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Cytotoxic activity and effect on nitric oxide production of tirucallane-type triterpenes

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Abstract

Hexane extract from the bark of *Amphipterygium adstringens*, as well as its principal constituents, masticadienonic acid (**1**) and 3α-hydroxymasticadienolic acid (**2**), inhibited the growth of five human cancer cell lines. Derivatives of **1**, namely 24,25*S*-dihydromasticadienonic acid (**3**) and masticadienolic acid (**4**), were also evaluated. The results showed that both **3** and **4** had greater activity than **1** on colon cancer cell lines. The effects of **1**–**4** on the production of nitric oxide (NO) from both resting and lipopolysaccharide-activated macrophages were determined. It was found that **1**, **2** and **4** caused an increase in NO release from resting macrophages; in lipopolysaccharide-activated macrophages, only **2** and **4** caused an increase in NO production.

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Introduction

Masticadienonic acid (**1**) (3-oxotirucalla-7,24Z-dien-26-oic acid, terebinthone) and 3α-hydroxymasticadienolic acid (**2**) (3-epimasticadienolic acid or schinol) were isolated as the main constituents of several species, including *Amphipterygium adstringens* (syn. *Juliania adstringens*, trivial name "cuachalalate") (Soriano-García et al 1987; Watson et al 1987; Navarrete et al 1989; Olivera et al 1999). This species has been widely used in traditional medicine as a gastroprotective (Navarrete et al 1998; Arrieta et al 2003), hypocholesterolaemic (Mata et al 1991), anti-inflammatory (Oviedo-Chávez et al 2004), antiprotozoal (Del Rayo et al 2000) and cytotoxic (Makino et al 2004) agent. Many of these properties have been attributed to the presence of **1** and **2**.

Despite the fact that *A. adstringens* has been traditionally used in the treatment of gastrointestinal cancer (Olivera et al 1999), to our knowledge, there is only one study relating to its anticancer properties. Makino et al (2004) reported that seven tirucallane-type triterpenes isolated from the bark of *A. adstringens* inhibited the growth of mouse leukaemia cell lines (L-1210). However, there are no studies dealing with their probable cytotoxic activity in human cancer cell lines. This prompted us to evaluate the cytotoxic activity of the hexane extract, as well as both **1** and **2** on five human cancer cell lines. In addition, in order to determine if the presence of a double bond at C-24/C-25, as well as the carbonyl moiety at C-3, are involved in the cytotoxic activity of **1**,24,25*S*-dihydromasticadienonic acid (**3**) and masticadienolic acid (**4**), both derivatives of **1**, were also evaluated.

It is well known that macrophages play a prominent role in host defence by inducing cellular damage in infectious agents and tumours. One mechanism that the macrophages use to exert their cytotoxic and cytostatic effects on target cells is that of releasing nitric oxide (NO). NO is derived from L-arginine by the enzymatic activity of inducible nitric oxide synthase (iNOS), functioning as an antitumour and antimicrobial molecule in-vitro and in-vivo (Davis et al 2001). Thus, we evaluated the modulation of NO production by **1**–**4** in both resting and lipopolysaccharide (LPS)-activated macrophages.

Materials and Methods

Chemicals

Reagents and cell culture materials were obtained from the following sources: sulforhodamine B (SRB), *Escherichia coli* 055:B5 LPS, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), superoxide dismutase (SOD), RPMI-1640, dimethylsulfoxide (DMSO) and doxorubicin were from Sigma Chemical Co. (St Louis, MO, USA); phosphate-buffered saline, Dulbecco's modified essential medium (DMEM), fetal calf serum (FCS) and amphotericin B were from GIBCO (Grand Island, NY, USA); brewer thioglycollate medium was from BD Bioxon (Becton Dickinson de México). Other reagents were purchased from Sigma Chemical Co. Colon cancer (HCT-15), breast cancer (MCF-7), leukaemia (K-562 CML), central nervous system (U-251 Glio, CNS) and prostate cancer (PC-3) cell lines were supplied by the National Cancer Institute (Bethesda, MD, USA). The reference triterpene, ursolic acid, was isolated from *Salvia mexicana* L. var. *minor* Benth. (Argumedo et al 2003).

Isolation of 1 and 2

Compounds **1** and **2** were isolated from the hexane extract of *A. adstringens* as previously described (Oviedo-Chávez et al 2004). The structures of **1** and **2** are shown in Figure 1.

