MipLAAO, a new L-amino acid oxidase from the redtail coral snake *Micrurus mipartitus*

Paola Rey-Suárez¹, Cristian Acosta¹*, Uday Torres¹*, Mónica Saldarriaga-Córdoba²*, Bruno Lomonte³* and Vitelbina Núñez¹,4*

¹Programa de Ofidismo y Escorpionismo, Facultad de Ciencias Farmacéuticas y Alimentarias, Universidad de Antioquia, Medellín, Colombia
²Centro de Investigación en Recursos Naturales y Sustentabilidad, Universidad Bernardo O’Higgins, Santiago de Chile, Chile
³Instituto Clodomiro Picado, Facultad de Microbiología, Universidad de Costa Rica, San José, Costa Rica
⁴Escuela de Microbiología, Universidad de Antioquia, Medellín, Colombia

*These authors contributed equally to this work.

**ABSTRACT**

L-amino acid oxidases (LAAOs) are ubiquitous enzymes in nature. Bioactivities described for these enzymes include apoptosis induction, edema formation, induction or inhibition of platelet aggregation, as well as antiviral, antiparasite, and antibacterial actions. With over 80 species, *Micrurus* snakes are the representatives of the Elapidae family in the New World. Although LAAOs in *Micrurus* venoms have been predicted by venom gland transcriptomic studies and detected in proteomic studies, no enzymes of this kind have been previously purified from their venoms. Earlier proteomic studies revealed that the venom of *M. mipartitus* from Colombia contains \( \sim 4\% \) of LAAO. This enzyme, here named MipLAAO, was isolated and biochemically and functionally characterized. The enzyme is found in monomeric form, with an isotope-averaged molecular mass of 59,100.6 Da, as determined by MALDI-TOF. Its oxidase activity shows substrate preference for hydrophobic amino acids, being optimal at pH 8.0. By nucleotide sequencing of venom gland cDNA of mRNA transcripts obtained from a single snake, six isoforms of MipLAAO with minor variations among them were retrieved. The deduced sequences present a mature chain of 483 amino acids, with a predicted pI of 8.9, and theoretical masses between 55,010.9 and 55,121.0 Da. The difference with experimentally observed mass is likely due to glycosylation, in agreement with the finding of three putative N-glycosylation sites in its amino acid sequence. A phylogenetic analysis of MmipLAAO placed this new enzyme within the clade of homologous proteins from elapid snakes, characterized by the conserved Serine at position 223, in contrast to LAAOs from viperids. MmipLAAO showed a potent bactericidal effect on *S. aureus* (MIC: 2 \( \mu \)g/mL), but not on *E. coli*. The former activity could be of interest to future studies assessing its potential as antimicrobial agent.

**Keywords** Antibacterial activity, L-Amino acid oxidase, *Escherichia coli*, *Staphylococcus aureus*, *Micrurus mipartitus*, Snake venom, Protein sequence, Coral snake

**How to cite this article** Rey-Suárez et al. (2018), MipLAAO, a new L-amino acid oxidase from the redtail coral snake *Micrurus mipartitus*. PeerJ 6:e4924; DOI 10.7717/peerj.4924
INTRODUCTION

L-amino acid oxidases (LAAs, E.C. 1.4.3.2) are flavoenzymes that catalyze the stereospecific oxidative deamination of L-amino acid substrate to α-keto acid, producing ammonia and hydrogen peroxide (Izidoro et al., 2014). These enzymes are widely distributed in different organisms such as bacteria (Huang et al., 2011; Hossain et al., 2014) fungi (Davis, Askin & Hynes, 2005; Pišlar et al., 2016), birds (Struck & Sizer, 1960) mammals (Puiﬀe et al., 2013), and plants (Du & Clemetson, 2002). In snake venoms (svLAAs) they are present in the Viperidae and Elapidae families, in amounts between 0.1 and 30% of total protein content (Izidoro et al., 2014). Also, these enzymes have been found in non-venomous snakes such as Python regius and Pantherophis guttatus (Hargreaves et al., 2014). These proteins are responsible for the characteristic yellow color of many snake venoms (Izidoro et al., 2014).

svLAAs are generally homodimeric glycoproteins (with approximately 4% of carbohydrates), with molecular masses ranging between 120 and 150 kDa in native forms, and from 55 to 66 kDa in monomeric forms, possibly with a non-covalent association between the two subunits (Du & Clemetson, 2002). They have a wide range of isoelectric points (pI), from about 4.4 to 8.5, and they can bind either to ﬂavine mononucleotide (FMN) or to ﬂavine adenine dinucleotide (FAD) (Izidoro et al., 2014). Most svLAAs demonstrate a relatively high aﬃnity for hydrophobic and aromatic amino acids, including L-Phe, L-Met, L-Leu and L-Ile because of substrate speciﬁcity related to side-chain binding sites (Costa et al., 2014; Geueke & Hummel, 2002), and they are sensitive to temperature, pH changes and lyophilization (Du & Clemetson, 2002).

svLAAs are multifunctional enzymes exhibiting a wide range of biological activities including apoptosis induction (Tan et al., 2017; Carone et al., 2017; Costa et al., 2017), edema formation (Ali et al., 2000; Teixeira et al., 2016), inhibition or induction of platelet aggregation (Takatsuka et al., 2001; Samel et al., 2008; Naumann et al., 2011), leishmanicidal (Tempone et al., 2001; Ciscotto et al., 2009; Stábeli et al., 2007; Carone et al., 2017) and antibacterial functions, among other activities.

The antibacterial activity of svLAAs was reported more than forty years ago (Skarnes, 1970) in Crotalus adamanteus venom. Since then, other authors have also reported antibacterial activity against Gram-positive and Gram-negative bacteria (Toyama et al., 2006; Stábeli et al., 2007; Rodrigues et al., 2009; Zhong et al., 2009; Lee et al., 2011; Vargas et al., 2013; Vargas Muñoz et al., 2014; Lazo et al., 2017). However, such activity has not been reported for a Micrurus mipuritus venom enzyme. The aim of this work was to isolate and characterize the biochemical properties of LAAO from this venom, and its antibacterial activity.

