Trypanocidal Activity of Abietane Diterpenoids from the Roots of *Craniolaria annua*

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A chloroform extract from roots of *Craniolaria annua* provided six new C-11 unsubstituted abietanes, 14-hydroxy-6,12-dione-7,9(11),13-abietatriene (**1**), 14-hydroxy-12-oxo-7,9(11),13-abietatriene (**2**), 6,12,14-trihydroxyabieta-5,8,11,13-tetraen-7-one (**3**), ar-abietatriene-12-ol-6,7-dione-14,16-oxide (**4**), ar-abietatriene-12,16-diol-14,16-oxide (**5**) and ar-abietatriene-12-ol-7-one-14,16-oxide (**6**), and two known compounds, ferruginol and stigmasterol. The structures of both the new and known compounds were established by spectroscopic methods. Abietane **1** gave 14-hydroxy-6-oxoferruginol (**1A**) upon treatment with $NaBH_4$. Abietanes **1**, **1A**, **3**–**5** and ferruginol showed cytotoxic effects against trypomastigote and epimastigote forms of *Trypanosoma cruzi* and against fibroblastic Vero cells.

Key words: Abietane Diterpenoids, *Trypanosoma cruzi*, Cytotoxicity

Introduction

Craniolaria annua (Martyniaceae) is a herb, 0.4–0.6 m in height, that grows in American tropical areas (Bretting and Nilsson, 1988; Steyermark *et al.*, 2001). The plant is used in traditional medicine (Hernández-Cano and Volpato, 2004), and its roots are employed for the treatment of microbial infections in Venezuela. However, phytochemical data is not available for the genus.

In a previous work we studied the trypanocidal activity of 1,3-dienes (Cabrera *et al.*, 2005). In the search for new sources of bioactive compounds from Venezuelan plants we observed that a chloroform extract obtained from roots of *Craniolaria annua* had also significant trypanocidal activity. We describe here the isolation and identification of six abietane diterpenoids, **1**–**6**, and two known compounds, ferruginol and stigmasterol, and the synthesis of the abietane 14-hydroxy-6-oxoferruginol (**1A**) from **1**. We also report the *in vitro* cytotoxicity of the purified compounds against *Trypanosoma cruzi* trypomastigote and epimastigote forms and fibroblastic Vero cells.

Materials and Methods

General experimental procedures

Melting points were measured on a Thomas-Hoover 6427-H10 apparatus. Optical rotation was recorded on a Perkin Elmer 341 polarimeter. UV spectra were obtained on a Milton Roy Spectronic 3000 Array (Milton Roy Company, Ivyland, USA). IR spectra were recorded on a Nicolet Magna 550 FT spectrometer, using KBr disks. NMR experiments were recorded on a JEOL Eclipse+ spectrometer operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C NMR. HSQC, HMBC and NOESY spectra were obtained in the phase-sensitive mode. Chemical shifts were measured taking the solvent as internal standard. HREIMS (70 eV) was performed on a JEOL JMS-AX505WA double focus mass spectrometer. Column chromatography was performed with silica gel 60 (Merck, 5–25 μm). Analytical thin-layer chromatography was carried out on precoated silica gel 60 F_{254} aluminum plates (Macherey-Nagel) of 200 μm thickness, while preparative thin-layer chromatography was performed on precoated silica gel 60 plates (Merck) of 250 μm thickness. Komarowsky reagent, a 5:1 solution of 2 % 4-hydroxybenzaldehyde in methanol and 50 % H_2SO_4 solution (Tapondjou *et al.*, 2006), was used

for TLC detection. Stigmasterol was obtained from Supelco (Park Bellefonte, USA).

Plant material

The roots of *C. annua* were colleted in Cumaná, Venezuela, in February 2000. Dr. Luis José Cumana, Universidad de Oriente, Venezuela, identified the plant material, and a voucher specimen (HRBR 0180) has been deposited at the Isidro Bermúdez Herbarium.

Extraction and isolation

The fresh roots (5.9 kg) were extracted by maceration in $CHCl_3$ (3 × 24 h) at room temperature. The solvent was removed under reduced pressure to give a golden syrup (11 g). The syrup (10.2 g) was chromatographed on silica gel (150 g) with a stepwise gradient of hexane/$CHCl_3$ (1:1, 250 mL; 3:7, 250 mL), $CHCl_3$ (250 mL), $CHCl_3$/CH_3CN (9:1, 250 mL), CH_3CN (250 mL) and MeOH (250 mL) to yield 6 fractions (I–VI). Fraction II (2.24 g) was chromatographed on silica gel (100 g) with a stepwise gradient of hexane/$CHCl_3$ (7:3, 170 mL; 3:7, 170 mL), $CHCl_3$ (170 mL) $CHCl_3$/CH_3CN (9:1, 170 mL) and CH_3CN (170 mL) to yield 5 fractions (IIa–IIe). Fraction IIa (440 mg) afforded **1** (290 mg) upon crystallization with hexane/$CHCl_3$ (99:1). Fraction IId (847 mg) was chromatographed on silica gel (40 g) with a stepwise gradient of hexane/diethyl ether (9:1, 90 mL; 1:1, 90 mL) and diethyl ether (90 mL) to yield 3 fractions (IId1–IId3). Fraction IId1 was chromatographed by preparative TLC (hexane/diethyl ether, 9:1) to afford **2** (R_f = 0.75). Fraction III (2.80 g) was chromatographed on silica gel (100 g) with a stepwise gradient of chlorobutane/diethyl ether (99:1, 170 mL; 95:5 170 mL; 7:3, 170 mL) and CH_3CN (170 mL) to give 4 fractions (IIIa–IIId). Fraction IIIb (350 mg) was chromatographed on silica gel (30 g) with hexane/diethyl ether (19:1, 50 mL) to afford ferruginol (250 mg). Fraction IIId (820 mg) afforded **4** (185 mg) and stigmasterol (25 mg) upon selective precipitation using first hexane/ethyl acetate and second CH_3CN. Fraction IV (2.50 g) was chromatographed on silica gel (100 g) with a stepwise gradient of hexane/$CHCl_3$ (1:9, 170 mL), $CHCl_3$/CH_3CN (9:1, 170 mL; 9:5, 170 mL) and CH_3CN (170 mL) to yield 4 fractions (IVa–IVd). Fraction IVc (400 mg) was chromatographed on silica gel (20 g) with hexane/diethyl ether (10:3, 34 mL) to afford **3** (50 mg) upon crystallization from hexane/$CHCl_3$. Fraction V (1.50 g) was separated by column chromatography over silica gel (40 g) and eluted with a stepwise gradient of hexane/diethyl ether (1:1, 67 mL; 3:7, 67 ml; 1:5, 67 mL) and diethyl ether (67 mL) to yield 4 fractions (Va–Vd). Fraction Vb (430 mg) was chromatographed on silica gel (30 g) with hexane/ethyl acetate (17:3, 50 mL; 7:3, 50 mL) to afford **5** (200 mg). Fraction Vc (825 mg) was chromatographed on silica gel (40 g) with a stepwise gradient of hexane/diethyl ether (2:3, 67 mL; 3:7, 67 mL; 1:9, 67 mL) and diethyl ether (67 mL) to yield 4 fractions (Vc1–Vc4). Fraction Vc3 (284 mg) was subjected to preparative TLC (hexane/diethyl ether, 1:1) to afford **6** (R_f = 0.4).

