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Synthesis and evaluation of sesquiterpene lactone inhibitors of phospholipase A_2 from *Bothrops jararacussu*

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ABSTRACT

Several sesquiterpene lactone were synthesized and their inhibitive activities on phospholipase A_2 (PLA_2) from *Bothrops jararacussu* venom were evaluated. Compounds Lac01 and Lac02 were efficient against PLA_2 edema-inducing, enzymatic and myotoxic activities and it reduces around 85% of myotoxicity and around 70% of edema-inducing activity. Lac05–Lac08 presented lower efficiency in inhibiting the biological activities studied and reduce the myotoxic and edema-inducing activities around only 15%. The enzymatic activity was significantly reduced. The values of inhibition constants (K_I) for Lac01 and Lac02 were approximately 740 μM, and for compounds Lac05–Lac08 the inhibition constants were approximately 7.622–9.240 μM. The enzymatic kinetic studies show that the sesquiterpene lactones inhibit PLA_2 in a non-competitive manner. Some aspects of the structure–activity relationships (topologic, molecular and electronic parameters) were obtained using *ab initio* quantum calculations and analyzed by chemometric methods (HCA and PCA). The quantum chemistry calculations show that compounds with a higher capacity of inhibiting PLA_2 (Lac01–Lac04) present lower values of highest occupied molecular orbital (HOMO) energy and molecular volume (VOL) and bigger values of hydrophobicity (LogP). These results indicate some topologic aspects of the binding site of sesquiterpene lactone derivatives and PLA_2.

1. Introduction

PLA_2 are enzymes that hydrolyze glycerophospholipid membranes (PL) in the *sn*-2 position, releasing, among other fatty acids, arachidonic acid (AA). AA is involved in the inflammatory process, producing the pro-inflammatory prostaglandins (PGs) and leukotrienes (LTs). The excessive production of PGs and LTs is associated with many physiopathological processes such as asthma, cerebral illnesses, cancers, cardiovascular disorders, and inflammation (Funk, 2001). The inhibition of PLA_2 can prevent the excessive production of PGs and LTs, since the formation of AA is avoided (Yedgar et al., 2000; Balsinde et al., 2002). Venoms from different snake specimens are utilized as a PLA_2 source, due to the abundance of these materials. Thus, these enzymes are utilized as a tool for several pharmacological studies (Jabeen et al., 2005; Yedgar et al., 2006; Romero et al., 2010).

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Lactones are esters formed from the cyclisation reaction between a hydroxyl group and another acid in the same molecule. Lactones with 5 or 6 carbons are more stable due to their low tension energy in the ring. Some studies have demonstrated the capacity of different lactones to inhibit phospholipase A_2. The bromoenol lactone can inhibit calcium-independent PLA_2 (Balsinde and Dennis, 1996; Dentan et al., 1996; Jenkins et al., 2002; Da Silva et al., 2006; Song et al., 2006; Da Silva et al., 2007). In addition, wedelolactone and its derivatives from the class of coumestans, are capable of inhibiting the toxic action of both venom and PLA_2, isolated from *Bothrops jararacussu* and *Crotalus durissus terrificus* (Melo and ownby, 1999; Diogo et al., 2009; Melo et al., 2010).

In this study, we synthesized eight sesquiterpene lactone compounds and evaluated their ability to inhibit some of the toxic effects of both whole venom, and PLA_2 isolated from the venom of *B. jararacussu*. To analyze the toxic effects induced by this venom and provoked by PLA_2, edema-inducing, enzymatic and myotoxic activities of these substances were determined. After these experimental analyses, all lactones compounds were submitted to *ab initio* quantum calculations (DFT – Density Functional Theory – UB3LYP/6-31G*) and the values of their physical–chemistry properties were analyzed by chemometric methods, in order to recognize patterns that correlate the lactone structures with their biological activities. The results obtained may aid in the development of new selective inhibitors for phospholipases A_2 and, consequently, the treatment of poisoning by snake bites.

2. Material and methods

2.1. Chemicals

All reagents, including Lac01 (α-santonin), were purchased from Aldrich or Sigma Co (USA). *B. jararacussu* venom was purchased from a private serpentarium in Formiga, MG, Brazil.

2.2. Bothrops jararacussu *PLA_2 isolation*

B. jararacussu PLA_2 was isolated employing two chromatographic steps: first gel filtration on Sephadex G-75, followed by cation-exchange chromatography. The column was previously equilibrated with 0.05 M ammonium bicarbonate buffer, pH 8.0. Elution was carried out with a continuous gradient up to a concentration of 0.5 M ammonium bicarbonate. Absorbance of the effluent solution was recorded at a wavelength of 280 nm. PLA_2 homogeneity was assessed by native and SDS-PAGE and reverse-phase HPLC. Fraction II, known as Asp49 BthTX-II, was used in this study. This phospholipase will be denominated in this paper as just PLA_2 (Da Silva et al., 2008a,b).

2.3. Animals

Male Swiss mice, 6–8 weeks old, were matched for body weight (18–22 g). The animals were housed for at least one week before the experiment in laminar-flow cages maintained at a temperature of 22 ± 2 °C and a relative humidity of 50–60%, under a 12:12 h light–dark cycle. The animal experiments were carried out with the approval of the institutional committee of ethics, in accordance with protocols following the recommendations of the Canadian Council on Animal Care. The mice used in this study were kept under specific pathogen-free conditions.