MATERIALS AND METHODS

Isolation of MipLAAO

MipLAAO, the fraction identified as LAAO in proteomic analysis of M. mipuritus venom (Rey-Suárez et al., 2011), was isolated using size exclusion chromatography in a high performance liquid chromatographic system (SEC-HPLC). For this, two mg of lyophilized
venom (obtained from specimens maintained in Serpentarium of Universidad de Antioquia from the western region of Antioquia, Colombia), were dissolved in PBS (phosphate-buffered saline; 0.12 M NaCl, 40 mM sodium phosphate, pH 7.2) and separated on a Biosec S-2000 column (Phenomenex, 5 µm particle diameter; 300 × 7.8 mm), using a Shimadzu Prominence-20A chromatograph, monitored at 215 nm. Elution was performed at 0.7 mL/min using the same buffer. Five chromatographic separations were repeated and the LAAO fraction was collected, immediately concentrated, and finally desalted with Amicon Ultra filters (MWCO 10,000 membrane).

To assess the purity of MipLAAO, the protein was submitted to analytical reverse-phase high performance liquid chromatographic (RP-HPLC) using a C18 column (Pinnacle, 5 µm particle diameter; 250 × 4.6 mm), eluted at 1 mL/min with a linear gradient from 0 to 70% solution B (0.1% TFA, 99.9% acetonitrile) in 25 min. Electrophoretic homogeneity was evaluated by SDS-PAGE (Laemmli, 1970) after reduction with 5% 2-mercaptoethanol at 100 °C for 5 min.

**Molecular mass determination**
The molecular mass of MipLAAO was determined by MALDI-TOF mass spectrometry. The protein (1 µg) was diluted in water-TFA (0.1%), mixed at 1:1 with saturated sinapinic acid in 50% acetonitrile, 0.1% TFA, and spotted (1 µL) onto an OptiToF-384 plate for MALDI-TOF MS analysis. Spectra were acquired on an Applied Biosystems 4800-Plus instrument (Foster City, CA, USA), using 500 shots/spectrum and a laser intensity of 4,200, in linear positive mode, over the m/z range 10,000–80,000.

**Protein identification by MALDI-TOF/TOF peptide sequencing**
For MS peptide sequencing, MipLAAO (40 µg) was reduced with dithiothreitol (10 mM), and alkylated with iodoacetamide (50 mM), followed by digestion with sequencing grade trypsin for 24 h at 37 °C, as described (Rey-Suárez et al., 2012). The resulting peptides were separated by RP-HPLC on a C18 column (2.1 × 150 mm; Phenomenex), eluted at 0.3 mL/min with a 0–70% acetonitrile gradient over 40 min, manually collected, and dried in a vacuum centrifuge (Vacufuge, Eppendorf). Peptides were redissolved in 50% acetonitrile, 0.1% TFA, and analyzed by MALDI-TOF/TOF (4,800 Plus Proteomics Analyzer; Applied Biosystems, Foster City, CA, USA) using α-cyano-hydroxycinnamic acid as matrix, at 2 kV in positive reflectron mode, 500 shots/spectrum, and a laser intensity of 3,000. Fragmentation spectra were initially searched using the Paragon® algorithm of ProteinPilot 4.0 (AB; Sciex, Framingham, MA, USA) against the UniProt/SwissProt serpentes database, and further they were manually interpreted de novo.

**Snake venom gland cDNA synthesis and sequencing**
mRNA was extracted from M. mipartitus venom gland of a specimen which died in captivity in the Serpentarium of Universidad de Antioquia, using the trizol method (Life Technologies, Carlsbad, CA, USA). The cDNA was obtained using Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). The conditions used in thermocycler were 95 °C for 8 min, followed by 10 min at 95 °C, 10 min at 53 °C and 2 min at 72 °C and 7 min at 72 °C to complete the cycle.
The LAAO-cDNA was obtained using the forward primer 5′-GAT GAA TGT CTT CTT TAT GTT CTC-3′, and the reverse 5′-GCA AGA GAT GTG AAT CGT GCT-3′ and PCR Supermix (Invitrogen), and conditions described by Pierre et al. (2005). The purified product was ligated to the pGEM-T Easy cloning vector (Promega, Madison, WI, USA) and used to transform Escherichia coli competent cells DH5-α and TOP10. The cells were cultured on LB (Luria-Bertani) agar, and transformed colonies were used to obtain the plasmid using the QIA prep Spin Miniprep kit (QIAGEN, Hilden, Germany). The extracted product was sent to Macrogen Korea, specifying that it corresponded to complete plasmids with gene inserted at the multiple cloning site, to be sequenced from the vector promoter T7 and SP6 in order to confirm the direction in which the construct was inserted into the vector.

Bioinformatics procedures

The edition of cDNA sequences was performed in BioEdit version 7.0 (Hall, 1999). Nucleotide sequences were translated into amino acids to evaluate the reading frame and ensure the absence of premature stop codons or other nonsense mutation using GeneDoc software (Nicholas, Nicholas & Deerfield, 1997). SignalP 4.01 server available at http://www.cbs.dtu.dk/services/SignalP/ was used for signal peptide prediction (Petersen et al., 2011) and the posterior analyses were performed only with the predicted mature protein. NetNglyc (http://www.cbs.dtu.dk/services/NetNGlyc/) was used to predict the glycosylation sites.

Phylogenetic relationships and genetic distance

Bayesian inference (BI) algorithms implemented in MrBayes v3.0B4 (Ronquist & Huelsenbeck, 2003) was used to infer phylogenetic trees. A total of 48 related amino acid sequences of venom LAAOs, including 17 from Elapidae, 30 from Viperidae and one from Polychrotidae were obtained in two protein databases, BLAST (BLASTp http://blast.ncbi.nlm.nih.gov/Blast.cgi) and Uniprot (http://www.uniprot.org/blast/). These sequences were selected with E-values close to zero and with percentage of identity >30% (Pearson, 2013) (Supplementary material S1).

The multiple alignment of amino acid sequences of mature chains was performed in PRALINE (Heringa, 1999) using default parameters. After including gaps to maximize alignments, the final number of amino acid positions was 500. For phylogenetic analysis we used Anolis carolinensis sequence (R4GD21) as outgroup. The best-fitting model of amino acid substitution was selected using Bayesian Information Criterion (BIC) (Neath & Cavanaugh, 2012) implemented in the program MEGA version 7 (Kumar, Stecher & Tamura, 2016). These results gave a best fit for the JTT + Γ (Jones, Taylor & Thornton, 1992) amino acid substitution model.

