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Antifungal and Insect Antifeedant 2-Phenylethanol Esters from the Liverwort *Balantiopsis cancellata* from Chile

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A chemical study of a dichloromethane extract of *Balantiopsis cancellata* led to the isolation of four known 2-phenylethanol esters (**1**−**4**) and a phenylethanediol benzoate (**5**). Antifeedant activity toward *Spodoptera littoralis* (disk-choice bioassay) and growth inhibition of the phytopathogen *Cladosporium herbarum* in TLC−bioautography assays were determined. The results show that the antifeedant and antifungal activity of the extract is attributable mainly to the *trans*-β-methylthioacrylate **4**.

KEYWORDS: ***Balantiopsis cancellata*; Hepaticae; liverwort; *Spodoptera littoralis*; *Cladosporium herbarum*; antifeedant activity; antifungal activity; bioautography; (*R*)-2-hydroxy-2-phenylethyl benzoate**

INTRODUCTION

Liverworts (phylum Marchantiophyta) produce in their oil bodies an array of secondary metabolites with structures often unique within the land plants (*1*). They normally grow in humid habitats, coexisting with rot fungi, slugs, and other plant predators without suffering adverse effects. In contrast to higher plants, dry liverwort specimens in herbaria are characteristically not attacked by rodents, insects, or fungi. This suggests a high stability and a wide range of action of the defensive compounds they produce. They are therefore suitable subjects in a search for natural substances that could be used as part of an integrated management of pest control, and many liverworts have been shown to contain compounds with antifeedant and antimicrobial activity (*2*).

In continuation of our search for natural pesticides from native Chilean species (*3*), we are now investigating the rich but poorly known liverwort flora of southern Chile, which includes many endemic species. The small size of these plants and difficulties with botanical identification have hindered chemical studies of liverworts from Chile until now, as they require suitable techniques to deal with tiny amounts of extracts. Thus, for bioassay-directed chemical studies, we first submit a dichloromethane extract of a species to the brine shrimp (*Artemia salina*) lethality bioassay (*4*). Extracts showing LC_{50} values lower than 50 ppm (0.05 mg/mL) are tested with more specific bioassays using insect larvae with nonspecific feeding habits of the genus *Spodoptera* (*5*) and bioautography on TLC plates with *Cladosporium herbarum*, a rot fungus (*6*).

Additionally, to characterize a liverwort species chemically and gain insight into the type of compounds it produces, it is useful to obtain GC−MS and ^{1}H NMR profiles of an extract (*7*), which may be simpler in composition than those of higher plants. This method along with the usual chromatography techniques is very helpful in selecting strategies toward the isolation of the bioactive compounds.

Following these procedures, we report in this paper results obtained in a bioassay-directed study of *Balantiopsis cancellata* (Nees) Steph. (Balantiopsidaceae), a liverwort commonly found on soil or on the bark of native trees in southern Chile. Previous chemical studies of *Balantiopsis rosea*, *Balantiopsis erinacea*, and *B. cancellata* show that species of this genus are characterized by the elaboration of benzyl benzoate as a main compound, alongside cinnamate, benzoate, or thioacrylate esters (*1*).

MATERIALS AND METHODS

General Procedures. GC−MS of the hexane-soluble fraction used a Fisons gas chromatograph coupled to a Fisons MD-800 mass detector, at 70 eV. The source and injector temperatures were set at 200 and 250 °C, respectively. Separations used an HP-5 capillary column [poly-

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(5%-diphenyl-95%-dimethyl)siloxane, 25 m × 0.2 mm i.d. and 0.33 μm] (carrier gas He at 1−1.2 mL/min; temperature program, 80 °C held for 1 min and then 7 °C/min till 300 °C). GC−MS of compound **5** was performed generally as described previously (*8*). Melting points were taken using a Gallen III apparatus and are uncorrected.

Plant Material. *B. cancellata* (Nees) Steph. (Balantiopsidaceae) was collected in July 2001 (winter) at Aituy (Isla Grande de Chiloé, Chile). A voucher specimen (CL19) is retained by DSR. Further collections of this species were made at Parque Nacional Puyehue (Osorno, Chile) in November 2003 (spring) for comparison purposes.

Extraction and Isolation. Air-dried and powdered whole plants (13.7 g) were extracted with dichloromethane (room temperature, 1 h) to give 0.5 g of crude extract after evaporation of solvent under reduced pressure. Low-pressure column chromatography over silica gel (G 60, Merck) using a hexane−EtOAc gradient afforded a mixture of **1**−**4** (180 mg) in the fraction eluted with 10−15% EtOAc−hexane. Preparative TLC (three runs in 10% EtOAc−hexane) of 50 mg of this mixture gave 2-phenylethyl benzoate (**1**; 12.5 mg), 2-phenylethyl *cis*-cinnamate (**2**; 2.5 mg), 2-phenylethyl *trans*-cinnamate (**3**; 4.2 mg), and isotachin B (**4**; 6.2 mg). Additionally, the fraction eluted with 30% EtOAc−hexane afforded compound **5** (25 mg), which was purified by recrystallization from hexane−CH_2Cl_2.

