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Modified secoiridoid from *Acicarpha tribuloides* and inhibition of nitric oxide production in LPS-activated macrophages

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Abstract

Bioassay-guided fractionation of *Acicarpha tribuloides* Juss. resulted in the isolation of an uncommon non-glycosylated secoiridoid, tribulolide (**1**), two known secoiridoid glycosides named secologanic acid (**2**) and vogeloside (**3**) as well as two natural chromones, 6,7-dimethoxychromone (**4**) and 7-hydroxy-6-methoxy-chromone (**5**). Compounds **1**–**3** showed inhibition of nitric oxide production in lipopolysaccharide-activated macrophages; their activity is comparable to that of aminoguanidine, a classic inhibitor.

Keywords: *Acicarpha tribuloides*; Calyceraceae; Inhibition nitric oxide production; Macrophages; Secoiridoids; Tribulolide

1. Introduction

Macrophages are important cell effectors involved in antigen presentation and in microbicidal and tumoricidal activities. Early in the innate immune response macrophages synthesize bioactive molecules that orchestrate the inflammatory reaction (Nathan, 1987). Lipopolysaccharides (LPSs) and cytokines, including tumor necrosis factor-α (TNF-α), interleukin-1 and tumor necrosis factor (TNF-γ), activate macrophages through the classic pathway, stimulating production of inflammatory mediators and upregulating expression of inducible nitric oxide synthase (iNOS). This enzyme catalyses nitric oxide (NO) production from L-arginine, and the cytotoxic, microbicidal and tumoricidal effects of macrophages are associated with NO and other short-lived nitrogenated molecules (Rodriguez-Galán et al., 2001). Overproduction of NO has also been associated with oxidative stress (Ji et al., 1999; Sies and Mehlhorn, 1986) and with the pathophysiology of various diseases, such as arthritis, diabetes, stroke, septic shock, chronic inflammation and neurodegenerative and autoimmune diseases (Carreras et al., 2002; Akaike and Maeda, 2000; Szabó, 2000; Schmidt and Walter, 1994; Moncada et al., 1991). Because of the pivotal role of NO in regulation of the immune response, a significant effort has been focused on developing therapeutic agents that regulate its production and secretion (Poderoso et al., 1999). Moreover, natural product sources have yielded interesting, novel inhibitors (Lee et al., 2005; Kita et al., 2002; Wang et al., 2002; Green et al., 1996).

As part of a program aimed at the discovery of new inhibitors of production of NO in LPS-activated macrophages, an extract of *Acicarpha tribuloides* Juss. (Calyceraceae) was selected after a screening of several endemic

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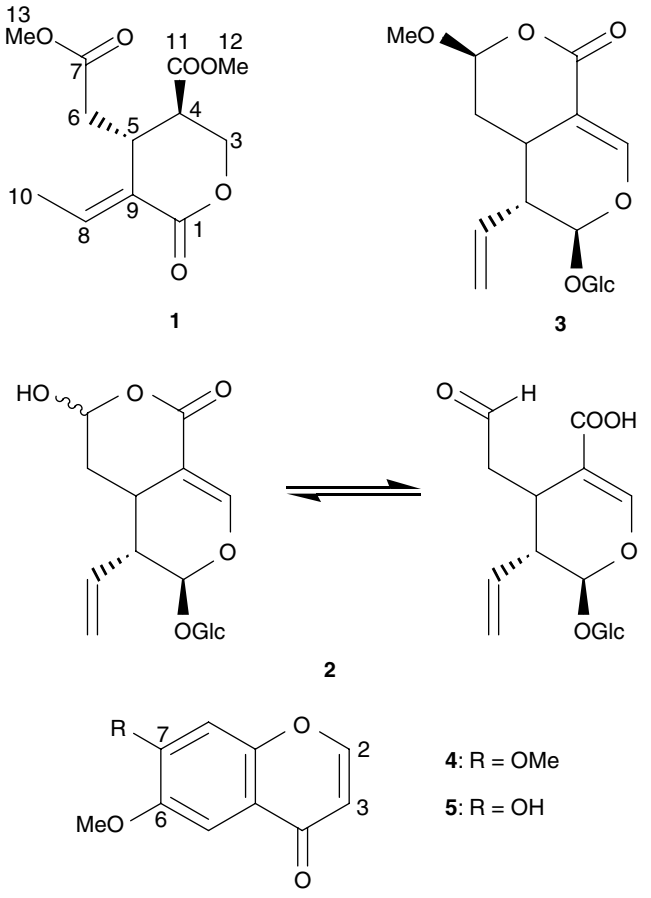
Argentinian plant extracts. *A. tribuloides* is an annual herb that grows in north and central Argentina, and it is reputed to be a medicinal plant (Chiapella, 1999), used in folkloric medicine to treat respiratory and urinary tract infections (Capasso et al., 1996). Previous studies of *A. tribuloides* showed analgesic and spasmolytic activity of the polar extracts. Five iridoids and iridoid diglycosides were isolated from these extracts. However, the activity of the pure compounds was not tested (Capasso et al., 1996). Screening of the flavonoids present in this species has also been published (Bohm et al., 1995).