We executed three parallel MCMC runs simultaneously, each one was run for $30 \times 10^6$ generations with four Markov chains (one cold and three heated chains). We used Tracer v1.6 (Rambaut & Drummond, 2013) for visualizing output parameters to ascertain stationarity and whether or not the duplicated runs had converged on the same mean likelihood. Nodes were considered supported if posterior probabilities (PP) > 0.95. Trees were visualized using FigTree v1.1 (Rambaut, 2009) available at http://tree.bio.ed.ac.uk/software/figtree/.
In order to establish the genetic distance among LAAOs obtained in this study with LAAOs corresponding to *Micrurus* species used in the phylogenetic analysis, a matrix distance from aligned *Micrurus* sequences was obtained in Mega Version 7 (Supplemental Information 2).

**Enzymatic characterization**

LAAO activity was determined using the method of Kishimoto & Takahashi (2001). In brief, increasing doses (0.9 µg–3.0 µg) of MipLAAO (in 10 µL of water) were added to 90 µL of a reaction mixture (250 µM L-Leucine, 2 mM o-phenylenediamine, and 0.8 U/ml horseradish peroxidase) in 50 mM Tris pH 8.0 buffer. After incubation at 37 °C for 60 min, the reaction was stopped with 50 µL of 2 M H₂SO₄, and absorbances were recorded at 492 nm.

To determine the substrate specificity of MipLAAO, L-Leu was replaced with other L-amino acids (L-His, L-Ser, L-Arg, L-Ala, L-Trp, L-Glu, L-Cys, L-Lys, L-Tyr, L-Val, L-Ile, L-Thr), under standard assay conditions. The amount of MipLAAO in the reaction mixture was 1.5 µg. LAAO activity on L-Leu was also evaluated at different pH values (5.0–11.0).

**Antimicrobial assay**

Antimicrobial assays were performed according to the National Committee for Clinical Laboratory Standards (Clinical and Laboratory Standards, 2009) using two methods: the first, agar diffusion assays, in which 5 µL (10 µg) of MipLAAO was added to media (Muller-Hinton medium) with a suspension of 1.5 × 10⁶ colony forming units (CFU)/mL of *E. coli* (ATCC 25922) or *S. aureus* (ATCC 25923) and incubated at 37 °C for 24 h. Sterile saline solution and chloramphenicol (10 µg; Phyto Technology Laboratories, Lenexa, KS, USA) were used as negative and positive controls, respectively. Diameters of the bacterial growth inhibition zones were measured (Vargas et al., 2013). Each assay was performed in triplicate.

The second method was the broth microdilution in 96-well plates (Clinical and Laboratory Standards, 2009). Inoculum suspensions of *S. aureus* cultures were prepared and adjusted to a density of 1.5 × 10⁸ CFU/mL. In each well, 50 µL of the bacterial suspension and 50 µL of MipLAAO (0.01–7.0 µg/mL) were mixed. Plates were incubated at 37 °C for 24 h, and the minimum inhibitory concentration (MIC) was defined as the lowest concentration of enzyme that prevented visible growth (absence of turbidity) in the broth. Assays were performed in triplicate. Sterile saline solution and chloramphenicol were used as controls.

**Statistical analysis**

Results were expressed as mean ± SD. Analysis of variance (ANOVA- Kruskal-Wallis test) followed by Bonferroni post-test was employed to evaluate the statistical significance of data on LAAO activity, substrate preference, and pH effects. Differences were considered statistically significant when *p* < 0.05.
RESULTS
Isolation, determination of molecular mass, cloning and amino acid sequencing of MipLAAO

The fraction identified as LAAO in *M. mipartitus* venom (Rey-Suárez et al., 2011), here named MipLAAO, was isolated using SEC-HPLC, with an retention time of 4.5 min, (Fig. 1A). This fraction corresponds to 6% of the chromatogram with a final yield of 100 µg per run. The protein eluted as a homogeneous peak by RP-HPLC analysis, at 19.6 min (Fig. 1B). SDS-PAGE of MipLAAO under reducing (Fig. 1B, insert), or non reducing conditions (not shown) both presented a single band, with a migration at ~57 kDa, indicating that it is a monomeric protein.

The cDNA obtained from the venom gland mRNA of *M. mipartitus* evidenced a product of ~1,503 bp, corresponding to the expected molecular mass for the LAAO coding sequence. The primers amplified a DNA fragment, which was purified, ligated and used to transform *E. coli* cells. Eight positive clones, randomly selected, were sequenced and six coding sequences, including signal peptide, were obtained and designated as MipLAAO-1 to MipLAAO-6 (Fig. 2). These sequences were deposited in Genbank, with access codes (MH010800, MH010801, MH010802, MH010803, MH010804, MH010805).

Protein sequences deduced from the six cDNA clones consist of 501 amino acids, with a segment of 18 amino acids corresponding to signal peptide, and 483 amino acids corresponding to mature chain (Fig. 2). Few changes among the sequences were observed. MALDI-TOF/TOF peptide sequences obtained *de novo* matched the amino acid sequences deduced from cDNA (Fig. 2). These sequences evidenced high identity values compared to other LAAOs from elapid and viperid snakes (Supplemental Information 1). Highest identities were obtained against elapid LAAOs, especially from other *Micrurus*, the LAAOs of which have been predicted by transcriptomic studies, such as *M. spixi* LAAO (89% identity; Supplemental Information 1).

The observed isotope-averaged molecular mass of the isolated MipLAAO was 59,100.6 Da, by MALDI-TOF MS (Fig. 1C). The theoretical molecular masses predicted (https://web.expasy.org/peptide_mass/) from the nucleotide-sequenced clones MipLAAO-1 to -6 vary from 55,121.02 Da to 55,010.94 Da. These variations correspond to few amino acid substitutions among them (Fig. 2). The difference between these theoretical mass values and that determined for the isolated protein are suggestive of post-translational modification of the enzyme, for example glycosylation. MALDI-TOF/TOF peptide sequencing matched the predicted MipLAAO cDNA-deduced sequence, covering 56% (Fig. 2). In addition, the deduced amino acid sequences of MipLAAO isoforms predict these to be basic proteins, according to the Compute Mw/pI tool at https://web.expasy.org/compute_pi/, with pI values within the range of 8.85–8.93.

