Antifungal Activity of Chlororinated Bibenzyl Compound on the Dermatophytic Fungus *Trichophyton mentagrophytes*

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The chlororinated bibenzyl compound (**1**) inhibited the growth of the Gram positive bacterium *Bacillus subtilis* ATCC 19659, (2 mm inhibition zone at 30 μg/disc), *Candida albicans* ATCC 14053, (2 mm inhibition zone at 30 μg/disc), and the dermatophytic fungus *Trichophyton mentagrophytes* ATCC 28185, (3 mm inhibition zone at 30 μg/disc).

Key words : Chlororinated bibenzyl compound (1), *Bacillus subtilis*, *Candida albicans*, *Bacillus subtilis*, *Trichophyton mentagrophytes*, antifungal activity

Introduction

Liverworts are the only class of the Bryophytes that contain complex oil bodies[1], and so are capable of synthesising a vast range of lipophilic aromatic and terpenoid compounds; it is perhaps because of this that liverworts have been more thoroughly investigated than mosses or hornworts for their rich and varied chemistry[2,3]. A recent review by Asakawa states more than 700 terpenoids and 220 aromatic compounds (excluding flavonoids) have been isolated from or detected in the Hepaticae[4]. It is possible that the chemicals synthesised by these primitive soft-bodied plants protect them from external attack, whereas higher plants protect themselves physically with thorns, and spikes and thick bark (as well as chemical forms of defence)[3]. Perhaps the best evidence for this chemical form of defence is that liverworts are rarely found damaged by insects, bacteria, fungi or mammals. *Riccardia marginata* (Colenso) Pearson (family Aneuraceae) is endemic to New Zealand[5], which it is found growing on old logs in wet forests[6]. No previous chemistry of *R. marginata* has been reported, but the genus *Riccardia* has yielded a range of compounds[2], including sesquiterpene-linked phenol derivatives[7,8], indole alkaloids[9,10] and bis-bibenzyls[11,12,13]. Brominated bibenzyls have previously been found in the red alga *Polysiphonia urceolata*[14]. The ether extract of the New Zealand liverwort Radula marginata isolated a new cannabinid type bibenzyl compound named perrottetinenic acid, and two new bibenzyls, together with a known cannabinoid, perrottetinene. The structure of perrottetinenic acid was a similar to that of delta-1-tetrahydrocannabinol, a known hallucinogen[15]. A crude extract of one of our collections of *R. marginata* showed activity against *Bacillus subtilis, Candida albicans* and *Trichophyton mentagrophytes* in our antimicrobial screening[16]. We now report the chlororinated bibenzyl compound (1) as the antifungal compound from *Riccardia marginata* (Colenso) Pearson (family Aneuraceae).

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Materials and Methods

1. General experimental procedures.

Solvents for extraction and chromatography were distilled prior to use. Preparative silica gel TLC was carried out using Merck DC-plastikfolien Kieselgel 60 F_{254}, visualized with an UV lamp then by dipping in a vanillin solution (1% vanillin, 1% H_2SO_4 in EtOH) and heating. UV spectrum was recorded with a Jasco V-550 UV spectrophotometer. IR spectrum was obtained with a Perkin-Elmer 1600 FTIR as a film on a NaCl disk. NMR spectra were recorded at 25℃ on a Varian INOVA 500 NMR spectrometer operating at 500 MHz for ^1H and 125 MHz for ^{13}C, using solvent signals as references ($CHCl_3$ at 7.25 ppm, $CDCl_3$ at 77.0 ppm). DEPT, HSQC, CIGAR and NOESY experiments were run at 25℃. EIMS was obtained on a VG70-250S double-focusing magnetic sector mass spectrometer. Column chromatography used octadecyl-functionalized sillica gel (Aldrich C_{18}) and 40-63 μm silica gel 60 (Merck). TLC was

carried out on silica gel F254 plates (Merck), with the solvent system hexane - ethyl acetate (9 : 1).