(*R*)-2-Hydroxy-2-phenylethyl Benzoate (5). White crystals (ex. hexane−CH_2Cl_2). Mp 74 °C. $[\alpha]_D$ −33° (*c* 0.7, $CHCl_3$). GC−MS: *m*/*z* (rel intens): 224 $[M - 18]^+$ (1), 120 (41), 107 (39), 105 (100), 91 (11), 79 (19), 77 (58), 51 (18). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.58 (1H, d, *J* = 3.0 Hz, OH), 4.44 (1H, dd, *J* = 11.6, 8.2 Hz, H-1a), 4.55 (1H, dd, *J* = 11.6, 3.4 Hz, H-1b), 5.13 (1H, ddd, *J* = 8.2, 3.4, 3.0 Hz, H-2), 7.3−7.5 (7H, m), 7.59 (1H, tt, *J* = 7.5, 1.4 Hz, H-4″), 8.07 (2H, obsc d, *J* = 7.5 Hz, 2H-2″). ^{13}C NMR (75 MHz, $CDCl_3$): δ 69.8 (C-1), 72.6 (C-2), 126.2 (2C-2′,6′), 128.3 (2C-4′,4″), 128.4 and 128.6 (2C-3″,5″ and 2C-3′,5′), 129.7 (2C-2″,6″), 129.8 (C-1″), 139.8 (C-1′), 166.7 (C═O).

Brine Shrimp (*A. salina*) Lethality. Toxicity toward *A. salina* was tested following the method described by Meyer et al. (*4*). Briefly, 10 one-day nauplii were placed in 10 mL of seawater solution containing the sample. Experiments were performed at different concentrations (0, 2.5, 5, 10, 25, and 50 ppm) with three replicates in each case. A solution of lindane (Asuntol, Bayer) was used as the control. Survivors were counted after 24 h. The LC_{50} values (lethal concentration for half of the population) were calculated using Finney's Probit Analysis program (*9*).

***Spodoptera littoralis* Feeding Deterrence.** Feeding deterrence toward *S. littoralis* (fifth-instar larvae) was tested with the disk-choice method described by Bellés et al. (*5*), placing five larvae in a Petri dish containing eight lettuce disks (1 cm^2) placed alternately (control, treated) and leaving the larvae to feed in darkness at 22 °C. The feeding ratio (FR_{50}) was calculated as the ratio consumed (treated disks/control disks) when half of the control disks had been eaten. Extracts were tested at 20 μg/disk, and compounds **1**−**5** were tested at 10 μg/disk. Experiments were performed in triplicate or, if necessary, in quintuplicate to reduce the standard deviation.

Growth Inhibition of *C. herbarum*. Isolates of *C. herbarum* (Pers.) Link were obtained from Dr. J. Guerrero (Universidad de La Frontera, Temuco, Chile). TLC−bioautography bioassays were performed in duplicate as described by Hadacek and Greger (*6*). Compounds **1**−**4** and the extract were dissolved in DMSO at concentrations of about 20 mg/mL as a stock solution. Ketoconazole and Captan (ANASAC, 13.5%) were used as standards at the same concentrations. Test solutions and a DMSO control were applied (10 μL each) as small spots (spacing 4 cm) on TLC plates (Si60, 0.25 mm, Merck). Plates were sprayed with a spore solution of 10^5 cfu and kept in a humid chamber at 23 °C. Observations were made after 3 days (72 h) and 6 days (144 h) of incubation, and the appearance of blank zones (halos) in the mycelium layer indicated antifungal activity. MICs were estimated by diluting the stock solutions until no inhibition halos were observed after 72 h.

RESULTS AND DISCUSSION

Analysis of the results of bioassays (**Table 1**) performed with the dichloromethane extract of *B. cancellata* showed that it was unusually active toward *A. salina* with LC_{50} < 2.5 ppm, similar in activity to a lindane-based commercial acaricide (Asuntol, Bayer). It also showed on bioautography plates strong inhibition of growth of *C. herbarum*, a fungus that attacks senescent grapes. The effect did not decay after a week, implying that the compounds were stable to humidity and temperature. The extract was more effective than either Captan or ketokonazole, standard fungicides tested at the same concentration. Antifeedant activity toward *S. littoralis* larvae at 20 μg/cm^2 (ca. 50%), although moderate, was also consistent with the presence of bioactive compounds in the extract.

