Biological Activities of Methyl-4-[[(2*E*)-3,7-dimethyl-2,6-octadienyl] oxy]-3-hydroxybenzoate

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Methyl-4-[[(2*E*)-3,7-dimethyl-2,6-octadienyl]oxy]-3-hydroxybenzoate (**5**) has been identified from the New Zealand liverwort *Trichocolea hatcheri* (*T. hatcheri*) on the basis of spectroscopic evidence. This compound was tested for its growth inhibitory effects against tumor cell lines, using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay. It showed growth inhibition activity against *Staphylococcus epidermidis* (MIC, 1,000 μg/mL). These results suggest that compound **5** possesses antitumoral , antimicrobial and antioxidative activities.

Key Words : *Trichocolea hatcheri*, MTT assay, Antitumoral, Antimicrobial and antioxidative activities

Introduction

Trichocolea hatcheri which grows throughout New Zealand, is distinguished from *T. mollissima* by its smaller size, dark green color and prostrate habit.[1] An extract of *T. hatcheri* showed cytotoxic effects (P 388 > 150 μg/disk) against monkey kidney (BSC) cell lines.[2] Reverse-phase flash chromatography concentrated the cytotoxic activity in fractions eluted with MeCN-H_2O 3 : 1 and 9 : 1. Si-gel column chromatography spread cytotoxic activity across ethyl acetate - cyclohexane 3 : 97, 5 : 95, 7 : 93 and 10 : 90 fractions containing geranyl phenyl ethers. These geranyl phenyl ethers were obtained pure in quantities too small for biological assays.[2] We investigated the inhibitory effects of methyl-4-[[(2*E*)-3,7-dimethyl-2,6-octadienyl] oxy]-3-hydroxybenzoate (**5**) against tumor cell lines, gram-positive bacteria, gram-negative bacteria and fungus. The effects of reference compounds were also examined for comparison.

Experimental Section

Chemicals and apparatus. All solvents were distilled before use. Removal of solvents from chromatography fractions was accomplished by rotary evaporation at up to 40 °C. Intial fractionation of crude plant extract, using reverse phase column chromatography was performed with octadecyl-functionalized silica gel (C-18, Aldrich) as the adsorbent. Further column fractionation was performed using Davisil silica 60 A^0 (35-70 μm silica gel, Alltech) as the adsorbent. Thin-layer chromatography was carried out using Merck DC-plastikfolien Kieselgel 60 F_{254} visualized first with a UV lamp, then by dipping in a vanillin solution (1% vanillin, 1% H_2SO_4 in EtOH) followed by heating. MS, UV and IR spectra were recorded on Kratos MS-80, Shimadzu UV 240, and Perkin-Elmer 1600 FT-IR instruments respectively. NMR spectra of $CDCl_3$ solutions at 25 °C were recorded at 300 MHz for ^{1}H-NMR and 75 MHz for ^{13}C-NMR on a Varian VXR-300 spectrometer. Chemical shifts are given in parts per million on the δ scale referenced to the solvent peak $CHCl_3$ at 7.27 and $CDCl_3$ at 77.08.

Cell culture. KB, SK-MEL-3, and NIH 3T3 cells were grown at 37 °C in RPMI medium supplemented with 10% FBS penicillin (100 units/mL) and streptomycin (100 μg/mL). The cells were grown in a humidified atmosphere of 95% air/5% CO_2. Cells were dissociated with 0.25% trypsin and were counted using a Hemacytomer just before transferring them for the experiment.

Plant material. *T. hatcheri* was collected from a steep earth bank in the Morrisons Creek area, Dunedin, New Zealand, in Februrary 1996 [University of Otago Herbarium (OTA) specimen no, 048094]. It was air dried (30 °C). The dry plant (13.2 g of roots, stems and leaves) was ground in a blender and extracted with ethanol (3×200 mL) and chloroform (200 mL) and filtered. The filtered extract was rotary evaporated to a green gum (0.443 g).

