BIOACTIVE FLAVANONES FROM *Luma chequen*

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Dedicated to the memory of Dr Václav Černý.

A bioassay-guided chemical study of a methanolic extract of fresh leaves of *Luma chequen* led to the isolation of lumaflavanones A (**1**), B (**2**) and C (**3**) whose structures are proposed on the basis of NMR spectroscopic data. The structure of lumaflavanone A was confirmed by X-ray analysis. Antifeedant (*Spodoptera littoralis*), brine shrimp (*Artemia salina*) and fungistatic (*Botrytis cinerea*) bioassays showed that while **3** was the most active in the first two assays the mixture of **1** and **2** was more effective as a fungistatic.

Keywords: Natural products; Flavanoids; NMR spectroscopy; Myrtaceae; Antifungal action; Antifeedant; Isolation and identification; X-ray diffraction.

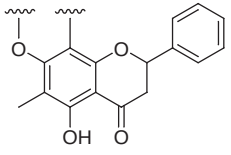
In the search for new biopesticides for a rational and integrated management of pest control, field observation of plants with natural resistance towards predation can provide a good lead. Extracts of these species may then be submitted to bioassays with test organisms such as unspecialised larvae or phytopathogenic fungi to guide a chemical study leading to the isolation of the bioactive compounds. *Luma chequen* (Myrtaceae) is a native aromatic evergreen shrub, frequently found in humid places of Central Chile[1] and in gardens as an ornamental plant, where it does not suffer from predation. The small coriaceous leaves are used in traditional medicine[1] in the treatment of gout, rheumatism, cough and diarrhoea. Earlier studies on extracts of this species reported that it did not inhibit xanthine oxidase[2], a bioassay that correlates with anti-inflammatory activity.

As part of a screening programme of the Chilean flora searching for new sources of bioactive compounds, we submitted the methanolic extract of

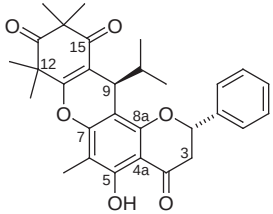
dried leaves of *L. chequen* to brine shrimp toxicity (*Artemia salina*) and feeding deterrence (disk-choice, 5th instar *Spodoptera littoralis* larvae at a dose of 20 μg/cm^2) bioassays, finding activity in both cases. A bioassay-directed chemical study of the extract afforded three new flavanone derivatives whose structures are proposed here on the basis of spectroscopic data. In order to explore the specificity of action of these compounds we tested their fungistatic activity towards *Botrytis cinerea*, a pathogenic fungus of various important cultivars in Chile.

RESULTS AND DISCUSSION

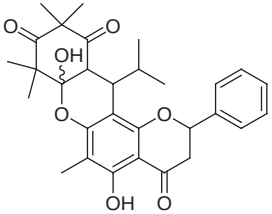
The dried methanolic extract was sequentially dissolved in hexane and dichloromethane to give extracts A and B, respectively and a polar residue (extract C). The results of bioassays (Table I) led us to combine A and B and submit them to column chromatography, to give five fractions (F1–F5) of increasing polarity. Further column chromatography of the most active fractions (F1 and F2) afforded two crystalline compounds. Their ^{1}H and ^{13}C NMR spectra indicated that both solids were complex mixtures of several closely related flavanone derivatives. Separation of the main compounds in each case could only be achieved after multiple preparative TLC and careful recrystallisation of enriched mixtures. In this way crystals of lumaflavanones A (**1**), B (**2**) and C (**3**) were obtained.