Phylogenetic relationships

The deduced protein sequences of six MipLAAO isoforms obtained in this study and 48 other related LAAOs were aligned to infer phylogenetic relationships. Our analysis recovered a deep split of LAAOs into two lineages (Clades A and B, Fig. 3), separating all Elapidae family enzymes in Clade A from those of Viperidae family in Clade B, except for the
Figure 1  Isolation of MipLAAO from *M. mipartitus* venom. (A) SEC-HPLC separation of *M. mipartitus* venom (2 mg) on a Biosec S-200 column eluted at 0.7 mL/min with PBS. MipLAAO (shadowed area) was collected in the peak eluting at 4.58 min, indicated with an arrow. (B) Protein homogeneity was observed for RP-HPLC on C\textsubscript{18} column eluted with a gradient of 0 to 70% solution B (Acetonitrile). The protein was analyzed by SDS-PAGE (15% gel) under reducing conditions (insert in B). MM, molecular mass standards, in kDa. (C) Molecular mass of the isolated MipLAAO by MALDI-TOF analysis.
King Cobra (*Ophiophagus hannah*) LAAO, which shares between 49.0% and 50.8% identity with other Elapidae LAAOs, and looped out with *Anolis carolinensis* LAAO (outgroup). This split, in clear agreement with snake taxonomy, is associated to differences in the amino acid residue at position 223, occupied by Ser in all LAAOs from clade A (with the exception of *M. surinamensis* and *Bungarus sp.*, having Asp and Asn, respectively) or by His in all members of clade B.

Within Clade A (Elapidae family), all LAOOs from *Micrurus* conform a well-supported group (*PP = 1.00*) with genetic distance range between 12% with *M. spixii* and 18.9% with *M. tener* (*Supplemental Information 2*). MipLAAO sequences here obtained present present variations with other *Micrurus* LAAOs at positions 175 (His instead of Tyr), 301 (His instead of Arg) and 384 (His instead of Leu).

**Functional characterization of MipLAAO**

MipLAAO was obtained in active form, presenting a conspicuous concentration-dependent enzymatic activity upon L-Leu (Fig. 4A). This enzyme only oxidized hydrophobic amino acids corresponding to the mature chains is shown. Polymorphic sites are highlighted in blue. Tryptic peptide sequences confirmed by MALDI-TOF/TOF MS are shown in bold and underlined in the MipLAAO-1 sequence. The three domains of LAAO are highlighted in colors: FAD-binding domain (FAD-BD in pink), substrate binding domain (S-BD in blue) and helical domain (H-BD in yellow). For reference to colors in this figure legend, the reader is referred to the web version of this article.

King Cobra (*Ophiophagus hannah*) LAAO, which shares between 49.0% and 50.8% identity with other Elapidae LAAOs, and looped out with *Anolis carolinensis* LAAO (outgroup). This split, in clear agreement with snake taxonomy, is associated to differences in the amino acid residue at position 223, occupied by Ser in all LAAOs from clade A (with the exception of *M. surinamensis* and *Bungarus sp.*, having Asp and Asn, respectively) or by His in all members of clade B.

Within Clade A (Elapidae family), all LAOOs from *Micrurus* conform a well-supported group (*PP = 1.00*) with genetic distance range between 12% with *M. spixii* and 18.9% with *M. tener* (*Supplemental Information 2*). MipLAAO sequences here obtained present present variations with other *Micrurus* LAAOs at positions 175 (His instead of Tyr), 301 (His instead of Arg) and 384 (His instead of Leu).

**Functional characterization of MipLAAO**

MipLAAO was obtained in active form, presenting a conspicuous concentration-dependent enzymatic activity upon L-Leu (Fig. 4A). This enzyme only oxidized hydrophobic amino acids corresponding to the mature chains is shown. Polymorphic sites are highlighted in blue. Tryptic peptide sequences confirmed by MALDI-TOF/TOF MS are shown in bold and underlined in the MipLAAO-1 sequence. The three domains of LAAO are highlighted in colors: FAD-binding domain (FAD-BD in pink), substrate binding domain (S-BD in blue) and helical domain (H-BD in yellow). For reference to colors in this figure legend, the reader is referred to the web version of this article.

King Cobra (*Ophiophagus hannah*) LAAO, which shares between 49.0% and 50.8% identity with other Elapidae LAAOs, and looped out with *Anolis carolinensis* LAAO (outgroup). This split, in clear agreement with snake taxonomy, is associated to differences in the amino acid residue at position 223, occupied by Ser in all LAAOs from clade A (with the exception of *M. surinamensis* and *Bungarus sp.*, having Asp and Asn, respectively) or by His in all members of clade B.

Within Clade A (Elapidae family), all LAOOs from *Micrurus* conform a well-supported group (*PP = 1.00*) with genetic distance range between 12% with *M. spixii* and 18.9% with *M. tener* (*Supplemental Information 2*). MipLAAO sequences here obtained present present variations with other *Micrurus* LAAOs at positions 175 (His instead of Tyr), 301 (His instead of Arg) and 384 (His instead of Leu).

**Functional characterization of MipLAAO**

MipLAAO was obtained in active form, presenting a conspicuous concentration-dependent enzymatic activity upon L-Leu (Fig. 4A). This enzyme only oxidized hydrophobic amino acids corresponding to the mature chains is shown. Polymorphic sites are highlighted in blue. Tryptic peptide sequences confirmed by MALDI-TOF/TOF MS are shown in bold and underlined in the MipLAAO-1 sequence. The three domains of LAAO are highlighted in colors: FAD-binding domain (FAD-BD in pink), substrate binding domain (S-BD in blue) and helical domain (H-BD in yellow). For reference to colors in this figure legend, the reader is referred to the web version of this article.
acids like L-Trp, L-Tyr, and L-Leu (Fig. 4B). Furthermore, the enzyme maintained its activity within the pH range 7.0–10.0, being optimal at pH 8.0 (Fig. 4C).

**Antibacterial activity of MipLAAO**

MipLAAO exhibited antibacterial activity against *S. aureus*, but not against *E. coli*. Against the former, the enzyme produced a bacterial growth inhibition halo of 19.8 ± 0.6 mm, while chloramphenicol used as a control produced a halo of 12 ± 1 mm. The MIC against *S. aureus* evaluated by the broth microdilution method was 2 µg/mL.