2. Plant materials.

Riccardia marginata (*R. marginata*) was collected from New Zealand's sub-Antarctic Auckland Islands in January 1994 (OTA046755), and near the Kiahoka Lakes, Cape Farewell, West Coast, New Zealand (OTA 050850) in March 1999. Collections were identified by R. Tangney and voucher specimens are deposited in the University of Otago Herbarium. The samples were air-dried (30℃).

3. Preparation of the extract.

Air-dried *R. marginata* was macrate in redistilled chloroform in a Waring Blender, and then filtered. the residual marc was reextracted in the same way with chloroform. The combined filtrates were evaporated under reduced pressure to give a dark green gum which was stored at 4℃ until tested.

4. Extraction and isolation.

The remaining plant material (46.05 g) from the two *R. marginata* collections was combined, extracted with $CHCl_3$ (4 x 460 mL), and concentrated under vacuum to give a deep green solid residue (1.67 g). The residue was chromatographed on C_{18} using a steep, stepped solvent gradient from H_2O to MeOH to $CHCl_3$ to hexane. The fraction that eluted at 100% MeOH (221 mg) was chromatographed further on silica gel, eluting with hexane - ethy acetate mixtures. The first two fractions (14 mg), eluted between hexane and 10% ethyl acetate, were further purified by silica gel TLC (plate thickness, 0.2 mm) using 10% ethy acetate in hexane (developed twice, drying the plates between) to give 2-chloro-3-hydroxybibenzyl (**1**, 1 mg, R_f 0.25): yellow gum; UV (MeOH) λ_{max} (log ε) 212 (4.0), 276 (3.2), 282 (3.1) nm; IR (film) ν_{max} 3523, 2917, 2845, 1580, 1450, 1292, 1190, 788, 757, 699 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ (1H, 7.20 m, 2-H), (1H, 7.28 m, 3-H), (1H, 7.21 m, 4-H), (1H, 7.28 m, 5-H), (1H, 7.20 m, 6-H), (2H, 2.89 m, α), (2H, 3.00 m, α'), (1H, 6.88 dd, J=1.5, 8.0 Hz, 4'-H), (1H, 7.07 t, J=7.5Hz, 5'-H), (1H, 6.74 dd, J=1.5, 7.5 Hz, 6'-H), (OH, 5.61 s,); ^{13}C-NMR ($CDCl_3$) δ 140.0 (C-1), 128.4 (C-2), 128.4 (C-3), 126.1 (C-4), 128.4 (C-5), 128.4 (C-6), 36.0 (C-α), 36.0 (C-α'), 141.3 (C-1'), 119.8 (C-2'), 151.6 (C-3'), 113.8 (C-4'), 127.5 (C-5'), 122.0 (C-6'); EIMS m/z 235 (1), 234 (7), 233 (3), 232 [M^+] (18), 141 (15), 91 (100); HREIMS m/z 232.0651 (calcd for $C_{14}H_{13}ClO$, 232.0655).

5. Screening for antiviral activity

The extract was applied (30 μL of a 5 mg/mL solution) to a small filter-paper disc, dried, and assayed for antiviral activity using Schroeder et al., methods[17]. 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), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), and *Candida albicans* (ATCC 14053) and *Cladosporium resinae* (ATCC 52833). 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. Solutions of compound for assay were dried onto 6 mm filter paper disks, which were then placed onto seeded agar Petri dishes and incubated. Activity was observed as a zone of inhibition around the disk, with its width recorded from the edge of the disk in mm. HM and SM refer to the observed margin surrounding the zone of inhibition. (H= hazy, S= sharp).

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 oraganisms were applied to dextrose agar plates. Aliquots of the extract solutions were applied to filter paper discs, at 30 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 30 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 nm 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).