2.4. Synthesis of sesquiterpene lactones

The compounds employed in this study are shown in Fig. 1. Lactones 2, 3, 5, 6, 7, and 8 were prepared by procedures described in the literature (Arantes et al., 2009; De Alvarenga et al., 2009). Lac04 was prepared as described below. To characterization of Lac04: IR spectra were recorded on a Perkin Elmer Paragon 1000 FTIR spectrophotometer, KBr, ν_{max}, cm^{-1}. ^1H and ^{13}C NMR spectra were obtained on a Bruker AVANCE DRX400 spectrometer at 400 and 100 MHz, respectively, and a Varian Mercury spectrometer observing ^1H at 300 MHz and ^{13}C at 75 MHz. All ^1H and ^{13}C spectra were obtained using $CDCl_3$ as solvent and TMS as internal standard. Low resolution mass spectra were obtained on a SHIMADZU GC MS-QP5050A instrument by direct injection. The microanalysis was obtained on a PERKIN ELMER *2400* instrument. HRMS data were recorded under conditions of chemical ionization (CI) on a Fisons Autospec– oaTof (resolution = 10,000 FWHM) in CI^+ mode using NH_3 as the ionization gas. All reagents and solvents used were previously purified and dried, as reported in the literature (Perrin et al., 1980).

2.4.1. *(3S)-5a-(1-bromo-1-methylethyl)-3-methyl-3,3a,5,5a,8,9b-hexahydro-4H-furo[2,3-f]chromene-2,7-dione (Lac04)*

To isofotosantonic acid (50 mg, MW 264 g/mol, 0.189 mmol) in dichloromethane (20 mL) was added a solution of bromine (38 mg, 0.238 mmol) in dichloromethane (3 mL) drop wise. The solvent was removed under vacuum to afford a yellow solid. This residue was recrystallized in a mixture of hexane/dichloromethane to give pale white crystals (48 mg, MW 424 g/mol, 60%). Mp = 176–177.3 °C IR ν_{max} 2976, 2935, 2903, 1782, 1734, cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ: 1.25 (d, 3H, $J_{13,11}$ = 6.9, H13), 1.70–1.75 (m, 1H, H6), 1.85 (s, 3H, H15), 1.88–1.94 (m, 1H, H7′), 1.97 (s, 3H, H14), 2.06–2.12 (m, 2H, H8), 2.39–2.50 (m, 1H, H11), 2.75–2.80 (m, 1H, H7), 3.13–3.16 (m, 2H, H2 H2′), 5.03–5.08 (m, 1H, H5), 6.06–6.09 (m, 1H, H3); ^{13}C NMR (75 MHz, $CDCl_3$): 12.7 (C13), 25.5 (C14), 30.2 (C15), 30.8 (C7), 31.0 (C8), 36.6 (C2), 42.1 (C11), 52.7 (C6), 70.4 (C10), 80.8 (C9), 90.0 (C5), 116.2 (C3), 133.5 (C4), 167.7 (C12), 177.9 (C1); MS, m/z (%): 424 – Br_2 [M^+.], 221 (100), 203 (15), 175 (10), 123 (11), 91 (13), 69 (14), 55 (16). (**found**: C, 52.16; H, 5.52. $C_{15}H_{19}BrO_4$ **requires**, C, 52.49; H, 5.58).

2.5. Edema-inducing activity

Male Swiss mice (18–22 g) were used for inducing edema. The edema was induced in the right foot pad by i.d. injection of 50 μL of a solution containing 50 μg of PLA_2, purified from *B. jararacussu* venom dissolved in 1% DMSO (Dimethyl Sulfoxide) in PBS (phosphate-buffered saline –

