COLUBRINIC ACID ISOLATED FROM A PHARMACOLOGICAL ACTIVE EXTRACT OF *TREVOA TRINERVIS* MIERS

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ABSTRACT

From *Trevoa trinervis* Miers (Rhamnaceae), a plant used in Chilean folklore medicine, a triterpenoid called colubrinic acid, has been isolated from a pharmacological active extract. Its structure has been established by an array of spectroscopic techniques, mainly two-dimensional NMR methods.

Key Words: *Trevoa trinervis*, Rhamnaceae, triterpenoid, colubrinic acid, 2D-NMR

RESUMEN

Trevoa trinervis Miers (Rhamnaceae) es una especie usada por la medicina folklórica chilena. De un extracto farmacológicamente activo se aisló un triterpenoide llamado ácido colubrínico. Su estructura se estableció en base a técnicas espectroscópicas, principalmente métodos de RMN bidimensional.

Palabras Claves: *Trevoa trinervis*, Rhamnaceae, triterpenoide, ácido colubrínico, RMN-2D

INTRODUCTION

Chilean folklore medicine uses *Trevoa trinervis* Miers (Rhamnaceae), "trevo", for the treatment of inflammation caused by wounds and burns[1)]. The antiinflammatory and antipyretic activities of its infusion and different extracts from the aerial part, along with their respective cytotoxicities, have been reported by us recently[2)]. Previous research on the secondary metabolites of this plant led to the isolation of friedelin, ursolic acid, oleanolic acid, betulinic acid and β-sitosterol[2,3)] and, after hydrolysis of an alcoholic extract, to the dammarane sapogenins trevoagenins A, B, C and D[4,5,6)]. In this paper we report the identification of a triterpenoid isolated during a bioguided study of the most potent antiinflammatory and antipyretic of the dichloromethane extract of this plant.

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EXPERIMENTAL

NMR spectra were run in DMSO solutions with TMS as internal standard and recorded at 400 MHz (^{1}H) and 100 MHz (^{13}C) using available Bruker DISNMR pulse programs. IR: in KBr disc. GC/MS was recorded on a Fisons md 800. The molecular modeling was performed on a Silicon Graphics Indigo computer.

TLC spots were detected under UV (254 and 365 nm) and heating the plates to 110°C after spraying with Liebermann-Burchard reactive. Silica gel 60 was used for TLC and flash-column chromatography.

Plant material

The aerial part of *Trevoa trinervis* Miers was collected in the Cuesta La Dormida, Puente La Laja, V Región, Chile, in the early summer (December), and identified by Professor Raúl Peña. A voucher specimen is kept in the herbarium of the Escuela de Química y Farmacia (SQF: 19753), Universidad de Chile.

Extraction and isolation

Air dried and powdered of plant material (4 kg) was extracted successively at room temperature with hexane, CH_2Cl_2 and MeOH, yielding respectively and after removal of the solvents in vacuo, 49 g of hexane extract, 112 g of dichloromethane extract and 1100 g of methanol extract. All three extracts were activities. The CH_2Cl_2 extract proved to be the most active and therefore an amount (60 g) was submitted to CC, eluting with hexane and hexane-CH_2Cl_2 mixtures of increasing polarity.

Most of the eluted fractions was used for the bioactivity evaluation while from an aliquot of those fractions eluted with hexane-CH_2Cl_2, after repeated chromatographic separations and crystallization, were isolated in a previous research **1** (400 g), a 2:1 mixture of **2** and **3** (400 g), β-sitosterol (10 mg)[2] and this work, an impure compound **4** (50 mg), which was finally purified through chromatography on Sephadex LH-20 eluted with hex:CH_2Cl_2:MeOH (1.5:1:1) and recrystallization.

Colubrinic acid, **4**, 2β-formyl-3α-hydroxy-A(1).*nor*-lup-20(29)-en-28-oic acid. Mp = 265°C. TLC on silica gel, Rf = 0.40 (CH_2Cl_2-EtOAc, 9:1). EIMS, m/z (rel. abund. %): 470(2), 452(100), 437(71), 419(73), 406(28), 391(50), 328(26), 259(76), 213(53). IR (KBr, cm^{-1}) 3480, 2750, 1740-1670, 1640, 1170, 890. ^{1}H and ^{13}C-NMR data (Table I).