*14-Hydroxy-6,12-dione-7,9(11),13-abietatriene (**1**)*

Yellow amorphous powder, m.p. 144–145 °C. – $[\alpha]_D^{20.0}$ –15.0° (*c* 0.5, $CHCl_3$). – UV (MeOH): λ_{max} (log ε) = 317 (3.9), 330 (4.4), 403 (3.6) nm. – IR (KBr): ν_{max} = 3328, 2960, 2933, 1672, 1618, 1544, 1467, 1417 cm^{-1}. – ^1H and ^{13}C NMR ($CDCl_3$): see Tables I and II. – HREIMS: *m/z* = 314.1877 (calcd. for $C_{20}H_{26}O_3$ 314.1882). – EIMS: *m/z* (rel. int.) = 314 [$M^{+\bullet}$] (100), 299 (58), 271 (64), 245 (26), 229 (38), 217 (32), 190 (35), 175 (17), 137 (17), 109 (30), 55 (50), 41 (48).

*14-Hydroxy-12-oxo-7,9(11),13-abietatriene (**2**)*

Yellow oil. – $[\alpha]_D^{20.0}$ +23.0° (*c* 0.5, $CHCl_3$). – UV (MeOH): λ_{max} (log ε) = 290 (3.8), 316 (3.9), 400 (3.3) nm. – IR (KBr): ν_{max} = 3300, 2965, 1640, 1612, 1560, 1320 cm^{-1}. – ^1H and ^{13}C NMR ($CDCl_3$): see Tables I and II. – HREIMS: *m/z* = 300.2081 (calcd. for $C_{20}H_{28}O_2$).

6,12,14-Trihydroxyabieta-5,8,11,13-tetraen-7-one (***3***)

Pale green plates, m.p. 175–176 °C. – UV (MeOH): λ_{max} (log ε) = 255 (3.7), 283 (3.9), 321 (3.9), 415 (3.6) nm. – IR (KBr): ν_{max} = 3537, 1660, 1623, 1218 cm^{-1}. – ^1H and ^{13}C NMR ($CDCl_3$): see Tables I and II. – HREIMS: *m/z* = 330.1825 (calcd. for $C_{20}H_{26}O_4$ 330.1831). – EIMS: *m/z* (rel. int.) = 330 [$M^{+\bullet}$] (100), 315 (25), 287 (40), 261 (40), 231 (25), 219 (20), 84 (48), 66 (52).

*ar-Abietatriene-12-ol-6,7-dione-14,16-oxide (**4**)*

Orange amorphous powder, m.p. 192–193 °C. – $[\alpha]_D^{20.0}$ +20.0° (*c* 0.5, $CHCl_3$). – UV (MeOH): λ_{max} (log ε) = 255 (3.9), 284 (3.8), 373 (3.4) nm. – IR (KBr): ν_{max} = 3205, 1710, 1650, 1620 1605, 1480, 1218 cm^{-1}. – ^1H and ^{13}C NMR ($CDCl_3$): see Tables I and II. – HREIMS: *m/z* = 328.1667 (calcd. for $C_{20}H_{24}O_4$ 328.1675). – EIMS: *m/z* (rel. int.) = 328 [$M^{+\bullet}$] (100), 313 (20), 295 (45), 285 (45), 245 (20), 218 (27), 69 (20), 55 (40), 41 (35).

*ar-Abietatriene-12,16-diol-14,16-oxide (**5**)*

White needles, m.p. 167–168 °C. – $[\alpha]_D^{20.0}$ –8° (*c* 0.5, $CHCl_3$). – UV (MeOH): λ_{max} (log ε) = 211 (3.8), 288 (3.0) nm. – IR (KBr): ν_{max} = 3600, 2941, 1629, 1492, 1321, 1197, 1078 cm^{-1}. – ^1H and ^{13}C NMR (DMSO-d_6): see Tables I and III. – HREIMS: *m/z* = 316.2035 (calcd. for $C_{20}H_{28}O_3$ 316.2038). – EIMS: *m/z* (rel. int.) = 316 [$M^{+\bullet}$] (82), 301 (100), 231 (18), 219 (21), 205 (33).

*ar-Abietatriene-12-ol-7-one-14,16-oxide (**6**)*

Pale yellow amorphous powder, m.p. 171–172 °C. – $[\alpha]_D^{20.0}$ –1° (*c* 0.5, $CHCl_3$). – UV (MeOH): λ_{max} (log ε) = 217 (4.0), 236 (4.1), 277 (3.9), 320 (3.5) nm. – IR (KBr): ν_{max} = 3300, 3100, 1664, 1603, 1596, 1272, 1170 cm^{-1}. – ^1H and ^{13}C NMR (DMSO-d_6): see Tables I and III. – HREIMS: *m/z* = 330.1827 (calcd. for $C_{20}H_{26}O_4$ 330.1831). – EIMS: *m/z* (rel. int.) = 330 [$M^{+\bullet}$] (65), 313 (100), 312 (60), 302 (50), 287 (25), 269 (20), 227 (25), 217 (38), 205 (35), 175 (25), 131, (20), 55 (28).