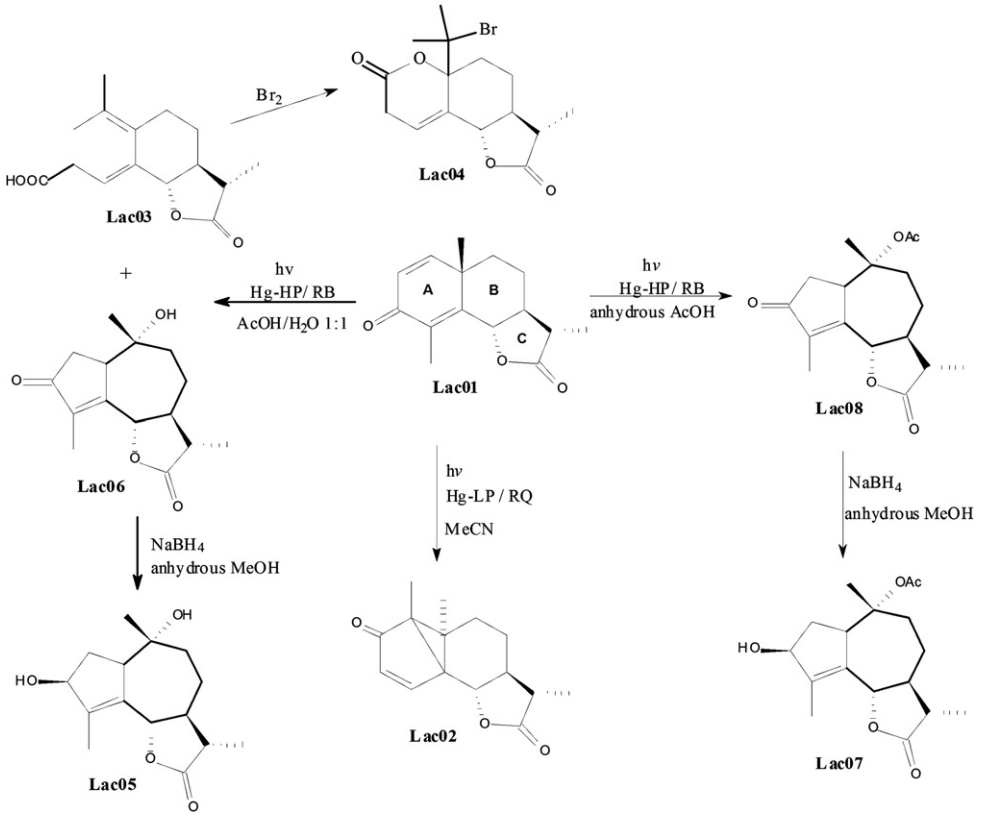

Fig. 1. Synthesis scheme and structures of sesquiterpene lactones.

pH 7.2). Injection (i.d.) of 50 μL of a solution containing a mixture of 50 μg of PLA_2 and 20 μg of each sesquiterpene lactone derivative compound dissolved in 1% DMSO in PBS (pH 7.2) was used in the inhibition studies. Prior to the injections, the mixtures containing PLA_2 and the inhibitors were pre-incubated for 10 min at 37 °C. The progression of edema was evaluated with a low pressure pachymeter (Mitutoyo, Japan) at various time intervals after injection (0.5, 1, 2, 4, 6, 24 h). Negative control groups were injected with 50 μL of 1% DMSO in PBS (pH 7.2). Control groups for each nitrostyrene compound were obtained through the i.d. injection of 50 μL of a solution containing only 25 μg of each sesquiterpene lactone derivative compound dissolved in DMSO in PBS (pH 7.2) (Soares et al., 2000; Calgarotto et al., 2008).

2.6. Myotoxic activity

Swiss male mice (18–22 g) were used to analyze the myotoxic activity. Mice were injected, intramuscularly, in the right gastrocnemius muscle with 50 μL of a solution containing 25 μg of PLA_2, purified from *B. jararacussu*. Inhibition studies were performed by injecting 50 μL of a mixed solution composed of 25 μg of PLA_2 and 20 μg of each sesquiterpene lactone derivative compound, dissolved in 1% DMSO in PBS (pH 7.2). Prior to the injections, the mixtures containing PLA_2 and the inhibitors were pre-incubated for 10 min at 37 °C. Mice were bled from the tail at 3 h after injections and blood was collected into heparinized capillary tubes. Plasma creatine kinase activity was determined using the CK-UV Kit (Bioclin, Brazil). Activity was expressed in units/L, with one unit corresponding to the production of 1 μmol of NADH per min at 30 °C. Negative controls received 50 μL of 1% DMSO-PBS alone. The control group for each sesquiterpene lactone compound received an intramuscular injection of 50 μL of a solution containing just 20 μg of each sesquiterpene lactone derivative compound, dissolved in 1% DMSO in PBS (pH 7.2) (Souza et al., 2008; Da Silva et al., 2008a).

2.7. Enzymatic activity

The measurement of the enzymatic activity using the micellar substrate, 1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphoglycerol (HPGP), was performed through the microtiter plate assay. Each sesquiterpene lactone derivative compound was tested in final concentrations of 0.0, 1.0, 2.0, 3.0 and 4.0 μM. Seven wells of a 96-well microtiter plate were used for each assay, resulting in six measurement repetitions of the enzymatic activity for each final concentration of the inhibitor. Thus, for each assay with different concentrations of the inhibitor, 100 μL of solution A in assay buffer (27 μM bovine serum albumin, 50 mM KCl, 1 mM $CaCl_2$, 50 mM Tris– HCl, pH 8.0) was added to seven wells, followed by the addition of a volume of lactone derivative compound (the volume used was

according to the assay concentration, 0.0–4.0 μM) dissolved in DMSO. For control reactions, the same volume of DMSO was used alone. Solution B had the same composition as that of Solution A, but contained PLA_2 (0.5 μg/mL) and was delivered in 100 μL volumes to seven wells, except for the first well. Instead of Solution B, an additional 100 μL-portion of Solution A was added to the first of the seven wells in the assay. Solution B was prepared immediately before each set of assays to avoid loss of enzymatic activity. Quickly, after the addition of Solution B, the assay was initiated by the addition of 0.5–50 μL of Solution C to the seven wells (53 mM HPGP vesicles in assay buffer), with a repeating pipette. The final concentration of HPGP varied from 0.125 to 10 mM. The final volume of the assay was 265 μL and, when necessary, the wells received an extra volume of solution A in order to complete this volume. The fluorescence (excitation = 342 nm, emission = 395 nm) was read with a microtiter plate spectrophotometer (Fluorocount, Packard Instruments). Control reactions without enzyme or inhibitor were run for all assays and the initial velocity was calculated from the initial slope of fluorescence versus time, for each concentration of the substrate used. The significance of differences between groups was evaluated using the Student's *t*-test. A *P*-value <0.05 was considered to be significant (Da Silva et al., 2008b).