RESULTS AND DISCUSSION

Repeated chromatography of the CH_2Cl_2 extract and crystallization had led to the isolation of the well known triterpenoids betulinic (**1**), ursolic (**2**) and oleanolic (**3**) acids, which were previously identified by comparison with authentic samples and bibliographic data[7], along with β-sitosterol[2] together with a small amount of a triterpenoid **4** now reported, called colubrinic acid (zizyberanalic acid)[8,9] and similar to the bicarboxylic triterpene acid ceanothic acid[10]. This compound is minority and has been determined in other Rhamnaceae but not reported in this species.

Compound **4**, in its MS, showed a M^+ at m/z 470, which in combination with DEPT and BB ^{13}C-NMR data allowed to determine its molecular formula as $C_{30}H_{46}O_4$. The IR spectrum of **4**, along with absorptions for hydroxyl (3380 cm^{-1}) and vinylidenic unsaturation (1640, 890 cm^{-1}), displayed a broad and intense band in the carbonyl region (1660-1740 cm^{-1}). Its ^{1}H-NMR spectrum (Table I) showed a fair similarity with that of betulinic acid (**1**). The presence of two broad singlets at 4.55 and 4.66 ppm, characteristics for the vinylidene group, together with a methyl singlet at 1.63 ppm corresponding to a methyl, attached to the vinylidene, along with the presence of other five methyl

singlets, supported this assumption. The differences observed in the proton spectrum of **4** in relation with that of **1** corresponded to the existence of a doublet at δ 9.73 ppm (J = 4.7 Hz), assigned to the proton of an alpha monosubstituted aldehyde and of another doublet at δ 4.16 ppm (*d*, J = 8.5 Hz), which was assumed to correspond to the signal ordinarily expected for the proton geminal to the 3β-hydroxyl group, typical for most triterpenoids. The ^{13}C-NMR spectrum of **4** (Table I) showed a set of signals whose chemical shifts closely corresponded to those of the upper half of the structure of betulinic acid, e.g. those corresponding to atoms in rings C, D and E, with the exception of carbons 9 and 11. Most other remaining signals of rings A and B, were shifted with respect to those of **4**. The presence of six methyl signals, the same number as for betulinic acid, together with the presence of the signal for a methine aldehyde at 72.3 ppm, and the multiplicity of the rest of the signals compelled us to propose for this substance the structure of a pentacyclic triterpene of the type A-*nor*-α-lupene, supporting the aldehyde function on the cyclopentane ring A.

TABLE I. ^{1}H and ^{13}C-NMR spectral data for compounds **1** and **4**.

C	**1**		**4**	
	^{13}C	H	^{13}C	H
1	38.2		206.6	9.73 *d*(4.7)
2	27.1		72.3	2.03 *dd*(8.5, 4.7)
3	76.7	2.98 *m*	79.7	4.16 *d*(8.5)
4	38.4		40.4	
5	54.8		61.8	1.09 *m*
6	17.9		17.7	1.31 *m*
7	33.9		33.7	
8	40.1		41.4	
9	49.9		49.5	1.55 *dd*(10.8, 2.0)
10	36.6		47.4	
11	20.4		24.7	1.55 *m*
12	25.0		23.8	1.39 *m*
13	37.5	2.25 *m*	37.5	2.17 *dd*(11.6, 4.5)
14	41.1		42.2	
15	29.1	1.30 *m*	29.3	1.35 *m*
		1.36 *m*		
16	31.6	1.38 *m*	31.8	2.10 *m*
		2.13 *m*		1.35 *m*
17	55.3		55.3	
18	48.5		48.5	1.47 (11.3)
19	46.5	2.95 *m*	46.7	2.9 *dt*
20	150.2		150.3	
21	30.0	1.08 *m*	30.0	1.79 *m*
		1.78 *m*		
22	36.2	1.42 *m*	36.4	
		1.82 *d*		
23	28.0	0.87 *s*	25.7	0.80 *s*
24	15.6	0.65 *s*	25.1	0.87 *s*
25	15.9	0.76 *s*	14.5	0.92 *s*
26	15.7	0.87 *s*	16.3	0.80 *s*
27	14.3	0.92 *s*	14.3	0.93 *s*
28	177.1		177.3	
29	109.5	4.56 *s*	109.8	4.55 *s*
		4.69 *s*		4.66 *s*
30	18.9	1.64 *s*	18.9	1.63*s*

(DMSO δ values in ppm, *J* in Hz, TMS int. std.

The overlapping in the ^{1}H-NMR spectrum of **4** did not allow the observation of several signals of significance for the structure assignment and the complete interpretation of spectra.

