Sesquiterpene Lactones from *Dimerostemma* Species (Asteraceae) and *in vitro* Potential Anti-Inflammatory Activities

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Two Brazilian species of *Dimerostemma* (Asteraceae) were chemically investigated. Two known sesquiterpene lactones (STLs), a germacrolide and an eudesmanolide, were isolated from *D. episcopale* while *D. brasilianum* afforded the new germacranolide 1β,5β,10α-trihydroxy-8α-angeloyloxy-germacra-3,11(13)-dien-6α,12-olide in addition to a known one. Structure identification based on NMR and MS analyses. 1β,10α,4α,5β-Diepoxy-8α-angeloyloxy-costunolide isolated from *D. brasilianum* was studied for its anti-inflammatory activity. This STL completely inhibited DNA binding of the transcription factor NF-$\varkappa$B at a concentration of 5 μM and 10 μM in Jurkat T and Raw 264.7 cells, respectively. Elastase release from human neutrophils was reduced to 50% at a concentrations of 23 μM after stimulation with PAF and of 27 μM after stimulation with fMLP without showing cytotoxic effects. Additionally, elastase was also directly inhibited.

Key words: Dimerostemma, Germacranolides, Anti-Inflammatory Activity

Introduction

Dimerostemma Cass. (Asteraceae, tribe Heliantheae) is a South American genus with 12 species (Karis and Ryding, 1994). So far five of them have been chemically investigated (Bohlmann *et al.*, 1981a, b, 1982, 1984; Stefani *et al.*, 2003). This genus is characterized by the biosynthesis of sesquiterpene lactones (STLs) of the eudesmanolide type with unusual substitution patterns that are not observed in any other genus, such as 4,15- or 3,4-epoxy rings together with 1α-OR and 8α-OR groups. These chemical features can be considered as chemotaxonomic markers for this genus. Some of these eudesmane derivatives were named as dimerostemmolides (Bohlmann *et al.*, 1981a, b, 1982). In this work, an additional Brazilian member of this genus, *D. episcopale* Blake, was investigated and afforded a known germacrolide (**1**) along with a known eudesmanolide (**2**) (Fig. 1). Reinvestigation of *D. brasilianum* Blake afforded a known (**3**) and a new germacranolide (**4**) (Fig. 1), and both of them are new for the genus. The three germacranolides are costunolide derivatives and unusual for the genus *Dimerostemma.*

Compound **3**, which was isolated in a higher amount, was further investigated for its anti-inflammatory activity using the transcription factor NF-$\varkappa$B and elastase as molecular targets.

Experimental

General experimental phytochemical procedures

IR spectra were recorded on a Nicolet Protégé 520 infrared spectrometer. NMR spectra were recorded on a Bruker ARX 400 spectrometer. Samples were dissolved in $CDCl_3$ and the spectra were calibrated at the solvent signals at δ 7.26 (^{1}H) or δ 77.0 (^{13}C). ESIMS data were obtained using a Micromass Quattro LC system (Waters, Milford, USA). HRMS data were obtained on Finnigan FTQ FT equipment (Thermo Electron Corp., Waltham, USA). Vacuum-liquid chromatography (VLC) was carried out using silica gel 60 H

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(Merck KGaA, Darmstadt, Germany), in a stepwise gradient system (100% hexane to 100% EtOAc) on glass columns with 5–10 cm i.d. Flash chromatography was carried out using silica gel 60 230–400 mesh ASTM (Merck) in a 450 × 25 mm glass column, isocratic gradient, *n*-hexane/EtOAc, 5 mL/min.

Plant material

Leaves of *D. brasilianum* were collected by F. B. Da Costa in February 1998 at Tangará da Serra, MT, Brazil (S 14° 27′ 698″ and W 57° 59′ 115″), having the voucher number FBC #59. Leaves of *D. episcopale* were collected by W. Vichnewski in February 1996 at Chapada dos Guimarães, BA, Brazil, having the voucher number WV #390. The material was identified by the taxonomist Prof. Dr. J. N. Nakajima, Universidade Federal de Uberlândia, Uberlândia, MG, Brazil. A voucher of each specimen is deposited at the Herbarium SPFR of the Departamento de Biologia, FFCLRP, Universidade de São Paulo, Ribeirão Preto, SP, Brazil.