The methanol extract of *A. tribuloides* inhibited production of NO in LPS-activated macrophages. Bioassay-guided fractionation of this extract led to isolation of a new modified secoiridoid called tribulolide (**1**), two known secoiridoid glycosides, secologanic acid (**2**) and vogeloside (**3**), together with two new natural chromones (**4** and **5**). The inhibitory activity of the pure compounds **1**–**3** toward NO production is also reported.



2. Results and discussion

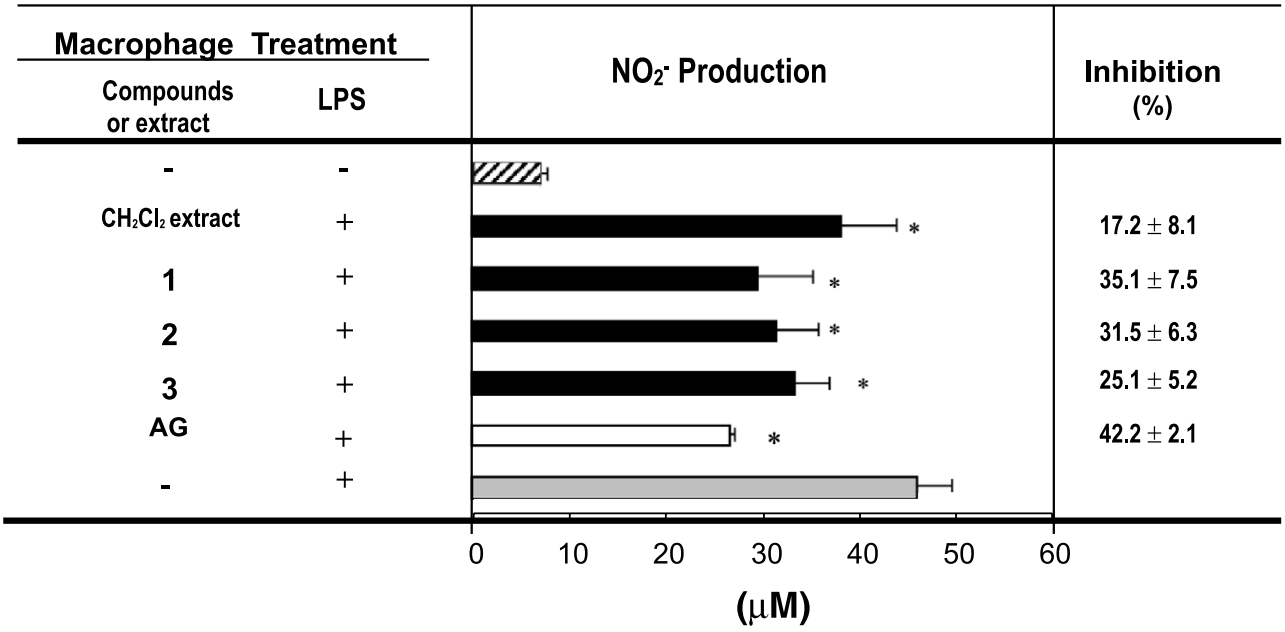
Tribulolide (**1**) have a molecular formula of $C_{12}H_{16}O_6$ as revealed by HREIMS, and also showed an UV absorption maximum at 216 nm, suggesting the presence of an α,β-unsaturated carbonyl group. Although the IR spectrum had a very strong single carbonyl absorption at 1733 cm^{-1}, the ^{13}C NMR spectrum (Table 1) showed three carbonyl resonances at δ 166.4 (α,β-unsaturated-γ-lactone), δ 171.1 and δ 171.2 (ester), respectively. The presence of the conjugated double bond was supported by the resonances at δ 142.1 and δ 128.5 in the ^{13}C NMR spectrum, and the IR absorptions at 1638 and 735 cm^{-1}. Apart from the carbonyl and the sp^2 carbon resonances, the combined analysis of the ^{13}C NMR spectrum and the DEPT-135 experiment identified two methoxy groups, one carbinolic methylene, two aliphatic methines, one aliphatic methylene, and one aliphatic methyl group. The ^1H NMR spectrum and the COSY-90 experiment showed an allylic methyl signal at δ 1.91 (H-10) coupled to an olefinic proton resonance at δ 7.03 (H-8); two methoxy group signals at δ 3.69 and δ 3.76; and methylene proton resonances at δ 4.36 (1H, *dd*, $J = 11.7$ and 6.9 Hz) and δ 4.50 (1H, *dd*, $J = 11.7$ and 4.0 Hz), which were coupled to a methine proton signal resonating at δ 3.08 (H-4). This proton was, in turn, coupled to the CH signal at δ 3.72 (H-5), which was also coupled to the non-equivalent CH_2 protons located at δ 2.60 (H-6a) and δ 2.56 (H-6b). The methylene protons H_2-6 appeared as a strongly coupled AB system at 600 MHz, but were not resolved at 200 MHz. The H-5 multiplet showed long-range coupling with the signals corresponding to H_3-10 and H-8. The resonance of H-5 was not well defined because it overlapped with the signal corresponding to one of the methoxy groups. The relative stereochemistry of **1** was determined by application of NOESY. The signal of both H-6 methylene protons showed NOE cross-peaks with the resonances of H-4 and H-3a (α). The NOESY spectrum not only confirmed the relative stereochemistry of C-4 and C-5 but also indicated the geometry of the double bond. The signal of H_3-10 showed NOE cross-correlation peaks with H-5 and H_2-6, while H-8 did not show correlation with any of the other resonances, indicating the *E* configuration of the double bond. HSQC and

