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Withanolid amine and nicotine from *Dunalia spinosa* (Solanaceae)

[Amino-witanólido y nicotina de *Dunalia spinosa* (Solanaceae)]

Javier ESPINOZA[1], Javier ECHEVERRÍA[1], Alejandro URZÚA[1] & Hermann M. NIEMEYER[2]

[1]Facultad de Química y Biología, Universidad de Santiago de Chile, Casilla 40, Correo 33, Santiago, Chile
[2]Departamento de Ciencias Ecológicas, Facultad de Ciencias, Universidad de Chile, Casilla 653, Santiago, Chile
Contactos | Contacts: Alejandro URZÚA - E-mail address: alejandro.urzua@usach.cl
Contactos | Contacts: Hermann M. NIEMEYER - E-mail address: niemeyer@abulafia.ciencias.uchile.cl

Abstract
A new withanolid amine was isolated from *Dunalia spinosa* (Solanaceae). Its relative stereochemistry was determined using FT-IR, ^{1}H and ^{13}C NMR spectroscopies and high resolution mass spectrometry. Nicotine was also isolated; chemotaxonomic and archaeological implications are discussed.

Keywords: *Dunalia spinosa*; Solanaceae; alkaloidal extract; GC-MS analysis; withanolide; nicotine

Resumen
Un nuevo amino-witanólido fue aislado de *Dunalia spinosa* (Solanaceae). Su estereoquímica relativa fue determinada usando espectroscopías FT-IR y RMN de ^{1}H y ^{13}C, y espectrometría de masas de alta resolución. También fue aislada nicotina; se discuten las implicancias quimotaxonómicas y arqueológicas.

Palabras Clave: *Dunalia spinosa*; Solanaceae; extracto alcaloidal; CG-EM

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INTRODUCTION

The consumption of psychotropic alkaloids during ritual ceremonies is a long-lived and standing tradition among native peoples of the Americas (Schultes *et al*., 1998; Torres, 2001; Dante and Capriles, 2004). A general assessment of alkaloid presence in the American flora indicates that plants from at least 200 genera from 20 families are potential sources of American hallucinogens (Schultes and Hofmann, 1980). Despite this prediction and the uniqueness of Chilean flora associated to its insular position given by the Pacific Ocean, the Northern desert and the Andes range, the flora of Chile has not been sufficiently explored for the occurrence of alkaloids. We have undertaken the analysis of native plants from Northern Chile in search for alkaloids. Plants were collected during the flowering season and their basic extracts assessed for presence of alkaloids using TLC plates sprayed with Dragendorff reagent. Alkaloid-positive extracts were further studied. This paper reports on alkaloids found in *Dunalia spinosa* (Solanaceae), a small tree growing in Southern Peru and Northern Chile used in folk medicine against toothaches, coughing and high altitude sickness (Villagrán and Castro, 2003).

RESULTS AND DISCUSSION

The methanolic extract of aerial parts of *D. spinosa* subjected to acid-base treatment yielded compound 1 and nicotine as main alkaloids in the basic extract and in the mother liquor from the isolation of compound 1, respectively. High resolution mass spectrometric analysis of compound 1 showed a molecular ion of 487.2930 g/mol corresponding to the molecular formula $C_{28}H_{41}NO_6$. Infrared spectroscopic data of compound 1 (Table 1) showed the presence of a ketonic carbonyl group, a hydroxyl group and an amino group. Nuclear magnetic resonance analysis of compound 1, which included ^{1}H and ^{13}C NMR, DEPT-135, HMBC and HSQC-ed experiments (Table 2), showed that the structure corresponds to (4β, 5β, 6β)-3-amino-4,27-dihydroxy-5,6-epoxy-1-oxo-witha-24-enolide (Figure 1). Stereochemistry at C-17 was determined by comparison with NMR data of withaferine A, whose X-ray structure is known (McPhail and Sim, 1968). Stereochemistry at C-3 was determined from J_{C-4} = 5 Hz, consistent with an angle of 60º between H-3 and H-4 and hence a *cis* orientation of the amino group at C-3 with respect to the hydroxyl group at C-4. Withanolides have been found to occur mainly in the tribe Solanoideae of the Solanaceae but occasional occurrences have been demonstrated in the Fabaceae, Lamiaceae, Myrtaceae and Dioscoreaceae (Chen *et al*., 2010; Misico *et al*., 2011). So far, withanolid amines (with an amino group attached to the skeletal carbons) had only been reported as synthetic derivatives (Keihan *et al*., 1983; García *et al*., 2012); hence, this is the first report of a naturally occurring withanolide amine.

