Bioactive Polychlorinated Bibenzyls from the Liverwort *Riccardia polyclada*

Cecilia Labbé,*[,†] Francesca Faini,[†] Carolina Villagrán,[‡] Josep Coll,[§] and David S. Rycroft[⊥]

Departamento de Química, Facultad de Ciencias, Universidad de Chile, Casilla 653, Santiago, Chile, Departamento de Biología, Facultad de Ciencias, Universidad de Chile, Casilla 653, Santiago, Chile, Química Orgànica Biològica, IIQAB, CSIC, Jordi Girona 18-26 08034 Barcelona, Spain, and Department of Chemistry, University of Glasgow, Glasgow G12 8QQ, Scotland, U.K.

Received April 26, 2007

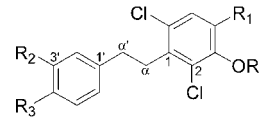
GC-MS and ^{1}H NMR spectroscopic profiling of a $CDCl_3$ extract of the liverwort *Riccardia polyclada* (syn. *R. umbrosa*) revealed the presence of four main compounds bearing several chlorine atoms on a bibenzyl skeleton. Separation of a CH_2Cl_2 extract was achieved using preparative TLC, and structures **1**–**4** were proposed on the basis of spectroscopic evidence. Compounds **1**–**4** were active in a brine shrimp lethality bioassay (*Artemia salina*). In addition, **2** and **4** displayed moderate antifeedant activity in disk-choice bioassays with *Spodoptera littoralis* larvae and inhibited the growth of *Cladosporium herbarum* cultures on TLC plates.

In a search for new pesticides from native plants, we have turned our attention to a little investigated group, the Chilean liverworts. $CDCl_3$ extracts of small samples from voucher specimens of 20 species collected at Chiloé Island were analyzed by GC-MS and ^{1}H NMR spectroscopy according to a profiling method described by Rycroft.[1] Additionally, CH_2Cl_2 extracts were screened for activity toward *Artemia salina* (brine shrimp lethality bioassay[2]), *Spodoptera littoralis* larvae (disk-choice feeding deterrence[3]), and a rot fungus, *Cladosporium herbarum* (TLC-bioautography[4]). Among the species collected, the CH_2Cl_2 extract of *Riccardia polyclada* was one of the most active toward the three organisms tested.

The large and complex genus *Riccardia* (Metzgeriales, Aneuraceae) is well represented in Chile with 45 species recognized in the taxonomic revision for the region,[5] of which most are endemic to southern Chile and Argentina. Among these is *R. umbrosa* (Schiffn. & Gottsche) Hässel, which was determined recently to be synonymous with *R. polyclada* (Mitt.) Hässel.[6] This taxon commonly forms dark green cushions within the native rainforests distributed from Valdivia to Magallanes.[7] Chemical studies of 14 *Riccardia* species from different countries have shown that this genus is characterized by the elaboration of macrocyclic bis-bibenzyl derivatives and sesquiterpenoids.[8] More recently, a study of *R. marginata*[9] from New Zealand led to the isolation of rather unusual, but simple, chlorinated bibenzyls with antibacterial and antifungal activity.

It was evident from the GC-MS profile of the $CDCl_3$ extract of *R. polyclada* that polychlorinated compounds were present, because the MS of the four main components (**1**–**4**, representing almost 90% of the total volatiles of the extract) contained peaks with chlorine isotope patterns. To establish that the chlorinated compounds were not artifacts of an extraction process involving a chlorinated solvent, a CD_3CN extract was prepared similarly.[1] The GC-MS profile of this extract when acetylated also showed four major peaks derived from the same four chlorinated compounds present in the $CDCl_3$ extract (a different instrument was used for this GC-MS profile and acetylation was necessary to avoid adsorption problems).

