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Reversal of P-Glycoprotein-Mediated Multidrug Resistance by Terpenoids from *Maytenus* Species

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The phytochemical analysis of the root bark extracts of the Chilean *Maytenus*, *M. chubutensis* and *M. magellanica* (Celastraceae) led to the isolation of one phenolic nor-triterpene (**1**) and one diterpene with a nor-*ent*-kaurene skeleton (**2**). In addition, four known compounds were isolated, among which compound **3** has been isolated for the first time from a natural source. Their structures were elucidated by spectroscopic methods, including 1D and 2D (COSY, ROESY, HSQC and HMBC) experiments, comparison with data reported in the literature and chemical correlations. The isolated compounds were assayed for their reversal activity against a multidrug-resistant *Leishmania tropica* line. Compound **1** showed moderate activity with a growth inhibition of 82% at 30 μM.

Sudanese medicine used for the treatment of tumors, dysentery and snake bites, whereas, *M. ilicifolia* has been used for anticancer and contraception **Introduction.** - Medicinal plants play an important role in public health, especially in developing countries. In fact, more than a quarter of all the medicines used in the world today contain ingredients derived from plants [1]. Drug resistance has emerged in the last few years as one of the major impediments for the successful treatment of cancer and diseases caused by protozoan parasites like *Leishmania* spp. One of the most important drug resistance mechanisms is the overexpression of the ABC efflux pump P-glycoprotein (Pgp) and other related drug transporters. Therefore, Pgp-related proteins are promising targets for anti-infectious therapy [2].

Maytenus species (Celastraceae family) are used in traditional medicine around the world. The stem bark of *M. senegalensis* is a traditional as a medicinal folk plant in South America. In the sub-Andean rain forest, a plant known as “chuchuhuasha” (a plant confirmed as belonging to the genus *Maytenus*) is used for the treatment of rheumatism, asthma, fertility-regulating agent, to treat stomach problems and skin cancer [3].

Maytenus chubutensis (chaurilla) and *M. magellanica* (maitén negro) are found in the Antarctica Andean woodland covering parts of Argentina and Chile. We have reported previously on the roots of both species, the isolation of dihydro-β-agarofuran sesquiterpenes [4], potent and specific modulators of *Leishmania* Pgp-like transporter [5], and triterpene dimers [6].

This paper reports the isolation of one phenolic nor-triterpene (**1**) and one nor-*ent*-kaurene diterpene (**2**), along with four known compounds from the root bark extracts of *M. chubutensis* and *M. magellanica*. Their structures were elucidated by comprehensive NMR analyses and spectrometric methods. The known compounds were identified by comparison with authentic samples or reported data. In our program for developing bioactive natural products from the Celastraceae as reversal agents of the multidrug resistance (MDR) phenotype, we have carried out the biological evaluation of the isolated compounds as potential modulators of the MDR in a *Leishmania tropica* line overexpressing a Pgp-like transporter.

Results and Discussion. - Repeated chromatography of the hexanes/Et_2O (1:1) extracts of the root bark of *M. chubutensis* and *M. magellanica* afforded the new compounds **1** and **2**, in addition to the known triterpenes 28-methyl ester of the acid 3,4-secolupa-4(23):20(29)-dien-3-oic (**3**), obtained for the first time as a natural product [7], 6-oxo-pristimerol (**4**) [8], pristimerin (**5**) [9] and the diterpene (-)-pimara-9(11), 15-dien-19-ol (**6**) [10].

3-*O*-Methyl-6-oxo-pristimerol (**1**), obtained as a pale yellow lacquer, was shown to have the molecular formula $C_{31}H_{42}O_5$ by analysis of its HREIMS and ^{13}C NMR data. The IR spectrum exhibited absorption bands at 3518 cm^{-1} (hydroxyl group), 1727 cm^{-1} (ester carbonyl), 1642 cm^{-1} (α,β-unsaturated carbonyl) and 1571 and 756 cm^{-1} (aromatic ring). This was confirmed by its UV spectrum, showing absorption maxima at 239 and 303