Extraction and isolation

300 g of dried and powdered leaves of *D. brasilianum* were exhaustively extracted with CH_2Cl_2 at room temperature to obtain 15 g of crude extract after solvent evaporation. This material was dissolved in MeOH/H_2O 4:1 to obtain 12.5 g of residue after wax precipitation and solvent evaporation. It was partitioned with *n*-hexane and $CHCl_3$ to give 3.5 g and 4.8 g, respectively, of residue after solvent evaporation. The $CHCl_3$ phase was then fractionated through VLC to give nine fractions of 500 mL each. Fraction 8 (208 mg) was found to contain STLs via IR spectral analysis. This fraction was further separated by repeated flash chromatography. The resulting fractions furnished the pure STLs **3** (40 mg) and **4** (8 mg).

2.1 kg of dried and powdered leaves from *D. episcopale* were exhaustively extracted with CH_2Cl_2 at room temperature to obtain 31 g of crude extract after solvent evaporation. This material was dissolved in MeOH/H_2O 4:1 to obtain 25 g of residue after wax precipitation and solvent evaporation. The extract was partitioned with *n*-hexane and $CHCl_3$ to give 7.8 g and 12.8 g, respectively, of residue after solvent evaporation. The $CHCl_3$ phase was then separated through VLC to give nine fractions of 600 mL each. Fraction 5 (1.2 g) contained STLs and was further separated by VLC to give six fractions of 300 mL each. Fraction 2 (246 mg) was separated by repeated flash chromatography. The resulting fractions gave the pure STLs **1** (10 mg) and **2** (3 mg).

*1β,5β,10α-Trihydroxy-8α-angeloyloxy-germacra-3,11(13)-dien-6α,12-olide (**4**)*

Amorphous powder. – *UV:* λ_{max} = 212 nm. – IR: ν_{max} = 3407, 2968, 2934, 1769, 1714, 1650, 1573, 1454, 1384, 1235, 1146, 1043, 970, 901, 817 cm^{-1}. – ^1H and ^{13}C NMR: see Table I. – HRMS: *m/z* = 381.19078 $[M + H]^+$, calcd. for $C_{20}H_{28}O_7$. – ESIMS, LC MS: *m/z* (rel. int) = 381.34 (100) $[M + H]^+$.

Cell culture

Raw 264.7 cells in DMEM (Dubelco's Modified Eagle Medium, Gibco) and Jurkat T cells were maintained in RPMI (Rochester Polytechnical Medicinal Institute, Gibco) 1640 medium. All media were supplemented with 2 mM glutamine (Sigma Aldrich Chemie, Munich, Germany), 10% fetal calf serum, 100 IU/mL penicillin (F. Hoffmann-La Roche Ltd., Basel, Switzeland), and 100 μg/mL streptomycin (Roche). Solutions of 5 μg/μL LPS (lypopolysaccharide; Sigma) and 1000 U/μL TNF-α (tumor necrosis factor-α; Roche) were used for stimulation.

Electrophoretic mobility shift assay (EMSA)

This assay was carried out as previously described (Müller *et al.*, 2004).

Elastase release assay

Fresh heparin blood was used for erythrocyte sedimentation with Dextran T500 (10%; Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). Neutrophil granulocytes were isolated through gradient centrifugation with Ficoll-Paque™ Plus (Amersham Pharmacia Biotech) and suspended at a concentration of approx. 15×10^6 cells/mL in PBS (phosphate buffer salt) containing Ca^{2+}/Mg^{2+}. The cells were incubated at 37 °C with cytochalasin B (5 μg/mL final concentration; Sigma), SAAVNA (*n*-succinyl-ala-ala-val-*p*-nitroanilide, 0.8 mM final concentration; Bachem; Bioscience Inc., King of Prussia, USA) and with compound **3** at different concentrations for 5 min and subsequently stimulated with fMLP (*N*-formyl-methionyl-leucyl-phenylalanine, 0.1 mM; Bachem AG, Bubendorf, Switzerland) or PAF (platelet-activating factor, 0.1 mM; Bachem) for 20 min at 37 °C. The reaction was stopped with cit-

ric acid (5.2 mg/mL final concentration; Sigma). The samples were centrifuged for 5 min at room temperature, and the supernatant was photometrically measured at 405 nm. In the negative controls, albumin buffer replaced the stimulus.