Preparation of compound 3 (Figure 2)

A suspension of **1** (160 mg, 0.352 mmol) and palladium-carbon 5% (60 mg) in EtOAc (20 mL) was stirred for 48 h, under hydrogen atmosphere. When thin-layer chromatography (hexane/EtOAc 1:1) indicated complete reaction, the mixture was diluted with EtOAc, filtered through Celite, and the filtrate was evaporated under reduced pressure. The residue was chromatographed on SiO_2 (hexane/EtOAc as mobile phase). After the usual workup, compound **3** (88 mg, 55%, mp 140–142°C) was obtained as a colourless solid. The structure of **3** (Figure 1) was established by comparing the physical data obtained with data reported in the literature (Barton & Seoane 1956) and by the analysis of ^{13}C and ^{1}H NMR data.

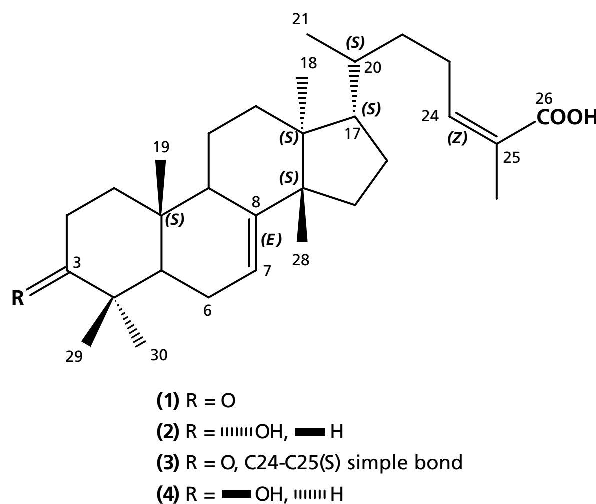

Figure 1 Tirucallane triterpenoid compounds: masticadienonic acid (**1**), 3-α-hydroxymasticadienolic acid (**2**), 24,25*S*-dihydromasticadienonic acid (**3**) and masticadienolic acid (**4**).

Preparation of compound 4 (Figure 2)

A solution of $NaBH_4$ (260 mg) in H_2O (3 mL) was added to a solution of **1** (217.4 mg, 0.598 mmol) in MeOH (20 mL). The mixture was stirred at 20°C for 18 h. It was then diluted with H_2O and extracted with EtOAc. The organic layer was dried and evaporated under reduced pressure. Crystallization from EtOAc afforded a white amorphous powder (234.6 mg, 86%, mp 160–170°C), which was identified as compound **4** (Figure 1) by comparing the spectral and physical data obtained with data reported in the literature (Barton & Seoane 1956; Monaco et al 1974; Papageorgiou et al 1997).

The purity of **1**–**4** was determined by high-performance liquid chromatography analysis under the following conditions: acetonitrile/water (80:20) as mobile phase, flow rate 1 mL min^{-1}, 24 min, UV 220 nm (spectra and chromatograms of compounds **1**–**4** are not shown).

Cell culture and assay for cytotoxic activity

The cytotoxic activity of **1**–**4** and the hexane extract on tumour cells was determined using the protein-binding dye SRB (Monks et al 1991). Cell lines were cultured in RPMI-1640, supplemented with 10% FCS, 2 mM L-glutamine, 100 IU mL^{-1} penicillin G, 100 $\mu g\,mL^{-1}$ streptomycin sulfate, and 0.25 $\mu g\,mL^{-1}$ amphotericin B. They were maintained at 37°C in a 5% CO_2 atmosphere. For the assay, 100 μL/well of 5×10^4 cell mL^{-1} (K562, MCF-7), 7.5×10^4 cells/well (U251, PC-3) and 10×10^4 cells/well (HCT-15) were seeded in 96-well microtitre plates and incubated for 24 h. In addition, 100 μL of each test compound (hexane extract, compounds **1**–**4**), ursolic acid and doxorubicin (reference substances) were added to the respective wells. All the test substances were dissolved in DMSO. The equivalent volume of DMSO added to control dishes had no effect on the cell lines.

After 48 h, adherent cell cultures were fixed in-situ by adding 50 μL of cold 50% (wt/vol) trichloracetic acid and incubated for 60 min at 4°C. The supernatant was discarded and the plates were washed three times with water and air-dried. Cultures fixed with trichloracetic acid were stained for 30 min with 100 μL of 0.4% SRB solution. Protein-bound dye was extracted with 10 mM unbuffered Tris base and the optical density (OD) was read on a microplate reader (El_x 808; Bio-Tek Instruments, Inc., Winooski, VT, USA) with a test wavelength of 515 nm. Percentage growth inhibition was calculated by the following equation: growth inhibition (%) $= 100 - [B \times 100/A]$, where A is the OD of cells only, and B is the OD of cells plus test sample. A dose–response curve was plotted for each compound, and the 50% inhibitory concentration (IC50) was estimated from

