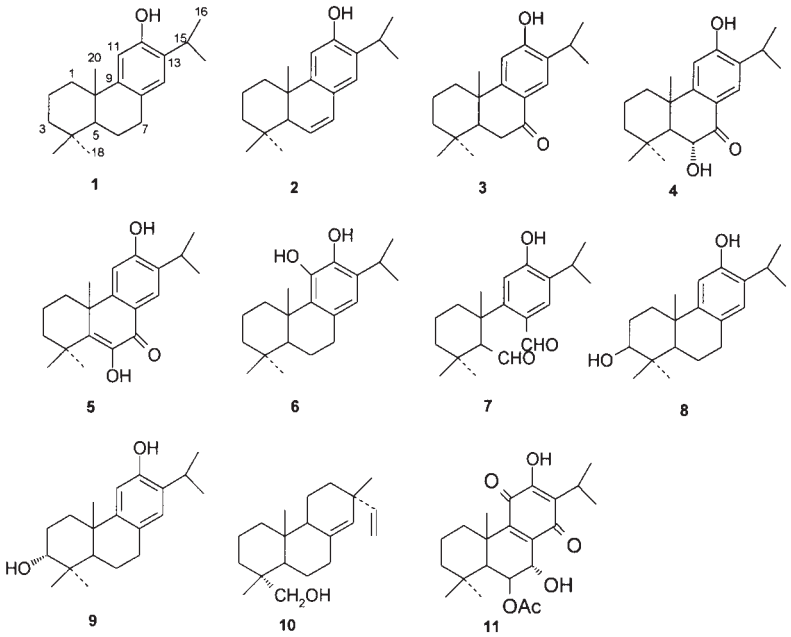


**Fig. 1.** Diterpenes isolated from *T. cryptomerioides.* Ferruginol (**1**), 6,7-dehydroferruginol (**2**), sugiol (**3**), 6α-hydroxysugiol (**4**), 5,6-dehydro-6-hydroxysugiol (**5**), 11-hydroxyferruginol (**6**), secoabietane dialdehyde (**7**), hinokiol (**8**), isohinokiol (**9**), isopimarinol (**10**) and 6β-acetoxy-7α-hydroxyroyleanone (**11**).

iology of trees. It is now well-established that the isoprene rule and its evolved version, the biogenetic isoprene rule, are of fundamental importance in terpene biosynthesis (Dev 1989). The starting substrate of the proposed diterpenes synthesis pathway is suggested to be acetyl-CoA (Higuchi 1997). From it, biosynthesis of isopimarane-type and abietane-type diterpenes proceed stepwise *via* mevalonate, isopentenyl pyrophosphate (IPP), dimethylallyl pyrophosphate (DMAPP), farnesyl pyrophosphate (FPP) and geranylgernyl pyrophosphate (GGPP) in similar biochemical processes to produce monoterpenes and sesquiterpenes. Croteau and Johnson (1985) have found that two tricyclic diterpenes in *Pinus pinaster*, *e.g.*, abietic acid and pimaric acid, are synthesized at the base of conifer leaves, while dextropimaric acid, which is a dicyclic diterpene, is synthesized by whole leaves. Meanwhile, xylem contains monoterpenes and tricyclic diterpenes. It has also been demonstrated that the principal resin acid of grand fir, (-)-abietic acid, originates by cyclization of the corresponding $C_{20}$ isoprenoid precursor, followed by sequential oxidation of A-ring α-methyl of the olefin to a carboxyl func-