(**a**)



1 (racemic)



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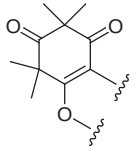
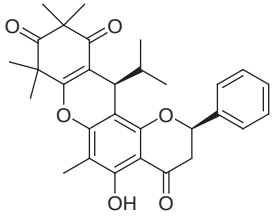


(**b**)



2 (racemic)

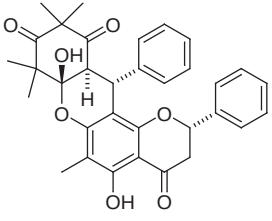


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TABLE I
Bioassay results

	A. salina (LC_{50}, ppm)	*S. littoralis* (FR_{50})	*B. cinerea*
Extract (MeOH)	87 (148–65)	0.52±0.07	–
A (hexane)	53(134–33)	0.26±0.06	
B (CH_2Cl_2)	25(34–19)	0.43±0.11	
C (MeOH)	60(153–38)	0.55±0.13	
F-1 (20% EtOAc in hexane)		0.30±0.11	
F-2 (40% EtOAc in hexane)		0.26±0.07	
F-3 (60% EtOAc in hexane)		0.60±0.18	
F-4 (80% EtOAc in hexane)		0.65±0.17	
F-5 (EtOAc)		0.42±0.05	
1 + **2**	>100	0.68±0.11	+
3	1.97(2.74–0.79)	0.11±0.02	–

TABLE II
^{1}H NMR chemical shifts (in ppm) of lumaflavanones A, B and C

H	**1**	**2**	**3**
2	5.43 dd 3,13	5.37 dd 3, 13	5.41 dd 3, 13
3a	3.09 dd 13, 17	3.10 dd 13, 17	3.06 dd 13, 17
3b	2.90 dd 3, 17	2.87 dd 17, 3	2.88 dd 3, 17
9	4.28 d 4	4.23 d 3	4.08 dd 4, 6
10			3.67 d 6
16	1.47	1.47 s	1.46
17	1.41	1.40 s	1.41
18	1.62	1.63 s	1.60
19	1.37	1.34 s	1.30
6-Me	2.18	2.18 s	2.05
i-Pr	1.83 m; 0.69 d (7); 0.75 d (7)	1.93 m; 0.85 d (7); 0.75 d (7)	2.53 m; 0.84 d (7); 0.62 d (7)
2′,6′	7.45	7.45 (5 H, m)	7.47
C-5–OH	12.17	12.21	12.16

The ^{1}H NMR spectrum of **1** (Table II) showed signals for five tertiary methyl groups, an isopropyl substituent coupled to a deshielded doublet, a phenyl residue, an ABX system and a deshielded hydroxyl group consistent with a 5-hydroxyl in a flavanone system. These features suggested the presence of a flavanoid moiety with a fully substituted ring A. The ^{13}C NMR spectrum (Table III) showed resonances for three carbonyl groups, two of them conjugated, a fully substituted aromatic ring, two quaternary aliphatic carbons and a tetrasubstituted double bond. Long-range proton–

TABLE III
^{13}C NMR chemical shifts (in ppm) of lumaflavanones A, B and C

C	1	2	3
2	79.2	79.5	78.9
3	43.7	44.2	43.8
4	197.0	197.1	196.8
4a	103.3	103.9	104.3
5	159.5	159.4	159.4
6	105.8	105.6	106.7
7	156.8	156.7	156.9
8	105.3	105.2	107.7
8a	157.2	157.1	157.1
9	34.7	35.1	29.4
10	112.3	112.1	45.2
11	167.9	167.9	99.0
12	47.5	47.5	54.5
13	212.0	212.1	214.1
14	56.1	56.1	58.0
15	197.5	197.6	205.3
16	24.6	24.8	24.2
17	18.5	18.9	16.2
18	25.0	25.0	27.2
19	24.4	24.2	21.7
1′	138.1	138.3	138.6
2′,6′	125.9	125.9	125.5
4′	128.8	128.8	128.6
3′,5′	128.9	128.9	128.8
6-Me	7.2	7.2	7.1
i-Pr	32.1; 19.4; 25.3	32.2; 19.5; 25.3	31.1; 18.1; 20.1

