Further Sesquiterpene Lactones from Viguiera robusta and the Potential Anti-Inflammatory Activity of a Heliangolide: Inhibition of Human Neutrophil Elastase Release

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This work is dedicated to the late Prof. Silvio J. Sarti (FCFRP-USP)

In addition to known heliangolides, a new eudesmanolide was isolated from the leaf rinse extract of \textit{Viguiera robusta} (Asteraceae). Structural elucidation was based on spectral analysis. It is the first report on eudesmanolides in members of the subgenus \textit{Calanticaria} of \textit{Viguiera}. In this work, the main isolated compound, the furanoheliangolide budlein A, besides its previously reported \textit{in vitro} and \textit{in vivo} anti-inflammatory activities, inhibited human neutrophil elastase release. The inhibition was at the concentration of \((16.83 \pm 1.96) \mu M\) for formylated bacterial tripeptide (fMLP) stimulation and \((11.84 \pm 1.62) \mu M\) for platelet aggregation factor (PAF) stimulation, being slightly less active than the reference drug parthenolide. The results are important to demonstrate the potential anti-inflammatory activities of sesquiterpene lactones and corroborate the previous studies using other targets.

\textit{Key words:} Asteraceae, Sesquiterpene Lactones, Human Neutrophil Elastase

Introduction

\textit{Viguiera} Kunth is a paraphyletic genus from the tribe Heliantheae with about 200 species (Schilling \textit{et al.}, 2000; Ambrósio \textit{et al.}, 2004). This genus contains the greatest diversity and the highest number of species in the subtribe Helianthinae (Romo de Vivar and Delgado, 1985). It is widespread from south-western North America to southern South America, and about 35 species are endemic in the Brazilian “cerrado” (Schilling \textit{et al.}, 2000).

A survey on the secondary metabolite chemistry of this genus points out that sesquiterpene lactones (STLs) and diterpenes are its main constituents. Several \textit{Viguiera} species have been chemically investigated during the last three decades and numerous articles focusing mostly on Mexican species were published (Romo de Vivar and Delgado, 1985; Bohlmann, 1990). So far, only a few Brazilian members have been studied, most of them by our research group. The great majority of these species showed as main constituents STLs of the heliangolide-type and diterpenes of the kaurane-type; guaianolides, germacrolides and pimpinellane diterpenes have also been isolated (Ambrósio \textit{et al.}, 2004; Da Costa \textit{et al.}, 1996, 2001; Schorr \textit{et al.}, 2002). In a previous work (Da Costa \textit{et al.}, 1996), the STLs budlein A tiglate and atriplicolide tiglate were isolated from the crude extract of the leaves of \textit{V. robusta} Gardn. Later, budlein A and its analogues were detected by HPLC in the glandular trichomes of the leaves of different populations of \textit{V. robusta} (Da Costa \textit{et al.}, 2001).

In this work, besides budlein A tiglate, we describe the isolation of large amounts of budlein A as well as the heliangolide niveusin A and a new eudesmanolide from the leaf rinse extract of \textit{V. robusta}.

\textsuperscript{1} Authors contributed equally to this work.
The STLs comprise an important class of secondary metabolites responsible for several pharmacological or toxicological activities displayed by several plants of the family Asteraceae. The α-methylene-γ-butyrolactone moiety is a relevant feature associated with a wide range of biological properties displayed by these compounds (Fuchino et al., 2001; Picman, 1986; Schmidt, 1999), since this group reacts with sulfhydryl groups of cysteine residues in a Michael-type addition. An example of an important effect displayed by several STLs is their anti-inflammatory activity due to the fact that some of these compounds usually occur in traditional medicinal plants like Arnica montana (arnica) and Tanacetum parthenium (feverfew).

Recently, the main STL isolated in this work, the furanoheiangolide budlein A (1), was shown to completely inhibit DNA binding of the transcription factor NF-αB at a concentration of 5 μM in Jurkatt T-cells (Siedle et al., 2004). STLs are well known NF-αB inhibitors, probably by selective alkylation of a cysteine sulfhydryl group in its p65 subunit (Siedle et al., 2004). The transcription factor NF-αB serves as a central regulator of the human immune and inflammatory response by regulating the transcription of pro- and inflammatory mediators such as cytokines, nitric oxide synthase, cyclooxygenase-II, immunoreceptors, haematopoietic growth factors and cell adhesion molecules (Rüngeler et al., 1998; Schorr et al., 2002; Siedle et al., 2004).

Consequently, budlein A also inhibits the interleukin 8 (IL-8) production in HeLa229 cells using ELISA, showing an IC50 value of 0.84 μM. IL-8 is a cytokine which directs the recruitment of leucocytes from blood to the inflammation site, stimulating particularly neutrophils (Lindenmeyer et al., 2006).