Assay with isolated elastase

After incubation of human granulocytes with cytochalasin B (5 μg/mL final concentration) for 5 min at 37 °C, PAF (0.1 mM) was added for 20 min to stimulate the release of elastase. Then, the elastase-containing supernatant was incubated with SAAVNA (0.8 mM final concentration) and compound **3** for 30 min at 37 °C. The reaction was stopped with citric acid (5.2 mg/mL final concentration) and measured at 405 nm.

Vitality test

The cell viability was studied by the MTT [3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyltetrazolium bromide] test with stimulated Raw 264.7 and Jurkat T cells. The cells were plated, respectively, incubated for 1 h with the test compound and stimulated for 1 h with LPS or TNF-α. Subsequently, the cells were incubated with MTT (3.4 mg/mL final concentration; Roth Chemicals, Karlsruhe, Germany) for 3 h at 37 °C. After centrifugation the pellet which contained the colored formazan was dissolved in DMF (*N,N*-dimethylformamide, 50%; Roth Chemicals) and SDS (sodium dodecyl sulphate, 10%; Boehringer Ingelheim, Ingelheim, Germany) solution for 16 h. The solution was transferred to a microplate and photometrically measured at 595 nm.

To investigate the cell viability of compound **3** for the neutrophils, the cells were treated as described for the elastase release assay, but incubated with MTT before the addition of citric acid. Subsequent steps were done as described above.

Statistical analysis

All assays were performed at least three times. Inhibition rates were calculated to controls. Statistical analysis was performed using GraphPad Prism 4 Version 4.02 software.

Results and Discussion

The CH_2Cl_2 extract of the leaves of *D. episcopale* afforded the germacrolide 15-hydroxy-8α-hydroxymethacryloyloxy-costunolide (**1**) (Neves *et al.*, 1999) and the eudesmanolide 1α-hydroxymethacryloyloxy-8α-hydroxy-eudesm-4(15),11(13)-dien-6α,12-olide (**2**) (Miski *et al.*, 1987), while the CH_2Cl_2 extract of the leaves of *D. brasilianum* afforded the germacranolides 1β,10α,4α,5β-diepoxy-8α-angeloyloxy-costunolide (**3**) (Gershenzon *et al.*, 1984) and the new 1β,5β,10α-trihydroxy-8α-angeloyloxy-germacra-3,11(13)-dien-6α,12-olide (**4**). The structures of all compounds were elucidated by means of MS and NMR data analyses as well as comparison with data in the literature (Fig. 1).

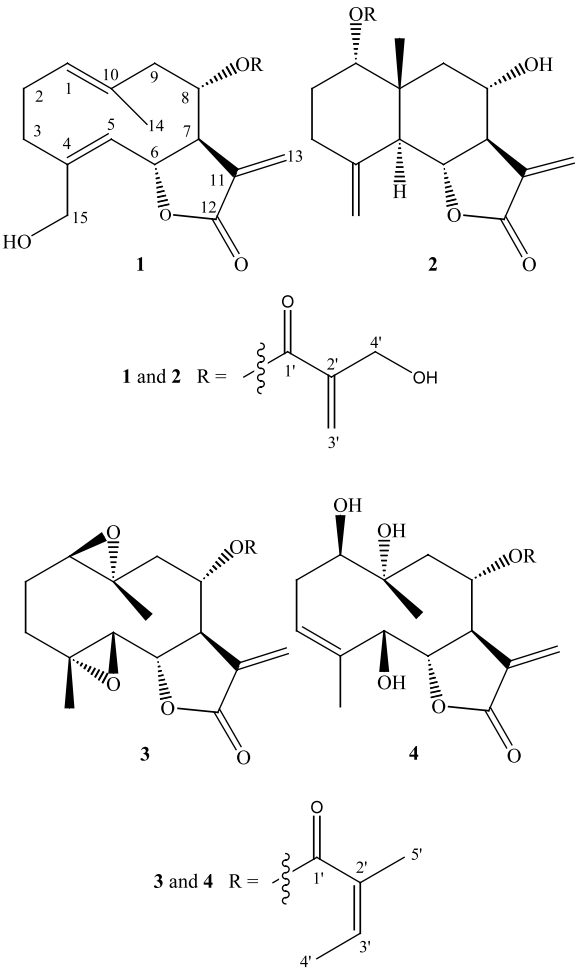

Fig. 1. Structures of the isolated sesquiterpene lactones from *Dimerostemma* species.