*Reduction of **1** to 14-hydroxy-6-oxoferruginol (**1A**)*

1 (24.9 mg, 0.0795 mmol) was dissolved in 300 μL of CH_3CN, and 10 mL of 95 % EtOH were added. Then $NaBH_4$ (10.0 mg, 0.265 mmol) was added slowly. The mixture was placed in an ice bath and stirred overnight under N_2 atmosphere with protection from light. The solvent was evaporated under reduced pressure, and 5 mL 10 % HCl were added. The mixture was extracted with chlorobutane (3 × 2 mL). The organic fractions were pooled and dried over Na_2SO_4. The solvent was evaporated under reduced pressure to afford 23.6 mg. – $[\alpha]_D^{20.0}$ +13° (*c* 0.5, $CHCl_3$). – ^1H and ^{13}C NMR ($CDCl_3$): see Tables I and III. – HREIMS: *m/z* = 316.2032 (calcd. for $C_{20}H_{28}O_3$ 316.2038). – EIMS: *m/z* = 316 [M^+] (100), 301

Table I. ^1H spectral data [δ_H values in ppm, (*J* values in Hz)] of **1**–**6** and **1A** (400 MHz).

H	1	2	3	4	5	6	1A
1α	1.21 (dd, 4.1, 13.7)	1.19 (m)	1.59 (m)	1.69 (m)	1.25 (m)	1.4 m	1.65 (m)
1β	1.45 (dbr, 13.7)	1.47 (m)	2.20 (dd, 7.0, 12.1)	2.03 (dbr, 8.8)	2.10 (dbr, 12.7)	2.15 (d, 12.6)	2.18 (dbr, 11)
2α	1.65 (m)	1.62 (m)[a]	1.65 (m)	1.69 (m)[a]	1.51*	1.39 m	1.65 (m)[a]
2β	1.75 (dbr, 13.9)	–	1.82 (m)	–	1.69 (dbr, 13.6)	1.58 (dbr, 14.1)	–
3α	1.65 (m)	1.45 (m)	1.36 (m)	1.17 (m)	1.20*	1.25 (ddbr, 3.1, 13.3)	1.13 (dd, 3.3, 12.8)
3β	1.975 (dbr, 13.5)	1.97 (dbr, 12.1)	1.77 (m)	1.45 (dbr, 13.6)	1.42 (dbr, 13.2)	1.46 (m)	1.42 (m)
5	2.47 (s)	1.53 dd (4.4, 11.7)		2.60 (s)	1.21	1.74 (dd, 4.0, 13.6)	2.45 (s)
6	–	2.44 (ddd, 3.3, 11.7, 2.1)[b]	–	–	1.51[b]	2.47 (m)	–
		2.60 (dd, 4.4, 6.6)[c]			1.81 (dd, 6.0, 13.2)[c]		
7	6.60 (d, 1.2)	7.16 (dd, 4.4, 12.1)	–	–	2.61 (m)	–	3.40 (d, 13.5)[b] 3.60 (d, 13.5)[c]
11	6.39 (d, 1.2)	6.30 (s)	6.87 (s)	6.38 (s)	6.55 (s)	6.71 (s)	6.74 (s)
15	3.05 (sept, 7.0)	3.07 (sept, 7)	4.58 (sept, 7.0)	3.24 (dq, 4.4, 7.0)	2.96 (q, 7.3)	2.96 (q, 7.3)	3.05 (sept, 7.0)
16	1.29 (d, 7.0)	1.29 (d, 7)	1.33 (d, 7.0)	4.21 (dd, 5.1, 11.0)[b]	5.42 (d, 4.4)	5.50 (s)	1.33 (d, 7.0)
				4.31 (dd, 4.0, 11.0)[c]			
17	1.35 (d, 7.0)	1.34 (d, 7)	1.26 (d, 7.0)	1.33 (d, 7.0)	1.09 (d, 7.0)	1.05 (d, 7.0)	1.35 (d, 7.0)
18	1.15 (s)	0.91 (s)	1.37 (s)	1.13 (s)	0.93 (s)	0.89 (s)	1.06 (s)
19	1.24 (s)	0.98 (s)	1.32 (s)	1.27 (s)	0.88 (s)	0.93 (s)	1.31 (s)
20	1.23 (s)	1.06 (s)	1.38 (s)	1.29 (s)	1.08 (s)	1.13 (s)	1.09 (s)
6-OH	–	–	8.06 (s)	–	–	–	–
12-OH	–	–	8.29 (s)	7.11 (s)	8.72 (s)	–	5.41 (s)
14-OH	7.12 (s)	7.47 (s)	10.45 (s)	–	–	–	5.57 (s)
16-OH	–	–	–	–	7.00 (d, 5.5)	–	–

[a] 2H.
[b] α position.
[c] β position.
* Overlapped.

Table II. ^{13}C NMR spectral data[a] (δ_C values in ppm) and key HMBC correlations of compounds **1**–**4**.

