Antifungal Activity of 4-Geranyloxy Compound on the Dermatophytic Fungus

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4-Geranyloxy compound (1) has elucidated by spectroscopic analysis. This compound (1) inhibited the growth of the dermatophytic fungus *Trichophyton mentagrophytes* ATCC 28185, (2 mm inhibition zone at 15 μg/disc), cytotoxic to P388 murine leukaemia cell lines ATCC CCL 46 P388D1, (IC_{50} 1,125 ng/ml at 7.5 μg/disc) and BSC monkey kidney cell lines (100% of well at 15 μg/disc).

Key words : 4-Geranyloxy compound(1), *Trichophyton mentagrophytes*, P388, cytotoxic activity.

Introduction

Liverworts are generally located in damp forested areas, growing on trees, rotting logs and rocks or on the ground. Frequently, several species of liverwort will be found growing intertwined, and some species grow to resemble a carpet covering large areas of the forest floor. In New Zealand, liverworts can be found throughout the country, generally in rainforest areas, growing on the ground, or on rotting logs in damp and humid regions[1].

There are approximately 20 species of the genus *Trichocolea* (family Trichocoleaceae, order Jungermanniales) found in the world. In New Zealand, the family Trichocoleaceae is represented by two genera; *Trichocolea*, which contains five species, of which three have been studied, and *Eotrichocolea*, which has one species, that has yet to be investigated. Three New Zealand *Trichocolea* species (*T. hatcheri*, *T. mollisima* and *T. lanata*) were found to contain prenylated benzyl ethers. Prenylated benzyl ethers are characteristic of *Trichocolea*, having been identified in many *Trichocolea* species from different parts of the world[2-4]. Liverworts of the genus *Trichocolea* are a treasury of isoprenyl phenyl ethers. *Trichocolea hatcheri* Hodgs, which grows throughout New Zealnd, is distinguished from *T. mollissima* by its smaller size, dark green color, and prostrate habit[1]. A chemical abstracts search of *Trichocolea* revealed that only *T. tomentella*[2,5-6], *T. lanata*[2], *T. mollisima*[2,7], *T. hatcheri*[4], and *T. pluma*[8] have been studied for their chemical constituents.

In this study, the antiviral, antimicrobial activities and cytotoxicity of methyl-4-(geranyloxy)-3-hydroxybenzoate (**1**) from *T. hatcheri* were examined and have investigated its antifungal activity.

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Materials and Methods

1. Chemicals and apparatus.

All solvents were distilled before use. Removal of solvents from chromatography fractions were removed by rotary evaporation at temperature up to 40^0. Intial fractionaiton 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 adsorbent. TLC 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 ℃, were recorded at 300 MHz for ^1H and 75 MHz for ^{13}C 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.

2. Plant material

T. hatcheri was collected from a steep earth bank in the

Morrisons Creek area, Dunedin, New Zealand, in Fabruary 1996 [University of Otago Herbarium (OTA) specimen no. 048094].

3. Preparation of the extract

Air-dried *T. hatcheri* (13. 2 g) was macrate in redistilled ethanol (300 ml) in a Waring Blender, and then filtered. the residual marc was reextracted in the same way with more ethanol (2 x 150 ml) and chloroform (200 ml). The combined filtrates were evaporated under reduced pressure to give a dark green gum (0.443 g). The crude ethanol extract was subjected to flash column chromatography on C-18 (10 g) with a H_2O : CH_3CN : $CHCl_3$ gradient. The bioactive subfractions were chromatographed on silica gel with an ethyl acetate : cyclohexane. These fractions and subfractions were stored at 40 ℃ until tested.

4. Cell culture.

P388 murine leukaemia cells (ATCC CCL 46 P388D1) were grown at 37^{0}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.