Finally, we demonstrated that this compound has in vivo anti-inflammatory and antinociceptive activity at 1–10 mg/kg p. o. in mice, respectively (Valério et al., 2007). The mechanism involved is dependent on the inhibition of cytokines release and action. These findings corroborate the previous in vitro studies reporting the anti-inflammatory activity of budlein A and other STLs.

As part of our continuing efforts to study the anti-inflammatory properties of STLs and based on our previously reported results, in this work we explored the effects of budlein A in the release of human neutrophil elastase (HNE) using an assay which has been recently optimized (Schorr et al., 2005). HNE is a serine protease and the main enzyme of the granules in neutrophils. It can degrade fibrous elastin, cartilage proteoglycans, several collagens and fibronectin, resulting in an abnormal turnover of connective tissue proteins. Cytokines, endotoxins, PAF and fMLP can stimulate the release of HNE (Schorr et al., 2005; Siedle et al., 2007).

Experimental

Plant material

Leaves of V. robusta were collected by F. B.C. in April 2000 in Batatais (km 35, SP-351 highway), state of São Paulo, Brazil. The plant material was identified by E. E. Schilling (Department of Botany, University of Tennessee, Knoxville, TN, USA) and J. N. Nakajima (Institute of Biology, University of Uberlândia, Uberlândia, MG, Brazil). A voucher specimen (FBC # 105) is deposited at the herbarium of the Department of Biology, Universidade de São Paulo, Ribeirão Preto, SP, Brazil, under the code SPFR 07155.

Extraction and isolation

Air-dried intact leaves (2.5 kg) were rinsed with 18 L of CH2Cl2 at room temperature (ca. 26 °C) for 10 min yielding 14 g of a crude extract after solvent evaporation. This material was re-suspended in MeOH, diluted with H2O (4:1, v/v), and successivelypartitioned with n-hexane, CH2Cl2 and MeOH to give 3.1, 4.0 and 6.5 g of organic soluble residues, respectively, after solvent evaporation. The residues were further analyzed by IR spectroscopy. The IR spectra of the CH2Cl2 residue showed strong carbonyl stretching bands of γ-lactones around 1760 cm⁻¹.

The CH2Cl2 residue was chromatographed over silica gel 60 H (Merek, art. no. 7736) using vacuum liquid chromatography (VLC) (Pelletier et al., 1986) with increasing amounts of EtOAc in n-hexane. The solvent was removed under reduced pressure in a rotatory evaporator. Nine fractions (500 mL each) were obtained and fractions 6 (1.0 g) and 7 (0.7 g) showed, via IR analysis, the presence of carbonyl groups of γ-lactones.

Fraction 6, which contained a great amount of solid mass, was washed with cold Et2O, and compound 1 (500 mg) was obtained. Fraction 7 was fractionated by medium pressure chromatography – “flash” chromatography (Still et al., 1978) –
using silica gel 60 (Merck, art. no. 9385, 0.040–0.063 mm) and an isocratic mixture consisting of n-hexane/CHCl₃/acetone (5:5:1:0.3:5) as mobile phase. Fraction 7.2 (30 mg) was analyzed by HPLC [Shimadzu liquid chromatograph SCL 10 AVP, Shimadzu ODS column, 4.6 × 250 mm, 5 μm, MeOH/H₂O (45:55), flow rate 1.0 mL/min, UV 225 nm] in portions of 0.5 mg each furnishing compounds 2 (2 mg), 3 (1 mg) and 4 (1 mg).

The structures of the known compounds were determined by 1H and 13C NMR spectroscopy as well as comparison with data from authentic samples, while the structural elucidation of 4 was made through the analysis of one- and two-dimensional NMR data (1H and 13C NMR, 1H-1H COSY, HMQC, HMBC and NOESY) as well as high-resolution electrospray ionization mass spectrometry (HR-ESIMS).

**Neutrophil isolation**

Fresh heparin blood was used for erythrocyte sedimentation with 6 mL of Dextran T500. The neutrophil granulocytes were isolated through gradient centrifugation with Ficoll-Paque™ Plus and suspended at a concentration of approx. 15 · 10⁶ cells/mL in PBS medium containing Ca²⁺/Mg²⁺.