Table 1
^1H and ^{13}C NMR spectroscopic data for compound **1** in $CDCl_3$[a] recorded at 600 and 150 MHz, respectively

Position	δ_H (int., mult., *J* in Hz)	δ_C[b]	HMBC
1		166.4 *s*	
3a (α)	4.50 (1H, *dd*, $J = 11.7$; 4.0)	65.3 *t*	C-1, C-5, C-11
3b (β)	4.36 (1H, dd, $J = 11.7$; 6.9)		
4	3.08 (1H, *ddd*, $J = 11.0$; 6.9; 4.0)	43.1 *d*	
5	3.72 (1H, *m*)	32.1 *d*	C-4, C-6
6	2.60 (1H, *d*, $J = 5.4$)	37.5 *t*	C-4, C-5, C-7, C-9
	2.56 (1H, *dd*, $J = 5.4$; 2.1)		
7		171.1 *s*	
8	7.03 (1H, *dq*, $J = 7.3$; 1.4)	142.1 *d*	
9		128.5 *s*	
10	1.91 (3H, *d*, $J = 7.3$)	14.1 *q*	C-8, C-9
11		171.2 *s*	
12	3.69 (3H, *s*)	52.0 *q*	C-11
13	3.76 (3H, *s*)	52.5 *q*	C-7

[a] TMS as internal standard.
[b] Multiplicities of carbon signals were determined by DEPT.

Table 2
Effect of *Acicarpha tribulolides* compounds and extract on NO production in LPS-activated macrophages



Normal peritoneal macrophages were pretreated with compounds or extract during 2 h and then activated with LPS (1 μg/ml) for 48 h. After this period, supernatants were collected and assayed for NO production by Griess reaction. Results are given as the means ± SD of at least six independent wells. Results of three different experiments were pooled.

*, Difference between the treatment and LPS-activated macrophages, $p < 0.05$.
AG: aminoguanidine as positive inhibition control in this system (Correa et al., 2003).