The ^{1}H NMR spectroscopic profile[1] of the $CDCl_3$ extract of *R. polyclada* was relatively simple. The main resonances were consistent with several nonequivalent aromatic protons, two methoxyl groups (δ 3.8 and 3.9) and two multiplets (δ 3.1 and 2.8) arising from the CH_2-CH_2 bridge in a substituted bibenzyl skeleton. The structural relationship between chlorinated bibenzyls and well-known antiseptics and pesticides led us to isolate and identify these liverwort compounds.



	R	R_1	R_2	R_3
1	H	H	H	OMe
2	H	H	Cl	OMe
3	H	Cl	Cl	OMe
4	H	Cl	Cl	OH
4 Ac	Ac	Cl	Cl	OAc

On TLC (hexane−EtOAc, 5:1) the CH_2Cl_2 extract of the bulk of the plant material showed a major spot under UV light that turned light blue, with two purple spots partially overlapping it, on spraying with $FeCl_3$−HCl reagent and heating. Repeated column chromatography of the extract, using different combinations of solvents, failed to separate the mixture. Preparative TLC of enriched fractions, with multiple developments, led to the isolation of four compounds (**1**–**4**) in small amounts. GC-MS confirmed that these compounds were the same as the four compounds observed in the GC-MS profiles of the $CDCl_3$ and CD_3CN extracts. The coupling constants and chemical shifts of the aromatic protons in the ^{1}H NMR spectra (Table 1) confirmed that **1**–**4** differ in the number or type of substituents attached to both aromatic rings. Additionally, as the main fragment in the EIMS of bibenzyls arises from cleavage of the CH_2-CH_2 bridge, the mass of each resulting benzyl radical provided information about the type and number of substituents present on the phenyl group.

The MS of the compound isolated in largest amount (**1**) showed an isotope pattern of parent ions (*m*/*z* 296/298/300) characteristic of a dichloro derivative. The molecular formula was deduced to be $C_{15}H_{14}O_2Cl_2$ from HRCIMS. The ^{1}H NMR spectrum exhibited, in addition to OMe (δ_H 3.81) and hydroxyl (δ_H 5.59) resonances, overlapping doublets at δ_H 7.2 (3H) and at δ_H 6.9 (3H) arising from six aromatic protons. Selective irradiations at the OMe signal and one of the CH_2 signals (δ_H 2.78) showed NOE effects that identified an AA′XX′ coupling system of four protons in a *p*-methoxy-substituted benzyl moiety (Table 1). This is consistent with the base peak (*m*/*z* 121) in the EIMS and was further corroborated by ^{13}C and HETCOR NMR spectra. Thus, the other benzyl moiety of **1** was deduced to carry the two chlorine atoms and the hydroxy group. Irradiation at δ_H 3.14 (CH_2) did not produce a NOE effect on either of the *ortho*-coupled protons (δ_H 6.87 and 7.21, $J = 8.6$ Hz), suggesting that C-2, C-3, and C-6 are substituted. The relatively high shielding of one of the aromatic protons is consistent with an *ortho*-hydroxy group attached to C-3 or C-6. Placement of the hydroxy group at C-3 was based on the chemical

* To whom correspondence should be addressed. Tel: 56 02 6787336. Fax: 56 02 2713888. E-mail: clabbe@uchile.cl.
[†] Departamento de Química, Universidad de Chile.
[‡] Departamento de Biología, Universidad de Chile.
[§] IIQAB, CSIC, Barcelona.
[⊥] University of Glasgow.

10.1021/np070192c CCC: $37.00
Published on Web 11/20/2007

Table 1. ^{1}H NMR Spectroscopic Data (400 MHz, $CDCl_3$) for Bibenzyls **1**–**4** and **4 Ac**