C	**1**		**2**		**3**		**4**	
	δ_C	HMBC	δ_C	HMBC	δ_C	HMBC	δ_C	HMBC
1	37.3 (t)	$H_{3\beta}$ H_{20}	37.0 (t)	H_{20}	35.2 (t)	–	36.8 (t)	$H_{3\beta}$ H_{20}
2	18.3 (t)	–	18.8 (t)	–	18.1 (t)	–	18.2 (t)	–
3	42.5 (t)	$H_{1\beta}$ H_{18}	41.5 (t)	H_{18}	38.7 (t)	H_{18}	42.4 (t)	$H_{1\beta}$ H_{19}
4	33.1 (s)	H_5 H_{18}	33.5 (s)	H_5 H_{18}	35.4 (s)	$H_{3\beta}$ H_{18}	33.2 (s)	H_5 H_{18}
5	62.1 (d)	$H_{1\beta}$ H_{19} H_{20}	48.8 (d)	H_7 $H_{6\alpha}$ H_{18} H_{20}	136.4 (s)	6-OH H_{18} H_{19}	63.8 (d)	$H_{1\beta}$ $H_{3\beta}$ H_{18}
6	199.8 (s)	H_5	26.5 (t)	H_5	145.2 (s)	6-OH	193.9 (s)	H_5
7	131.1 (d)	–	144.8 (d)	OH H_{11} H_{15}	181.7 (s)	6-OH H_{11}	180.5 (s)	–
8	140.5 (s)	H_{11} H_{15}	130.4 (s)	$H_{6\alpha}$ H_{11} H_{15}	118.9 (s)	H_{11} H_{15}	116.0 (s)	H_{11} $H_{16\alpha}$
9	161.7 (s)	H_7 H_{20}	143.5 (s)	$H_{6\alpha}$	149.1 (s)	H_{20}	160.0 (s)	H_5 H_{20}
10	43.1 (s)	$H_{1\beta}$ H_5	38.4 (s)	H_5 H_{20}	40.2 (s)	H_{11} H_{20}	42.9 (s)	$H_{1\alpha}$ H_5 H_{20}
11	120.0 (d)	–	117.2 (d)	–	109.5 (d)	–	118.3 (d)	
12	181.5 (s)	–	181.8 (s)	OH	144.7 (s)	12-OH H_{11} H_{15}	143.5 (s)	12-OH H_{11}
13	126.4 (s)	14-OH H_{15} H_{16}	127.3 (s)	14-OH H_{15} H_{16}	135.5 (s)	12-OH H_{15} H_{17}	116.8 (s)	H_{11} H_{15}
14	146.4 (s)	14-OH H_{11} H_{15}	162.3 (s)	H_7	150.2 (s)	14-OH H_{11}	152.9 (s)	H_{15} $H_{16\alpha}$
15	26.8 (d)	H_{16} H_{17}	26.6 (d)	H_{16} H_{17}	26.9 (d)	H_{17}	27.6 (d)	H_{17}
16	20.2 (q)	H_{17} H_{15}	20.5 (q)	H_{15} H_{17}	20.8 (q)	H_{17}	73.4 (t)	H_{17}
17	20.2 (q)	H_{16} H_{15}	20.5 (q)	H_{16} H_{15}	21.0 (q)	H_{16} H_{15}	16.3 (q)	$H_{16\alpha}$
18	32.9 (q)	H_5 H_{19}	32.5 (q)	–	29.3 (q)	H_{19}	32.8 (q)	H_5 H_{19}
19	21.7 (q)	H_3 H_5 H_{18}	22.1 (q)	H_5 H_{19}	27.6 (q)	H_{18}	21.6 (q)	H_5 H_{18}
20	26.1 (q)	H_5 H_{11}	21.8 (q)	H_5	37.1 (q)	–	27.2 (q)	H_5 $H_{3\beta}$

[a] Assignments based on DEPT and HSQC spectral data.

Table III. ^{13}C NMR spectral data[a] (δ_C values in ppm) and key HMBC correlations of compounds **5**, **6** and **1A**.

	5		**6**		**1A**	
C	δ_C	HMBC	δ_C	HMBC	δ_C	HMBC
1	39.5 (t)	H_{20}	38.5 (t)	H_{20}	39.4 (t)	–
2	19.6 (t)	–	19.2 (t)	–	18.9 (t)	–
3	41.7 (t)	H_{18}	41.4 (t)	$H_{1\beta}$ H_{18}	42.6 (t)	$H_{1\beta}$ H_{18}
4	33.7 (s)	$H_{3\alpha}$ H_{19}	33.4 (s)	H_5 H_{18}	32.5 (s)	H_5
5	51.1 (d)	$H_{6\beta}$ H_{18}	49.4 (d)	H_{19} H_{20}	62.1 (d)	H_5 $H_{7\alpha}$
6	19.2 (t)	–	36.8 (t)	–	210.6 (s)	H_5 $H_{7\alpha}$
7	27.4 (t)	–	197.4 (s)	$H_{6\beta}$	43.6 (t)	–
8	122.0 (s)	$H_{6\beta}$ H_{11}	119.6 (s)	H_{11}	122.5 (s)	H_{11} $H_{7\beta}$
9	139.9 (s)	H_{11}	152.3 (s)	H_{20}	141.0 (s)	$H_{7\beta}$ H_{20}
10	37.6 (s)	H_{11} H_{20}	38.2 (s)	H_{11} H_{20}	40.6 (s)	H_5 H_{20}
11	112.0 (d)	12-OH	111.0 (d)	–	108.5 (d)	–
12	142.9 (s)	12-OH H_{15}	144.1 (s)	H_{11} H_{15}	141.9 (s)	H_{11}
13	129.9 (s)	H_{17} H_{16}	134.0 (s)	H_{15} H_{17}	131.5 (s)	12-OH $H_{7\alpha}$ H_{16}
14	143.0 (s)	H_{16}	147.4 (s)	H_{11}	142.1 (s)	–
15	44.4 (d)	16-OH H_{16} H_{17}	45.9 (d)	H_{17}	28.1 (d)	H_{16}
16	107.7 (d)	16-OH H_{17}	107.4 (d)	H_{15} H_{17}	20.3 (q)	–
17	17.0 (q)	H_{15} H_{16}	17.7 (q)	H_{15} H_{16}	20.4 (q)	–
18	33.6 (q)	H_{19}	32.8 (q)	H_5 H_3	32.4 (q)	H_{19}
19	22.0 (q)	H_{18}	21.7 (q)	H_3 H_{18} H_5	21.7 (q)	H_5 H_{18}
20	25.8 (q)		23.9 (q)	H_5	26.2 (q)	H_5

[a] Assignments based on DEPT and HSQC spectral data.

(63), 285 (28), 273 (20), 259 (32), 233 (12), 232 (12), 217 (19), 189 (20).