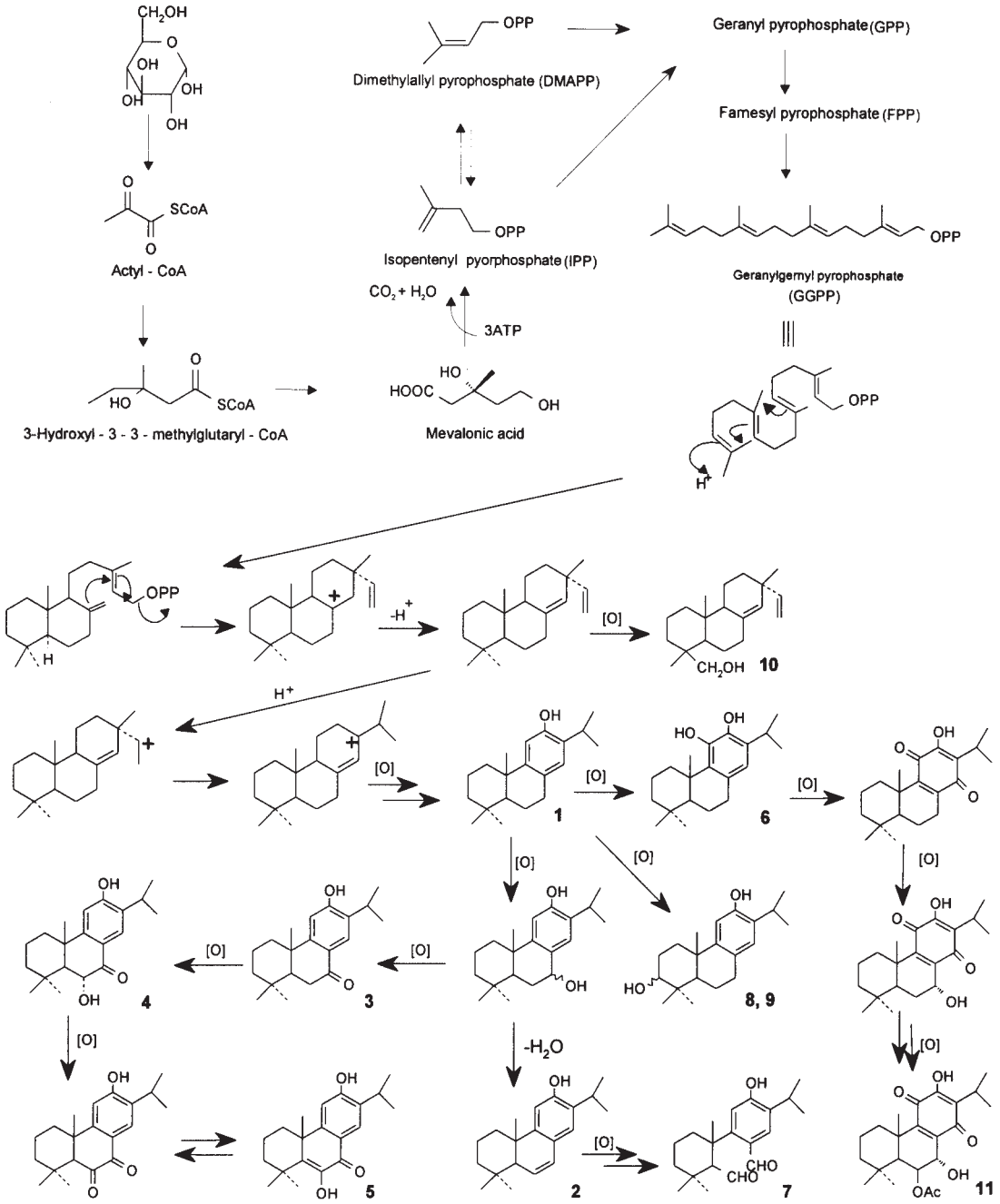


**Fig. 2.** Possible biosynthesis pathway of diterpenes in *T. cryptomerioides* heartwood. Ferruginol (**1**), 6,7-dehydroferruginol (**2**), sugiol (**3**), 6-hydroxysugiol (**4**), 5,6-dehydro-6-hydroxysugiol (**5**), 11-hydroxyferruginol (**6**), secoabietane dialdehyde (**7**), hinokiol (**8**), isohinokiol (**9**), isopimarinol (**10**) and 6β-acetoxy-7α-hydroxyroyleanone (**11**).

tion involving two distinct cytochrome $P_{450}$-dependent hydroxylase and an aldehyde dehydrogenase (Steele *et al.* 1995). Thus, considering the diterpenes isolated from the heartwood of *T. cryptomerioides*, a reasonable biosynthesis pathway for diterpenes in *T. cryptomerioides* heartwood is proposed. The intermediate substrate, GGPP, leads to the synthesis of the diterpenes. In *T. cryptomerioides* heartwood, abietane-type and isopimarane-type diterpenes are proposed to be biosynthesized *via* the enzymatic, stepwise processes proposed in Figure 2.