5. Screening for antiviral activity

The extract was applied (15 $\mu\ell$ of a 5 mg/ml solution) to a small filter-paper disc, dried, and assayed for antiviral activity using Schroeder et al.'s methods[9]. The results were observed either cell death (cytotoxicity), inhibition of virus replication, no effect (i.e., all of the cells show viral infection), or a combination of all three. The results were noted as the approximate size of the circular zone, radiating from the extract sample, from 1+ to 4+ representing 25 % through to whole well sized zones. The notation used is inhibition/antiviral activity. The type of antiviral effect, indicated by a number after the size of the zone, was also considered important and may give some indication as to the mode of cytotoxic action.

6. Screening for antibacterial and antiyeast activities

Activity against the following bacterial strains and yeast was tested: multiresistant *Bacillus subtilis* (ATCC 19659), and *Candida albicans* (ATCC 14053). Extracts were dissolved and diluted in an appropriate solvent (usually ethanol : water) to a concentration of 5 mg/ml. Test plates are prepared from Mueller Hinton agar containing extract to give a final concentration of 100 μg extract/ml agar. Activity growing cultures of the test strains were diluted in saline so as to deliver 10^4 colony forming units onto the test, control (solvent), and blank (agar only) plates with a multipoint inoculator. Inoculated plates were incubated overnight at 37 ℃. Growth on the blank and control plates was checked and, if satisfactory, growth on the test plates was scored for each test strain as follows: (-) inhibition, no reduction in growth compared with the control, (+) inhibition, no growth.

7. Screening for antifungal activity

Activity against the following fungal strain was tested: *Trichophyton mentagraphytes* (ATCC 28185) local strain]. Fungal spore suspensions of the test organisms were applied to dextrose agar plates. Aliquots of the extract solutions were applied to filter paper discs, at 15 μg extract/disc, and dired at 37 ℃ for two hours. These discs were applied to the agar plates, two per plate, and incubated at 28 ℃.

8. Screening for cytotoxic activity

This is a measure of the ability of a sample to inhibit the multiplication of murine leukaemia cells. The sample was dissolved in a suitable solvent, usually ethanol, at 5 mg/ml, and 15 μl of this solution was placed in the first well of a multiwell plate. Seven two-fold dilutions were made across the plate. After addition of the cell solution, the concentration range in the test wells was 25,000 down to 195 ng/ml. After incubation for three days, the plates were read using an Elisa palte reader at 540 ㎚ wavelength. Automated reading of the plates was possible with the addition of a MTT tetrazolium salt (yellow color). Healthy cells reduce this salt to MTT formazan (purple color).

9. Isolation of methyl-4-[[(2E)-3,7-dimethy l-2,6-octadienyl] oxy] -3-hydroxybenzoate (1).

Dried T. hatcheri (13.2 g) was extracted With EtOH (2 x 300 ml) and $CHCl_3$ (200 ml) by homogenizing and filtering to give a dark green gum (443 ml, 50 % BSC cytotoxicity at 150 μg/disc). Reversed-phase flash chromatography over C18 (443 mg precoated on 1.0 g C18, loaded on a 10 g C18 column) was developed in 20 ml steps from H_2O through CH_3CN to $CHCl_3$. Fractions eluted with H_2O - CH_3CN 1 : 3 and 1 : 9 (86 mg, brown oil, 75 % cytotoxicity at 60 μg/disc) were developed in steps from EtOAC - cyclohexane 3 : 97 to EtOAC - cyclohexane 20 : 80. Si-gel column fractions eluted with EtOAC - cyclohexane 3 : 97 and 5 : 95 showed the same UV active spot on TLC (lilac with vanillin - H_2SO_4). These were combined and the solvent removed to produce a residue (14