Table 1. Results of Bioassays with *A. salina* (Toxicity), *S. littoralis* (Feeding Deterrence) and *C. herbarum* (Growth Inhibition Halos)

compound or extract	*A. salina* LC_{50}[a] (ppm)	*S. littoralis* FR_{50}[b]	*C. herbarum* MIC[c] (mg/spot)
CH_2Cl_2 extract	<2.5	0.42 ± 0.12	0.01
1	<2.5	0.56 ± 0.08	0.1
2	<2.5	0.50 ± 0.05	nd[d]
3	<2.5	0.96 ± 0.17	nd
4	<2.5	0.44 ± 0.06	0.006
5	7.4 (9.4–6.3)	0.73 ± 0.04	0.05
ketoconazole	na[e]	na	0.01
Captan	na	na	0.02
lindane	7.2 (9.7–4.9)	na	na

[a] LC_{50} = lethal concentration for half of the nauplii after 24 h (confidence interval in parentheses). [b] FR_{50} = feeding ratio in disk-choice assay = area consumed of treated disks per area consumed of the control disk using a dose of 10 μg/disk (except for the extract, where the dose was 20 μg/disk); values are presented as means (n = 3) ± SD. [c] MIC for no halo formation determined by dilution experiments with bioautography on TLC plates; halos were measured after 72 h at 23 °C. [d] nd = not determined; no inhibition was observed with 0.003 mg/spot, and the material available was insufficient for further experiments. [e] na = not applicable.

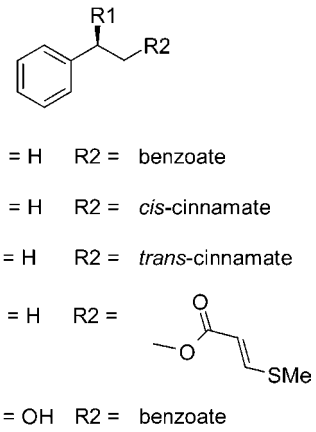

Figure 1. Compounds isolated from a dichloromethane extract of the liverwort *B. cancellata*.

Low-pressure column chromatography on the extract and preparative TLC of the fraction eluted with 10−15% ethyl acetate in hexane led to the isolation of compounds **1**−**4**. Compound **5** precipitated from the fraction eluted with 30% ethyl acetate in hexane (**Figure 1**). Comparison of GC−MS profiles and ^{1}H NMR spectra of the extract and pure compounds indicated that the main compounds were **1** and **4** (both with R_t = 19.2 min and 62% area) in almost equal amounts, as deduced from relative integrals in the ^{1}H NMR spectra of the extract and relative areas in the GC−MS profile. **5** (R_t = 22.7 min, 20% area), **2** (R_t = 23.1 min, 3% area), and **3** (R_t = 25.3 min, 9% area) were present in smaller amounts.

Comparison of the ^{1}H NMR spectra of the pure compounds **1**−**4** and fragmentation pattern of MS spectra easily led to the assignment of structures. Thus, all spectra showed signals

consistent with the presence of a 2-phenylethoxyl group, esterified with benzoic acid (**1**), with cinnamic acid (**2** and **3**, *cis* and *trans*, respectively), and with *trans*-β-methylthioacrylic acid (**4**) (*10*). MS spectra of **1**−**4** had base peaks at $m/z = 104$ or 105, in good agreement with the proposed structures. Compounds **1**−**4** have been isolated previously from *B. rosea* (*10*) and *Isotachis japonica* (*11*). Compounds **1** and **3** were also reported to occur in *B. erinacea* along with benzyl benzoate, the last being the main component of *B. cancellata* (*12*).

The ^{1}H NMR spectrum of **5** only differed from that of **1** in the loss of one and deshielding of the other proton at the benzylic position, consistent with the presence of a hydroxyl group at this position. GC−MS analysis and comparison of ^{1}H NMR data with those reported for the synthetic benzoate (*13*) were in good agreement with structure **5**. A negative optical rotation suggested that it is the *R*-isomer, in contrast to the positive rotation displayed by the (*S*)-benzoate. This seems to be the first report of the isolation of **5** from natural sources.

Comparison of the results of bioassays performed with the pure compounds (**Table 1**) shows that all are very toxic toward *A. salina*, at the same level ($LC_{50} < 2.5$ ppm) as displayed by the extract. On the other hand, **1**, **2**, and **4** inhibited feeding of *S. littoralis* moderately (ca. 50% deterrence) at 10 μg/cm^2, while **3** and **5** were almost inactive. The most striking results were obtained with TLC−bioautography, where isotachin B (**4**) produced a huge inhibition of *C. herbarum* growth and was active even at a concentration of 0.006 mg/spot, which is lower than that required with either pure ketoconazole or commercial Captan as observed in dilution experiments. The benzoate esters **1** and **5** were also slightly active in this bioassay at concentrations higher than 0.1 mg/spot.