Structure **4** was elucidated by means of ESIMS, HRMS, ^{1}H and ^{13}C NMR as well as ^{1}H-^{1}H COSY, HMQC and HMBC (8.5 Hz) studies. The ESIMS data showed a $[M + H]^+$ ion at *m/z* 381.34 and the HRMS a $[M + H]^+$ ion at *m/z* 381.19078 corre-

Table I. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data for compound **4** in $CDCl_3$.

Position	δ_H	δ_C	COSY	HMQC	HMBC
1	4.26 (2.3, 9.8) br dd	73.6	2.72 (H-2a); 2.76 (H-2b)	73.6	74.0 (C-10); 31.4 (C-2)
2a	2.72 (2.3, 3.8, 11.4) br ddd	31.4	2.76 (H-2b); 4.26 (H-1); 5.70 (H-3)	31.4	125.4 (C-3); 134.2 (C-4)
2b	2.76 (9.8, 5.0, 11.4) br ddd	–	2.72 (H-2a); 4.26 (H-1); 5.70 (H-3)	31.4	125.4 (C-3); 134.2 (C-4)
3	5.70 (3.8, 5.0) dd	125.4	2.72 (H-2a); 2.76 (H-2b)	125.4	–
4	–	134.2	–	–	–
5	4.95 (3.4) br d	78.8	1.67 (H-15); 4.43 (H-6)	78.8	22.7 (C-15); 81.6 (C-6); 134.2 (C-4)
6	4.43 (3.4, 10.6) dd	81.6	3.60 (H-7); 4.95 (H-5)	81.6	–
7	3.60 (3.3, 3.5, 5.6, 10.6) dddd	45.6	4.43 (H-6); 5.38 (H-8); 5.62 (H-13a); 6.25 (H-13b)	45.6	43.5 (C-9); 71.6 (C-8); 122.2 (C-13)
8	5.38 (5.6, 8.6, 9.0) ddd	71.6	2.17 (H-9a); 2.37 (H-9b); 3.60 (H-7)	71.6	–
9a	2.17 (8.6, 16.4) dd	43.5	2.37 (H-9b); 5.38 (H-8)	43.5	–
9b	2.37 (9.0, 16.4) dd	–	2.17 (H-9a); 5.38 (H-8)	43.5	–
10	–	74.0	–	–	–
11	–	n.o.	–	–	–
12	–	n.o.	–	–	–
13a	5.62 (3.3) d	122.2	3.60 (H-7)	122.2	45.6 (C-7)
13b	6.25 (3.5) d		3.60 (H-7)	122.2	45.6 (C-7)
14	1.26 (3H) s	24.1	–	24.1	73.6 (C-1); 74.0 (C-10); 43.5 (C-9)
15	1.67 (3H) s	22.7	4.95 (H-5)	22.7	125.4 (C-3); 134.2 (C-4)
1′	–	n.o.	–	–	–
2′	–	139.2	–	–	–
3′	6.18 (1.3, 3.3) dq	125.8	1.93 (H-4′); 2.03 (H-5′)	125.8	139.2 (C-2′)
4′	1.93 (3H; 1.3) d	15.8	6.18 (H-3′)	15.8	–
5′	2.03 (3H; 1.3) d	20.4	6.18 (H-3′)	20.4	–

n.o., not observed.