Methyl-4-[[(2E)-3,7-dimethyl-2,6-octadienyl]oxy]-3-hydroxybenzoate (5). Colorless oil; ^{1}H-NMR spectrum and EIMS matched those reported previously.[1] Methyl-3,4-dihydroxy-benzoate (**2**, 278 mg, 1.65 mmol); geranyl bromide (434 mg, 2.00 mmol); NaH (60%, 66 mg, 1.65 mmol) in dry DMF (2 mL); 0 °C; 17 hrs; flash chromatography (5%

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EtOAC - hexane) gave **5** (220 mg, 44%). Also isolated was methyl-3,4-di-[[(2*E*)-3,7-dimethyl-2,6-octadienyl]oxy]-benzoate (**7**) (130 mg, 18%). Compounds (**5**, **7**) were identified by comparing their spectral data (TLC, MS, NMR and IR) with those published or by direct comparison with that of an authentic sample.[2]

Methyl-3-[[(2E)-3,7-dimethyl-2,6-octadienyl]oxy]-4-hydroxybenzoate (6). Methyl- 3,4-dihydroxybenzoate (**2**, 278 mg, 1.65 mmol); geranyl bromide (358 mg, 1.65 mmol); NaH (60%, 66 mg, 1.65 mmol) in dry DMF (2 mL); 20 °C; 48 hrs; flash chromatography (5% EtOAC - hexane) gave **6** (196 mg, 27%). Also isolated was methyl-3,4-di-[[(2*E*)-3,7-dimethyl-2,6-octadienyl]oxy]-benzoate (**7**) (245 mg, 49%). Compounds (**6**, **7**) were identified by comparing their spectral data (TLC, MS, NMR and IR) with published data or by direct comparison with that of an authentic sample.[2]

Microorganisms. The microorganisms used included: *Streptococcus aureus* (ATCC 29213), *Streptococcus mutans* (JC-2), *Staphylococcus epidermidis* (ATCC 12228), *Pseudomonas aeruginosa* (KCTC 1636). *Pseudomonas putida* (KCTC 8729), *Candida albicans* (KCTC 1940).

Screening for antimicrobial activities. The dried plant extract was dissolved in 10% aqueous dimethyl sulfoxide (DMSO) to a final concentration 2,000 μg/mL and sterilized by filtration through a 0.45 μm membrane filter. Antimicrobial tests were then carried out by the Agar serial dilution method.[3,4] Each of several concentrations of a tube of molten agar was mixed, poured into a petri plate, and allowed to solidify. The organisms containing 10^6 bacterial cells/mL or 10^8 yeast cells/mL were inoculated in the petri plates. After the plates were incubated 24 hrs at 37 °C for bacteria and for five to seven days at 22 °C for fungi, the lowest concentration of ethanolic extract that inhibits growth of the organisms was determined as the minimum inhibitory concentrations (MICs) of the antimicrobial agents. Ampicillin served as positive controls for *S. aureus, S. mutans* and *S. epidermidis*, whereas, ketoconazole served as a negative control for *P. aeruginosa* and *P. putida*. Each test was carried out in triplicate.

4,5-Dimethylthiazol-2-yl-2,5-diphenyl-2H-tetrazolium-bromide (MTT) assay. The assay is dependent on the cellular reduction of water-soluble MTT (Sigma Chemical Co. St. Louis, M.O.) by mitochondrial dehydrogenase of vial cells to a blue water-insoluble formazan crystal product that can be measured spectrophotometrically.[5,6] KB, SK-MEL-3, and NIH 3T3 fibroblasts were cultured in RPMI-1640 medium (Gubco Laboratories) containing 10% fetal bovine serum. Exponentially growing tumor cells (5×10^5) were cultured for 48 hrs at 37 °C in a humidified 5% CO_2 incubator in the presence or absence of sample.

Evaluation of toxicity: Cytotoxicity assay. To determine the cytotoxicity mediated by **5**, **6**, **7**, **8** and **9**, the colorimetric assay was used. These compounds were serially diluted in EMEM (eagle's minimum essential medium) with 10% FBS and mixed with an equal volume of NIH 3T3 fibroblasts (5×10^5 cells/mL). After one hour, fresh culture medium was supplied to a total volume of 1-100 μM. On the third day of incubation in an 37 °C incubator, MTT (5 mg/mL; 20 μL/well) terazolium dye (Polyscience, Inc., Warrigton, PA) were added to the cells. After 3 hrs, the absorbance was measured at 540 nm, using ELISA reader.