A wide range of pharmacological effects have been described for withanolides, e.g. antitumor, anti-inflammatory, antimicrobial, cytotoxic, immunoregulatory and cholinesterase inhibitory activities (Chen *et al*.,, 2010; Misico *et al*., 2011); they have also been shown to affect the central nervous system (Singh *et al*., 2010). Moreover, compound 1, being an alkaloid in a broad sense (Pelletier, 1983), is likely to be an agonist or antagonist of neurotransmitters and neuroreceptors (Wink and Schimmer, 1999; Wink, 2000). Hence, compound 1 is a putative source of psychotropic effects. Whether *D. spinosa* has been utilized on account of such properties remains to be established.

The GC-MS analysis of the mother liquor from the crystallization of compound 1 (Table 5), showed the presence of the alkaloid nicotine, whose identification was confirmed by comparing its mass spectrum and retention index with values from the literature (Adams, 2007). The genus *Dunalia*, e.g. *D. tubulosa*, has yielded mainly tropane alkaloids, as many other members of the subfamily Solanoideae (Wink, 2003); to date, neither pyridine nor pyrrolizidine alkaloids have been reported in species of the genus *Dunalia*. A study of the distribution of alkaloids within the family Solanaceae showed that nicotinic derivatives are mainly found in the subfamily Nicotianoideae, with isolated occurrences as minor components in a few genera of the subfamily Solanoideae (Wink, 2003), Thus, although the occurrence of nicotine in *Dunalia* is not entirely surprising, the finding calls for a more thorough study of alkaloid presence in the Solanaceae.

Studies which have detected nicotine or nicotine derivatives in residues from archaeological objects such as smoking pipes have led to the proposal of use of *Nicotiana* spp. (Rafferty 2002, 2006). The present work shows, on one hand, that other candidates should be considered as potential sources of nicotine, particularly plants used in other cultural contexts, e.g. as medicinal plants and, on the other,

that additional analytical methods should be employed to ascertain the plant of origin of alkaloids, for example, the study of seeds, microfossils or additional molecular markers accompanying nicotine in the archaeological residues. It would be interesting to analyze residues present in smoking pipes and snuffing tablets from Northern Chile to assess the use of *D. spinosa* as source of hallucinogens, bearing in mind that an eventual finding of nicotine should be followed by more thorough analyses, as mentioned above.

TABLE 1
IR spectral data for compound 1

Frequency (cm^{-1})	Intensity	Assignment	Functional group
1704.6	s	stretching C=O	ketonic C=O
2950.8 and 2879.0	m	stretching C-H	CH_3, CH_2 and CH
3498.7	m	stretching O-H	OH
3359.4 and 3291.4	m	stretching N-H	NH_2

EXPERIMENTAL

Plant material

Aerial parts of *Dunalia spinosa* (Meyen) Dammer which included twigs with leaves, flowers and fruits were collected near Putre, Chile (18º20' S, 69º56' W, 3520 masl) in may 2010. Voucher specimens were deposited in the herbarium at Universidad de Concepción.