carbon chemical shift correlations in a 2D NMR spectrum suggested two part structures, (**a**) derived from 5-hydroxy-6-methylflavanone and (**b**) from syncarpic acid, together with an isopropyl group. Assembly of these structural units led to structure **1** for lumaflavanone A. Confirmation was obtained by an X-ray structure determination which revealed the structure as **1** with the phenyl and isopropyl substituents *trans*. The space group of the crystal lattice, *P*-1, showed that the compound was racemic, suggesting that it had arisen by non-enzymatic cyclisation of a chalcone precursor. Except for small differences in the isopropyl signals, lumaflavone B (**2**) has virtually identical spectroscopic properties to those of lumaflavone A suggesting that the two compounds are epimers. Thus lumaflavanone B is the corresponding *cis* isomer (**2**) and may reasonably be assumed to be racemic, too.

In the ^{13}C NMR spectrum of lumaflavanone C (**3**) a new singlet resonance at 99.0 ppm (characteristic of a hemiacetal), a methine signal at 45.2 ppm, and the observed deshielding of the carbonyl and C-12 resonances suggested that structure **3** was formally related to structures **1** or **2** by hydration of the double bond of the unsaturated ketone. The ^{1}H NMR spectrum of **3** only differed from those of **1** and **2** in the presence of a new doublet at δ_H 3.67 (J = 6 Hz) coupled to H-9, which was shielded as expected on hydration of the double bond. The stereochemistry of the chiral centres of **3** could not be determined.

The lumaflavanones represent further examples of natural products containing a modified acylphloroglucinol moiety. This particular type of cyclic triketone occurs commonly in *Eucalyptus* and other members of the Myrtaceae[3]. Recently a group of related flavanones, leucadenones A–D [*e.g.* A (**4**)], has been reported[4] from *Melaleuca leucadendron*. Although the report[4] implies that the leucadenones are homochiral, the space group of **4** reported in the deposited data (Cambridge Structural Database, Version 5.22) is *P*-1, the same as that of **1**, so that **4** should be racemic. The reported space group of the leucadenone B structure is *Cc*, a space group that has an internal mirror (glide) plane, and so this compound is also racemic.

The results of the bioassays (Table I) indicate that the natural resistance of *L. chequen* can be attributed in part to the compounds isolated in this study. At natural concentrations, higher than the inhibitory concentration, they should deter herbivory and fungal attack.

EXPERIMENTAL

The plant material was collected at Cuesta La Dormida (V Region, Chile) in spring (September 1996) and identified as *Luma chequen* (Mol.) A. Gray by Dr S. Teillier. A voucher speci-

men (M96-54) is kept at the Natural Products Herbarium, Facultad de Ciencias, Universidad de Chile. NMR spectra (including DEPT and two-dimensional direct and long-range proton–carbon chemical shift correlation experiments) were recorded in $CDCl_3$ at *ca* 25 °C using a Bruker AM 360 instrument (^1H at 360 MHz and ^{13}C at 90 MHz). Chemical shifts are reported in ppm relative to $CHCl_3$ at δ_H 7.25 and $CDCl_3$ at δ_C 77.0. Coupling constants (*J*) are given in Hz. Mass spectra (EI) were measured at 70 eV.

Isolation of Compounds

Fresh leaves (1 kg) were extracted with MeOH (3 l, room temperature, 2 h) and the residue (98 g) obtained after evaporation of solvent under reduced pressure was submitted to sonication (30 min, 500 ml × 2) using hexane, dichloromethane and finally methanol. Evaporation of the solvent as above, afforded extracts A (29 g), B (26 g) and C (43 g). A and B were submitted to reduced pressure column chromatography in silica gel (G-60 MERCK) using 100 ml aliquots of hexane–EtOAc mixtures of increasing polarity to give five fractions: F1 (20% EtOAc), F2 (40%), F3 (60%), F4 (80%), F5 (100% EtOAc). From fractions F1 and F2, two crystalline solids (560 and 150 mg) precipitated. These were further purified by multiple preparative TLC (5% EtOAc in hexane) and fractional crystallization (MeOH– CH_2Cl_2–hexane) of enriched mixtures, to give pure **1** (15 mg), **2** (10 mg) and **3** (9 mg), respectively. Recrystallization of **1** (MeOH) afforded crystals suitable for X-ray analysis.