### *DPPH radical scavenging activity of diterpenes from Taiwania*

The oxidation of lipids, DNA, protein and carbohydrates by toxic reactive oxygen species (ROS) may often cause DNA mutation, damage target cells or tissues and result in cellular senescence and death (Halliwell 1997; Chang *et al.* 2001c). Recently, the potential for antioxidant activities to reduce oxidative stress *in vivo* have prompted investigators to search for effective antioxidants from various plant sources (Zi *et al.* 1997; Hu *et al.* 2000; Liu and Ng 2000; Pietta *et al.* 2000). In previous reports (Chang *et al.* 1998, 1999b, 2000a, c, 2001a), we have demonstrated the antifungal, antimite, antitermitic, antibacterial and antitumor activities of *T. cryptomerioides* extractives. Here, we also evaluated the antioxidant activity of the specific diterpenes from *T. cryptomerioides* heartwood, employing the DPPH radical scavenging activity assay. Due to the limited diterpene yield, we selected only four abietane-type diterpenes, *i.e.* ferruginol, hinokiol, secoabietane dialdehyde and sugiol for this study. DPPH is a stable radical, which has been used to evaluate the total antioxidant activity of plant and microbial extracts (Halliwell 1997). Another two diterpenes, isopimarinol (**10**) and 6β-acetoxy-7α-hydroxyroyleanone (**11**), isolated from heartwood and bark of *T. cryptomerioides* (Kuo *et al.* 1987; Chang *et al.* 2000a), respectively, were also employed for the antioxidant activity assays. Figure 3 shows the DPPH radical scavenging activity of the tested diterpenes. The results reveal that ferruginol possesses a significant inhibitory activity against the DPPH radical, followed by hinokiol, secoabietane dialdehyde, 6β-acetoxy-7α-hydroxyroyleanone and isopimarinol, with sugiol showing the least radical scavenging activity. These results implied that the phenol structure present in the abietane-type diterpene is essential to its radical scavenging activity. Furthermore, as an electron-withdrawing group nears the aromatic ring, the observed antioxidant activity decreases (*e.g.*, in the case of sugiol and secoabietane dialdehyde). Isopimarinol, which has a primary alcohol structure, does not show significant antioxidant activity compared to other abietane-type diterpenes from *T. cryptomerioides* heartwood. The results obtained in this study indicate that ferruginol has potential for use as a natural food preservative.

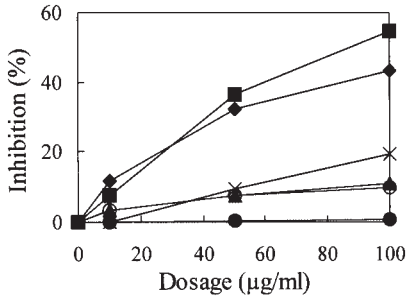


**Fig. 3.** 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of diterpenes isolated from *T. cryptomerioides*. ■: ferruginol; ◆: hinokiol, ×: secoabietane dialdehyde; ▲: 6β-acetoxy-7α-hydroxyroyleanone; ○: isopimarinol; and ●: sugiol.

## Conclusions

Although the plant kingdom represents an extraordinary reservoir of novel bioorganic molecules, this has been under-appreciated until the recent worldwide surge of interest in herbal medicine research. In this study, we have investigated the more significant constituents from *T. cryptomerioides* heartwood. The capacity of these compounds to act as antioxidants *in vitro* were also evaluated. Among the diterpenes isolated from the heartwood of *T. cryptomerioides*, ferruginol exhibits the strongest radical scavenging. ROS-induced damage has been implicated in the development of chronic degenerative disease and in the aging process; thus, it is important to discover effective antioxidants from natural sources, especially from plant species, to reduce ROS activities. It has also been proven that ferruginol is an effective antifungal compound of *T. cryptomerioides* heartwood (Chang *et al.* 1999b). Since both white-rot and brown-rot fungus release oxidase to cleave the cellulose or lignin of wood, it is plausible that ferruginol inhibits the growth of fungus by blocking the radical transition. However, this inference requires further elucidation in future studies.

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