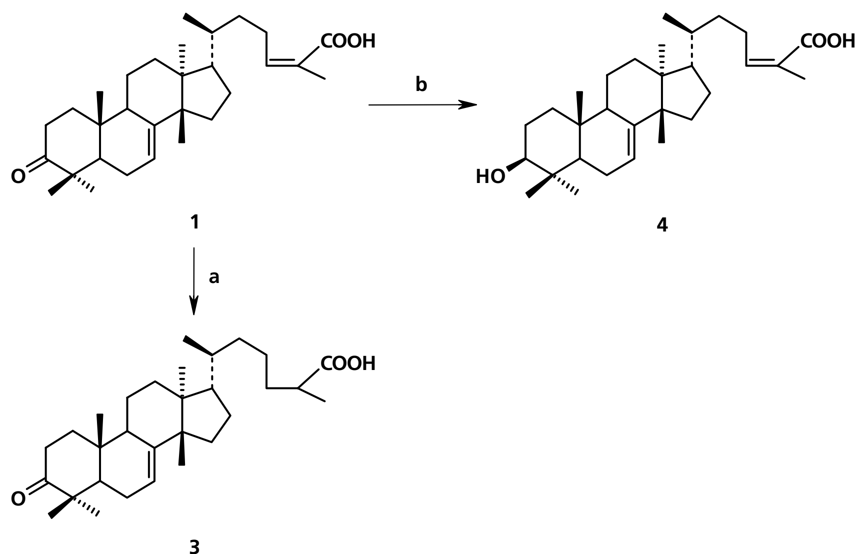

Figure 2 Reactions of masticadienonic acid (**1**) to produce 24,25*S*-dihydromasticadienonic acid (**3**) (55% yield), and masticadienolic acid (**4**) (86% yield). a, 5% Pd-C/EtOAc; b, $NaBH_4$/MeOH.

non-linear regression equations using JMP software (version 3.2.1.; SAS Institute Inc., Cary, NC, USA).

Determination of NO concentration

Isolation and culture of primary peritoneal macrophages (Fushiya et al 1998)

Swiss female mice, 25–30 g, were treated in accord with the Animal Care and Use Committee (PROY-NOM 087-ECOL-SSA1–2000). Mice were injected intraperitoneally with 1 mL of 3% (wt/vol) thioglycollate 3 days before harvesting. Peritoneal exudate cells were harvested, washed and suspended in DMEM. Peritoneal exudate cells were seeded into 24-well plates (Becton Dickinson, Oxnard, CA, USA) at a concentration of 1×10^6 cells mL^{-1}, and then incubated for 2 h at 37°C in a 5% CO_2 incubator. Non-adherent cells were washed off and cultured in DMEM supplemented with 10% FCS. Cells were treated for 24 h at 37°C, with 60 U mL^{-1} SOD, with or without 10 μg mL^{-1} LPS, both in the absence and presence of test compounds (**1**–**4**) and ursolic acid. Concentrations of 0.001–10 μM for **1**–**4**, and 0.001–1 μM for ursolic acid were used. All the test substances were dissolved in EtOH. The final concentration of EtOH in the cell culture supernatant was $\leq$0.1%. This concentration did not have any effect on the assays.

Nitrite production in peritoneal macrophages (Dirsch et al 1998)

Nitrite accumulation was determined by mixing equal volumes (100 μL each) of cell culture medium and Griess reagent (1% sulfanilamide/0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride/5% H_3PO_4) at 20°C for 10 min, and the OD was read on a microplate reader at 515 nm. The nitrite concentration (μM) was determined by interpolation of standard curves constructed with known concentrations of $NaNO_2$.

Cell viability

Cell respiration, an indicator of cell viability, was determined in the remaining macrophages by the modified MTT method (Mosmann 1983). Briefly, 10 μL of MTT (5 mg mL^{-1} in phosphate-buffered saline) was added to the remaining cells in 96-well plates and incubated for 4 h at 37°C. Incorporated dye was dissolved in DMSO (100 μL). Absorbance was measured on a microplate reader at 515 nm. Results were expressed as the percentage of MTT incorporated into treated cells compared with control cells.

Statistical analysis

All experiments were performed at least three times in four replicates. For the cytotoxicity assay, IC50 results are presented as the mean ± s.e. For multiple comparisons, a one-way analysis of variance was carried out followed by post-hoc Dunnett's test. For NO production, results are presented as mean ± s.e. For multiple comparisons, a one-way analysis of variance was carried out, followed by post-hoc Dunnett's test. Values of $P < 0.05$ and $P < 0.01$ were considered significant. Statistical analysis was carried out using JMP software (version 3.2.1.; SAS Institute Inc.).