Compound **1**: colourless crystals from $CHCl_3$–MeOH, m.p. 214–215 °C.

Compound **2**: colourless crystals from $CHCl_3$–MeOH, m.p. 208–209 °C. EI MS, *m/z* (%): (mixture of **1** and **2**) $C_{30}H_{32}O_6$: 487 (0.1), 447 (5.5), 446 (29.4), 445 (M – i-Pr) (100), 375 (M – i-Pr – 70) (13.7), 341 (30.4), 271 (31.4), 243 (11.4), 215 (5.3), 91 (4.8), 43 (6.2), 41 (7.4).

Compound **3**: colourless crystals from $CHCl_3$–hexane, m.p. 184–185 °C. EI MS, *m/z* (%): $C_{30}H_{34}O_7$: 506 (0.7), 463 (8.2), 447 (0.2), 446 (1.4), 445 (4.8), 324 (8.8), 271 (16.2), 270 (74.3), 269 (30.8), 193 (73.7), 166 (100), 138 (78.6), 123 (50.4), 70 (79.3), 41 (89.1).

Bioassays (Table I)

Toxicity towards *A. salina* (brine shrimp) was tested following the method described by Meyer *et al.*[5]. Briefly, ten one-day nauplii were placed in 10 ml of sea-water solution containing the sample. Experiments were performed at different concentrations (1, 5, 10, 25, 50, 100 and 200 ppm) and a blank, with three replicates in each case. 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[6].

Feeding deterrency towards *S. littoralis* (5th instar larvae) was tested with the disk-choice method described by Belles *et al.*[7], placing 5 larvae in a Petri dish containing 8 lettuce disks (1 cm^2) placed alternately (control, treated) and leaving them to feed at 22 °C in darkness. The feeding ratio (FR_{50}) was calculated as the ratio consumed (treated disks/control disks) when half of the control disks had been eaten. Extract and fractions were tested at a dose of 20 μg/disk and compounds were tested at 10 μg/disk. Experiments were performed in triplicate and, when standard deviations were too high, two more replicates were used.

Fungistatic activity towards *Botrytis cinerea* (400 μl of spores, 8.5×10^8 cells/ml) was tested by the agar diffusion method (Petri dishes with potato-dextrose agar acidified with lactic acid) using sensidiscs (1 cm diameter) impregnated with 90 μl of a concentrated methanolic solution (2 mg/sensidisc). After 96 h at 23 °C, observation of growth inhibition areas around

the sensidiscs was taken as a positive result. Experiments were done in triplicate using sensidiscs with methanol and ketoconazole (1%) as controls.

Experimental Details of X-Ray Crystallographic Structure Determination

Details of the data collection procedures and structure are given in Table IV. A single crystal of suitable size was attached to a glass fibre using acrylic resin, and mounted on a goniometer head in a general position. Data were collected at ambient temperature in bisecting mode on an Enraf–Nonius TurboCAD4 diffractometer, running under CAD4-Express software, and using graphite-monochromated X-radiation (λ = 0.71073 Å). Precise unit cell dimensions were determined by refinement of the setting angles of 25 high-angle reflections that were flagged during data collection. Standard reflections were measured every 2 h during data collection, and a small variation in intensities was corrected by linear interpolation. No absorption correction was deemed necessary. The structure was solved by direct methods (SIR97)[8]. All non-H atoms were allowed anisotropic thermal motion. Aliphatic and aromatic C–H hydrogen atoms were included at calculated positions, with C–H = 0.96 Å, and were refined with a riding model and with U_{iso} set to 1.2 or 1.5 times that of the attached C-atom. Refinement (SHELXL97-2)[9] was by full-matrix least-squares on F^2, using all the unique data and the weighting scheme $w = [\sigma^2(F_o)^2 + (AP)^2 + BP]^{-1}$, where $P = [F_o^2/3 + 2F_c^2/3]$ and A = 0.0663, B = 0.3149. $\sigma(F_o)^2$ was estimated from counting statistics. Neutral atom scattering factors, coefficients of anomalous dispersion and absorption coefficients were obtained from ref.[10]. Calculations using PLATON (ref.[11]) indicated that there were no voids in the lattice capable of containing any solvent molecules. Thermal ellipsoid plots were obtained using the program ORTEP-3 for Windows[12] (Fig. 1). All calculations were carried out using the WinGX package[13] of crystallographic programs.