Extraction of alkaloids and analysis

Oven dried plant material (90 g) was degreased with petroleum ether and extracted with 500mL of MeOH at room temperature during 24 h. The suspension obtained was filtered through a frit funnel and the resulting methanolic extract was evaporated under reduced pressure on a rotary evaporator. The syrupy residue was agitated with 250 ml 5% HCl for 1 h, allowed to stand for 24 h at 35º C, and filtered through paper. The clear filtrate was washed with CH_2Cl_2 (4x40 mL). The aqueous phase was adjusted to pH 10 with NH_4OH and extracted with $CHCl_3$ (4x25 mL) until the extracts gave negative Dragendorff reaction. Finally, the organic extract was dried with anhydrous Na_2SO_4 and evaporation of the solvent yielded an extract potentially containing alkaloids (0.24 g).

Analysis of the extract was performed in two ways:

Procedure A

The alkaloidal extract of *D. spinosa* (0.24 g) was suspended in 5 mL of MeOH. The suspension obtained was heated to solubilize the alkaloids and the hot solution was filtered under reduced pressure. The filtrate was cooled to 0-5° C for 24 h and filtered cold under reduced pressure; the solid isolated was crystallized from MeOH. The crystalline precipitate obtained (compound 1, 80.2 mg, 0.089% dry weight yield, melting point: 190 - 191 º C) was filtered and dried. Ten mg of compound 1 were analyzed by Fourier-transform infrared spectroscopy (Bruker model IFS66V: frequency range of 4000 - 400 cm^{-1}, KBr tablet), high-resolution mass spectrometry (JEOL GCmate: electron impact ionization at 70 eV, sample placed directly into the chamber), and mono and bidimensional ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy (Varian Mercury 300: 300 MHz, compound dissolved in $CDCl_3$).

TABLE 2
NMR data for compound 1

Position	DEPT 135	^{13}C Chemical shift (ppm)	^{1}H Chemical shift (ppm)	Integration, multiplicity and coupling constant (Hz)
1	C	210.96	-	-
2	CH_2	42.35	3.16	1H; *dd*; J = 14.9, 6.3
			2.24	1H; *dd*; J = 14.9, 5.7
3	CH	50.14	3.55	1H; *dd*; J = 11.2, 5.8
4	CH	77.29	3.26	1H; *d*; J = 5.0
5	C	64.91	-	-
6	CH	60.72	3.27	1H; *s*
7	CH_2	30.96	2.16	1H; *dt*; J = 13.9, 3.0
			1.31	1H; *m*
8	CH	28.98	1.38	1H; *m*
9	CH	42.66	1.32	1H; *m*
10	C	50.36	-	-
11	CH_2	21.99	1.57	1H; *m*
			1.33	1H; *m*
12	CH_2	27.21	1.69	1H; *m*
			1.36	1H; *m*
13	C	42.51	-	-
14	CH	55.95	0.98	1H; *m*
15	CH_2	24.24	1.67	1H; *m*
			1.15	1H; *m*
16	CH_2	39.21	1.92	1H; *m*
			1.19	1H; *m*
17	CH	51.96	1.11	1H; *m*
18	CH_3	11.61	0.68	3H; *s*
19	CH_3	16.35	1.35	3H; *s*
20	CH	38.75	2.00	1H; *m*
21	CH_3	13.32	1.00	3H; *d*; J = 6.7
22	CH	78.71	4.42	1H; *dt*, J = 13.2, 3.5
23	CH_2	29.79	2.50	1H; *dd*; J = 17.6, 13.2
			1.99	1H; *m*
24	C	152.83	-	-
25	C	125.67	-	-
26	C	166.97	-	-
27	CH_2	57.40	4.39	1H; *s*
			4.37	1H; *s*
28	CH_3	19.99	2.05	3H; *s*

s = singlet, *d* = doublet, *t* = triplet, *td* = doublet of triplets, *dd* = doublet of doublets, *m* = multiplet.