The difference between *cis*- and *trans*-isomers **2** and **3** in antifeedant activity is significant and indicates the relative importance of the spatial arrangement of the molecule in the activity displayed. The small differences in activity observed between **1** and **5** in the three bioassays could only be attributed to the extra OH in the structure of **5**, which seems slightly to increase the antifungal activity.

The results of bioassays described in this paper would indicate that the high natural concentrations of **1** and **4** in *B. cancellata* (ca. 0.5% dry weight) would protect the plant against fungal attack and predation, **4** being mainly responsible for the activity displayed by the extract. A further collection of *B. cancellata* at a different location and season (see the Materials and Methods) yielded only compounds **1** and **4**, in higher yields (0.86% dry weight). Further work is required to investigate whether environmental conditions influence the relative amounts of aromatic esters produced in this species. However, *B. cancellata* has been treated under a broad species concept and displays a considerable range of morphological variation in common with other *Balantiopsis* species (*14*); it is possible that the chemical variability observed is simply a consequence of the presence of a diverse gene pool within the species.

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LITERATURE CITED

(1) Asakawa, Y. Chemosystematics of the Hepaticae. *Phytochemistry* **2004**, *65*, 623−669.

(2) Asakawa, Y. Terpenoids and aromatic compounds with pharmacological activity from bryophytes. In *Bryophytes: Their Chemistry and Chemical Taxonomy*; Zinsmeister, H. D., Mues, R., Eds.; Clarendon Press: Oxford, U.K., 1990; pp 369−410.

(3) Labbé, C.; Coll, J.; Connolly, J. D.; Faini, F.; Farrugia, L. J.; Rycroft, D. S. Bioactive flavones from *Luma chequen*. *Collect. Czech. Chem. Commun.* **2002**, *67*, 115−123.

(4) Meyer, B. N.; Ferrigni, N. R.; Putnam, J. E.; Jacobsen, L. B.; Nichols, D. E.; McLaughlin, J. L. Brine shrimp: a convenient general bioassay for active plant constituents. *Planta Med*. **1982**, *45*, 31−34.

(5) Bellés, X.; Camps, F.; Coll, J.; Piulachs, M. D. Insect antifeedant activity of clerodane diterpenoids against larvae of *Spodoptera littoralis* (Boisd.) (Lepidoptera). *J. Chem. Ecol*. **1985**, *11*, 1439−1445.

(6) Hadacek, F.; Greger, H. Testing of antifungal natural products: methodologies, comparability of results and assay choice. *Phytochem. Anal.* **2000**, *11*, 127−147.

(7) Rycroft, D. S. Fingerprinting of plant extracts using NMR spectroscopy: application to small samples of liverworts. *Chem. Commun. (Cambridge)* **1996**, 2187−2188.

(8) Rycroft, D. S.; Cole, W. J.; Rong, S. Highly oxygenated naphthalenes and acetophenones from the liverwort *Adelanthus decipiens* from the British Isles and South America. *Phytochemistry* **1998**, *48*, 1351−1356.

(9) Finney, D. J. *Probit Analysis*; Cambridge University Press: Cambridge, U.K., 1971.

(10) Asakawa, Y.; Takikawa, K.; Tori, M.; Campbell, E. O. Isotachin C and balantiolide, two aromatic compounds from the New Zealand liverwort *Balantiopsis rosea*. *Phytochemistry* **1986**, *25*, 2543−2546.

(11) Asakawa, Y.; Toyota, M.; Harrison, L. J. Isotachin A and isotachin B, two sulphur-containing acrylates from the liverwort *Isotachis japonica*. *Phytochemistry* **1985**, *24*, 1505−1508.

(12) Asakawa, Y.; Inoue, H. Chemical constituents of selected Chilean liverworts. In *Studies on Cryptogams in Southern Chile*; Inoue, H., Ed.; Kenseisha: Tokyo, Japan, 1984; pp 109−115.

(13) Iwasaki, F.; Maki, T.; Onomura, O.; Nakashima, W.; Matsumura, Y. Chemo- and stereoselective monobenzoylation of 1,2-diols catalyzed by organotin compounds. *J. Org. Chem.* **2000**, *65*, 996−1002.

(14) Engel, J. J. A taxonomic monograph of the genus *Balantiopsis* (Hepaticae). *Nova Hedwigia* **1968**, *16*, 83−130, with plates 27-(1)−59(33).

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