Assay to test the trypanocidal activity

Epimastigote forms of *T. cruzi* (strain isolated in 1967 from an acute pediatric case of Chagas' disease by the late Jose W. Torrealba in Carabobo

State, Venezuela) were grown at 27 °C in liver infusion tryptose (LIT) medium (Difco, Detroit, USA), supplemented with 10 % fetal bovine serum and 2 % w/v of antibiotics (gentamicins and penicillins). Once in the logarithmic phase, they were collected and seeded into 96-well plates (Costar) ($23 \cdot 10^6$ parasites/well) in minimum essential medium (MEM), with and without the test compounds, and incubated at 27 °C during 16 h. Trypomastigote forms were obtained from infected Vero cells as described by Piras *et al.* (1982). Once in the logarithmic phase, they were seeded ($30 \cdot 10^6$ parasites/well) in MEM, with and without the test compounds, and incubated at 37 °C under humidified 5 % CO_2/95 % air atmosphere during 16 h. All assays were carried out three times, three replicates per assay.

Cytotoxicity assay

The cytotoxicity of the isolated abietane diterpenoids was examined against fibroblastic Vero cells (INHRR < ATCC N° CCL-81, Venezuelan Institute of Hygiene, Caracas, Venezuela) as previously described (Cabrera *et al.*, 2005).

MTT assays

Cell respiration, an indicator of cell viability, was assessed by the reduction of 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan. The abietane diterpenoids were dissolved in DMSO, and solutions with phosphate-buffered saline were prepared to obtain different test concentrations ranging from 0.2 to 200 μM. Benznidazole (Roche, São Paulo, Brazil) was also tested as positive control. The final content of DMSO (less than 0.5 %) did not affect the parasite forms or cell viability. 50 μL of each solution were added to the wells and, after the incubation period (16 h) with the compounds, 35 μL of MTT and phenazine methosulfate (PMS) solution (Muelas-Serrano *et al.*, 2000) (2.5 mg/mL of MTT with 0.22 mg PMS/mL) were added to each well, followed by an additional incubation time (75 min). Then 50 μL of sodium dodecyl sulfate solution (20 % in 0.01 M HCl) were added to dissolve formazan crystals, followed by another incubation period (75 min). The sample absorbance was read on a scanning multiwell spectrophotometer (Bio-Rad model 450, Hercules, CA, USA) at 595 nm. Bioactivity of the compounds is expressed as the concentration of the tested compound required to reduce the cell survival fraction to 50 % of the control (IC_{50}).

Results and Discussion

Roots of *Craniolaria annua* were macerated in chloroform to produce a crude chloroform extract. The cytotoxic effect of the chloroform extract was determined by the MTT assay (Muelas-Serrano *et al.*, 2000) using the epimastigote form and benznidazole, a reference compound in cytotoxicity studies against *T. cruzi*, as positive control. The extract showed an IC_{50} value of (5.4 ± 0.5) μg/mL while benznidazole showed (26.5 ± 0.9) μg/mL. So we considered the extract as highly cytotoxic and proceeded with the fractionation through chromatographic methods. The fractionation was monitored by the MTT bioassay and yielded 6 new compounds, **1**–**6**, and two known compounds, ferruginol and stigmasterol.

Compound **1** was isolated as an amorphous yellow solid with the molecular formula $C_{20}H_{26}O_3$ (HREIMS). Its IR spectrum showed absorption bands for a hydroxy (3328 cm^{-1}) and two carbonyl groups (1672 and 1618 cm^{-1}). Three UV maxima at 317, 330 and 403 nm indicated extensive conjugation. The ^{13}C (Table II) and DEPT spectra revealed the presence of 5 methyl, 3 methylene, 4 methine groups (2 sp^2) and 8 quaternary carbon atoms (6 sp^2, including 2 carbonyls). An aromatic carbon bearing a hydroxy group located at C-14 was observed at δ 146.4. The HSQC spectrum allowed the unambiguous assignment of C–H bonding. The ^1H NMR spectrum (Table I) further suggested that **1** is a compound with an abietane skeleton. It showed signals for a isopropyl group at δ_H 1.29 and 1.35 (each 3H, d, J = 7 Hz, H-16 and H-17) and 3.05 (1H, sept, J = 7 Hz, H-15) and three methyl groups at δ_H 1.15 (3H, s, H-18), δ_H 1.24 (3H, s, H-19) and δ_H 1.23 (3H, s, H-20). Downfield signals were observed for a hydroxy group, 14-OH, at δ_H 7.12 (1H, s, D_2O exchangeable) and for two coupled protons (COSY experiment) at δ_H 6.39 (1H, d, J = 1.2 Hz, H-11) and 6.60 (1H, d, J = 1.2 Hz, H-7). The ^1H-^1H COSY spectrum showed the existence of a -CH_2-CH_2-CH_2- spin system due to positions 1, 2 and 3 of ring A. The HMBC experiment showed correlations for δ_H 2.47 (H-5) and a carbonyl signal at δ_C 199.8 (C-6) and correlation for 14-OH resonance at δ_H 7.12 with aromatic signals at δ_C 146.4 (C-14) and

126.4 (C-13). Additionally H-11 showed HMBC correlation with a quaternary carbon atom at δ_C 43.1 (C-10). NOESY experiments showed the spatial proximity between H-11 and H-1β and H-20, supporting the assignment of the OH group at position C-14. The stereochemistry of **1** was confirmed by the NOESY experiment. Therefore compound **1** was assigned as 14-hydroxy-6,12-dione-7,9(11),13-abietatriene (Fig. 1).