Procedure B

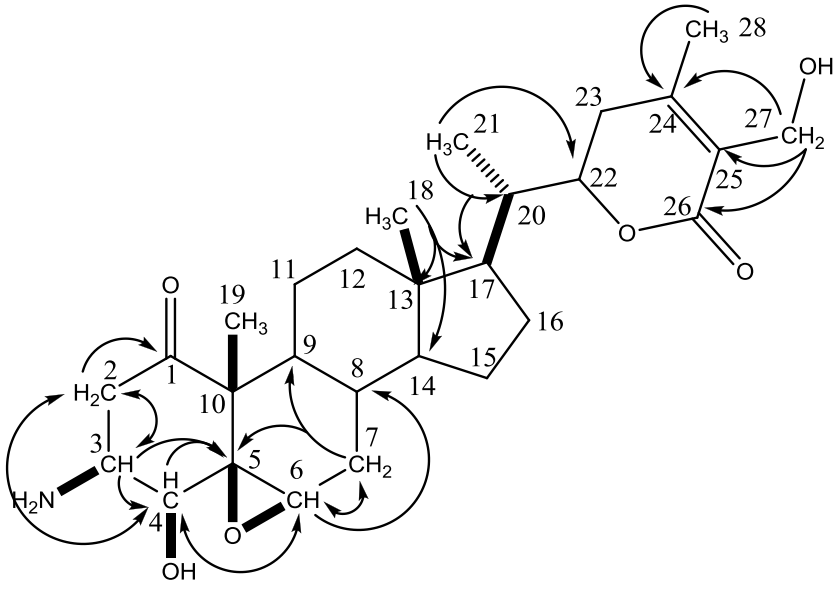
The mother liquor from the crystallization of compound 1 was seeded onto four 20 x 20 cm preparative glass chromatoplates coated with 1 mm silica gel (Merck, 60 F_{254}). The plates were developed three times using CH_2Cl_2 - MeOH (85:15) and examined under UV light at 254 and 365 nm. The eight bands selected were removed from the chromatoplates and extracted using CH_2Cl_2 - MeOH (9:1). Subsequently, the suspensions were vacuum filtered and the filtrates dried with anhydrous Na_2SO_4. Finally, each extract was evaporated to dryness using a rotary evaporator, labeled and stored for subsequent analysis. These fractions of basic compounds were analyzed by thin layer chromatography using silica gel coated aluminum chromatofolios (Merck, GF_{254}) developed using CH_2Cl_2 - MeOH (95:5) and were examined under UV light at 254 and 365 nm. Dragendorff reagent and *p*-anisaldehyde were used to assess for presence of alkaloids. The alkaloid-containing fractions (from fractions with Rf= 0.160 and 0.093 of the original preparative TLC) were analyzed by gas chromatography/mass spectroscopy (GC-MS).

TABLE 3
Gas chromatographic data for main compounds in extracts processed by Procedure B

Retention time (min)	Retention index	Compound	%*
22.08	1105	Methyl benzoate	2.3
28.32	1319	p-Menthane-1,8-diol	7.9
29.60	1366	**Nicotine**	43.2
41.91	1901	Methyl 3,4-dimethoxycynnamate	3.8

% *: considering only compounds detected.

FIGURE 1
Structure of compound 1 showing major Heteronuclear Multiple Bond Correlations.



GC/MS analysis was performed with a Thermo Scientific Trace GC Ultra linked to a ISQ quadrupole mass spectrometric detector with an integrated data system (Xcalibur 2.0, Thermo Fisher Scientific Inc. USA). An Rtx-5MS capillary column was used (film thickness 0.25 µm, 60m x 0.25 mm, Restek Corporation, Bellefonte, PA, USA): Operating conditions were as follows: on-column injection; injector temperature, 250° C; detector temperature, 280° C; carrier gas, He at 1.25 ml/min; oven temperature program: 40º C for 5 min, increase to 260º C at 5º C/min, and then 260º C for 5 min. The mass detector ionization employed an electron impact of 70 eV. Recording conditions employed a scan time of 1.5 s and a mass range of 40 to 400 amu. Compounds in the chromatograms were identified by comparison of their mass spectra with those in the NIST98 library database, and by comparison of their retention index for the same type of column with those reported in the literature (Adams, 2007), or those of commercial standards when available.

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