2.8. Ab initio *quantum calculations*

The structures of each sesquiterpene lactone derivative compound were submitted to *ab initio* quantum calculations. In order to select the best conformations, the HyperChem 7.51 software was utilized. The final geometry optimization was carried out using the GAUSSIAN03 package, applying the DFT (Density Functional Theory) methodology with the use of functional UB3LYP with 6-31G* basis set (Hariharan and Pople, 1973). The following molecular and electronic properties (descriptors) were calculated: total non-relativistic electronic energy (E_T), dipole moment (μ), Highest Occupied Molecular Orbital energy (HOMO), Lowest Occupied Molecular Orbital energy (LUMO), surface area (A), molecular volume (VOL), logarithm of partition coefficient (Log P), polarizability (POL), molecular refractivity (MR), the difference between the energy values of HOMO and LUMO (GAP; GAP = LUMO – HOMO), Mullikan electronegativity (ξ – eq. (1)), hardness (η – eq. (2)), electronegativity (χ – eq. (3)), softness (S – eq. (4)), electrophilicity index (ω – eq. (5)), ionization potential (IP – eq. (6)), electron affinity (EA – eq. (7)), Partial Atomic Charges (Q_n, where *n* corresponds to the atom number, according to Fig. 1) on the carbon, nitrogen, oxygen and chlorine atoms. The atom numbering shown in Fig. 1 does not correspond to that recommended by the IUPAC, and was elaborated aiming to standardize the chemometric analysis of the partial atomic charge (Q_n). The numbering, in agreement with UIPAC, is that used in item 2.3 (Material and methods) and reports the structural elucidation of the compounds synthesized.

$$\xi = \frac{(-HOMO - LUMO)}{2} \tag{1}$$

$$\eta = \frac{(LUMO - HOMO)}{2} \tag{2}$$

$$\chi = \frac{(IP/EA)}{2} \tag{3}$$

$$S = \frac{1}{2\eta} \tag{4}$$

$$\omega = \frac{\mu^2}{2\eta} \tag{5}$$

$$IP = [(TE_{CATION} + TCE_{CATION}x0.9806) - (TE_{NEUTRAL} + TCE_{NEUTRAL}x0.9806)]x27.2114 \tag{6}$$

$$EA = [(TE_{NEUTRAL} + TCE_{NEUTRAL}x0.9806) - (TE_{ANION} + TCE_{ANION}x0.9806)]x27.2114 \tag{7}$$

where TE is the total electronic energy and TCE is the total energy, corrected for zero-point vibrational energy (ZPVE) for both neutral and ionic (positive and negative) species. The correction factor of the ZPVE is 0.9806 for the B3LYP/6-31G* model and 1 Hartree = 27.2114 eV (Parr and Pearson, 1983; Chattaraj et al., 1991; Scott and Radom, 1996; Kohn et al., 1996; Da Silva et al., 2009; Parr et al., 1999; Sinha et al., 2004).

In addition to the molecular and electronic descriptors, sixteen topological descriptors were also calculated through the use of the program, Dragon: total structure connectivity index, polarity number log of the product of row sums (PRS), average vertex distance degree, mean square distance index (Balaban), Schultz Molecular Topological Index (MTI), Wiener-type index from van der Waals weighted distance matrix, Wiener-type index from electronegativity weighted distance matrix, Wiener-type index from polarizability weighted distance matrix, Balaban distance connectivity index, Balaban-type index from electronegativity weighted distance matrix, Balaban-type index from polarizability weighted distance matrix, Balaban-type index from mass weighted distance matrix, Balaban-type index from van der Waals weighted distance matrix, maximal electrotopological negative variation, maximal electrotopological positive variation, molecular electrotopological variation, sum of topological distances between different heteroatom pairs.

In addition, the Pirouette program was used to perform the Principal Component Analysis (PCA) and Hierarchical Clustering Analysis (HCA). The chemometric methods are particularly appropriate to provide insight into the Structure–Activity Relationships (SAR) when one is dealing with systems depending on many variables (Beebe and Pell, 1988). (PCA) and (HCA) are statistical methods used in the recognition of standards in multivaried studies (Da Silva et al., 2004; Weber et al., 2005; Calgarotto et al., 2007). The properties calculated by DFT were auto-scaled using the Fisher weight (Costa and Takahata, 2003). The differences in the calculated properties are able to better discriminate the relationship between the structures of the sesquiterpene lactone derivative compounds and their biological activities.

2.9. Statistical analysis

Results are presented as the mean values ± S.D., obtained from the indicated number of tested animals. The statistical significance of differences between groups was evaluated using Student's unpaired *t*-test. A *P*-value < 0.05 was considered to indicate significance.