nm, characteristic of an aromatic ring and a conjugated ketone, respectively. The ^{1}H NMR spectrum of **1** (*Table 1*), showed signals for six methyls at δ(H) 2.66, 1.52, 1.29, 1.17, 1.09 and 0.57, attributed to H_3-23, H_3-25, H_3-26, H_3-30, H_3-28 and H_3-27, respectively. The phenolic moiety was characterized by two singlet protons at δ(H) 6.24 and 6.95, which were assigned to a proton in α position to a conjugated ketone and an aromatic proton, respectively. The unusual downfield chemical shift at δ(H) 2.66, assigned to the methyl group attached to a *sp*^{2} carbon (H_3-23), is due to anisotropic effects produced by its periplanar position with the carbonyl group located on C-6. Further oxygenated functional groups were attributed to two methoxyl groups at δ(H) 3.53 and 3.79 and one phenolic proton at δ(H) 6.08 (interchangeable with D_2O). The ^{13}C NMR and DEPT experiments (*Table 1*) showed signals for 31 carbons. The most significant being those at δ(C) 187.2 and 178.8, attributed to C-6 and C-29, respectively, and signals for two *sp*^{3} carbons belonging to methoxyl groups (δ 61.1 and 51.5). These data suggested that **1** was a phenolic nor-triterpene of pristimerin-type.

The full assignments and connectivities were based on COSY, HSQC, HMBC and ROESY experiments and comparison with published data for 6-oxo-pristimerol [8]. The long-range correlation observed in the HMBC spectrum (*Figure*) from the singlet at δ(H) 3.79 (3H) to the aromatic carbon at δ(C) 132.9 (C-3) confirmed the attachment of the methoxyl group at C-3. The ester group was located at C-29 by correlations of the methyl group at δ(H) 1.17 (C-30) and the methoxyl group at δ(H) 3.53 with the carbonyl carbon of the ester group at δ(C) 178.8 (C-29). Furthermore, this

was confirmed by the ROE effects observed between the signals at δ(H) 3.79 (3H, *s,* OMe) and δ(H) 2.66 (3H, *s*, H_3-23) and between the signals at δ(H) 3.53 (3H, *s*, OMe) and δ(H) 0.57 (3H, *s*, H_3-27) in a ROESY experiment. All these data allowed the unequivocal assignment of the structure of **1** as 3-*O*-methyl-6-oxo-pristimerol, which was further confirmed by chemical correlations with 6-oxo-pristimerol (**4**) [8]. Thus, when compounds **1** and **4** were methylated with dimethyl sulphate in the usual way, they afforded the same compound, 2,3-*O*-dimethyl-6-oxopristimerol [11].

Compound **2** was isolated as a colorless lacquer with the molecular formula $C_{19}H_{28}O_2$ determined by HREIMS and ^{13}C NMR analysis. The presence of carboxylic acid functionality was evident from IR absorptions (370-2700 and 1703 cm^{-1}). The ^{13}C NMR spectrum exhibited 19 carbons, attributable to one methyl, ten methylenes, four methines and four quaternary carbons, indicating a nor-diterpene structure. The most significant were three down-field signals at δ(C) 103.1 (*t*), 155.6 (*s*) and 182.0 (*s*) attributed to two carbons of an exocyclic double bond and one carboxylic acid, respectively. Comparison with related kauranoid diterpenes suggested that **2** possess an *ent*-kaurane-type skeleton with a carboxylic acid located at C-17 [12]. The ^{1}H NMR spectrum revealed the presence of a tertiary methyl at δ(H) 0.97 (3H, *s*, Me-20) and a methylene group at δ(H) 4.75 and 4.80 (each 1H, *br s*, 2H-19). The full assignments were made by careful analysis of 1D and 2D spectra. The connectivity of the units and the position of the groups were determined from an HMBC experiment (*Figure*). Thus, the long-range correlation observed between the proton

signals at δ(H) 2.33 (H-13), 2.65 (H-16) and 1.44, 1.57 (H-15) and the carbon signal at δ(C) 182.0 (C-17) supported the location of the carboxylic acid group at C-17. Moreover, cross-peak between the proton signals at δ(H) 4.75 and 4.80 (H_2-19) and the carbon signals at δ(C) 155.6 (C-4), 49.1 (C-3) and 48.7 (C-5) located the double bond between C-4 and C-18. The signal at δ(H) 0.97 (H_3-20) was correlated with the carbons at δ(C) 38.8 (C-1), 48.7 (C-5), 53.5 (C-9) and δ(C) 38.1 (C-10). All these data established the structure of compound **2** as 18-nor-*ent*-kaur-4(19)-en-17-oic acid.

The known seco lupane triterpene 28-methyl ester of the acid 3,4-secolupa-4(23):20(29)-dien-3-oic (**3**) was isolated from a natural source for the first time, and its ^{1}H and ^{13}C NMR (*Table 1*) assignments, not previously reported, were achieved by 1D and 2D NMR techniques.

Biological Activity. In the search for reversal agents of MDR phenotype from Celastraceae, the isolated compounds **1**, **2**, **4** and **6** were assayed against a MDR *Leishmania tropica* overexpressing a Pgp-like transporter, in order to determine their ability to revert the resistance phenotype. The results showed that only compound **1** had a moderate activity with a growth inhibition of 82% at 30 μM (*Table 2*).