The spectroscopic data indicated that **1** is a structural isomer of the diterpene quinone methide taxodione (Kupchan *et al.*, 1968) differing in the OH position. The main difference between the ^{1}H NMR spectrum of the taxodione and **1** was the upfield shift of H-1β to 1.45 ppm which confirmed

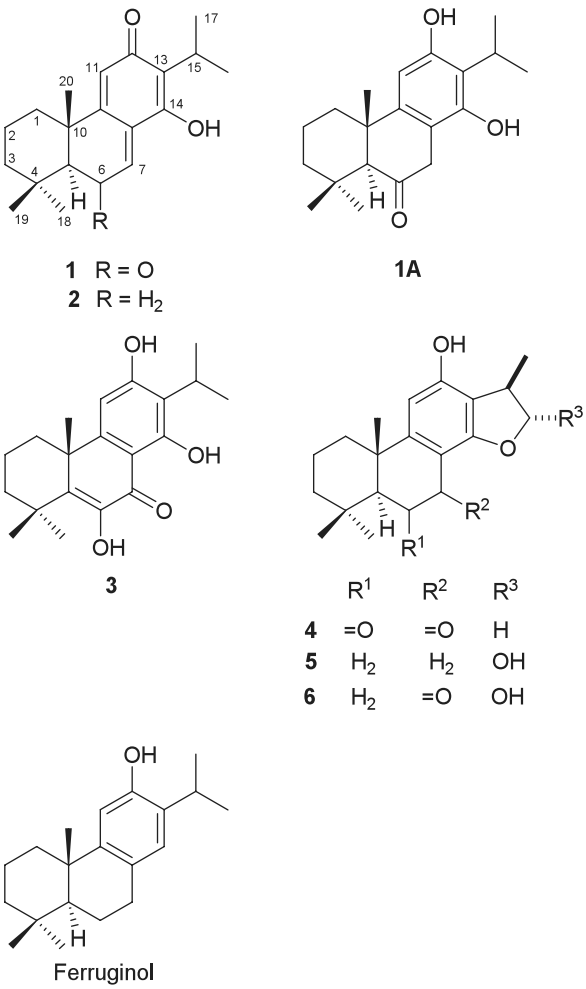

Fig 1. Chemical structures of six novel abietane diterpenoids and ferruginol isolated from *Craniolaria annua.*

that the hydroxy group in **1** was not bound to C-11 (Kuo *et al.*, 1998).

Compound **2** was isolated as yellow oil with the molecular formula $C_{20}H_{28}O_2$ (HREIMS). The infrared spectrum indicated hydroxy (3300 cm^{-1}) and conjugated carbonyl groups (1640, 1612 cm^{-1}) and the UV spectrum the occurrence of an extensive chromophore. The ^{13}C (Table II) and DEPT spectra revealed the presence of 5 methyl, 4 methylene, 4 methine groups (2 sp^2) and 7 quaternary carbon atoms (5 sp^2 including 1 carbonyl). The ^{1}H NMR data of compound **2** (Table I) were similar to those of compound **1** except for the presence of a coupled olefinic proton to be part of a -CH-CH$_2$-HC= spin system due to positions 5, 6 and 7 of ring B. The HMBC spectrum (Table II) showed correlations for the 14-OH group at δ_H 7.47 with a carbonyl group at δ_C 181.8 (C-12) and an aromatic carbon atom at δ_C 127.3 (C-13). An aromatic methine resonance at δ_H 6.30 (H-11) showed HMBC correlation with the quaternary carbon signal at δ_C 38.4 (C-10). These couplings required the placement of the phenolic hydroxy group at C-14 and the carbonyl group at C-12. The assignments were further supported by NOESY correlations (Table IV) for H-11 with H-1β and H-20. The stereochemistry of **2** was confirmed by NOESY experiments. Thus the structure of **2** was determined as 14-hydroxy-12-oxo-7,9(11),13-abietatriene (Fig. 1). Compound **2** is a structural isomer of 11-hydroxy-12-oxo-7,9(11),13-abietatriene, isolated from *Plectranthus elegans* (Dellar *et al.*, 1996).

Compound **3** was isolated as greenish crystals with the molecular formula $C_{20}H_{26}O_4$ (HREIMS). The IR spectrum showed signals for phenol (3537 and 1218 cm^{-1}) and aryl ketone groups (1660 and 1623 cm^{-1}). A UV maximum at 321 nm indicated a conjugated aromatic ring system. The ^{13}C NMR (Table II) and DEPT spectra exhibited resonances for 5 methyl, 3 methylene, 2 methin groups (1 sp^2) and 10 quaternary carbon atoms including a carbonyl group. The ^{1}H and ^{13}C NMR spectra of **3** were similar to that of the abietane 6-hydroxysalvinolone (Topcu and Ulubelen, 1996). However, the signal at δ_H 2.20 (1H, dd, J = 12.1, 7.0 Hz, H-1b) suggested a methine group at C-11 instead of C-14. The HMBC correlations (Table II) between the 6-OH signal at δ_H 8.06 and the resonance for the quaternary carbon atom and carbonyl group at δ_C 136.4 and 181.7, respectively, confirmed the position of the 6-OH group. The NOESY spectrum

(Table IV) allowed the assignment of the stereochemistry of **3**. Additionally it revealed that H-11 is sterically close to H-20 and H-1β. Thus compound **3** was assigned as 6,12,14-trihydroxyabieta-5,8,11,13-tetraen-7-one (Fig. 1).

Compound **4** was isolated as orange powder with the molecular formula $C_{20}H_{24}O_4$ (HREIMS). The infrared spectrum showed phenol peaks (3205 and 1218 cm^{-1}), an aryl-ketone peak (1650 cm^{-1}) and a six-membered ring ketone band (1710 cm^{-1}). The UV maximum at 373 nm indicated an aromatic conjugated system. The ^{13}C (Table II) and DEPT spectra showed the presence of 4 methyl, 4 methylene (1 with an oxygen-bound group), 3 methine groups (1 sp^2) and 9 quaternary carbon atoms (7 sp^2). Two carbonyl carbon atoms at δ_C 193.9 (C-6) and 180.5 (C-7) suggested an α-dicarbonyl moiety. The ^1H NMR spectrum had characteristic signals for an abietane-type compound. A typical methyl dihydrofuran system was observed at δ_H 1.33 (3H, d, J = 7.0 Hz, H-17), 3.24 (1H, dq, 4.4, J = 7.0 Hz, H-15), 4.21 (1H, dd, J = 5.1, 11.0 Hz, H-16a) and 4.31 (1H, dd, J = 4.0, 11.0 Hz, H-16b) (Gonzalez *et al.*, 1992). The signal at δ_H 2.60 (1H, s, H-5) indicated the presence of a carbonyl group at C-6. The assignment of the dihydrofuran ring at C-13 and C-14 was based on HMBC correlations between H-15 and H-17 and a quaternary carbon atom at δ_C 116.8, and H-16α and a quaternary carbon atom at δ_C 152.9. A HMBC correlation between H-5 and carbonyl C-6 corroborated the position of the α-dicarbonyl group. NOESY correlations revealed that H-11 was sterically close to H-20 and H-1β. The stereochemistry and structure of **4** was further confirmed by NOESY. Accordingly, compound **4** was assigned as ar-abietatriene-12-ol-6,7-dione-14,16-oxide.