3. Results and discussion

Myonecrosis, muscle tissue damage, is a common consequence of envenoming by snakes of the Bothrops genus and this effect is, partially, caused by PLA_2 (Gutiérrez, 2002; Soares et al., 2004). Compounds Lac01 and Lac02 reduced myotoxicity by approximately 70%, when compared to the PLA_2 control assay (Fig. 2). Compounds Lac03 and Lac04 reduced the myotoxic activity by approximately 56%, while compounds Lac05–Lac08 did not demonstrate any activity against myotoxic effects.

Edema-inducing activity is a pharmacological activity that depends upon the combined action of various toxins, including PLA_2 (Soares and Giglio, 2003; Soares et al.,2004). Fig. 3 shows that, after a 2 h period, Lac01–Lac04 reduces the levels of edema-inducing activity of PLA_2 to 40–50%, when compared to the PLA_2 control experiment. In this same period, the compounds Lac05–Lac08 reduced edema levels to only 90%.

The action of PLA_2 from *B. jararacussu* on the micellar substrate, HPGP, reflects the classical behavior of a Michaelian enzyme, not only in the presence of small concentrations of the lactone compounds (1–4 μM), but also in their absence (graphics not show) (Souza et al., 2008; Da Silva et al., 2008). The kinetic parameters obtained in this study are shown in Table 1 and Fig. 4.

Fig. 4 demonstrates that, in all the tests, the maximum velocity of the enzyme (V_{max}) varied in function of the presence of growing concentrations of inhibitor compounds. Lac01 and Lac02 were the more efficient inhibitors and reduce the enzymatic activity around 80–90% (Fig. 4A). The compounds, Lac05–Lac08, did not effectively inhibit the PLA_2

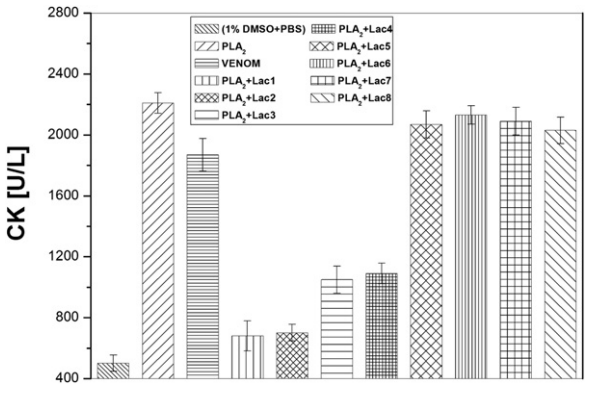

Fig. 2. Effect of lactone compounds on the myotoxic activity induced by purified PLA_2 from *B. jararacussu* venom. Mice were injected (i.m.) with 25 μg of PLA_2 and 20 μg of each inhibitor. After 3 h of inoculation, the blood was collected and the kinase activity was determined. The PBS control solution contains 1% of DMSO. Results are expressed as the means ± S.D. ($n = 6$).

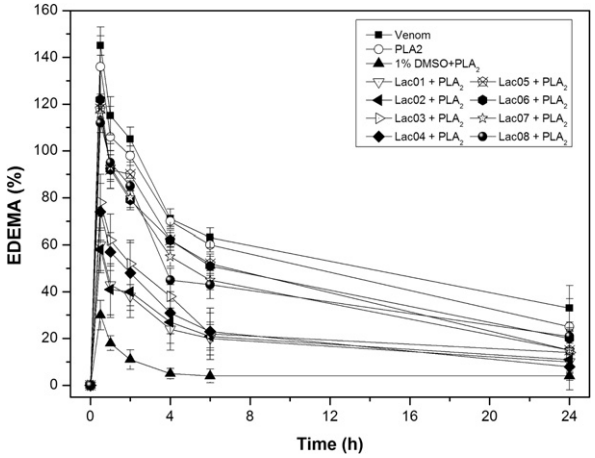

Fig. 3. Effect of lactone compounds on edema induced by PLA_2, purified from *B. jararacussu* venom. Mice were injected (i.d) with solution (50 μL) containing 25 μg of PLA_2 and 20 μg of each inhibitor. Edema progression was evaluated in intervals: 0.5, 1, 2, 4, 6, 24 h. The PBS control solution contains 1% of DMSO. Results are expressed as the means ± S.D. ($n = 6$).

enzymatic activity, reducing V_{max} (approximately 30% reduction, Fig. 4A). On the other hand, the presence of the inhibitors did not induce any alteration in K_M values (Fig. 4B). The K_M values were around 740 μM (Table 1). In addition, the inhibition constant values (K_I) were between 0.075 and 9.240 μM (Table 1). K_I reflects the dissociation of enzyme-inhibitor and the smaller its value, the greater its ability to bind the inhibitor, which can be observed that Lac01 and Lac02 presented the best capacity to inhibit the enzymatic activity of PLA_2 from *B. jararacussu*, while Lac05–Lac08 presented low inhibition capacity (Fig. 4). This set of results shows that the enzymatic inhibition provoked by lactone derivatives is non-competitive and that these compounds might be bound to a site different from that of the enzyme active site and do not compete with HPGP.