Results and Discussion

Riccardia marginata (Colenso) Pearson (family Aneuraceae) grows throughout New Zealnd. Foliage plant collected from sub-Antarctic Auckland Islands and near the Kiahoka Lakes, Cape Farewell, West Coast, New Zealands. An crude extract of *R. marginata* was prepared by grinding dried plant material and extracted with chloroform. A crude extract was not cytotoxic to P388 murine leukaemia cells ATCC CCL 46 P388D1, (IC_{50} 25,000 µg/mL) and BSC monkey kidney cells (150 µg/mL). Table 1 does not show the antiviral activity against *Herpes simplex* Type I virus (ATCC VR 733) and *Polio* Type I virus (Pfizer vaccine strain) (@ 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 *R. marginata*. As indicated in Table 1, this crude extract inhibited the growth of the Gram-positive bacterium *Bacillus subtilis* ATCC 19659, (4 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) and *Cladosporium resinae* (ATCC 52833), against the Gram-negative bacteria *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853) at 150 µg/disc. This extract showed weaker antimicrobial activity than chloramphenicol and nystatin (Tables 1 and 2)[18].

2-Chloro-3-hydroxybibenzyl (**1**) was purified by chromatography on reversed-phase C_{18} followed by normal-phase silica. The ^{1}H-NMR spectrum of this compound (**1**) showed -CH_2-CH_2- signals (4H multiplets, δH 2.89 - 3.00 ppm) and phenolic OH signals (D_2O - exchangeable broad singlet, δH 5.61 ppm). The other signals were of aromatic protons, which were overlapping, especially for the minor compound (**1**). Negative electrospray ionization (-ESI) MS gave rather confusing result for **1**, but did suggest the presence of chlorine atom. Electron-impact MS gave molecular ions corresponding to $C_{14}H_{13}ClO$ for **1**. This spectrum showed strong *m/z* 91 ions, due to $C_6H_5CH_2^+$. These results, along with the NMR data above, showed that this compound was one bibenzyl, with one unsubstituted ring and one ring substituted with -OH and -Cl (s). The ^{1}H-NMR spectrum of the monochloro compound (**1**) was best dispersd and showed a three-proton spin system due to a 1,2,3-trisubstituted aromatic ring. The shielded position of one of the CH signals (δ_H 6.89, δc 113.8) suggested that this was *ortho* to the OH, with another CH signal (δ_H 6.74, δc 122.0) showing NOESY correlations to the -CH_2-CH_2-bridge protons, giving structure **1**. This was confirmed by 2D-NMR correlations (Fig. 1) and by ^{1}H and ^{13}C shifts very similar to the known compound bitungolide A (**2**)[19].

Table 1. Biological activities of the crude extract from *R. marginata*

Assay	Tested material			
	Crude extract	Chloramphenicol	Nystatin Gentamycin	Mitomycin C
Cytotoxicity BSC-1 cells[a]	-			
P388 IC_{50}	5,000[b]			59.7[c]
Antiviral activity[d]				
Herpes simplex virus	-			
Polio virus	-			
Antimicrobial activity[e]				
B. subtilis	SM 4	SM 12	0	0
E. coli	-	0	0	SM 9
P. aeruginosa	-	0	0	SM 10
C. albicans	-	0	SM 11	0
C. resinae	-	0	SM 10	0
T. mentagrophytes	SM 6	0	HM 8	0

[a]% of well showing cytotoxic effects, with virus growing in cytotoxic zone. @ 5 mg/mL, 150 µg/disc; -: no activity. BSC-1 cells; African green monkey kidney cells. [b]Toxicity of sample to P388 murine leukaemia cells (ATCC CCL 46 P388D1) in ng/mL at 150 µg/disc. [c]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. [d]Antiviral assays. @ 5 mg/mL, 150 µg/disc: Zone of cytotoxic activity: -: no activity. [e]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)

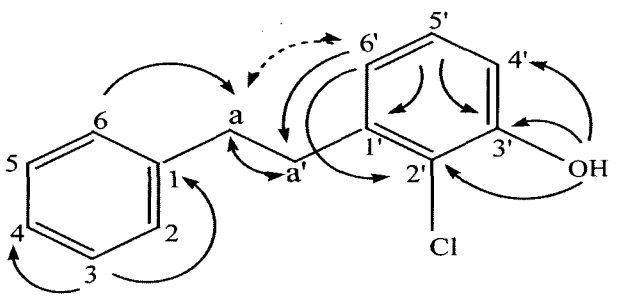

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Fig. 1. Selected CIGAR (solid line) and NOESY (dotted line) correlations for **1**.