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Experimental part

General. Column chromatography (CC): Silica gel 60 (SiO_2; 15-40 and 63-200 μm, Macherey-Nagel), TLC: Polygram Sil G/UV_{254} (Macherey-Nagel), HPTLC-Platten: Nano-Sil 20 UV_{254} (Macherey-Nagel), Sephadex LH-20 (Pharmacia Biotech). The spots were visualized by UV light and heating silica gel plates sprayed with H_2O-H_2SO_4-AcH (1:4:20). Optical rotations: Perkin Elmer 241 automatic polarimeter, the $[\alpha]_D$ values are given in units of 10^{-1} deg cm^2 g^{-1}. UV Spectra: JASCO V-560. IR Spectra: Bruker IFS 55 spectrophotometer. ^1H (400 MHz) and ^{13}C (100 MHz) NMR and 2D-NMR Spectra: Bruker Avance 400 spectrometer; chemical shifts δ are given in ppm and coupling constants in Hz, with residual $CDCl_3$ as internal reference (δ_H 7.26, δ_C 77.0); DEPT COSY, ROESY, HSQC, and HMBC experiments were carried out with the pulse sequences given by Bruker. EIMS and HREIMS: Micromass Autospec spectrometer in *m/z*. All solvents used were analytical grade from Panreac. Reagents were purchased from Sigma Aldrich and used without further purification.

Plant Material. Maytenus chubutensis (Speg.) Lourt., O'Don. et Sleum was collected in December 2004, in the Séptima Región in Talca province, Chile. The plant was identified by Prof. José San Martín. *Maytenus magellanica* (Lam) Hook. f. was collected in December 2003, in the Novena Región in Temuca province, on the slopes of the volcano Osorno in Chile and identified by Prof. Sebastián Teyller. The voucher specimens are 93-5200 and 93-5342-A, respectively, on file with the Facultad de Ciencias, Universidad de Chile, Chile.

Extraction and Isolation. Air dried, chopped root bark of *M. chubutensis* (390 g) was extracted with hexanes-Et_2O (1:1) in a Soxhlet apparatus. Evaporation to dryness under reduced pressure gave 7.8 g of residue, which was fractionated by vacuum liquid chromatography on silica gel and eluted with hexanes (0.5 l) followed by a gradient of hexanes-EtOAc up to 100% EtOAc (0.5 l of each solvent mixture). Fifty-two fractions were collected and combined on the basis of their TLC profiles to afford nine main fractions (A-I). Further flash chromatography of fraction D (492 mg) on silica gel, using a gradient elution from hexanes-Et_2O 20% up to 100% Et_2O, yielded six fractions (D1-D6) after TLC analysis. Compound **1** (4.1 mg) was obtained by purification of fraction D2 (14 mg) by

preparative HPTLC, using hexanes-Me_2CO (8:2) as eluent. The rest of the fractions were subjected to preparative TLC, using a mixture of hexanes-Me_2CO, hexanes-Et_2O or hexanes-EtOAc affording 6-oxo-pristimerol (**4**) and pristimerin (**5**), which were identified by means of spectroscopic methods and comparison with authentic samples or reported data.

The root bark of *M. magellanica* (570 g) was extracted with *n*-hexane-Et_2O (1:1) in a Soxhlet apparatus. Evaporation to dryness under reduced pressure gave 13.5 g of residue. The residue was fractionated by CC over Sephadex LH-20 (8 x 70 cm) eluting with hexanes-$CHCl_3$-MeOH (2:1:1) to afford 55 fractions which were combined in eleven fractions (A-K) on the basis of their TLC profiles. Further flash chromatography of fractions E (180 mg) and F (137 mg) on silica gel, using a gradient elution from hexanes-EtOAc 20% up to 100% EtOAc, yielded eight fractions (1-8) after TLC analysis. Compound **2** (8.2 mg) was isolated from fraction E2 (15.1 mg) after purification by preparative TLC, using hexanes-Me_2CO (7:3) as eluent. Compound **3** (7.3 mg) was obtained by purification of fraction F3 (14.8 mg) by preparative TLC, using hexanes-Me_2CO (6:4) as eluent. The rest of the fractions were subjected to preparative TLC using mixtures of hexanes-Me_2CO, hexanes-Et_2O or hexanes-EtOAc affording: 6-oxo-pristimerol (**4**), pristimerin (**5**) and (-)-pimara-9(11)-15-dien-19-ol (**6**), which were identified by spectroscopic methods and comparison with authentic samples or reported data.