The structures of the sesquiterpene lactone derivative compounds were submitted to quantum chemistry calculations and chemometric studies (PCA and HCA). PCA (Principal Components Analyze) is a multivariate statistical technique that reduces the data dimensionality by the linear transformation of the original data set in a new and

Table 1
K_M and K_I constant values,[a] obtained on the reaction of PLA_2 from *B. jararacussu* in the presence of sesqueterpen lactone compounds (0.0–4.0 μM) using the substrate HPGP. K_M and K_I are in μM.

Compounds	K_M (μM)	K_I (μM)
Lac01	734 ± 112	0.075 ± 0.051
Lac02	746 ± 132	0.075 ± 0.023
Lac03	742 ± 125	0.887 ± 0.093
Lac04	738 ± 90	0.833 ± 0.103
Lac05	744 ± 91	7622 ± 0.123
Lac06	744 ± 148	7.913 ± 0.083
Lac07	738 ± 88	8.834 ± 0.098
Lac08	742 ± 101	9.240 ± 0.158

[a] Average of three independent determinations and six replicates for each inhibitors concentration (0.0, 1.0, 2.0, 3.0, 4.0 μM); values are mean ± SD.

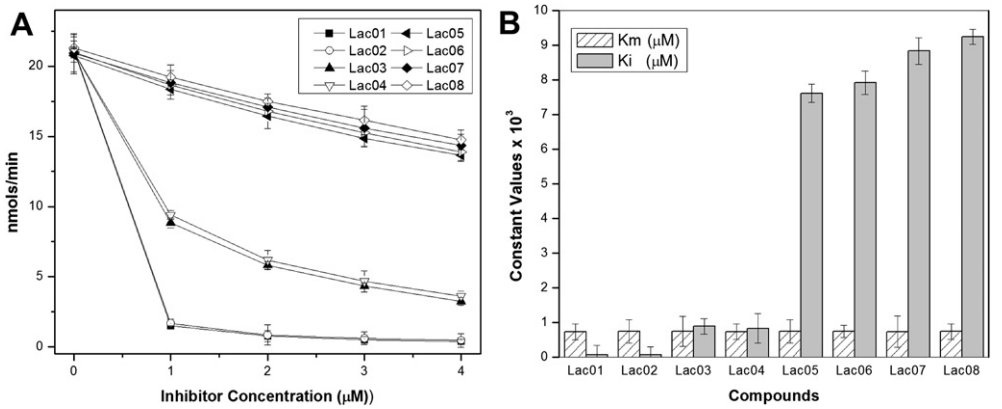

Fig. 4. Kinetic parameters obtained in this study. A) V_{max} varies as a function of concentration of the inhibitors. Increasing the concentration of inhibitors (0.0–4.0 uM) decreases the V_{max} values. B) The K_M values were approximately constant regardless of the concentration of the inhibitors. The values of K_I show that Lac01 Lac01–Lac02 compounds are more efficient in the inhibition of PLA_2. Together, these data indicate that the lactones inhibit phospholipase A_2 in a non-competitive. The values are average of three independent determinations and six replicates for each inhibitors concentration.

smaller set of uncorrelated variables (Beebe and Pell, 1988). This technique has been widely applied in the chemometric studies of bioactive compounds (Da Silva et al., 2004; Weber et al., 2005; Calgarotto et al., 2007). Fisher weight was used to analyze the auto-scaled values for all the calculated properties (molecular, electronic and topological). Fisher weight revealed six descriptors, whose variances may be responsible for the differences observed in the biological activities of the sesquiterpene lactone derivative compounds indicated (HOMO, VOL, GAP, IP, Log P, Balaban-type index from polarizability weighted distance matrix). The chemometric analyses used these six descriptors, selected by Fisher weight.

When the PCA technique was applied to the auto-scaled values of the selected properties obtained from the *ab initio* quantum calculations (DFT - UB3LYP/6-31G*) of the lactone compounds, the best separation was obtained using the values of three variables (VOL, Log P, HOMO energy) (Fig. 5A).

Fig. 5B shows that, utilizing values of proprieties selected by PCA (VOL, Log P, HOMO energy), all sesquiterpene lactone derivative compounds may be grouped in three distinct regions: Group 1 (Lac01–Lac02, high activity in all tests); Group 2 (Lac03–Lac04, intermediate activity in all tests); Group 3 (Lac05–Lac08, low (or no) activity in all tests). PCA results showed that the first component (PC1) is responsible for 75.78% of the data variance and that the second one (PC2) is responsible for 22.29% (data not shown). Considering the first and second principal components (PC1 and PC2), the accumulated variance increased to 98.07%.

The Hierarchical Cluster Analysis (HCA) technique verifies the distance among the samples in a data set and is a tool for preliminary data analysis that can be used to inspect data sets for expected or unexpected clusters (Beebe and Pell, 1988; Da Silva et al., 2004; Weber et al., 2005; Calgarotto et al., 2007). In the HCA analysis, the same six descriptors, selected according to Fisher weight and used in the PCA, were utilized. Similarly to the PCA, the HCA algorithm also permits different combinations among the descriptors selected to describe the best multivariate system, based on the degree of similarity of their variances.