Results and Discussion

Cytotoxic activity

Compounds **1** and **2** were isolated and identified as previously described (Oviedo-Chávez et al 2004). Evaluation

Table 1 Cytotoxic effects of hexane extract (IC50 μg mL^{-1}), compounds **1**–**4** and reference drugs (IC50 μM) on five cancer cell lines

Compound	HCT-15 (Colon)	MCF-7 (Breast)	U251 (CNS)	PC-3 (Prostate)	K562 (Leukaemia)
Hexane extract	13.7 ± 1.0	7.9 ± 1.9	35.3 ± 1.5	38.3 ± 2.0	8.4 ± 0.1
1	42.1 ± 6.4	18.4 ± 1.0	20.4 ± 2.2	27.4 ± 3.9	36.8 ± 9.4
2	27.6 ± 6.0	31.5 ± 6.8*	31.6 ± 5.4*	29.2 ± 0.3	24.2 ± 0.7*
3	22.9 ± 5.2*	26.0 ± 2.9*	48.2 ± 13.5*	29.2 ± 2.9	33.1 ± 5.6
4	20.2 ± 8.4*	28.0 ± 14.0	29.1 ± 6.8	32.6 ± 4.0	24.9 ± 6.3
Ursolic acid	17.5 ± 6.8	17.5 ± 1.1	15.1 ± 4.9	30.5 ± 0.5	17.2 ± 2.3
Doxorubicin[a]	0.23 ± 0.01	0.14 ± 0.01	0.09 ± 0.02	0.32 ± 0.02	0.28 ± 0.01

1, Masticadienonic acid; **2**, 3-α-hydroxymasticadienolic acid; **3**, 24,25*S*-dihydromasticadienonic acid; **4**, masticadienolic acid. Results are expressed as the mean ± s.e., n = 3–5. *$P < 0.05$ compared with **1** (Dunnett's test). [a]Data from Martínez et al (2002).

of the cytotoxic activity of the hexane extract and **1**–**4** was performed by the SRB test on five tumour cell lines. The IC50 values are shown in Table 1. The hexane extract was more effective against breast cancer and leukaemia cell lines (IC50: 7.9 ± 1.9 and 8.4 ± 0.1 μg mL^{-1}, respectively) than the other cell lines tested. Compound **1** was most active on breast and CNS cancer cell lines (IC50: 18.4 ± 1.0 and 20.4 ± 2.2 μM, respectively), and **2** was effective on leukaemia cell lines (IC50: 24.2 ± 0.7 μM). To determine if the presence of the double bond at C-24/C-25 and the carbonyl moiety at C-3 were involved in the cytotoxic activity of **1**, compounds **3** and **4** (derivatives of **1**) were also evaluated (Figure 2; Table 1).

On colon cancer cell lines, both **3** (IC50 22.9 ± 5.2 μM) and **4** (IC50 20.2 ± 8.4 μM) inhibited growth by roughly 2-fold compared with the parent compound **1** (IC50: 42.1 ± 6.4 μM). However, **1** was more active on breast cancer (IC50: 18.4 ± 1.0 μM) and CNS cancer (IC50: 20.4 ± 2.2 μM) cell lines than compounds **2**–**4**. Although the presence of the hydroxyl group at C-3, as in **3**, or the sp^3 bond in C-24/C-25, as in **4**, could be important for the cytotoxic effect on the colon cancer cell line, the presence of these moieties are not important for the cytotoxic activity of **1** on breast cancer and CNS cancer cell lines.

On leukaemia cells, **2** and **4** were more active (IC50: 24.2 ± 0.7 μM and 24.9 ± 6.3 μM, respectively) than **1** and **3**. The cytotoxic effects of **1**–**4** on prostate cancer cell lines were not statistically different. Furthermore, the cytotoxic effects of epimers **2** and **4** were similar on the five tumour cell lines. Hence, the configuration of the hydroxyl group at C-3 was not considered relevant.

Ursolic acid was used as the reference drug since it is one of the best known pentacyclic triterpenes with cytotoxic and antiproliferative properties (Chattopadhyay et al 2002). Our results are in agreement with those previously published (see Table 1).