	1	**2**	**3**	**4**	**4 Ac**
position	δ_H (*J* in Hz)	δ_H (*J* in Hz)	δ_H (*J* in Hz)	δ_H (*J* in Hz)	δ_H (*J* in Hz)
4	6.87, d (8.7)	6.88, d (8.8)			
5	7.21, d (8.7)	7.22, d (8.8)	7.35, s	7.35, s	7.45, s
α	3.14, m	3.13, m	3.12, m	3.11, m	3.17, m
α′	2.78, m	2.76, m	2.74, m	2.73, m	2.79, m
2′	7.19, d (8.6)	7.28, d (2.2)	7.27, d (2.2)	7.21, d (2.1)	7.35, d (2.0)
3′	6.86, d (8.6)				
5′	6.86, d (8.6)	6.87, d (8.4)	6.86, d (8.4)	6.95, d (8.3)	7.07, d (8.2)
6′	7.19, d (8.6)	7.10, dd (8.4, 2.2)	7.09, dd (8.4, 2.2)	7.05, dd (8.3, 2.1)	7.16, dd (8.2, 2.0)
OH	5.59, s	5.59, s	5.88, s	5.88, s; 5.43, s	
OAc					2.41, s; 2.36, s
OMe	3.81, s	3.90, s	3.90, s		

Table 2. Biological Activity of Bibenzyls **1**–**4**

compound	*Artemia salina* (LC_{50} in ppm)[a]	*Spodotera littoralis* (FR_{50})[b]	*Cladosporium herbarum* (diameter of inhibition zone in cm)[c]
1	0.44 (0.54–0.34)	nd[d]	2.2 (0.05 mg)
2	0.42 (0.57–0.06)	0.63 ± 0.07	1.2 (0.07 mg)
3	1.71 (4.05–1.28)	nd[d]	0.0 (0.05 mg)
4	2.39 (3.03–1.98)	0.43 ± 0.08	2.0 (0.07 mg)
ketoconazole	14.9 (20.5–9.8)	nd[d]	1.5 (0.25 mg)
Asuntol	10.8 (16.7–6.8)	nd[d]	nd[d]

[a] Confidence interval in parentheses by probit analysis. [b] FR_{50} = treated area eaten (when 50% of the untreated area had been eaten)/50% untreated area, at a dose of 10 μg/disk. [c] Dose applied per spot in parentheses. [d] nd = not determined.

shifts of the CH_2 (δ_H 3.14, δ_C 34.4), C-2 (δ_C121.1), and C-6 (δ_C 125.9) resonances. These are almost identical to those reported for 2,4,6-trichloro-3-hydroxybibenzyl, where the additional *meta*-chlorine substituent has little effect, as can be seen by comparison of the data for 2,4-dichloro-3-hydroxybibenzyl and 2,4,6-trichloro-3-hydroxybibenzyl.[9] Support for the assignment of the hydroxy group at C-3, with the *ortho* substituents chlorine and hydrogen, was provided by the observation that the ^{1}H NMR chemical shift of the hydroxy proton of **1** is very close to that of 2-chloro-3-hydroxybibenzyl,[9] where the substituents *ortho* to the hydroxy group are also chlorine and hydrogen. Accordingly, compound **1** was assigned as 2,6-dichloro-3-hydroxy-4′-methoxybibenzyl.

Compound **2** gave an isotope pattern of MS parent ions (*m*/*z* 330/332/334/336) indicating the presence of three chlorine atoms. The molecular formula was deduced to be $C_{15}H_{13}O_2Cl_3$ from HRCIMS. The base peak (*m*/*z* 155) of the EIMS and the resonances associated with H-4, H-5, and OH in the ^{1}H NMR spectrum showed that the more substituted benzyl group is identical to that in **1**. Attachment of the extra chlorine atom to C-3′ followed from the coupling pattern of the three-proton spin system and the NOE effects observed on H-2′ and H-6′ when the 2H-α′ resonance was irradiated and on H-5′ when the methoxy protons were irradiated. Compound **2** was therefore assigned as 2,6,3′-trichloro-3-hydroxy-4′-methoxybibenzyl.