Compound **5** was isolated as white needles with the molecular formula $C_{20}H_{28}O_3$ (HREIMS). The infrared spectrum showed phenol peaks (3600 and 1197 cm^{-1}). The UV maximum at 288 nm corroborated the presence of an aromatic system. The ^{13}C and DEPT spectra (Table III) revealed 4 methyl, 5 methylene, 4 methine groups (1 sp^2) and 7 quaternary carbon atoms (5 sp^2). An additional feature, not seen in **4**, was a hemiacetal carbon atom at δ_C 107.7 (C-16). The ^1H NMR data for compound **5** suggested an abietane-type skeleton. The ^1H-^1H COSY spectrum showed the coupling between δ_H 1.81 (1H, dd, J = 6.0, 13.2 Hz, H-6β) and δ_H 2.61 (2H, m, H-7). The HMBC correlation between the

Table IV. Key COSY and NOESY correlations of compounds **1**–**6** and **1A**.

H	**1**		**2**		**3**		**4**		**5**		**6**		**1A**	
	COSY	NOESY	COSY	NOESY	COSY	NOESY	COSY	NOESY	COSY	NOESY	COSY	NOESY	COSY	NOESY
1α	$H_{1\beta}$ $H_{2\alpha}$ $H_{2\beta}$		$H_{1\beta}$		$H_{1\beta}$ $H_{3\alpha}$		$H_{1\beta}$		$H_{1\beta}$		$H_{1\beta}$		$H_{1\beta}$	
1β	$H_{2\alpha}$ $H_{2\beta}$ $H_{3\beta}$	H_{11}	$H_{1\alpha}$	H_{11}	$H_{1\alpha}$ $H_{2\beta}$	H_{11}	$H_{1\alpha}$	H_{11}	$H_{1\alpha}$	H_{11}	$H_{1\alpha}$ H_5	H_{11}	$H_{2\alpha}$ $H_{2\beta}$ $H_{3\beta}$	
2α	$H_{2\beta}$ $H_{1\alpha}$ $H_{1\beta}$		$H_{1\alpha}$ $H_{1\beta}$ $H_{3\alpha}$		$H_{2\beta}$		$H_{3\alpha}$		$H_{2\beta}$		$H_{2\beta}$			
2β	$H_{3\beta}$				$H_{2\alpha}$ $H_{1\beta}$				$H_{2\alpha}$		$H_{2\alpha}$			
3α	$H_{3\beta}$		$H_{3\beta}$ H_2		$H_{3\beta}$ $H_{1\alpha}$		$H_{3\beta}$ $H_{2\alpha}$ $H_{2\beta}$		$H_{3\beta}$ H_{19}		$H_{3\beta}$ H_5		$H_{3\beta}$	
3β	$H_{3\alpha}$ $H_{2\beta}$ $H_{1\beta}$		$H_{3\alpha}$		$H_{3\alpha}$		$H_{3\alpha}$	H_{19}	$H_{3\alpha}$ H_{19}	H_{18}	$H_{3\alpha}$ H_5		H_{19}	
5	H_{18}	H_{18}	$H_{6\alpha}$ $H_{6\beta}$	H_{18}				H_{18}	H_{18} $H_{6\alpha}$		H_3 H_6	H_{18}	$H_{7\alpha}$ H_{19} H_{20}	H_{18}
6			$H_{6\alpha}$ $H_{6\beta}$ H_5						$H_{6\alpha}$ $H_{6\beta}$ $H_{7\alpha}$		H_5			
			$H_{6\beta}$ H_5 H_7						$H_{6\beta}$ $H_{6\alpha}$ $H_{7\alpha}$					
7	H_{11}	H_{15} H_{16}	$H_{6\beta}$						$H_{6\beta}$ $H_{6\alpha}$				H_5 H_{11}	
11	H_7	$H_{1\beta}$ H_{20}		$H_{1\beta}$ H_{20}		$H_{1\beta}$ H_{20}		$H_{1\beta}$		$H_{1\beta}$ H_{20}		$H_{1\beta}$ H_{20}		H_{20}
								12-OH		12-OH				
15	H_{16} H_{17}	H_7	H_{16} H_{17}		H_{16} H_{17}		$H_{16\alpha}$ $H_{16\beta}$ H_{17}		H_{17}		H_{17}		H_{16} H_{17}	
16	H_{15}	H_7	H_{15}	14-OH	H_{15}	H_{17}	$H_{16\alpha}$ $H_{16\beta}$ H_{15}		16-OH	H_{15}			H_{15}	H_{17}
							$H_{16\beta}$ $H_{16\alpha}$ H_{15}							
17	H_{15}		H_{15}		H_{15}	H_{16}	H_{15}	$H_{16\alpha}$	H_{15}	H_{16}	H_{15}		H_{15}	H_{16}
18	H_5	H_5		H_5				H_5		$H_{3\beta}$		H_5	H_{19}	H_5
19				H_{20}		H_{20}				H_{20}		H_{20}	$H_{3\alpha}$ H_5 H_{18}	
20		H_{11}		H_{19}		H_{11} H_{19}				H_{11} H_{19}		H_{11} H_{19}	H_5	H_{11}
12-OH								H_{11}		H_{11}				H_{11}
14-OH				H_{16}										
16-OH									H_{16}					

Compound	IC_{50} [μM]		
	Trypomastigotes	Epimastigotes	Vero cells
1A	0.85 ± 0.03	2.1 ± 0.1	5.6 ± 0.1
1	5.6 ± 0.3	5.7 ± 0.3	28.3 ± 0.8
5	5.8 ± 0.2	48 ± 1	90 ± 7
4	20 ± 1	90 ± 5	200 ± 10
3	25 ± 2	69 ± 2	54 ± 2
Ferruginol	52 ± 2	90 ± 5	51 ± 4
Benznidazole	29.3 ± 0.6	123 ± 7	[a]

Table V. Cytotoxicity of compounds **1A**, **1**, **3**–**5** and ferruginol to trypomastigote and epimastigote forms of *T. cruzi* and to fibroblastic Vero cells expressed as inhibitory concentration (IC_{50}). Benznidazole is included as positive control.