HMBC experiments allowed the full and unambiguous assignment of all the proton and carbon NMR signals. The most relevant HMBC correlations are included in Table 1. Most of the secoiridoids occur in nature as glycosides (Boros and Stermitz, 1991; El-Naggar and Beal, 1980). However, tribulolide (**1**) presents the unique structural feature of having a hemiketal function oxidized to a lactone.

Secologanic acid (**2**) and vogeloside (**3**) were previously isolated from *Anthocleista vogelii* (Chapelle, 1976); their structures were elucidated by spectroscopic means and the data were coincident with those recorded in the literature. Compounds **4** and **5** were identified as 6,7-dimethoxychromone (**4**) and 7-hydroxy-6-methoxy-chromone (**5**) by comparison of the spectroscopic data with those already published for the synthetic compounds (Romussi and Ciarallo, 1976). This is the first report of chromones in the genus and the first report of chromones **4** and **5** as plant natural products.

The NO inhibitory activity of the pure compounds was measured in purified rat macrophages stimulated by LPS. As shown in Table 2, non-stimulated macrophages produced a basal level of nitrite. In this system, the maximum production of the reactive nitrogen intermediate was observed 48 h after stimulation with LPS and the level of NO increased eightfold compared with the basal value ($p < 0.05$). The pretreatment of monolayer macrophages during 2 h with CH_2Cl_2 extract inhibited LPS-induced NO release by 17.2%. When the activity of three of the pure compounds was evaluated, the NO production was inhibited 35.1% by **1**, 31.5% by **2** and 25.6% by **3** ($p < 0.05$ as compared to macrophages + LPS alone) (Table 2). Interestingly, the maximum inhibition obtained with the pure compounds (~30–35%) was comparable to the effect of aminoguanidine, an specific NO inhibitor in this system (~40%) (Correa et al., 2003). When the compounds were added to macrophages without LPS pretreatment, they had not effect on NO production. The cell viability during the culture was greater than 95%, showing that these compounds were not toxic to the cells.

3. Concluding remarks

The results reported disclose a group of iridoids inhibiting production of NO at a level comparable to that of aminoguanidine, a classical NO inhibitor. Natural products may provide not only novel and potent inhibitors for NO production, but also they may serve as templates that can be modified to improve their pharmacological activity for use in therapeutics.

4. Experimental

4.1. General experimental procedures

IR spectra were obtained on an ATI Mattson Infinity Series FTIR spectrophotometer. The optical rotation

values were obtained on a Jasco P-1010 polarimeter. The 1D and 2D (COSY, NOESY, HSQC and HMBC) NMR experiments for compound **1** were recorded in $CDCl_3$, and they were collected by one of us (RRG) on a Demo Bruker Advance DRX-600, operating at 600.13 MHz for ^1H and 150.13 for ^{13}C (Bruker BioSpin, GmbH, Silberstreifen 4, 76287, Rheinstetten, Germany). The 1D ^{13}C NMR and DEPT-135 spectra of compound **1** were collected on a Bruker AC-200 at 50.32 MHz. Tetramethylsilane (TMS) was used as internal standard. The UV–Visible spectra were run on a Shimadzu UV-260 spectrophotometer using MeOH as solvent. HREIMS data were obtained with a Varian Mat CH-5 mass spectrometer. Chromatographic separations were achieved by column CC using silica gel 60 (40–63 μm, Merck). Preparative TLC was performed on silica gel 60 G F254, 16×5 cm (L × H) plates, 0.2 mm thick, 15 mg maximum sample loading. Analytical TLC was performed on precoated silica gel 60 F254 plates (Merck) and detection was achieved by spraying with sulfuric acid in EtOH, followed by heating. All solvents were distilled before use.

4.2. Plant material

Acicarpha tribuloides was collected in Pampa de Achala, Córdoba, Argentina, in April 2001 and it was identified by Dr. Luis Ariza Espinar. A voucher specimen has been deposited at the Museo Botánico (COR), Universidad Nacional de Córdoba, Argentina, under No. 846.