mg, yellow oil). Final purification was by Si-gel TLC with EtOAC - cyclohexane 20 : 80. A UV-active band at R_f 0.30 was eluted with Et_2O to give 1 (4.5 mg). Colorless oil; UV (MeOH) λmax (log ε) 262 (3.35) nm; IR (dry film) νmax 3408, 2956, 1716, 1622, 1513, 1436, 1382, 1355, 1284, 1213, 1126, 1033, 989, 886, 761 cm-1; ^{1}H-NMR ($CDCl_3$) δ 7.6 (2H, m, 2'-H, 6'-H), 6.87 (1H, d, J=7 Hz, 5'-H), 5.7 (1H, brs, OH), 5.47 (1H, brt J=7 Hz, 2-H), 5.07 (1H, m 6-H), 4.67 (2H, d=7 Hz, 1-H), 3.88 (3H, s, OMe), 2.1 (2 x 2H, m, 4-H, 5-H), 1.75 (3H, brs, 10-H), 1.68 (3H, brs, 8-H), 1.61 (3H, brs, 9-H); ^{13}C-NMR ($CDCl_3$) δ166.8 (C-7'), 149.7 (C-4'), 145.5 (C-3'), 142.7 (C-3), 132.0 (C-7), 123.5 (C-6), 123.2 (C-1'), 122.6 (C-6'), 118.4 (C-2), 115.5 (C-2'), 111.0 (C-5'), 65.9 (C-1), 51.9 (C-7'OMe), 39.5 (C-4), 26.2 (C-5), 25.7 (C-8), 17.7 (C-9), 16.7 (C-10). EIMS (30 ev) m/z 318.1475 [M]+ (calcd for $C_{18}H_{22}O_5$, 318.1467)[4].

10. Synthsis of methy l-4-[[(2E)-3,7-dimethyl-2,6-octadienyl] oxy]-3-hydroxybenzoate (1).

Methyl-3,4-dihydroxybenzoate (278 mg, 1.65 mmol); geranyl bromide (434 mg, 2.00 mmol); NaH (60 %, 66 mg, 1.65 mmol) in dry DMF (2 ml); 0oC; 17 hrs; flash chromatography (5 % EtOAC - hexane) gave 1 (220 mg, 44 %). Compound (1) was identified by comparing its spectral data (TLC, MS, NMR and IR) with those published or by directly comparison with an anthentic sample[4].

11. Statistical analysis.

All values, expressed as the mean ± S.D., were statistically analyzed through analysis of Student's t-test. The P value less than 0.05 was considered as significant.

Results and Discussion

1. Biological screenings of extract

Trichocolea hatcheri Hodgs (family Trichocoleaceae) grows throughout New Zealnd. Foliage plant collected from a steep earth bank in the Morrisons Creek area in Dunedin. An extract of *T. hatcheri* was prepared by grinding dried plant material and extracting separately with ethanol then chloroform. The two extracts were combined, as their ^{1}H-NMR spectra were similar. The crude extract was cytotoxic to P388 murine leukaemia cells ATCC CCL 46 P388D1, (IC_{50} > 12,500 μg/ml) and BSC monkey kidney cells (50 % of well at 150 μg/ml). Table I shows the mediocre antiviral activity against *Herpes simplex* Type I *virus* (ATCC VR 733) and *Polio Type* I *virus* (Pfizer vaccine strain) (50 % activity, @ 5 mg/ml at 150 μg/disc). The crude extract inhibited the growth of the Gram-positive bacteria and fungus of the extract prepared from New Zealand medicinal plant. As indicated in Table I, this crude extract inhibited the growth of the Gram-positive bacterium *Bacillus subtilis* ATCC 19659, (1 mm inhibition zone at 150 μg/disc) and the dermatophytic fungus *Trichophyton mentagrophytes* ATCC 28185, (6 mm inhibition zone at 150 μg/disc). No activity was observed against the fungus *Candida albicans* (ATCC 14053) at 150 μg/disc. This extract showed weaker antimicrobial activity than chloramphenicol and nystatin (Table 1). However, this crude extract is stronger antimicrobial activity than the crude extract of *B. monroi* and this extract is inactive against P388 murine leukaemia cells ATCC CCL 46 P388D1[10].