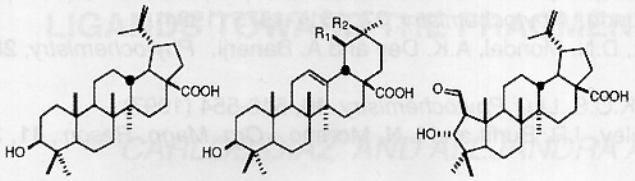
Consequently, several 2D-NMR spectra (COSY, HMQC and HMBC) were obtained and analyzed to overcome these difficulties. The results (Table II) served us to confirm the cyclopentane nature of ring A in **4** and to assign all the signals of both carbon and proton spectra. Thus the doublet at δ 9.73 ppm of the aldehyde proton, showed long-range correlations with the carbon signals assigned to its vicinal methine carbon (δ 72.3 ppm) and to the methine supporting the hydroxyl function (δ 79.7 ppm). The signal at δ 4.16 ppm, corresponding to the proton geminal to the hydroxyl group, was long-range coupled to that of the aldehyde carbonyl. These correlations permitted to establish the existence of a β-hydroxyaldehyde moiety. Additionally, the two methyl signals at 0.80 and 0.87 ppm, assigned to a gem-dimethyl moiety due to its mutual hydroxylated carbon signal at δ 79.7 ppm, as well as with the signal for the quaternary carbon (40.4 ppm) supporting both methyl groups. Finally, the splitting (*dd*, J = 8.5 and 4.7 Hz) of the proton geminal to the aldehyde group (2.02 ppm), along with the long-range heteronuclear correlation between the methine at δ 72.3 ppm and the proton signal for the angular methyl group at δ 0.93 ppm, confirmed the rearrangement of ring A in **4**.

TABLE II. 2D HMBC correlation data for compound **4**

C	δ (ppm)	H correlated
1	206.6	2, 3
2	72.3	1, 3, 25
3	79.7	1, 2, 23, 24
4	40.4	23, 24
5	61.8	7, 24, 25
6	17.7	5
7	33.7	5, 26
8	41.4	7, 26, 27
9	49.5	2, 25, 26
10	47.4	2, 25, 5
11	24.7	13
12	23.8	11
13	37.5	12, 18, 19, 27
14	42.2	13, 16, 27
15	29.3	27
16	31.8	18
17	55.3	15, 16
18	48.5	16, 19, 21, 22
19	46.7	21, 29, 30
20	150.3	18, 19, 30
21	30.0	19, 22
22	36.4	-
23	25.7	5.24
24	25.1	5.23
25	14.5	2
26	16.3	-
27	14.3	13
28	177.3	16, 22, 18
29	109.8	19, 30
30	18.9	18, 19, 29

The remaining correlations observed, similar to those found by us for betulinic acid, that also served to modify previous assignments[7] (Table I) and for colubrinic acid[8], allowed us to connect all the carbon and proton signals for the substance and hence to unambiguously establish its molecular constitution.

In order to define these changes several NOE difference experiments were carried out, whose main results are implemented in Figure 1.



1

2 R^1=Me, R^2=H
3 R^1=H, R^2=Me

4

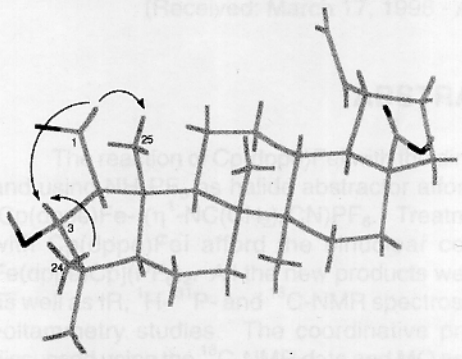


FIG. 1. Lower energy conformation and relevant NOEs for colubrinic acid (**4**)

On irradiation of the aldehyde proton, a NOE was observed for the signals assigned to H-25 methyl group and the H-3 geminal to the hydroxyl. This fact indicated a β arrangement for both protons, the aldehydic and that at position C-3; in consequence, the hydroxyl group must be α. Similar confirming results were obtained when irradiating the H-25 methyl signal and, finally, another NOE effect observed on the H-3 signal upon irradiation of one of the two germinal methyls allowed us to ascertain unequivocally which of both groups had the β disposition. All these facts allowed us to confirm structure **4** for colubrinic acid[9]. Compound **4** was built up using Macromodel v.4. facilities[11]. Conformational analysis of these bonds were searched with MM2 force field implemented in this package. The interprotonic distances were measured in the lowest energy conformer (E = 335.4 kJuls/mol) and compared with the NOE differences observed in this compound (Fig. 1).

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