3-O-Methyl-6-oxo-pristimerol (**1**). Pale yellow lacquer; $[\alpha]_D^{25}$: +1.9 (c = 0.19, $CHCl_3$). UV (EtOH): 239 (4.2), 303 (4.4). IR (film): 3518, 2930, 2870, 1727, 1642, 1571, 1480, 1454, 1313, 1262, 1100, 1027, 756. ^1H (400 MHz, $CDCl_3$) and ^{13}C (100 MHz, $CDCl_3$) NMR data: *Table 1*. EI-MS: 494 (M^+, 100), 479 (29), 435 (5), 299 (7), 232 (53), 203 (14), 105 (9). HR-EI-MS: 494.3023 (M^+), $C_{31}H_{42}O_5$; calc. 494.3032.

Methylation of compounds **1** *and* **4.** To a solution of 3-*O*-methyl-6-oxo-pristimerol (**1**, 2.2 mg) or 6-oxo-pristimerol (**4**, 2.9 mg) in acetone (1 ml), K_2CO_3 (9 mg) and dimethyl sulphate (20 μl) were added. The reaction was stirred for 14 h at room temperature. The reaction mixture was concentrated to yield an oil, which was purified by preparative TLC using a mixture of hexanes-EtOAc (8:2) as eluent to afford compound 2,3-*O*-dimethyl-6-oxo-pristimerol (1.9 mg and 2.3 mg, respectively).

18-nor-ent-Kaur-4(19)-en-17-oic acid (**2**). Colorless lacquer; $[\alpha]_D^{25}$: -23.8 (*c* = 0.82, $CHCl_3$). IR (film): 3700-2700, 2925, 2855, 1703, 1450, 1005. ^{1}H NMR (400 MHz, $CDCl_3$): 0.86, 1.84 (2H, H-1), 0.97 (*s*, 3H, H-20), 1.12 (1H, H-9), 1.13, 1.99 (*m*, 2H, H-14), 1.32 (*m*, 1H, H-5), 1.40 (2H, H-12), 1.44, 1.57 (2H, H-15), 1.50, 1.92 (2H, H-2), 1.51, 1.63 (2H, H-7), 1.58 (2H, H-6), 1.64 (2H, H-11), 2.03 (*m*, 2H, H-3), 2.33 (*m*, 1H, H-13), 2.65 (br *s*, 1H, H-16), 4.75 (br *s*, 1H, H-19), 4.80 (br *s*, 1H, H-19). ^{13}C NMR (100 MHz, $CDCl_3$): 15.0 (*q*, C(20)), 18.1 (*t*, C(11)), 20.2 (*t*, C(6)), 24.1 (*t*, C(12)), 30.2 (*t*, C(2)); 33.0 (*t*, C(7)), 38.1 (*s*, C(10)), 38.8 (*t*, C(1)), 39.7 (*t*, C(15)), 40.0 (*t*, C(14)), 43.8 (*d*, C(16)), 44.1 (*d*, C(13)), 44.4 (*s*, C(8)), 48.7 (*d*, C(5)), 49.1 (*t*, C(3)), 53.5 (*d*, C(9)), 103.1 (*t*, C(19)), 155.6 (*s*, C(4)), 182.0 (*s*, C(17)). EI-MS: 288 (M^+, 69), 273 (76), 255 (18), 245 (100), 229 (64), 199 (79), 149 (40), 117 (41), 91 (66), 55 (54). HR-EI-MS: 288.2110 (M^+), $C_{19}H_{28}O_2$; calc. 288.2089.

28-Methyl ester of the acid-3,4-secolupa-4(23):20(29)-dien-3-oic (**3**). Colorless lacquer; $[\alpha]_D^{25}$: +15.3º (c = 0.73, $CHCl_3$). IR ($CHCl_3$): 3520-2637, 2947, 2869, 1708, 1641, 1456, 1215, 1156, 890, 757. ^{1}H (400 MHz, $CDCl_3$) and ^{13}C (100 MHz, $CDCl_3$) NMR data: *Table 1*. EI-MS: 484 (M^+, 10), 425 (12), 403 (47), 343 (81), 273 (20), 248 (19), 201 (35), 189 (46), 175 (57), 133 (43), 121 (55), 107 (70), 95 (85), 81 (82), 69 (84), 55 (100). HR-EI-MS: 484.3550 (M^+), $C_{31}H_{48}O_4$; calc. 288.3553.