This compound (**1**) seem to represent successive chlorination of bibenzyl (**3**) by a haloperoxidase, similar to that recently found in the liverworts *Caldariomyces fumigo* and

Bazzania trilobata[20]. 3-Hydroxybibenzyl (**3**) has been rerported once as a natural product, in another liverwort, Radula frondescens[2], but we did not see NMR signals appropriate for **3** in any chromatographic fractions or in the crude extract of *R. marginata*. The *R. marginata* collection containing **1** gave an extract with antimicrobial activity against the Gram-positive bacterium *Bacillus subcillus* and the dermatophytic fungus *Trichophyton mentagrophytes*, whereas the Auckland Islands extract was inactive. Assay of **1** showed activity against *B. subcillus*, *Trichophyton mentagrophytes*, and the yeast *Candida albicans*, but not against the Gram-negative bacteria *Escherichia coli* an*Pseudomonas aeruginosa*. and against the plant pathogenic fungus *Cladosporium resinae* (Table 3).

As indicated in Table 2, the 2-chloro-3-hydroxybibenzyl (**1**) inhibited the growth of the Gram-positive bacterium *Bacillus subtilis* ATCC 19659, (2 mm inhibition zone at 30 μg/disc). Antiyeast activity was observed against the fungus *Candida albicans* ATCC 14053 (2 mm inhibition zone at 30 μg/disc). Antifungal activity was shown against the dermatophytic fungus *Trichophyton mentagrophytes* ATCC 28185, (3 mm inhibition zone at 30 μg/disc). This compound (**1**) showed weaker antimicrobial activity than chloramphenicol and nystatin (Tables 2 and 3).

Table 2. Antimicrobial activity of 2-chloro-3-hydroxybibenzyl (1) from *R. marginata*.

Tested material	Antimicrobial activity[a]					
	B.subtilis	*C.albicans*	*T.ment.*	*E.coli*	*C.resinae*	*P.aeruginosa*
1	HM 2	HM 2	HM 3	-	-	-
Chloramphenicol	SM 13	0	0	0	0	0
Nystatin	0	SM 12	SM 6	0	SM 10	0
Gentamycin	0	0	0	SM 9	0	SM 10

[a]Width of zone of inhibition in mm; 30 μg/disc; -: not detected, 0: not determined. Chloramphenicol: 30 μg/disc, Gentamycin: 30 μg/disc, Nystatin: 100 unit/disc. HM: Hazy margin, SM: Sharp margin, numbers refer to zone of inhibition (mm)

Table 3. List of microorganisms used for antimicrobial susceptibility test.

Gram-positive bacterium	
Bacillus subtilis	ATCC 19659
Gram-negative bacteria	
Escherichia coli	ATCC 25922
Pseudomonas aeruginosa	ATCC 27853
Fungi	
Cladosporium resinae	ATCC 52833
Candida albicans	ATCC 14053
Trichophyton mentagrophytes	ATCC 28185

In conclusion, the crude ethanol extract of *R. marginata* inhibited the growth of the Gram positive bacterium *Bacillus subtilis* ATCC 19659, (4 mm inhibition zone at 150 μg/disc) and the dermatophytic fungus *Trichophyton mentagrophytes* ATCC 28185, (6 mm inhibition zone at 150 μg/disc), and inactive to P388 murine leukaemia cells ATCC CCL 46 P388D1, (IC_{50} >25,000 μg/mL at 150 μg/disc). The chlororinated bibenzyl compound (**1**) has isolated from the whole plant of *R. marginata*, and its structure has determined by spectroscopic analysis. This compound (**1**) inhibited the growth of the Gram positive bacterium *Bacillus subtilis* ATCC 19659, (2 mm inhibition zone at 30 μg/disc). Antiyeast activity was observed against the fungus *Candida albicans* ATCC 14053 (2 mm inhibition zone at 30 μg/disc). Antifungal activity was shown against the dermatophytic fungus *Trichophyton mentagrophytes* ATCC 28185, (3 mm inhibition zone at 30 μg/disc).

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