The EIMS of compound **3** showed a base peak (*m*/*z* 155) from a chloromethoxybenzyl moiety, assigned the same substitution pattern as in **2** from NOE effects in the ^{1}H NMR spectrum. The molecular formula, deduced to be $C_{15}H_{12}O_2Cl_4$ from HRCIMS, therefore indicated that, compared to **2**, an extra chlorine atom was attached to either C-4 or C-5 of the other benzyl group. The *ortho*-coupled doublets (H-4, H-5) in the ^{1}H NMR spectrum of **2** were replaced by a proton singlet at δ_H 7.35, corresponding to H-5 deshielded by two *ortho*-chlorine atoms, as in 2,4,6-trichloro-3-hydroxybibenzyl, where the chemical shift is almost the same (δ_H 7.33).[9] The chemical shift of the OH proton is sensitive to the change of an *ortho*-substituent from hydrogen to chlorine and, at δ_H 5.88, is more deshielded than in **2**, but almost the same as in 2,4,6-trichloro-3-hydroxybibenzyl (δ_H 5.86).[9] Thus, compound **3** was assigned as 2,4,6,3′-tetrachloro-3-hydroxy-4′-methoxybibenzyl.

The ^{1}H NMR spectrum of compound **4** was almost identical to that of **3** but lacked a OMe resonance. The molecular formula was deduced to be $C_{14}H_{10}O_2Cl_4$ from HRCIMS, and the base peak in the EIMS (*m*/*z* 141) corresponded to a chlorohydroxybenzyl moiety. A resonance at δ_H 7.35 (s, H-5) indicated that the other benzyl moiety is the same as in **3**, leading to structure **4** (2,4,6,3′-tetrachloro-3,4′-dihydroxybibenzyl). GC-MS of the acetylation product (**4 Ac**) confirmed that two hydroxyl groups in **4** were acetylated.

Results of bioassays are in Table 2. Compounds **1**–**4** were active in the brine shrimp lethality bioassay and gave lower LC_{50} values than that for Asuntol (15% coumaphos, 56% hexachlorobenzene, Bayer), a commercial acaricide used as a reference in this bioassay. On TLC-bioautography with *C. herbarum* cultures, compounds **1** and **4** showed growth inhibition zones larger than those obtained with the fungicide ketoconazole; compound **2** was slightly active at the concentration tested, while **3** did not show any activity. Owing to the small amounts available, it was not possible to ascertain MIC values for these compounds. Differences in the concentrations used in this bioassay did not allow firm conclusions on the basis of the results obtained. However, the fungicidal activity of these compounds does not appear to be directly proportional to the number of chlorine atoms in their structures. Compounds **2** and **4** were tested in the *S. littoralis* antifeedant bioassay and displayed moderate activity.

Compounds **1**–**4** are all new. They are closely related to the three chlorinated bibenzyls reported from the New Zealand liverwort, *Riccardia marginata*,[9] but a clear difference between the two groups of compounds is that one of the benzyl groups is never substituted in the New Zealand compounds. The present study on *R. polyclada* confirms that some species of this genus elaborate high concentrations of bioactive polychlorinated bibenzyls that should protect them from pathogenic microorganisms and herbivores. Differences in the fungicidal activity of compounds do not appear to correlate with the number of chlorine atoms in the structure. The phytochemical affinity between *R. marginata* from New Zealand and *R. polyclada* from southern South America provides a potentially useful lead for studies of biogeographical and phylogenetic relationships between liverworts in the southern hemisphere. This topic, exploiting methods for phylogenetic reconstruction based on analysis of differences in DNA sequences, is of much current interest.[10]

Experimental Section

General Experimental Procedures. GC-MS and ^{1}H NMR spectroscopic profiles were obtained as described previously.[11] ^{1}H NMR spectra were measured using a Bruker DPX 400 spectrometer, and ^{13}C NMR spectra using a Varian Unity 300 spectrometer. EIMS data were derived from GC-MS. HRCIMS, using isobutane, was undertaken with a JEOL JMS-700 instrument. A fritted glass filter funnel packed with silica gel H (Merck) and connected to a water pump via a filtration assembly was used for reduced-pressure column chromatography. A Chromatotron model 7924T (Harrison Research, California) was used for circular chromatography. Silica gel 60G (F_{254}, Merck, 30 g per plate, 20 cm × 20 cm) was used for preparative TLC. Analytical grade solvents were used for extraction and chromatography.