**DISCUSSION**

LAAOs are widely found in snake venoms, both in Elapidae and Viperidae families. In *Micrurus*, this enzyme has been detected by proteomic or transcriptomic analyses in *M. corallinus* (*Corrêa-Netto et al., 2011; Aird et al., 2017; Morais-Zani et al., 2018), *M. altirostris* (*Corrêa-Netto et al., 2011), *M. fulvius* (*Margres et al., 2013*), *M. surinamensis* (*Olamendi-Portugal et al., 2008; Aird et al., 2017), *M. mipartitus* (*Rey-Suárez et al., 2011*), *M. nigrocinctus* (*Fernández et al., 2011*), *M. lemniscatus*, *M. paraensis*, *M. spixii* (*Rey-Suárez et al., 2018*).
**Figure 4** Functional characterization of MipLAAO. (A) Enzymatic activity on L-Leu. (B) Substrate preference: L-Leu was replaced with other L-amino acids (L-His, L-Ser, L-Arg, L-Ala, L-Trp, L-Glu, L-Cys, L-Lys, L-Tyr, L-Val, L-Ile, L-Thr) under standard assay conditions. (C) pH influence; the enzyme activity was tested at various pH conditions (5.0–11.0). Each point represents the mean ± SD (n = 3). * Represents statistical significant difference compared to the negative control.

Full-size DOI: 10.7717/peerj.4924/fig-4
M. clarki (Lomonte et al., 2016), M. tener (Rokyta, Margres & Calvin, 2015), M. mosquitensis (Fernández et al., 2015), M. alleni (Fernández et al., 2015), and M. tschudii (Sanz et al., 2016).

The abundance of this enzyme in venoms varies among species, from traces (0.15%) as in *Naja oxiana* (Samel et al., 2008) to major proportions (25%) as in *Bungarus caeruleus* (More et al., 2010). In *Micrurus* venoms, it represents a low abundance component, with a range of 0.7–4.0% of the total proteins. Interestingly, the highest proportion of LAAO in this genus has thus far been found in *M. mipartitus* venom (4%; Rey-Suárez et al., 2011).

This study reports the first LAAO isolated and characterized from *Micrurus* venoms. MipLAAO is a basic protein with 483 amino acid residues. The enzyme was obtained in active form, showing substrate specificity for hydrophobic amino acids, and optimal catalytic activity at basic pH. Sequencing of cDNA clones obtained from the venom glands of a single individual, evidenced the presence of at least six isoforms, with few conservative differences among them. Similarly, transcriptomic analyses of *M. fulvius* (Margres et al., 2013), *M. tener* (Rokyta, Margres & Calvin, 2015), and *M. lemniscatus* (Aird et al., 2017) venom glands reported three, two, and two LAAO isoforms, respectively.

Structurally, the obtained MipLAAO sequences conserve the three well-known domains named FAD-binding domain (Asp2 to Val72, Gly238 to Arg322 and Tyr425 to Ala483), substrate binding domain (Lys73 to Arg129, Arg232 to Val237 and Ser323 to Lys424), and helical domain (Val130 to Lys231) (Feliciano et al., 2017). Few sequence variations were observed between the LAAO isoforms of *M. mipartitus* venom. In the FAD-binding domain, MipLAAO-2 has one substitution at residue His35, and MipLAAO-6 presents two, Leu52Pro and Thr329Ala. In the substrate binding domain, MipLAAO-4 displays one modification, Pro351Ser, and MipLAAO-3, 5, 6 all present the same change, His342Arg. Finally, in the helical domain, only MipLAAO-6 varied from the others, presenting the substitution Ser220Gly. The role of isoform sequence substitutions in the enzymatic activity and substrate preferences remains to be explored.

Amino acids involved in catalytic activity of the enzyme (Arg90, Tyr372, Gly464, Ile430, Phe227, and Lys326) are conserved in the sequences obtained for *M. mipartitus* LAAO, with the exception of residue 374 that presents a conservative Ile/Leu substitution. This amino acid is of key importance given that it participates in the hydrophobic interactions that are formed with the side chains of the substrate in the catalytic mechanisms (Izidoro et al., 2014).

As for almost all Elapidae LAAOs, MipLAAO presents Ser at position 223, which is occupied by His in LAAOs from Viperidae. His223 has been shown to play an important role in the enzymatic reaction during the binding of substrate to the catalytic site (Moustafa et al., 2006). However, His223 is substituted by Ser in almost all sequences of venom LAAOs from Elapidae, except in *M. surinamensis* (Asp223), *B.multicinctus*, and *B. multifasciatus* (Asn223). Chen et al. (2012) observed that His223 was present in all viperid LAAOs, Ser223 in most of elapid LAAOs, while Asn223 is present in krait LAAOs, and Asp223 is found in king cobra LAAO. These authors suggested that the different residues at position 223 may regulate substrate specificities of LAAOs by expanding the substrate-binding pocket and reducing steric repulsion.
MipLAOO has three potential N-glycosylation sites at Asn145, Asn194, and Asn361, indicating that it possibly presents this post-translational modification, in similarity with other venom LAAOs (Chen et al., 2012). Furthermore, this could explain the difference between the calculated and observed molecular mass values. It has been suggested that carbohydrates in LAAOs could contribute to cytotoxicity by mediating the binding of the enzyme to the cell surface and local accumulation of H₂O₂ (Geyer et al., 2001).

Similar to other svLAAOs (Ponnudurai, Chung & Tan, 1994; Sakurai et al., 2001; Vargas et al., 2013; Zhong et al., 2009; Jin et al., 2007), MipLAOO oxidized a variety of L-amino acids, especially the hydrophobic L-Trp, L-Tyr and L-Leu, indicating its optical isomer selectivity. In contrast, positively charged amino acids such as L-Lys and L-Arg present unfavorable electrostatic interactions with the catalytic site of the enzyme and are not oxidized (Moustafa et al., 2006).

Snake venom LAAOs exhibit wide ranges of pI, from acidic (Alves et al., 2008; Tönismägi et al., 2006; Toyama et al., 2006; Izidoro et al., 2006; Rodrigues et al., 2009; Okubo et al., 2012; Vargas Muñoz et al., 2014) to basic (Lu et al., 2018; Zhang & Wei, 2007; Zhang et al., 2004; Vargas et al., 2013). According to its sequence, MipLAOO is predicted to be a basic protein, with a theoretical pI of 8.9. Differences in charge density may influence the pharmacological activities of LAAOs (Izidoro et al., 2014).