The HCA indicated that the best similarity degree among the most active and less active compounds is

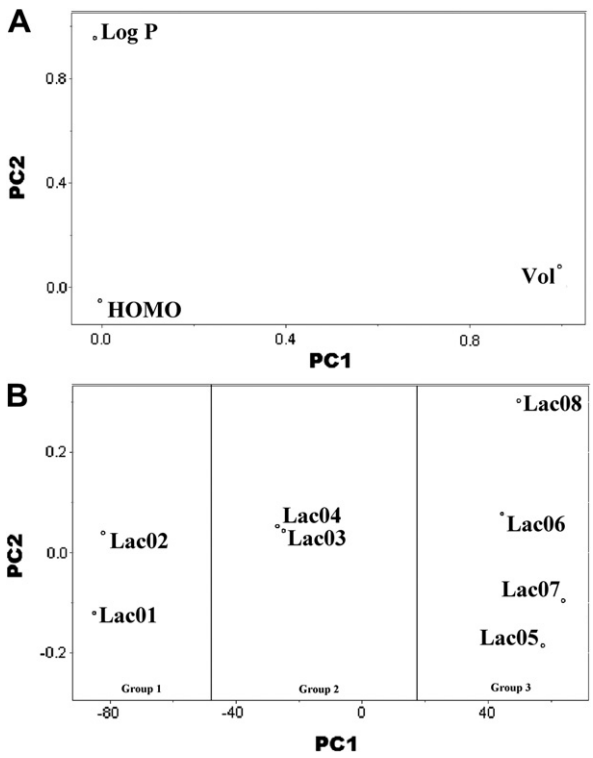

Fig. 5. Principal Component Analyses: A) Loading plot for the three variables responsible for the classification of the eight lactone compounds: HOMO energy (Highest Occupied Molecular Orbital energy); Vol (molecular volume); Log P (Logarithm of partition coefficient). B) Score plot for the eight sesquiterpene lactone derivative compounds.

reached through the combination of values of HOMO energy, Log P and VOL (same descriptors used in PCA). Fig. 6 indicates that HCA separates the sesquiterpene lactone compounds into two major blocks with zero similarity. One branch (branch A) of the dendrogram contains the active compounds of Groups 1 and 2 (Lac01–Lac04 – see Fig. 6B). Another distinct branch (branch B) grouped the compounds with low (or no) activity inside the concentration range used in the tests (Lac05–Lac08 – see Fig. 5B). This classification confirms the same pattern observed in PCA and indicates that HOMO energy, Log P and VOL could potentially be responsible for the biological activity shown by the lactone compounds used in this work.

4. Proposed model for PLA_2 sesquiterpene lactone compound binding sites

Table 2 shows that the more active compounds (Lac01–Lac04) present lower HOMO energy and volume (VOL), and higher values of Log P. The selection of these properties by PCA, confirmed by HCA, indicates that Lac01–Lac04: 1) can form transferring charge complexes during the inhibition process of PLA_2 (lower HOMO energy values); 2) the binding site has a limited volume (lower VOL values); and 3) the binding site has hydrophobic characteristics (higher Log *P* values). Lower HOMO values indicate that compounds Lac01-Lac04 might be receiving electrons from PLA_2 amino acids in an easier manner than the compounds Lac05–Lac08. Two interesting points of charge transferring in the lactones used in this study are the ketone groups in rings A and C (see Fig. 1) that can form a hydrogen or electrostatic bond in the binding site with PLA_2. In addition, Lac05–Lac08 are more voluminous molecules and are less hydrophobic than Lac01–Lac04 and these characteristics may decrease their efficiency of inhibition of PLA_2.

Table 1 and Fig. 4 shows that Lac01–Lac02 more efficiently inhibit the PLA_2 from *B. jararacussu* than Lac03–Lac04. Structural analyses demonstrate that the main difference between Lac01–Lac04 and Lac05–Lac08 is the B ring. The additional presence of a methyl group in ring B significantly increases the molecular volume of Lac05–Lac08 when compared with the volumes of Lac01–Lac04 (see Fig. 1 and Table 2). Apparently, there is an area on the lactone-binding site in PLA_2 that can receive a B structure with six carbons (Lac01–Lac04) but does not allow a structure B with seven carbons (Lac05–Lac08). The correct positioning of the B ring in the binding site may be responsible for the formation of the transferring charge complex, probably through the establishment of a hydrogen or electrostatic bond of the ketone group in the C ring with the same amino acid as that of PLA_2.

Table 2
Lactone compound property values selected by PCA and calculated by *ab initio* method quantum chemistry (DFT – UB3LYP/6-31G*).

Molecule	Vol (Á3)	Log P	HOMO energy (Ev)
Lac01	724.47	2.74	−9.1507
Lac02	726.91	2.88	−9.0433
Lac03	784.09	2.04	−9.1349
Lac04	781.93	2.09	−9.0239
Lac05	866.44	0.59	−8.4984
Lac06	853.56	1.12	−8.5083
Lac07	873.22	0.62	−8.6566
Lac08	859.48	1.25	−8.6335

The kinetic parameter values found in the enzymatic assays (Table 1 and Fig. 4) show that the lactone derivative compounds inhibit PLA_2 in a non-competitive manner, signifying that the binding site of these inhibitors might be different from the active site of the enzyme. The set of experimental evidences, as well as the structural information obtained with the *ab initio* calculations and the chemometric studies, allow the proposition of a model of the sesquiterpene lactone compound binding sites for PLA_2. The principal characteristics of these binding sites are: 1) the binding site is not able to support molecules with seven carbons in the ring B; 2) the ester carbonyl in the C ring may be the responsible for hydrogen or electrostatic interactions between the lactones and the PLA_2. Since Lac01 was the most active compound of all the analyzed molecules, we used this compound to propose a model for the binding site (Fig. 7).