Plant Material. *Riccardia polyclada* was collected on the main island of Chiloé in July 2001 and July 2002 alongside a path in forest at Alto Aytuy and was identified by C.V. and Dr. Gabriela Hässel de Menéndez (Argentina). Plants were cleaned, washed, and left to dry at room temperature (17 °C). Voucher specimens (CL-55A) are deposited in the herbarium (SGO) of the Museo Nacional de Historia Natural, Santiago, Chile, and in Glasgow (personal herbarium of D.S.R.).

Extraction and Isolation. Samples of the first (28 mg for $CDCl_3$; 25 mg for CD_3CN) and second collections (46 mg for $CDCl_3$) were checked piece by piece through a hand lens for the absence of contaminating plant material, ground with a glass rod and glass fragments, and then extracted with $CDCl_3$ or CD_3CN (1 mL) at room temperature (0.5 h) to give filtered solutions (0.7 mL) that were submitted directly to ^{1}H NMR spectroscopic and GC-MS analysis.

Plant material from the two collections was combined (55.7 g) and extracted with CH_2Cl_2 in a Soxhlet apparatus (24 h) to yield an extract (2.4 g). This was submitted to reduced-pressure column chromatography on silica gel using mixtures of EtOAc−hexane of increasing polarity as eluent. Fractions eluted with 5–15% EtOAc afforded a complex mixture (1 g), which was treated with hexane in an ultrasonic bath to give an oily fraction (180 mg), leaving a solid residue that was partially soluble in CH_2Cl_2. Both fractions were submitted separately to repeated column and circular chromatography on silica gel using different low-polarity solvent systems (CCl_4 or hexane with EtOAc or CH_2Cl_2) to yield enriched mixtures of compounds **1**–**4**, which were further purified by preparative TLC using CCl_4−CH_2Cl_2 (7:3) with three to five partial developments. In this way, compounds **1** (28 mg), **2** (18 mg), **3** (8 mg), and **4** (10 mg) were obtained almost pure. Acetylation (acetic anhydride−pyridine, 60 °C, 1 h) of a mixture enriched with **4** (20 mg) and preparative TLC afforded the diacetate, **4 Ac** (10 mg).

Bioassays. Experimental conditions were the same as described previously.[12] Feeding deterrence experiments were performed in Barcelona (IIQAB, CSIC) using fifth instar larvae of *Spodoptera littoralis* and concentrations of 10 μg/disk. *Artemia salina* (commercial batch) lethality was determined at concentrations of 0, 0.5, 1.5, 2.5, 5, 10, 25, and 50 ppm in all cases. TLC-bioautography was performed on precoated silica gel glass plates (0.25 mm, Merck) by applying 10 μL of standard solutions of the compounds in DMSO to form spots with 1 cm diameter; the dose applied per spot is shown in Table 2. Plates were sprayed with a spore suspension (5×10^5 cfu) of *Cladosporium herbarum* in malt extract (4%) medium, and inhibition zones were clearly observed after 72 h at 27 °C.

2,6-Dichloro-3-hydroxy-4′-methoxybibenzyl (1): colorless solid (GC-MS TIC showed also **3**, 1%); ^{1}H NMR ($CDCl_3$, 400 MHz) in Table 1; ^{13}C NMR ($CDCl_3$, 75 MHz) δ 158.1 (C, C-4′), 150.4 (C, C-3), 137.6 (C, C-1), 133.3 (C, C-1′), 129.3 (2CH, C-2′ and C-6′), 128.6 (CH, C-5), 125.9 (C, C-6), 121.1 (C, C-2), 114.5 (2CH, C-3′ and C-5′), 113.9 (CH, C-4), 55.3 (CH_3, OMe), 34.4 (CH_2, C-α), 33.3 (CH_2, C-α′); EIMS *m/z* 300 (1), 298 (8), 296 (12), 175 (2), 122 (9), 121 (100), 91 (5), 78 (6), 77 (6); HRCIMS *m/z* 297.0429 $[M + H]^+$ (100) (calcd for $C_{15}H_{15}O_2{}^{35}Cl_2$, 297.0449), 299.0414 $[M + H]^+$ (62) (calcd for $C_{15}H_{15}O_2{}^{35}Cl^{37}Cl$, 299.0420).