Table 2 Effects of compounds **1**–**4** and ursolic acid on the accumulation of nitrite in cell culture supernatants of mouse peritoneal macrophages

Compound	Dose (μM)	NO (μM)	
		Without LPS	With LPS
Control		24.4 ± 1.4	44.3 ± 1.7
1	0.001	42.7 ± 2.8**	54.3 ± 0.6
	0.01	38.4 ± 3.0**	54.7 ± 4.9
	0.1	35.9 ± 0.9*	47.2 ± 3.2
	1.0	39.2 ± 1.2**	54.5 ± 4.5
	10.0	40.1 ± 4.1**	51.1 ± 5.7
2	0.001	39.0 ± 0.3**	61.8 ± 2.7**
	0.01	39.4 ± 0.9**	58.0 ± 1.6*
	0.1	38.5 ± 2.6**	53.4 ± 5.2
	1.0	42.6 ± 4.5**	53.6 ± 1.8
	10.0	34.7 ± 2.6*	47.2 ± 4.9
3	0.001	30.5 ± 3.4	44.2 ± 4.2
	0.01	30.8 ± 4.2	45.5 ± 3.8
	0.1	27.6 ± 2.5	45.1 ± 2.5
	1.0	29.5 ± 3.7	45.7 ± 4.3
	10.0	26.4 ± 1.8	33.3 ± 3.7*
4	0.001	36.9 ± 5.7	50.8 ± 2.4
	0.01	36.3 ± 4.0	51.6 ± 2.2
	0.1	39.8 ± 2.4*	52.0 ± 2.4
	1.0	38.2 ± 3.1*	54.6 ± 3.0*
	10.0	36.7 ± 4.0	50.3 ± 1.9
Ursolic acid	0.001	33.8 ± 1.8	48.1 ± 5.8
	0.01	35.9 ± 1.8*	54.5 ± 4.4
	0.1	38.7 ± 0.9**	54.0 ± 5.1
	1.0	38.4 ± 3.6**	51.3 ± 3.9
	10.0	28.9 ± 2.3	38.0 ± 13.7

1, Masticadienonic acid; **2**, 3-α-hydroxymasticadienolic acid; **3**, 24,25*S*-dihydromasticadienonic acid; **4**, masticadienolic acid. LPS, lipopolysaccharide. Results are expressed as the mean ± s.e., n = 3–5. *$P < 0.05$ and **$P < 0.01$, significantly different compared with the control (Dunnett's test). Cell viability for triterpenes **1**–**4** was up to 95%; ursolic acid only showed cytotoxicity above 80% at 10 μM (MTT test).

NO production in peritoneal macrophages

We evaluated the effects of **1**–**4** on the production of nitric oxide from mouse peritoneal macrophages (Table 2). Our results showed that **1**, **2** and **4** significantly enhanced the nitrite release from resting macrophages. However, the effects shown by the different concentrations of **1** tested were not significantly different; the same was true for **2** and **4**. These results indicated a constant production of

NO, at least for **1**, **2** and **4** at the concentrations tested. Compound **3** did not show any significant effect. These results indicate that the sp^2 bond at C-24/C-25 in the triterpenoid structure could be required for the up-release of NO from resting macrophages.

The effects of **1–4** on NO production by LPS-treated macrophages were also evaluated. Macrophages showed enhanced nitrite release when they were activated with LPS (NO concentration: $44.3 \pm 1.7\ \mu\text{M}$). However, only **2** and **4** showed significant enhanced effects. Also, in both cases, the NO production was almost constant. These results suggest that a hydroxyl group at C-3 or double bond in C-24/C-25 could be necessary for the enhancement of NO production in LPS-activated cells.

Although the amount of the NO release by LPS-treated cells was significantly different in resting cells, the NO production was increased in both cases. A different behaviour was shown by ursolic acid, which stimulates the NO release in resting macrophages (You et al 2001) and reduces the NO production by LPS-activated macrophages (Suh et al 1998).

Nevertheless, in our hands, ursolic acid did not show any significant effect on nitrite release by LPS-activated macrophages (Table 2). This may indicate that at low concentrations, such as we used in the present study, the reduction of NO production by ursolic acid does not occur. However, the discrepancy may also be attributed to the different experimental conditions used here compared with those previously reported, such as different animal type, number of macrophages tested, and the use of SOD enzyme. The inclusion of this enzyme was meant to avoid the presence of the superoxide anion that could be produced when the macrophages are activated, avoiding the reaction with NO to generate peroxynitrite, which may also be responsible for the Griess reaction.

In conclusion, the hexane extract of *A. adstringens*, as well as its principal constituents **1** and **2**, showed moderate cytotoxic activity against several human cancer cell lines. In addition, we found that **1** and **2**, as well the derivative **4**, enhanced the NO production by both resting and LPS-activated macrophages in-vitro. These proprieties could account for the use of *A. adstringens* in Mexican traditional medicine.

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