4.3. Extraction and isolation

The air-dried powdered whole plants (612.8 g) were extracted with MeOH (3×1.5 l) at room temperature; the extracts were pooled and the solvent was evaporated in vacuo (<40 °C). The crude dry extract (53.6 g) was partitioned with hexane–MeOH–H_2O (4:4:1). The MeOH from the 'hydroalcoholic' layer was evaporated under reduced pressure and the resulting water fraction was successively extracted with Et_2O, CH_2Cl_2 and EtOAc. The organic solvents were evaporated to dryness and the dry extracts were tested in the NO inhibitory assay according to the protocol described below. The NO inhibitory activity for each extract was: Et_2O, 14.4%; CH_2Cl_2, 17.2%; EtOAc, 11.3% and aqueous, 14.8%. The active extract, CH_2Cl_2 (971.2 mg), was subjected to silica gel CC eluting with a step gradient of CH_2Cl_2–EtOAc (9:1; 7:3; 1:1). The combined fractions rendered four subfractions: S-1 consisted of pure compound **1** (4.0 mg); prep-TLC of S-2 (11.5 mg) with 1:1 CH_2Cl_2–EtOAc allowed the isolation of compounds **1** (8.1 mg) and **4** (2.6 mg); purification of S-3 (7.0 mg) by prep-TLC with 9:1 CH_2Cl_2–EtOAc (×3 developments) allowed the isolation of **4** (1.1 mg) and **5** (0.9 mg); S-4 (513.9 mg) was fractioned by CC over silica gel with a step gradient of CH_2Cl_2–acetone (1:1 to 1:4), subfraction S-4-38-15 gave compound **2** (37.6 mg); S-4-38-22 was purified by silica gel CC eluting with CH_2Cl_2–MeOH (87:13) yielding compound **3** (7.0 mg).

4.3.1. Tribulolide (*1*)

White amorphous powder; $[\alpha]_D^{25} - 8$ ($CHCl_3$, *c* 1.08); UV λ_{max}^{MeOH} nm: 216; IR ν_{max}^{KBr} cm^{-1}: 2917, 2849, 1733, 1638, 1273, 1152, 735 cm^{-1}; for ^1H NMR and ^{13}C NMR spectroscopic data, see Table 1; HREIMS *m/z* 256.0941 (calcd for $C_{12}H_{16}O_6$, 256.0947).

4.4. Animals

Female 8- to 12-week-old Wistar rats (weighing 150–200 g) were used in this study. Animals were housed and cared for at the Animal Resource Facilities, Department of Clinical Biochemistry, College of Chemical Sciences, National University of Córdoba, in accordance with institutional guidelines.

4.5. Cell preparation and stimulation procedures

Peritoneal cells were harvested by sterile lavage of peritoneal cavity with 25 ml RPMI without phenol red (Sigma), supplemented with 0.1% gentamicin and heparin (50 U/ml), washed twice and resuspended in the same medium supplemented with 10% (v/v) heat-inactivated FCS. To obtain macrophage-rich cultures, peritoneal cells were incubated in 96-well plastic tissue culture plates (TPP) for 24 h at 37 °C in humidified atmosphere of 5% CO_2 in air (2×10^6 cells/ml, 200 μl/well). Non-adherent cells were removed by three washes with warm RPMI 1640 medium and discarded. The resultant macrophage monolayers showed 96% purity, according to morphologic analysis by May Grundwald-Giemsa, or non-specific esterase (Sigma). Viability assessed by trypan blue exclusion test was always higher than 90% (Rabinovich et al., 1998).

To determine the effect of the different samples on the NO production, the macrophage monolayers were incubated with vehicle or different compounds at 37 °C for 2 h, washed and activated with 1 μg/ml LPS (LPS from *Escherichia coli* serotype 055:B5, Sigma, St. Louis, MO) during 48 h. The supernatant was collected and stored at −80 °C until used. Cell viability was assessed by the trypan blue exclusion test at different times of the cell culture treatment. The final concentration of extracts and pure compounds was fixed at 50 μg/ml.

4.6. NO determination

To measure the nitrite (NO_2^-) concentration, 100 μl of macrophage culture supernatant was mixed with an equal volume of Griess reagent (1% sulfinilamide/0.1% *N*-(naphthyl)-ethylenediamine dihydrochloride/2.5% H_3PO_4) and incubated 15 min at room temperature. Nitrite concentration was determined by measuring the absorbance at 540 nm in a microplate reader (BioRad, Richmond, CA);

$NaNO_2$ was used for external calibration (Rodriguez-Galán et al., 2003).

4.7. Statistical analysis

Statistical significance and differences between groups were determined by analysis of variance and Bonferroni test. Each point represents the means ± SD.

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