Table 1. Biological assays of the crude extract from *T. hatcheri*

Extract	Cytotoxicity		
	BSC[a]	*Herpes simplex virus*[b]	*Polio virus*[b]
	+ +	+ +	+ +
	Antimicrobial activity[c]		
	B. subtilis	*C. albicans*	*T. mentagrophytes*
Extract	SM 1	-	HM 6
Chloramphenicol	SM 12	0	0
Nystatin	0	SM 11	HM 8
		P388	
Mitomycin C		59.7[d]	
Extract		> 12,500[e]	

[a]% of well showing cytotoxic effects. @ 5 mg/ml, 150 μg/disc: + + : 50 % activity.
[b]Cytotoxicity in antiviral assays. @ 5 mg/ml, 150 μg/disc: Zone of cytotoxic activity: + + : 50 % activity.
[c]Width of zone of inhibition in mm: 150 μg/disc: -: no reduction in growth, 0: not determined. Chloramphenicol: 30 mcg/disc, Nystatin: 100 unit/disk. SM: Sharp margin, HM: hazy margin, numbers refer to zone of inhibition (mm)
[d]Toxicity of sample to P388 murine leukaemia cells ATCC CCL 46 P388D1 in ng/ml at 0.075 μg/disc. P388 : Concentration of the sample required to inhibit cell growth to 50 % of a solvent control.
[e]Toxicity of sample to P388 murine leukaemia cells ATCC CCL 46 P388D1 in ng/ml at 75 μg/disc.

2. Cytotoxic and antifungal activities of bioactive fractions

The crude extract (0.443 g) was fractioned into Fr. 1 - Fr. 10 using C-18 silica gel column chromatography as described previously[11]. Chromatography on C-18 (10.0 g) with a H_2O, MeCN, $CHCl_3$ gradient gave ten fractions. The column fractions were combined based on visually similar TLC results. These combined fractions were assayed against *Herpes simplex* Type I *virus* (ATCC VR 733) and the activity was found to be spread over two fractions that were eluted with 3 : 1, H_2O/CH_3CN, 1 : 1 H_2O/CH_3CN, 1 : 3 H_2O/CH_3CN and 1 : 9 H_2O/CH_3CN. Reverse-phase flash column chromatography of a crude extract which eluted with CH_3CN : $CHCl_3$ (3 : 1) and $CHCl_3$ gave most of the mass in the less polar fractions. Among them, the fractions Fr. 1 - Fr. 9 are antifungal activity to *Herpes simplex* Type I *virus* (ATCC VR 733). The Fr. 6 that eluted with 1 : 3 and 1 : 9 H_2O : CH3CN (85.5 mg, 75%

activity, @ 2 mg/ml at 60 μg/disc) is the most antifungal activity (Table 2).

Table 2. In vitro antifungal activity of bioactive fractions on *Herpes simplex virus*[a].

Fraction No.	Eluent	Vol. (ml)	Mass (mg)	Cytotoxicity[b]
1	H_2O, 9 : 1, 3 : 1 H_2O/CH_3CN	46	119.1	+ -
2	3:1 H_2O/CH_3CN	7	5.3	+ -
3	3:1, 1:1 H_2O/CH_3CN	14	3.4	+ +
4	1:1 H_2O/CH_3CN	7	6.9	+ -
5	1:1, 1:3 H_2O/CH_3CN	14	10.5	+ -
6	1:3, 1:9 H_2O/CH_3CN	27	85.5	+ + +
7	1:9 H_2O/CH_3CN, CH_3CN	20	29.0	+
8	CH_3CN, 3:1 $CH_3CN/CHCl_3$	14	11.0	+
9	3:1 $CH_3CN/CHCl_3$	34	141.3	+
10	CHC_3	20	13.3	-

[a]Each fraction was examined in ten concentrations in triplicate experiments.
[b]Cytotoxicity in antiviral assays. @ 2 mg/ml, 60 μg/disc: Zone of cytotoxic activity: -: no discernible antiviral effects, + -: minor effects located under the disc, + : 25 % activity, + + : 50 % activity, + + + : 75 % activity.