Measurement of antioxidant activity. A 4 mL of methanol solution of compounds at various concentrations (2.5-120 μg/mL) was added to a solution of DPPH (1,1-dephenyl-2-picrylhydrazyl) (1.5×10^{-4} M) in methanol (1 mL), and the reaction mixture was shaken vigorously. After storage at room temperature for 30 min in air, the remaining DPPH was determined by spectrophotometry at 517 nm, and the radical scavenging activity of each sample was expressed by the ratio of the lowering of the absorption of DPPH (%), relative to the absorption (100%) of DPPH solution in the absence of test sample (control). The mean values were obtained from triplicate experiments.

Results and Discussion

A portion of the plant extract (0.443 g) was absorbed on octadecylfunctionalized silica gel (1.0 g, Aldrich Cat. 37, 763-5) by slurrying the absorbent in a solution of the extract in EtOH-H_2O (Ca 1 mL, mainly EtOH) and removing the solvent under reduced pressure. The extract was then loaded on a cotton wool pad at the top of a column of the C18-silica gel (10 g, 8×2 cm) that had been dry-packed and then washed successively with CH_3CN, CH_3CN-H_2O (1 : 1), and H_2O (each *ca*. 20 mL). An extract of *T. hatcheri* showed cytotoxic effects (P $388 > 150$ μg/disk) against monkey kidney (BSC) cell lines and antifungal activity aganist the dermatophyte *Trichophyton mentagrophytes*. Reversed-phase flash chromatography concentrated the cytotoxic activity in fractions eluted with $CH_3CN : H_2O$, 3 : 1 and 9 : 1. This fraction contained one main UV-active compound by TLC. The unique absorption bands due to an ester carbonyl band (1,716 cm^{-1} and 1,212 cm^{-1}) were shown in the IR spectrum along with a hydroxyl group (3,412 cm^{-1}) as well as an aromatic group (1,600, 1,509 and 1,436 cm^{-1}). The IR spectra of the compound showed the presence of conjugated carbonyl and aromatic group. Si-gel column chromatography spread cytotoxic activity across ethyl acetate - cyclohexane 3 : 97 and 5 : 95 fractions containing methyl-4-[[(2*E*)-3,7-dimethyl-2,6-octadienyl]oxy]-3-hydroxy-benzoate (**5**).[2] This compound was obtained pure in quantities, but they were too small for the biological assays, synthesis and biological activity reported here. The least polar compound (**5**), purified by preparative TLC, had UV and IR spectra appropriate for a 3,4-dioxygenated benzoic acid derivative. The MS showed a molecule of $C_{18}H_{24}O_4$. The phenolic OH of **5** was observed in the ^{1}H-NMR spectrum as a broad exchangeable signal at 5.7 ppm. The ^{13}C-NMR spectrum of compound (**5**) showed signals appropriate for a trisubstituted aromatic ring, a geranyl group, an ester carbonyl, and methoxyl and hydroxyl groups, as expected for **5**. We rigorously assigned the ^{1}H and ^{13}C-NMR spectra with the aid of HMQC, HMBC, DEPT and NOE difference experiments. The NOE difference experiments produced a surprising result. Irradiation of the

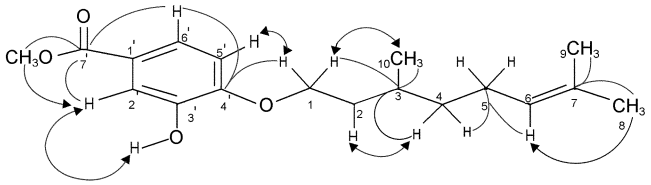

Figure 1. Important NMR correlations establishing the structure of

methoxyl and hydroxyl signals, which were barely resolved, only gave to enhancement of the H-2' aromatic proton signal. Structure **5** would be expected to give enhancements of both the H-2' and H-5'. An NOE interaction between H-5' and H-1 protons of the geranyl group was also consistent with structure **5** (Fig. 1). The critical features that distinguish this structure are the linking of the geranyl group to the aromatic ring via an ether linkage instead of an ester and the replacement of a methyl ether with a methyl ester. Unequivocal evidence of these features was obtained from the HMBC experiment, in which correlations were observed between the ester carbonyl (δ 166.8) and one hydroxyl proton signal (δ 5.70) and between a quaternary oxygenated aromatic signal (δ 149.7) and the H2-1 protons (δ 4.67, d, J = 7 Hz) of the geranyl group. A further NOE interaction between the geranyl H-10 and H-1 signals showed that the 2,3 double bond has *E* stereochemistry (Fig. 1).