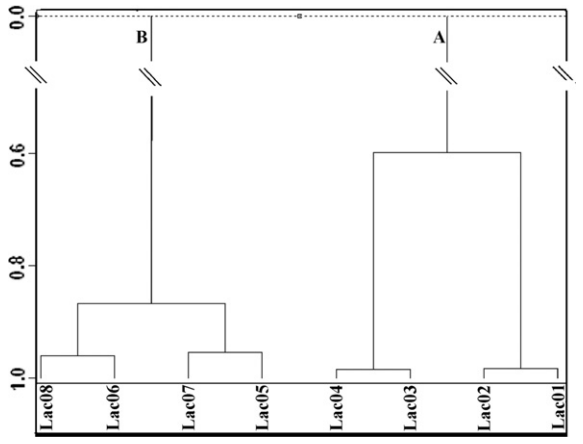

Fig. 6. Hierarchical Cluster Analysis (HCA): Dendrogram obtained for all the nitrostyrene derivative compounds.

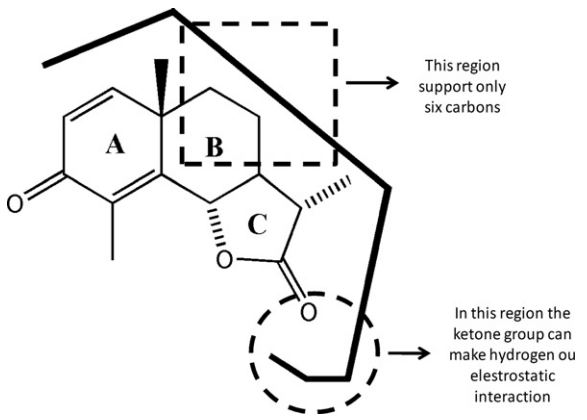

Fig. 7. Proposed model for the principal topological aspects of the PLA_2–binding site on the lactone compound. This model was established using Lac01, the compound with the highest PLA_2 inhibition capacity.

Conclusions

The search for new inhibitors of PLA_2 is an important strategy for the development of new anti-inflammatory drugs or as an adjunct in the treatment of poisonings from snake bites. In this strategy, the release of arachidonic acid is required to consequently decrease the activity of COX and LOX and its pro-inflammatory products. In the development of new PLA_2 inhibitors, many chemical substances (natural or synthetic) have been tested (Binisti et al.,1997; Sekar et al., 1997; Yedgar et al., 2000; Binisti et al., 2001; Chandra et al., 2002a,b; Soares and Giglio, 2003; Ticli et al., 2005; Yedgar et al., 2006; Lätting et al., 2007; Marcussi et al., 2007).

In this study, we showed that the lactones are able to inhibit several biological effects provoked by PLA_2 from the *B. jararacussu* venom. The ability of other lactone derivative compounds has already been demonstrated by other authors and our results follow the same trend (Balsinde and Dennis, 1996; Dentan et al., 1996; Melo and Ownby, 1999; Jenkins et al., 2002; Song et al., 2006; Cummings, 2007; Diogo et al., 2009; Melo et al., 2010). We verified that the compounds Lac01–Lac04 were able to inhibit the effects of PLA_2 from B. jararacussu and kinetic studies have shown that the compounds tend to non-competitively inhibit the enzyme activity, with respect to the substrate studied (1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphoglycerol - HPGP). The Lac05–Lac08 compounds did not demonstrate the capacity to inhibit the activity of PLA_2.

In addition, our studies of SAR (Structure Activity Relationship) showed that the most active lactones in the inhibition of edema-inducing activity, enzymatic activities and myotoxic activity, provoked by PLA_2, purified from venom of *B. jararacussu* are those that present the B ring with six carbons (see Fig. 1). Ring A, with five carbons, can increase the inhibitor activity, but is not essential. The electronic, molecular and topologic properties of Lac01–Lac08 were calculated using *ab initio* quantum calculations (DFT) and analyzed by chemometric methods (PCA and HCA). The proprieties of HOMO energy, Log P and molecular volume are probably responsible for the differences between the most and the less active compounds. One possible explanation for the inhibition effects on PLA_2 is the formation of transfer charge complexes between PLA_2 and the ketone group in Ring C. Thus, the most active compounds (Lac01–Lac04) present low HOMO energy values, which are favorable for PLA_2 electron reception by hydrogen or electrostatic bonds. The corrected position of the ketone group occurs when the B Ring has six carbons. Ring B, with seven carbons (Lac05–Lac08), may shift the correct positioning of the ketone group and prevent the inhibition of PLA_2.

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Conflict of interest statement

None declared.

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