**Elastase release assay**

The assay was done as described by Schorr et al. (2005). The cells were incubated for 5 min at 37 °C with 2.5 μL of cytochalasin B (5 μg/mL final concentration), 375 μL of N-succinyl-l-alanyl-l-alanyl-l-valine-p-nitroanilide (SAAVNA, 0.8 mM final concentration) and 1 μL of budlein A (1) or the STL parthenolide [reference drug; 4α,5β-epoxygermacra-1(10)-E,11(13)-dien-6a,12-olide; Sigma Aldrich] at different concentrations (1, 5, 10, 20, 50 and 100 μM) or DMSO and subsequently stimulated with 50 μL of formylated bacterial tripeptide (fMLP, 1 μM) or platelet aggregation factor (PAF, 0.1 mM) for 20 min at 37 °C. The reaction was stopped with 125 μL of citric acid (5.2 mg/mL final concentration). The samples were centrifuged (500 × g) for 5 min at room temperature and the released product was photometrically measured at 405 nm. In the negative controls, the stimulator was replaced by albumin buffer.

**Inhibition assay of the isolated elastase**

The inhibition assay was performed according to Schorr et al. (2005). Briefly, human granulocytes, isolated as described above, were maintained in PBS medium containing Ca²⁺/Mg²⁺. After incubation with cytochalasin B (5 mg/mL) for 5 min at 37 °C in a shaker water bath, PAF (0.1 mM) was added for 20 min to stimulate the release of HNE. Exocytosis of elastase was stopped by centrifugation (500 × g) for 10 min at room temperature. Then, the elastase-containing supernatant was incubated with SAAVNA in albumin buffer (0.8 mM) with budlein A or parthenolide (reference drug) for 30 min at 37 °C. The reaction was stopped with citric acid (5.2 mg/mL) and the released product measured at 405 nm.

**Statistical analysis**

All assays were performed at least three times in triplicate samples. Inhibition rates were calculated and normalized to control in percent. Statistical analysis was performed using the program Origin 7.0 (OriginLab Corporation, Northampton, USA). The results are expressed as mean values ± SD and analyzed using an independent t test (two groups); *p < 0.05 is considered statistically significant (*p < 0.05 and **p < 0.005 versus respective controls).

**Results and Discussion**

The phytochemical study of the leaf rinse extract of *V. robusta* led to the isolation of four sesquiterpene lactones, 1–4 (Fig. 1). The chemical structures of the known compounds 1–3 were established by 1H and 13C NMR data analysis and comparison with literature data as well as authentic compounds (Da Costa et al., 1996, 2001).

Compound 4 [1β,4β-dihydroxy-3β-acetoxy-8β-epoxyangeloyloxy-eudesm-11(13)-en-6α,12-transolidone] was found to be a novel STL with an eudesmanolide skeleton. The NMR data of 4 (Table I) showed many 1H and 13C signals similar to the already known natural compound tithofolinolide isolated from *Tithonia diversifolia* (Gu et al., 2002). The HR-MS data of 4 indicated the molecular formula C₃₂H₃₀O₉. The analysis of the 13C NMR spectrum led to the detection of 22 carbon atoms and the HMQC data gave the following information: five CH₃, three CH₂ and seven CH groups, remaining seven quaternary carbon atoms. The structure of 4 was determined by comparison of spectral data (Gu et al., 2002), except for the epoxyangelate (Epang) side chain ester attached at C-8. The epoxyangelate moiety was detected in
the $^1$H NMR spectrum by the typical methyl signals at $\delta$ 1.28 (doublet, $J = 5.4$ Hz, H-4') and 1.54 (singlet, H-5') as well as the H-3' quadruplet at $\delta$ 3.05 ($J = 5.4$ Hz). All the assignments were confirmed by $^1$H-$^1$H COSY, HMQC, HMBC and NOESY data (Table I).

Kaurane-type diterpenes and STLs, particularly heliangolides of the 1-keto-2,4-unsaturated-3,10-furano-type like budlein A (1), are usually found in many Viguiera species. Now we report for the first time the isolation of niveusin A from $V.$ robusta, a compound typically encountered in species from Helianthus. In addition, this is the first report on eudesmanolides like 4 in the subgenus Calanticaria of Viguiera. So far, this subclass of STLs has only been reported in V. laciniata (Gao et al., 1989), V. puruana (Spring et al., 2000) and V. potosina (Gao et al., 1985), all of them from the subgenus Amphilepis. It should be pointed out that the eudesmanolides from V. laciniata (tirontundifolin) and V. puruana (8$\beta$-2,3-epoxyangeloyloxybalchanin), which have been previously reported in the genus Tithonia, have the same skeleton as 4, including a $\beta$-orientated hydroxy group at C-1 and the epoxyangelate side chain ester at C-8. This observation corroborates the taxonomic relationship between Viguiera and Tithonia previously observed in other STL subtypes. In the case of the two above-mentioned eudesmanolides, they share with 4 the same skeletal subtype and main substituional features.