Many snake venom LAAOs have been shown to be bactericidal (Toyama et al., 2006; Stábelli et al., 2007; Rodrigues et al., 2009; Zhong et al., 2009; Lee et al., 2011; Vargas et al., 2013; Vargas Muñoz et al., 2014; Lazo et al., 2017). Bacteria inhibited by these enzymes include the Gram-positives Bacillus dysenteriae, B. megatherium, B. subtilis, S. aureus and S. mutans, and Gram-negative bacteria such as Aeromonas sp., E. coli, Pseudomonas aeruginosa, Salmonella typhimurium, Acinetobacter baumannii and Xanthomonas axonopodis pv passiflorae (Lee et al., 2011). According to literature, the most likely mode of action involved in the bactericidal activity of LAAOs involves oxidative stress in the target cell caused by hydrogen peroxide, triggering disorganization of the plasma membrane and cytoplasm, and consequent cell death (Toyama et al., 2006; Souza et al., 1999; Izidoro et al., 2014). These activities can be inhibited by the addition of catalase and other H₂O₂ scavengers (Torii, Naito & Tsuruo, 1997; Tempone et al., 2001; Suhr & Kim, 1999; Zhang et al., 2004). However, according to several studies, the role played by hydrogen peroxide in the biological activities of LAAOs is uncertain. It is likely that there are other mechanisms involved in their pharmacological and toxicological effects (Suhr & Kim, 1999; Zhang et al., 2004; Izidoro et al., 2014).

Lee et al. (2011) suggest that binding to bacteria is important for the activity of LAAO, as the concentration of H₂O₂ generated by the enzyme is not sufficient to kill bacteria. It was argued that binding of LAAO to the bacteria enables production of highly localized concentrations of H₂O₂ in or near the binding sites that will be sufficiently potent to kill the bacteria. This also explains why a very small amount of LAAO could effectively inhibit bacteria growth, as the MIC of King cobra LAAO against S. aureus was as low as 0.78 µg/mL (Lee et al., 2011). The interaction of LAAO with different cell membranes was demonstrated by Abdelkafi-Koubaa et al. (2016) in a study with CC-LAAO from Cerastes cerastes venom.
Present results showed that MipLAAO was effective against the Gram-positive *S. aureus*, with a low MIC value of 2 µg/mL, whereas the Gram-negative *E. coli* was not affected. This is in agreement with the selectivity of antibacterial action described for LAAOs of *B. schlegelii* (Vargas Muñoz et al., 2014), *O. hannah* (Lee et al., 2011), *C. durissus cumanensis* (Vargas et al., 2013), *D. russelli siamensis* (Zhong et al., 2009), *C. durissus cascavella* (Toyama et al., 2006), and *T. mucrosquamatus* (Wei et al., 2003) against gram positive bacteria. On the other hand, LAAOs from *B. pauloensis* (Rodrigues et al., 2009), *V. labetina* (Tõnismägi et al., 2006), *N. oxiana* (Samel et al., 2008) and *P. australis* (Stiles, Sexton & Weinstein, 1991) were more active against Gram-negative than Gram-positive bacteria, and LAAOs from *A. halys*, *B. alternatus*, and *B. moojeni* inhibited both Gram-positive and Gram-negative bacteria. These differences in the selectivity of the antibacterial action of LAAOs are presumably due to differences in their binding to bacteria.

**CONCLUSIONS**

MipLAAO, the first LAAO characterized from a coral snake venom, is a basic protein with 483 amino acid residues. The enzyme was obtained in active form, showing substrate specificity for hydrophobic amino acids, and optimal catalytic activity at basic pH. It showed a significant antimicrobial effect against *S. aureus*, a clinically relevant Gram-positive bacterium. It will be of interest to explore its potential applications as antimicrobial agent in future studies.

**ACKNOWLEDGEMENTS**

We thank the Ministry of Environment for the access to the genetic resources (151 RGE: 0205). This work was performed in partial fulfilment of the Ph.D. degree of Paola Rey-Suárez at the University of Antioquia, Colombia.

**ADDITIONAL INFORMATION AND DECLARATIONS**

**Funding**

This work was supported by Colciencias (111556933661 and 617), University of Antioquia UdeA, and Vicerrectoría de Investigación, University of Costa Rica (VI-741-B7608). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Grant Disclosures**

The following grant information was disclosed by the authors:

Colciencias: 111556933661 and 617.

University of Antioquia UdeA, and Vicerrectoría de Investigación, University of Costa Rica: VI-741-B7608.

**Competing Interests**

Bruno Lomonte is an Academic Editor with PeerJ. The authors declare that there are no competing interests.
Author Contributions

• Paola Rey-Suárez conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
• Cristian Acosta and Uday Torres performed the experiments, approved the final draft.
• Mónica Saldarriaga-Córdoba conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
• Bruno Lomonte analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
• Vitelbina Núñez conceived and designed the experiments, analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.

DNA Deposition

The following information was supplied regarding the deposition of DNA sequences:
These sequences were deposited in Genbank, with accession numbers: MH010800, MH010801, MH010802, MH010803, MH010804, MH010805.

Data Availability

The following information was supplied regarding data availability:
The raw data are provided in the Figures in the article.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.4924#supplemental-information.

REFERENCES

Abdelkafi-Koubaa Z, Aissa I, Morjen M, Kharrat N, El Ayeb M, Gargouri Y, Srairi-Abid N, Marrakchi N. 2016. Interaction of a snake venom L-amino acid oxidase with different cell types membrane. *International Journal of Biological Macromolecules* **82**:757–764 DOI 10.1016/j.ijbiomac.2015.09.065.

Aird SD, Da Silva NJ, Qiú L, Villar-Briones A, Saddi VA, Pires de Campos Telles M, Grau ML, Mikheyev AS. 2017. Coralsnake venomics: analyses of venom gland transcriptomes and proteomes of six Brazilian taxa. *Toxins* **9**:E187 DOI 10.3390/toxins9060187.

Ali SA, Stoeva S, Abbasi A, Alam JM, Kayed R, Faigle M, Neumeister B, Voelter W. 2000. Isolation, structural, and functional characterization of an apoptosis-inducing L-amino acid oxidase from leaf-nosed viper (*Eristocophis macmahoni*) snake venom. *Archives of Biochemistry and Biophysics* **384**:216–226 DOI 10.1006/abbi.2000.2130.

Alves RM, Antonucci GA, Paiva HH, Cintra AC, Franco JJ, Mendonça-Franqueiro EP, Dorta DJ, Giglio JR, Rosa JC, Fuly AL, Dias-Baruffi M, Soares AM, Sampaio SV.
2008. Evidence of caspase-mediated apoptosis induced by L-amino acid oxidase isolated from Bothrops atrox snake venom. *Comparative Biochemistry and Physiology* 151:542–550 DOI 10.1016/j.cbpa.2008.07.007.