Reverse-phase flash column chromatography concentrated the antifungal activity in fraction 6 eluted with H_2O - CH_3CN 1 : 3 and 1 : 9. Chromatography on silica gel (10.0 g) with an ethyl acetate - cyclohexane gradient gave eleven fractions. The second silica gel column chromatography of fractions (Fr. 6-5, 6-7 and 6-8) gave most of the mass in the polar fractions, eluted with 3 %, 5 % and 7 % ethyl acetate - cyclohexane. Among them, fr. 6 - 4 that eluted with 3 % ethyl acetate - cyclohexane is the most antifungal activity to *Herpes simplex* Type I *virus* ATCC VR 733, (0.3 mg, 50% activity, @ 1 mg/ml at 30 μg/disc). But the yield is too small (Table 3).

Table 3. In vitro antifungal activity of bioactive fractions on *Herpes simplex virus*[a].

Fraction No.	Eluent	Vol. (ml)	Mass (mg)	Cytotoxicity[b]
6-1	3 % EtOAC/Cyclohex	6	0.7	+
6-2	3 % EtOAC/Cyclohex	2	1.5	-
6-3	3 % EtOAC/Cyclohex	6	0.5	+
6-4	3 % EtOAC/Cyclohex	2	0.3	+ +
6-5	3 %, 5 % EtOAC/Cyclohex	12	13.5	+
6-6	5 % EtOAC/Cyclohex	8	0.8	+
6-7	5 % EtOAC/Cyclohex	16	12.5	+ -
6-8	5 %, 7 % EtOAC/Cyclohex	32	14.5	+ -
6-9	10 % EtOAC/Cyclohex	18	4.1	+ -
6-10	10 %, 13 % EtOAC/Cyclohex	38	2.6	+ -
6-11	13 %, 15 %, 20 % EtOAC/Cyclohex	20	5.3	+

[a]Each fraction was examined in eleven concentrations in triplicate experiments.
[b]Cytotoxicity in antiviral assays. @ 1 mg/ml, 30 μg/disc: Zone of cytotoxic activity: -: no discernible antiviral effects, + -: minor effects located under the disc, + : 25 % activity, + + : 50 % activity. EtOAC: ethyl acetate, Cyclohex: cyclohexane.

3. Identification and biological activity of methyl-4-(geranyloxy)-3-hydroxybenzoate(1).

Silica gel flash column chromatography concentrated the cytotoxic activity in fractions eluted with ethyl acetate - cyclohexane 3 : 97 and 5 : 95. This fraction contained one main UV-active compound by TLC. The unique absorption bands due to ester carbonyl band (1,716 cm^{-1} and 1,212 cm^{-1}) were shown in the IR spectrum along with hydroxyl group (3,412 cm^{-1}) as well as aromatic group (1,600, 1,509 and 1,436 cm^{-1}).

The IR spectrum of compound showed the presence of conjugated carbonyl and aromatic group. This compound (1) was obtained pure in quantities, but too small for biological assays, synthesis and biological activity report here. The least polar compound (1), purified by preparative TLC, had UV and IR spectra appropriate for a 3,4-dioxygenated benzoic acid derivative. The MS supported a molecular of $C_{18}H_{24}O_4$. the phenolic OH of 1 was observed in the ^{1}H-NMR spectrum as a broad exchangeable signal at 5.7 ppm. The ^{13}C-NMR spectrum of compound (1) showed signals appropriate for a trisubstituted aromatic ring, a geranyl group, an ester carbonyl, and methoxyl and hydroxyl groups, as expected for 1. We rigorously assigned the ^{1}H and ^{13}C-NMR spectra with the aid of HMQC, HMBC, DEPT and NOE difference experiments (Fig. 1).