2,6,3′-Trichloro-3-hydroxy-4′-methoxybibenzyl (2): colorless solid (GC-MS TIC showed also **1**, 1.7%, and **3**, 2.4%); ^{1}H NMR ($CDCl_3$, 400 MHz) in Table 1; EIMS *m/z* 336 (1), 334 (6), 332 (18), 330 (18), 175 (3), 157 (33), 155 (100), 112 (5), 105 (5), 77 (15); HRCIMS *m/z* 331.0029 $[M + H]^+$ (100) (calcd for $C_{15}H_{14}O_2{}^{35}Cl_3$, 331.0059), 333.0010 $[M + H]^+$ (96) (calcd for $C_{15}H_{14}O_2{}^{35}Cl_2{}^{37}Cl$, 333.0030).

2,4,6,3′-Tetrachloro-3-hydroxy-4′-methoxybibenzyl (3): colorless solid (GC-MS TIC showed also **1**, 2.3%); ^{1}H NMR ($CDCl_3$, 400 MHz) in Table 1; EIMS *m/z* 370 (2), 368 (7.5), 366 (16), 364 (11), 211 (3), 209 (3), 157 (32), 155 (100), 105 (5), 77 (15); HRCIMS *m/z* 364.9657 $[M + H]^+$ (54) (calcd for $C_{15}H_{13}O_2{}^{35}Cl_4$, 364.9670), 366.9653 $[M + H]^+$(79) (calcd for $C_{15}H_{13}O_2{}^{35}Cl_3{}^{37}Cl$, 366.9640).

2,4,6,3′-Tetrachloro-3,4′-dihydroxybibenzyl (4): oil (GC-MS TIC showed also **1**, 14.5%); ^{1}H NMR ($CDCl_3$, 400 MHz) in Table 1; EIMS *m/z* 356 (1); 354 (7); 352 (14); 350 (11); 211 (5); 209 (5); 177 (5); 175 (8); 143 (33); 141 (100); 109 (4); 77 (12); 51 (7); HRCIMS *m/z* 350.9498 $[M + H]^+$ (69) (calcd for $C_{14}H_{11}O_2{}^{35}Cl_4$, 350.9513), 352.9477 $[M + H]^+$ (84) (calcd for $C_{14}H_{11}O_2{}^{35}Cl_3{}^{37}Cl$, 352.9484).

Diacetate of compound 4 (4 Ac): oil; ^{1}H NMR ($CDCl_3$, 400 MHz) in Table 1; EIMS *m/z* 436 (1), 434 (1), 398 (5), 396 (20), 394 (40), 392 (30), 356 (6), 354 (26), 352 (50), 350 (39), 316 (5), 314 (5), 211 (7), 209 (7), 177 (11), 175 (16), 143 (33), 141 (100), 77 (8), 43 (47).

Acknowledgment. This research was supported by a grant from the Chemistry Department of the Facultad de Ciencias, Universidad de Chile, and by a Higher Education Link (SAN/984/117) from the British Council in Santiago. Financial support of CYTED (Spain) and Xarxa de Productes Naturals (Barcelona, Spain) is also acknowledged. We thank Mr. J. Tweedie and Dr. R. A. Anderson for GC-MS measurements, Dr. J. Guerrero for providing a cultivar of *C. herbarum*, and Dr. E. Barrera for her assistance and access to the herbarium of the National Museum of Natural History in Santiago.

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NP070192C