Carone SEI, Costa TR, Burin SM, Cintra ACO, Zoccal KF, Bianchini FJ, Tucci LFF, Franco JJ, Torqueti MR, Faccioli LH, Albuquerque S, Castro FA, Sampaio SV. 2017. A new L-amino acid oxidase from Bothrops jararacussu snake venom: isolation, partial characterization, and assessment of pro-apoptotic and antiprotozoal activities. *International Journal of Biological Macromolecules* 103:25–35 DOI 10.1016/j.ijbiomac.2017.05.025.

Chen HS, Wang YM, Huang WT, Huang KF, Tsai IH. 2012. Cloning, characterization and mutagenesis of Russell’s viper venom L-amino acid oxidase: insights into its catalytic mechanism. *Biochimie* 94:335–344 DOI 10.1016/j.biochi.2011.07.022.

Ciscotto P, Machado de Avila RA, Coelho EA, Oliveira J, Diniz CG, Farias LM, De Carvalho MA, Maria WS, Sanchez EF, Borges A, Chávez-Olórtegui C. 2009. Antigenic, microbial and antiparasitic properties of an L-amino acid oxidase isolated from Bothrops jararaca snake venom. *Toxicon* 53:330–341 DOI 10.1016/j.toxicon.2008.12.004.

Clinical and Laboratory Standards. 2009. Performance standards for antimicrobial susceptibility testing, nine-teenth informational supplement. In: *Approved standard M100-S19*. Wayne: National Committee for Clinical Standards.

Corrêa-Netto C, Junqueira-de Azevedo Ide L, Silva DA, Ho PL, Leitão-de Araújo M, Alves ML, Sanz L, Foguel D, Zingali RB, Calvete JJ. 2011. Snake venomomics and venom gland transcriptomic analysis of Brazilian coral snakes, Micrurus altirostris and M. corallinus. *Journal of Proteomics* 74:1795–1809 DOI 10.1016/j.jprot.2011.04.003.

Costa TR, Burin SM, Menaldo DL, De Castro FA, Sampaio SV. 2014. Snake venom L-amino acid oxidases: an overview on their antitumor effects. *Journal of Venomous Animals and Toxins including Tropical Diseases* 20:Article 23 DOI 10.1186/1678-9199-20-23.

Costa TR, Menaldo DL, Zoccal KF, Burin SM, Aissa AF, Castro FA, Faccioli LH, Greggi Antunes LM, Sampaio SV. 2017. CR-LAAO, an L-amino acid oxidase from Callotoxicus rhodostoma venom, as a potential tool for developing novel immunotherapeutic strategies against cancer. *Scientific Reports* 7:42673 DOI 10.1038/srep42673.

Davis MA, Askin MC, Hynes MJ. 2005. Amino acid catabolism by an area-regulated gene encoding an L-amino acid oxidase with broad substrate specificity in Aspergillus nidulans. *Applied and Environmental Microbiology* 71:3551–3555 DOI 10.1128/AEM.71.7.3551-3555.2005.

Du XY, Clemetson KJ. 2002. Snake venom L-amino acid oxidases. *Toxicon* 40:659–665 DOI 10.1016/S0041-0101(02)00102-2.

Feliciano PR, Rustiguel JK, Soares RO, Sampaio SV, Cristina Nonato M. 2017. Crystal structure and molecular dynamics studies of L-amino acid oxidase from Bothrops atrox. *Toxicon* 128:50–59 DOI 10.1016/j.toxicon.2017.01.017.
Fernández J, Alape-Girón A, Angulo Y, Sanz L, Gutiérrez JM, Calvete JJ, Lomonte B. 2011. Venomic and antivenomic analyses of the Central American coral snake, *Micrurus nigrocinctus* (Elapidae). *Journal of Proteome Research* **10**:1816–1827 DOI 10.1021/pr101091a.

Fernández J, Vargas-Vargas N, Pla D, Sasa M, Rey-Suárez P, Sanz L, Gutiérrez JM, Calvete JJ, Lomonte B. 2015. Snake venomics of *Micrurus alleni* and *Micrurus mosquitensis* from the Caribbean region of Costa Rica reveals two divergent compositional patterns in New World elapids. *Toxicon* **107**:217–233 DOI 10.1016/j.toxicon.2015.08.016.

Geuwe B, Hummel W. 2002. A new bacterial L-amino acid oxidase with a broad substrate specificity: purification and characterization. *Enzyme and Microbial Technology* **31**:77–87 DOI 10.1016/S0141-0229(02)00072-8.

Geyer A, Fitzpatrick TB, Pawelek PD, Kitzing K, Vrielink A, Ghisla S, Macheroux P. 2001. Structure and characterization of the glycan moiety of L-amino-acid oxidase from the Malayan pit viper *Calloselasma rhodostoma*. *European Journal of Biochemistry* **268**:4044–4053 DOI 10.1046/j.1432-1327.2001.02321.x.

Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. *Nucleic Acids Symposium Series* **41**:95–98.

Hargreaves AD, Swain MT, Logan DW, Mulley JF. 2014. Testing the Toxicofera: comparative transcriptomics casts doubt on the single, early evolution of the reptile venom system. *Toxicon* **92**:140–156 DOI 10.1016/j.toxicon.2014.10.004.

Heringa J. 1999. Two strategies for sequence comparison: profile-preprocessed and secondary structure-induced multiple alignment. *Computers and Chemistry* **23**:341–364 DOI 10.1016/S0097-8485(99)00012-1.

Hossain GS, Li J, Shin HD, Liu L, Wang M, Du G, Chen J. 2014. Improved production of α-ketoglutaric acid (α-KG) by a *Bacillus subtilis* whole-cell biocatalyst via engineering of L-amino acid deaminase and deletion of the α-KG utilization pathway. *Journal of Biotechnology* **187**:71–77 DOI 10.1016/j.jbiotec.2014.07.431.

Huang YL, Li M, Yu Z, Qian PY. 2011. Correlation between pigmentation and larval settlement deterrence by *Pseudoalteromonas* sp. sf57. *Biofouling* **27**:287–293 DOI 10.1080/08927014.2011.562978.

Izidoro LF, Ribeiro MC, Souza GRL, Sant’Ana CD, Hamaguchi A, Homsi-Brandebugro MI, Goulart LR, Beleboni RO, Nomizo A, Sampaio SV, Soares AM, Rodrigues VM. 2006. Biochemical and functional characterization of an L-amino acid oxidase isolated from *Bothrops pirajai* snake venom. *Bioorganic and Medicinal Chemistry* **14**(20):7034–7043 DOI 10.1016/j.bmc.2006.06.025.