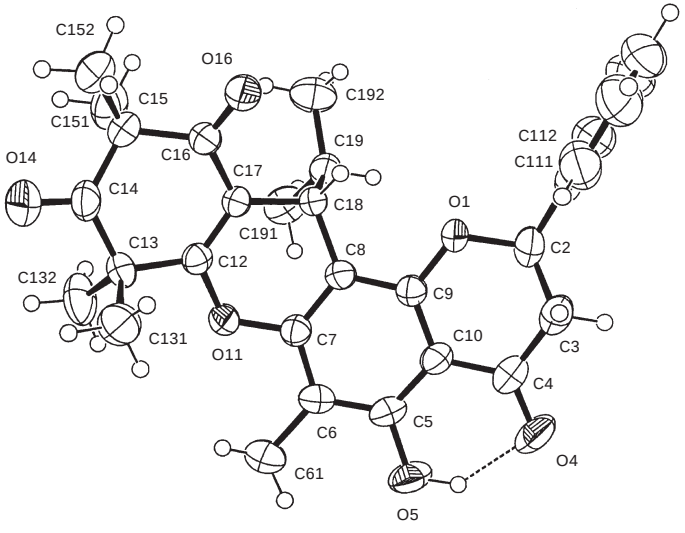

FIG. 1
Ortep diagram of lumaflavanone A (**1**)

TABLE IV
Experimental details of the crystallographic study of compound **1**

Compound formula	$C_{30}H_{32}O_6$
Compound colour	colourless
M_r	488.56
Space group	P-1
Crystal system	triclinic
a, Å	10.6523(7)
b, Å	11.0261(7)
c, Å	12.8962(12)
α, °	109.223(6)
β, °	113.018(6)
γ, °	92.863(5)
V, Å^{-3}	1 288.25(17)
θ range for cell	17.5–20.0
Z	2
D_{calc}, g cm^{-3}	1.259
F(000)	520
μ(MoKα), cm^{-1}	0.087
Scan mode	ω/2θ
θ range, °	2.6–25.0
Crystal size, mm	0.50 × 0.23 × 0.06
No. of data collected	5 236
No. of unique data	4 539
Range of *hkl*	–12→12; –13→12; –2→15
R_{int}	0.0263
No. of data in refinement	4 539
No. of refined parameters	320
Final $R[I>2\sigma(I)]$ (all data)	0.0515(0.113)
R_w^2 $[I>2\sigma(I)]$ (all data)	0.1239(0.1511)
Goodness of fit *S*	1.022
Largest remaining feature in electron density map, e Å^{-3}	0.35(max)–0.21(min)
Shift, esd in last cycle (max.)	0.001

$R = \Sigma(|F_o| - |F_c|)/\Sigma(F_o)$ $wR2 = \{\Sigma(w(F_o^2 - F_c^2)^2)/\Sigma(w(F_o^2)^2)\}^{1/2}$, $R_{int} = \Sigma|F_o^2 - F_o^2(\text{mean})|/\Sigma F_o^2$ (summation is carried out only where more than one symmetry equivalent is averaged).

Crystallographic data for the structure **1** reported in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication number CCDC-171599. Copies of the data can be obtained free of charge on application to CCDC, e-mail: deposit@ccdc.cam.ac.uk. Tables of observed and calculated structure factors are also available from L. J. Farrugia on request.

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