sponding to the proposed molecular formula of $C_{20}H_{28}O_7$. This compound differs from **3** by an additional double bond at C3 as well as by the lack of the two 1,10- and 4,5-epoxy rings. This information was easily taken from ^{13}C NMR spectral data, since no signals were observed between δ 56.0 and 67.0, and a pair of two extra olefinic signals appeared at δ 125.4 and 134.2 (Table I). The chemical shift of H-15 (δ 1.67 s 3H), which is typical of a methyl group attached to olefinic carbon atoms, along with its correlation with C-3 (δ 125.4) and C-4 (δ 134.2) in the HMBC spectrum supports the presence of the olefinic bond between C-3 and C-4. Additionally, this bond is also confirmed by the correlation observed between H-3 (δ 5.70 dd) and C-3 (δ 125.4) in the HMQC spectrum (Table I). The ^{1}H-^{1}H COSY spectrum showed a correlation of the signal of H-3 with two H signals, one at δ 2.72 (br ddd, J = 2.3, 3.8 and 11.4 Hz) and the other at δ 2.76 (br ddd, J = 9.8, 5.0 and 11.4 Hz), which belong to a $-CH_2-$ group (HMQC data, Table I). These signals were assigned to H-2a and H-2b, which in turn were also correlated with a signal (CHOH) at δ 4.26 (br dd, J = 2.3 and 9.8 Hz). This signal was assigned to H-1. Confirmation was based on the assignment of C-1, since two signals of C–OH were observed in the ^{13}C NMR spectrum, one at δ 73.6 and the other at δ 74.0. The HMBC spectrum showed that the CHOH signal at δ 4.26 correlates with the COH signal at δ 74.0. Based on this information, the former signal was assigned to H-1 and the latter to C-10, while the δ 73.6 signal refers to C-1 (Table I). Moreover, a signal at δ 4.95 (br d, J = 3.4 Hz) was assigned to H-5, which was correlated in the ^{1}H-^{1}H COSY with H-6 (δ 4.43 dd, J = 3.4 and 10.6 Hz) as well as with H-15 (δ 1.67 s, 3H). The HMBC data showed a correlation of H-5 with C-6 (δ 81.6) and corroborated the assignment of H-5. The signals of H-6 and H-7 were correlated among each other and all the remaining signals up to H-9 confirmed the proposed structure of **4**. Finally, the NMR data of **4** also indicated an angeloyloxy ester residue having α-orientation attached to C-8 (Table I). All ^{13}C signals were assigned with the aid of HMBC and HMQC data (Table I) as well as data from literature (Budešínský and Šaman, 1995).

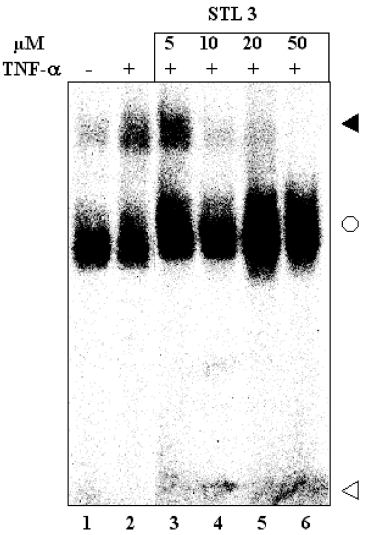

Fig. 2. Effect of germacrolide **3** on NF-$\varkappa$B DNA binding in Raw 264.7 cells. Lane 1 shows unstimulated control cells, in lane 2 cells were treated with 1 μg/mL of LPS. Lane 3 to 6 show the effect of different concentrations of compound **3** on cells which were incubated for 1 h and stimulated with LPS also for 1 h. The filled arrowhead indicates the position of NF-$\varkappa$B DNA complexes. The open circle denotes a non-specific activity binding to the probe. The open arrowhead shows unbound oligonucleotide. All experiments were carried out in duplicate.

STLs such as **4** have not been previously described in any other genus of the tribe Heliantheae. Nevertheless, other subtypes of germacranolides were found in *Dimerostemma* species, all in co-occurrence with eudesmanolides, which comprise the majority of the isolated STLs of this genus. In this study, we also report a known germacrolide (**1**) and a known germacranolide (**3**) that appear for the first time in this genus along with a known eudesmanolide (**2**). Interestingly, in a previous study of *D. brasilianum*, fourteen eudesmanolides and no germacranolides were reported (Bohlmann *et al*., 1982).