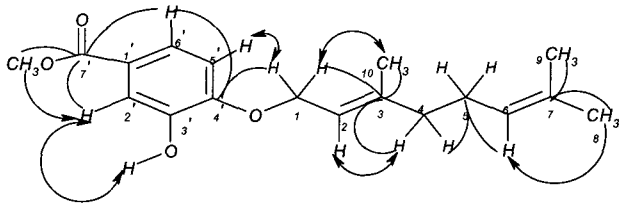

Fig. 1. Important NMR correlations establishing the structure of 1. ↔, selected NOE interactions; —, selected HMBC correlations.

Table 4. Biological activities of methyl-4-(geranyloxy)-3-hydroxybenzoate (1) from *T. hatcheri.*

	Cytotoxicity				
	BSC[a]	*Herpes simplex virus*[b]		*Polio virus*[b]	
1	+	+		+	
	Antimicrobial activity[c]				
	B. subtilis	*T. mentagrophytes*	*E. coli*	*C. resinae*	*P. aerug*
1	-	HM 1	-	-	-
Chloramphenicol	SM 13	0	0	0	0
	0	SM 7	0	0	0
	0	0	SM 10	0	SM 10
		P388			
Mitomycin C		62[d]			
1		21,623[e]			
1		> 6,260[f]			

[a]% of well showing cytotoxic effects. @ 2 mg/ml, 60 μg/disc and 0.5 mg/ml, 15 μg/disc: + : 25 % activity.
[b]Cytotoxicity in antiviral assays. @ 2 mg/ml, 60 μg/disc and 0.5 mg/ml, 15 μg/disc: Zone of cytotoxic activity: + : 25 % activity.
[c]Width of zone of inhibition in mm; 60 μg/disc: -: not detected, 0: not determined. Chloramphenicol; 30 mcg/disc, Gentamycin; 30 mcg/disc, Nystatin; 100 unit/disc. HM; Hazy margin, SM; Sharp margin, numbers refer to zone of inhibition (mm)
[d]Toxicity of sample to P388 murine leukaemia cell lines ATCC CCL 46 P388D1 in ng/ml at 0.075 μg/disc. P388 ; Concentration of the sample required to inhibit cell growth to 50% of a solvent control.
[e]Toxicity of sample to P388 murine leukaemia cells ATCC CCL 46 P388D1 in ng/ml at 30 μg/disc and [f]at 7.5 μg/disc .

Methyl-4-(geranyloxy)-3-hydroxybenzoate (1) which isolated from *T. hatcheri* is cytotoxic to P388 murine leukaemia cells (IC50 > 6,250 ng/ml at 7.5 μg/disc) and BSC monkey kidney cells (25 % activity, at 15 μg/disc). Table IV shows weak antiviral activity against *Herpes simplex* Type I *virus* ATCC VR 733 and *Polio* Type I *virus* (Pfizer vaccine strain) (25 % activity, @ 0.5 mg/ml at 15 μg/disc). As indicated in Table IV, the compound (1) inhibited the growth of the dermatophyte fungus *Trichophyton mentagrophytes* ATCC 28185, (1 mm inhibition zone at 60 μg/disc). The activities are expressed by the diameter of the developed inhibition zones and compared with those of the widely antibious chloramphenicol, gentamycin and nystatin (Table 4)[10] .

In conclusion, methyl-4-(geranyloxy)-3-hydroxybenzoate (1) has isolated from the whole plant of T. hatcheri, and its structure has determined by spectroscopic analysis. This geranyl phenyl ether (1) inhibited the growth of the dermatophytic fungus *Trichophyton mentagrophytes* ATCC 28185, (2 mm inhibition zone at 15 μg/disc), cytotoxic to P388 murine leukaemia cell lines ATCC CCL 46 P388D1, (IC_{50} 21,623 ng/ml at 30 μg/disc and IC_{50} > 6,260 ng/ml at 7.5 μg/disc) and BSC monkey kidney cell lines (25 % of well at 15 μg/disc).

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