Table 1 shows the potent cytotoxic activities of methyl-4-[[(2*E*)-3,7-dimethyl-2,6-octadienyl]oxy]-3-hydroxybenzoate (**5**), methyl-3-[[(2*E*)-3,7-dimethyl-2,6-octadienyl]oxy]-4-hydroxybenzoate (**6**), methyl-3,4-di-[[(2E)-3,7-dimethyl-2,6-octadienyl]oxy]-benzoate (**7**) adriamycin (**8**), and 5-fluorouracil (**9**) against KB cells, SK-MEL-3 cells and NIH 3T3 fibroblasts. In general, the cytotoxic activities of these compounds were in a dose-dependent manner, and the susceptibility of the tumor cell lines to methyl-4-[[(2*E*)-3,7-dimethyl-2,6-octadienyl]oxy]-3-hydroxybenzoate (**5**) was sensitive. The value of IC_{50} of these compounds showed that compound **5** exerts the potent cytotoxic activity. The values of IC_{50} on KB cells were determined at 111.63 μM and the values of IC_{50} on SK-MEL-3 cells were determined at 160.68 μM. However, compound **6** showed strong cytotoxicity on NIH 3T3 fibroblasts. References were evaluated for the strong cytotoxic activities against tumor cells.[7]

Table 2 summarizes the radical scavenging results of synthetic compounds on DPPH. Their antioxidant effects were much lower than those of L-ascorbic acid and BHA (butylated hydroxyanisole). Methyl-4-[[(2*E*)-3,7-dimethyl-2,6-octadienyl]oxy]-3-hydroxybenzoate (**5**) is not an effective antioxidative geranyl phenyl ether (IC_{50}, 580 μg/mL). It may not be usable as an antioxidant component.

We report here the results of screening methyl-4-[[2*E]*-3,7-dimethyl-2,6-octadienyl]oxy]-3-hydroxybenzoate (**5**) and references for antimicrobial activity against gram-positive bacteria, gram-negative bacteria and the fungus, *Candida albicans* (Table 3). This compound (**5**) showed weaker activity against gram-positive bacteria, gram-negative bacteria and fungus than ketoconazole and ampicillin. However, it has weak activity against *S. epidermidis* (MIC, 1,000 μg/mL).

Table 1. The cytotoxic activities of **5**, **6**, **7**, **8** and **9** by the MTT method

Compounds[a]	IC_{50} (μM)[b]		
	NIH 3T3	SK-MEL-3	KB
5	113.73	160.68	111.63
6	99.61	199.59	113.12
7	837.54	1261.03	1173.93
8	23.26	20.12	–
9	41.27	–	44.36

[a]Each compound was examined in four concentrations in triplicate experiments. [b]IC_{50} represents the concentration of a compound required for 50% inhibition of cell growth.

Table 2. The antioxidant activities of **5**, **6**, **7**, BHA and L-ascorbic acid on DPPH

Compounds[a]	RC_{50} (μg)[a]
5	580.38
6	NT
7	2907.38
BHA	14.0
L-ascorbic acid	12.0

[a]Amount required for 50% reduction of DPPH after 30 min. NT; not determined.

Table 3. Minimum inhibitory concentrations (MICs) of **5**, **6**, **7**, ampicillin and ketoconazole for the reference strains[a]

Microorganisms	MICs (μg/mL)				
	5	**6**	**7**	AP	KT
S. mutans	>1,000	>1,000	>1,000	3.125	50
S. epidermidis	1,000	>1,000	>1,000	50	50
S. aureus	>1,000	>1,000	>1,000	3.125	100
P. aeruginosa	>1,000	>1,000	>1,000	50	200
P. putida	>1,000	>1,000	>1,000	>200	50
C. albicans	>1,000	>1,000	>1,000	>200	25

MICs were examined in triplicate experiments (mean ± standard diaviation, n = 3). AP; Ampillin, KT; Ketoconazole.

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