It is already known that STLs partly exert their anti-inflammatory activity by inhibiting DNA binding of the transcription factor NF-$\varkappa$B (Rüngeler *et al*., 1999; Schorr *et al*., 2002; Siedle *et al*., 2004). NF-$\varkappa$B serves as a central regulator of the human immune and inflammatory response regulating the transcription of inflammatory mediators such as cytokines, cyclooxygenase-II, nitric oxide synthase, immunoreceptors, cell adhesion molecules and hematopoetic growth factors (Barnes and Karin, 1997).

A quantitative structure-activity relationship study recently undertaken has revealed that a strong NF-$\varkappa$B inhibitory activity of STLs mostly correlates with the number of unsaturated carbonyl functions in the molecule, the existence of an α-methylene-γ-lactone and the electron affinity (Siedle *et al.,* 2004). A STL with an unsaturated lactone moiety and two epoxy groups, such as in compound **3**, has not been studied up to now.

STL **3** completely inhibited NF-$\varkappa$B DNA binding at concentrations of 10 μM in Raw 264.7 cells (Fig. 2), and concentrations in Jurkat cells were even lower. Here only a concentration of 5 mM completely impaired NF-$\varkappa$B DNA (data not shown) without showing any cytotoxic effects in the MTT assay. It can be assumed that the angeloyloxy moiety located at C-8 as well as the two epoxy groups may contribute to the strong NF-$\varkappa$B DNA binding activity in addition to the unsaturated lactone moiety, because parthenolide with only one epoxy group or eupatoriopicrin with an unsaturated ester moiety were less active (20 μM, for a complete inhibition in Jurkat cells).

STLs were also shown to inhibit elastase release from human neutrophils (Schorr *et al.*, 2005; Siedle *et al*., 2003). Neutrophils play an important role on first line of defense against microorganisms and immunity (Burg and Pillinger, 2001). However, deregulation and release of the serine protease elastase can result in severe damage of adjacent nor-

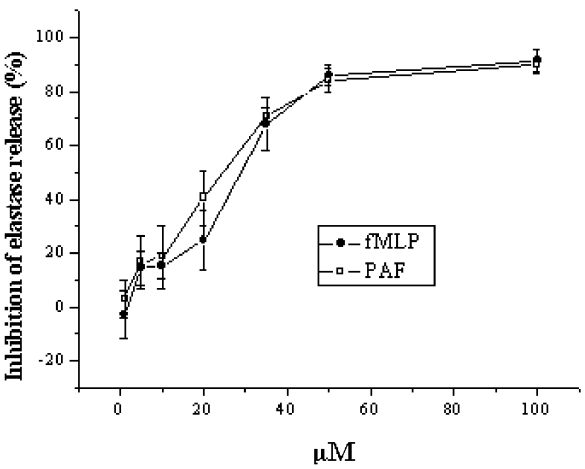

Fig. 3. Effect of the STL **3** on elastase release. Cells were incubated at 37 °C with cytochalasin B, SAAVNA and the STL **3** for 5 min and stimulated with fMLP or PAF for 20 min. The reaction was stopped with citric acid. The supernatant was measured at 405 nm.

mal tissues (Ohbayashi, 2002). To enhance our knowledge on structure-activity relationship STL **3** was also studied for its inhibition on elastase release from neutrophils, which were isolated from fresh human blood according to the assay previously reported (Schorr *et al*., 2005). Either PAF or fMLP were used to initiate the exocytosis of elastase. Concentration-response curves were recorded and IC_{50} values were calculated by nonlinear curve fitting (Fig. 3). STL **3** suppressed the PAF- as well as the fMLP-mediated elastase release in a dose-dependent manner resulting in IC_{50} values of $(27.45 \pm 1.24)\,\mu$M for fMLP and $(23.28 \pm 1.37)\,\mu$M for PAF (Fig. 3). These values were similar to those previously obtained from the germacrolide uvedalin possessing an unsaturated lactone moiety as well as an α-oriented C-8 acyl moiety. However, in contrast to uvedalin and other STLs (Schorr *et al*., 2005; Siedle *et al*., 2003) compound **3** directly inhibited elastase to 52.7% at $100\,\mu$M indicating that this STL probably acts through two mechanisms, by inhibiting directly the enzyme and by probably inhibiting the MAPK (mitogen activated protein kinase) pathways of activation of neutrophils (Schorr *et al*., 2005). It still remains to clarify which kinases are targeted by STLs.

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