In vitro assays for MDR reversion in Leishmania. Compounds **1**, **2**, **4** and **6** have been tested for efficacy as potential modulators of Pgp in a *Leishmania tropica* line according to an established protocol [13]. Briefly, we used a wild-type of *L. tropica* (LCR strain) and a MDR *L. tropica* line highly resistant to daunomycin (DNM), which was maintained in the presence of 150 μM DNM. This resistant line overexpress a Pgp-like transporter and possesses a MDR phenotype similar to that of tumor cells, with a cross resistance to several unrelated drugs. Promastigote forms were grown at 28 ºC in RPMI 1640-modified medium and supplemented with 20% inactivated foetal bovine serum. The reversal activity of isolated compounds was determined by the calculation of parasite growth inhibition using the MTT colorimetric assay after 72 h incubation at 28 ºC in presence of increasing concentrations of compounds.

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Table 1. *^{1}H and ^{13}C NMR Data of compounds **1** and **3***. Recorded at 400/100 MHz, respectively; in $CDCl_3$, δ in ppm, *J* in Hz. Assignment of ^{13}C NMR data were made with the aid of DEPT, HSQC and HMBC experiments.

Position	**1**		**3**	
	δ_H	δ_C	δ_H	δ_C
1	6.95 *s*	109.1 *d*	1.61	33.9 *t*
2		144.2 *s*	2.37, 2.22	28.1 *t*
3	OH-3, 6.08 *s*	132.9 *s*		179.1 *s*
4		132.0 *s*		147.5 *s*
5		154.9 *s*	1.92	50.4 *d*
6		187.2 *s*	1.39, 1.94	24.5 *t*
7	6.24 *s*	126.1 *d*	1.48*	32.8 *t*
8		151.9 *s*		40.9 *s*
9		40.4 *s*	1.48*	40.4 *d*
10		171.2 *s*		39.2 *s*
11	1.94, 2.21	34.0 *t*	1.37, 1.48	21.4 *t*
12	1.66, 1.72	29.8 *t*	1.05, 1.79	25.4 *t*
13		38.9 *s*	2.25	38.3 *d*
14		44.7 *s*		42.8 *s*
15	1.40, 1.80	28.5 *t*	1.49, 1.93	30.6 *t*
16	1.50, 1.80	36.4 *t*	1.47, 2.25	32.1 *t*
17		30.8 *s*		56.5 *s*
18	1.58	44.3 *d*	1.60	49.4 *d*
19	2.91 *d* (15.9), 1.66	30.5 *t*	2.99 *dt* (4.9, 10.8)	46.9 *d*
20		40.3 *s*		150.4 *s*
21	2.19, 1.37	29.7 *t*	1.40	29.7 *t*
22	2.04, 0.97	34.8 *t*	1.44, 1.90	36.9 *t*
23	2.66 *s*	14.7 *q*	4.65, 4.84 br *s*	113.4 *t*
24			1.72 *s*	23.2 *q*
25	1.52 *s*	37.6 *q*	0.83 *s*	20.1 *q*
26	1.29 *s*	20.8 *q*	0.97 *s*	15.9 *q*
27	0.57 *s*	18.3 *q*	1.00 *s*	14.6 *q*
28	1.09 *s*	31.6 *q*		176.6 *s*
29		178.8 *s*	4.61, 4.73 br *s*	109.7 *t*
30	1.17 *s*	32.7 *q*	1.68	19.3 *q*
OMe-3	3.79 *s*	61.1 *q*		
OMe-29	3.53 *s*	51.5 *q*		
OMe-28			3.66 *s*	51.3 *q*

* Overlapping signals.

Table 2. *Reversal activity in an MDR* L. tropica *line*[a].

	30 μM		15 μM		7.5 μM		3.75 μM	
Compd	Wt	MDR	Wt	MDR	Wt	MDR	Wt	MDR
1	38 ± 6	82 ± 7	8 ± 5	44 ± 1	1 ± 1	25 ± 4	0 ± 0	13 ± 0
2	2 ± 2	2 ± 3	6 ± 5	7 ± 5	4 ± 3	1 ± 1	0 ± 0	0 ± 0
4	11 ± 8	3 ± 3	6 ± 5	0 ± 0	4 ± 5	0 ± 0	4 ± 4	0 ± 0
6	3 ± 4	23 ± 8	2 ± 2	5 ± 2	0 ± 0	1 ± 1	0 ± 0	0 ± 0

[a]) Wild-type (Wt) and MDR parasites were exposed to compounds **1**, **2**, **4** and **6**, in the absence or presence of 150 μM DNM, respectively. Results are expressed as percentage growth inhibition relative to control growth in the absence of compounds. Data shown are the average of three independent experiments ± SD.

Figure: *Key HMBC correlations of compound **1**-**3***.