[a] Cytotoxic concentration > 0.85 mM.

methine resonance at δ_C 51.1 (C-5) and H-6β confirmed the assignment of the two methylenes C-6 and C-7. The HMBC spectrum revealed correlations between methine δ_C 44.4 (C-15) and δ_H 5.42 (1H, d, J = 4.4 Hz, H-16), δ_H 1.09 (3H, d, J = 7.0 Hz, H-17) and δ_H 7.0 (1H, d, J = 5.5 Hz, 16-OH), which supported the assignments of the cyclic hemiacetal. The NOESY spectrum showed that H-11 was sterically close to H-20. Additionally it confirmed the stereochemistry and structure of **5**. Thus compound **5** was assigned as ar-abietatriene-12,16-diol-14,16-oxide (Fig. 1).

Compound **6** was obtained as pale yellow amorphous powder with the molecular formula $C_{20}H_{26}O_4$ (HREIMS). The infrared spectrum indicated phenol (3300 and 1170 cm^{-1}) and α,β-conjugated carbonyl groups (1664 cm^{-1}). The UV spectrum showed the existence of an extensive chromophore. The ^{13}C and DEPT spectra (Table III) showed the presence of 4 methyl, 4 methylene, 4 methine groups (1 sp^2 and 1 hemiacetal carbon atom) and 8 quaternary carbon atoms. The ^1H NMR spectrum suggested that **6** is an abietane-type compound with a cyclic hemiacetal group as observed in **5**. An AB_2 spin system was observed at δ_H 1.74 (1H, dd, J = 4.0, 13.6 Hz, H-5) and δ_H 2.47 (2H, m, H-6). The HMBC correlation between δ_C 197.4 and H-6 required the placement of the carbonyl group at C-7. NOESY correlations revealed that H-11 was sterically close to H-1β. The stereochemistry and structure of **6** was further confirmed by NOESY. Accordingly, compound **6** was assigned as ar-abietatriene-12-ol-7-one-14,16-oxide.

Compound **1** was subjected to reduction with excess $NaBH_4$ in ethanol at 4 °C. One product was obtained, compound **1A**, which had the molecular formula $C_{20}H_{28}O_3$. The ^1H NMR spectrum of **1A** was very similar to that of **1**, the difference between them being the presence of coupled geminal signals, δ_H 3.40 (1H, d, J = 13.5 Hz, $H_{7\alpha}$) and δ_H 3.40 (1H, d, J = 13.5 Hz, $H_{7\beta}$). One- and two-dimensional NMR data (Tables III and IV) suggested that **1A** was a tautomer of the enol 6,12,14-trihydroxyabieta-6,8,11,13-tetraen isolated from *Salvia napifolia* (Ulubelen *et al.*, 1995). Thus the product **1A** was identified as 14-hydroxy-6-oxoferruginol (Fig. 1); it confirmed the assignments for compound **1**.

Additionally, two known compounds were identified: ferruginol by comparison of the spectroscopic data with literature values (Tsutomu *et al.*, 1983) and stigmasterol by comparison with an authentic reference sample.

The cytotoxicity of compounds **1**, **1A**, **3**–**6**, ferruginol and stigmasterol was assayed against *T. cruzi* epimastigote and trypomastigote forms and fibroblastic Vero cells. The dose-dependent assays were carried out between 0.2 and 100 μM and benznidazole was tested as positive control. Both **6** and stigmasterol were inactive against *T. cruzi* in the tested concentration range. The IC_{50} values of the cytotoxic compounds are shown in Table V. All bioactive compounds showed higher cytotoxicity against trypomastigotes, the infection form in the mammalian bloodstream, than against epimastigotes, the form present in the insect. The antitrypomastigote activity of compounds **1A**, **1**, **5**, **4** and **3** [IC_{50} (0.85 ± 0.03), (5.6 ± 0.3), (5.8 ± 0.2), (20 ± 1) and (25 ± 2) μM, respectively] were higher than that of benznidazole [IC_{50} (29.3 ± 0.6) μM]. The selectivity for the trypomastigotes was higher in relation to the Vero cells for all compounds except for ferruginol which had a similar cytotoxic effect against both organisms [IC_{50} (52 ± 2) μM against trypomastigotes and IC_{50} (51 ± 4) μM against Vero cells].

The reasons for these important cytotoxic effects of the tested compounds are unclear. However, the substitution of C-14 with an oxygen-bearing group seemed to increase the activity in comparison with ferruginol that had the lowest antitrypomastigostic

activity. In a previous study ferruginol has shown cytotoxicity against leukemia cells with an IC_{50} value of 118 μM (Rozalski *et al.*, 2006).

The presence of an alkylating group like a double bond or α,β-conjugated carbonyl system has been shown to be essential for the trypanocidal activity of withanolides (Abe *et al.*, 2006; Guerrero *et al.*, 2006). An alkylation process could explain, at least in part, the cytotoxicity of **3** and **1**. Compound **3** has a carbonyl group at C-7 and a double bond between C-5 and C-6 while **1** has a quinone methide group in ring C. Compound **1** is an isomer of taxodione which is known as a cytotoxic diterpene that may damage DNA (Topcu *et al.*, 2003).

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