In order to obtain information about the inhibitory potential of HNE by budlein A (1), fMLP and PAF were used to stimulate the neutrophils release, and the IC$_{50}$ values were calculated by non-linear curve fitting. Budlein A inhibited the release of elastase from fMLP- and PAF-stimulated human neutrophils at (16.83 ± 1.96) $\mu$m (IC$_{50}$-fMLP) and (11.84 ± 1.62) $\mu$m (IC$_{50}$-PAF). These results show that budlein A is slightly less active than parthenolide, an STL from Tanacetum parthenium, which responds to the secretion of elastase with an IC$_{50}$ value of (5.11 ± 0.29) $\mu$m and an IC$_{50}$-PAF value of (4.16 ± 0.06) $\mu$m. Using the same methodology and comparing to other previously investigated STLs (Schorr et al., 2005), that exhibit IC$_{50}$ values ranging from 4.6 to 82.4 $\mu$m (IC$_{50}$-fMLP) and 7.4 to 78.3 $\mu$m (IC$_{50}$-PAF), respectively, our results indicate that budlein A displays a good inhibitory activity. However, this STL displayed low efficiency in inhibiting the activity of elastase when evaluated in the isolated model, showing only 6% of enzymatic activity inhibition at 100 $\mu$m (data not shown). These results show that budlein A is a significant inhibitor of the elastase release from neutrophils, but it does not show significant direct inhibition of the enzyme.

Most of the previously tested STLs against HNE release have other carbon skeletons than budlein A (Schorr et al., 2005; Siedle et al., 2007). Only one of them (15-deoxygoyazensolide) has the same skeleton as 1 and showed the lowest IC$_{50}$ value, ca. two times lower than 1; nevertheless, that compound has an $\alpha$-oriented side chain ester at C-8 and lacks the hydroxy group at C-15.

Based on the significant inhibitory activity displayed by several STLs as well as the structure-activity relationship studies (Siedle et al., 2007), we can propose that such metabolites may serve as lead structures for further studies involving HNE release inhibitors. Moreover, the results show that budlein A and its furanoheliangolide derivatives can be used as starting materials for further struc-
Table I. NMR spectral data of compound 4 (400 MHz for $^1$H and 75 MHz for $^{13}$C NMR; $\delta$ in ppm; $J$ in Hz; CDCl$_3$).

<table>
<thead>
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<th>Position</th>
<th>$\delta_H$</th>
<th>$\delta_C$</th>
<th>COSY</th>
<th>HMBC</th>
<th>NOESY</th>
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<td>(400 MHz)</td>
<td>(75 MHz)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>1</td>
<td>3.64 (4.0, 11.5) dd</td>
<td>76.1</td>
<td>2.08 (H2α); 1.71 (H2β)</td>
<td>16.2 (C14)</td>
<td>1.90 (H5); 1.62 (H9a)</td>
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<td>33.2</td>
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<td>3.63 (H1); 1.72 (H2β); 4.85 (H3)</td>
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<tr>
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<td>–</td>
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<td>76.1 (C1); 55.1 (C5); 1.20 (C14); 1.45 (C15)</td>
<td>2.06 (H2α)</td>
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<td>3</td>
<td>4.86 (4.9, 12.5) dd</td>
<td>75.5</td>
<td>2.08 (H2α); 1.71 (H2β)</td>
<td>33.2 (C2); 73.5 (C4); 19.1 (C15); 170.4 (C1')</td>
<td>1.90 (H5); 3.63 (H1)</td>
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<td>–</td>
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<td>5</td>
<td>1.90 (11.3) d</td>
<td>55.1</td>
<td>4.63 (H6)</td>
<td>75.5 (C3); 73.5 (C4); 75.5 (C6); 53.2 (C7); 42.1 (C10); 16.2 (C14); 19.1 (C15); 21.1 (C2')</td>
<td>2.93 (H7); 4.85 (H3); 3.63 (H1)</td>
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<td>6</td>
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<td>75.5</td>
<td>1.90 (H5); 2.93 (H7)</td>
<td>53.2 (C7); 132.7 (C11); 120.9 (C13)</td>
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<td>4.63 (H6); 5.91 (H8); 6.22 (H13a); 5.52 (H13b)</td>
<td>75.5 (C6); 132.7 (C11); 120.9 (C13)</td>
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<td>66.8</td>
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tural modifications, either by synthetic or microbial transformation, with the aim to explore and better understand their anti-inflammatory activity. It should be pointed out that besides the potential activity of 1 against different anti-inflammatory targets, it is found in high amounts in V. robusta and can be easily extracted by rinsing dried leaves with an organic solvent.

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