Izidoro LF, Sobrinho JC, Mendes MM, Costa TR, Grabner AN, Rodrigues VM, Da Silva SL, Zanchi FB, Zuliani JP, Fernandes CF, Calderon LA, Stábeli RG, Soares AM. 2014. Snake venom L-amino acid oxidases: trends in pharmacology and biochemistry. *BioMed Research International* **2014**:Article 196754 DOI 10.1155/2014/196754.

Jin Y, Lee WH, Zeng L, Zhang Y. 2007. Molecular characterization of L-amino acid oxidase from king cobra venom. *Toxicon* **50**:479–489 DOI 10.1016/j.toxicon.2007.04.013.
Jones DT, Taylor WR, Thornton JM. 1992. The rapid generation of mutation data matrices from protein sequences. *Computer Applications in the Biosciences* **8**:275–282.

Kishimoto M, Takahashi T. 2001. A spectrophotometric microplate assay for L-amino acid oxidase. *Analytical Biochemistry* **298**:136–139 DOI 10.1006/abio.2001.5381.

Kumar S, Stecher G, Tamura K. 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution* **33**:1870–1874 DOI 10.1093/molbev/msw054.

Laemmli UK. 1970. Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature* **227**:680–685 DOI 10.1038/227680a0.

Lazo F, Vivas-Ruiz DE, Sandoval GA, Rodríguez EF, Kozlova EEG, Costal-Oliveira F, Chávez-Olórtegui C, Severino R, Yarlequé A, Sanchez EF. 2017. Biochemical, biological and molecular characterization of an L-Amino acid oxidase (LAAO) purified from *Bothrops pictus* Peruvian snake venom. *Toxicon* **139**:74–86 DOI 10.1016/j.toxicon.2017.10.001.

Lee ML, Tan NH, Fung SY, Sekaran SD. 2011. Antibacterial action of a heat-stable form of L-amino acid oxidase isolated from king cobra (*Ophiophagus hannah*) venom. *Comparative Biochemistry and Physiology Part C Toxicology & Pharmacology* **153**:237–242 DOI 10.1016/j.cbpc.2010.11.001.

Lomonte B, Sasa M, Rey-Suárez P, Bryan W, Gutiérrez JM. 2016. Venom of the coral snake *Micrurus clarki*: proteomic profile, toxicity, immunological cross-neutralization, and characterization of a three-finger toxin. *Toxins* **8**:E138 DOI 10.3390/toxins8050138.

Lu W, Hu L, Yang J, Sun X, Yan H, Liu J, Chen J, Cheng X, Zhou Q, Yu Y, Wei JF, Cao P. 2018. Isolation and pharmacological characterization of a new cytotoxic L-amino acid oxidase from *Bungarus multicinctus* snake venom. *Journal of Ethnopharmacology* **213**:311–320 DOI 10.1016/j.jep.2017.11.026.

Margres MJ, Aronow K, Loyacano J, Rokyta DR. 2013. The venom-gland transcriptome of the eastern coral snake (*Micrurus fulvius*) reveals high venom complexity in the intragenomic evolution of venoms. *BMC Genomics* **14**:531 DOI 10.1186/1471-2164-14-531.

Morais-Zani K, Serino-Silva C, Galizio NDC, Tasima LJ, Pagotto JF, Rocha MMTD, Marcelino JR, Sant’Anna SS, Tashima AK, Tanaka-Azevedo AM, Grego KF. 2018. Does the administration of pilocarpine prior to venom milking influence the composition of *Micrurus corallinus* venom? *Journal of Proteomics* **174**:17–27 DOI 10.1016/j.jprot.2017.12.010.

More SS, Kiran KM, Veena SM, Gadag JR. 2010. Purification of an L-amino acid oxidase from *Bungarus caeruleus* (Indian krait) venom. *Journal of Venomous Animals and Toxins including Tropical Diseases* **16**:60–76 DOI 10.1590/S1678-91992010005000002.

Moustafa IM, Foster S, Lyubimov AV, Vrieland A. 2006. Crystal structure of LAAO from *Calloselasma rhodostoma* with an L-phenylalanine substrate: insights into structure and mechanism. *Journal of Molecular Biology* **364**:991–1002 DOI 10.1016/j.jmb.2006.09.032.
Naumann GB, Silva LF, Silva L, Faria G, Richardson M, Evangelista K, Kohlhoff M, Gontijo CM, Navdaev A, De Rezende FF, Eble JA, Sanchez EF. 2011. Cytotoxicity and inhibition of platelet aggregation caused by an L-amino acid oxidase from Bothrops leucurus venom. Biochimica Et Biophysica Acta/General Subjects 1810:683–694 DOI 10.1016/j.bbagen.2011.04.003.

Neath AA, Cavanaugh JE. 2012. The Bayesian information criterion: background, derivation, and applications. WIREs Computational Statistics 4:199–203 DOI 10.1002/wics.199.

Nicholas KB, Nicholas Jr HB, Deerfield DW. 1997. GeneDoc: analysis and visualization of genetic variation. Embnewnews 4:Article 14.

Okubo BM, Silva ON, Migliolo L, Gomes DG, Porto WF, Batista CL, Ramos CS, Holanda HH, Dias SC, Franco OL, Moreno SE. 2012. Evaluation of an antimicrobial L-amino acid oxidase and peptide derivatives from Bothropoides mattrugrosensis pit viper venom. PLOS ONE 7:e33639 DOI 10.1371/journal.pone.0033639.

Olamendi-Portugal T, Batista CV, Restano-Cassulini R, Pando V, Villa-Hernandez O, Zavaleta-Martinez-Vargas A, Salas-Arruz MC, Rodriguez de la Vega RC, Becerril B, LD Possani. 2008. Proteomic analysis of the venom from the fish eating coral snake Micrurus surinamensis: novel toxins, their function and phylogeny. Proteomics 8:1919–1932 DOI 10.1002/pmic.200700668.

Pearson WR. 2013. An introduction to sequence similarity (“homology”) searching. Current Protocols in Bioinformatics 42:3–1 DOI 10.1002/0471250953.bi0301s42.

Petersen TN, Brunak S, Von Heijne G, Nielsen H. 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nature Methods 8:785–786 DOI 10.1038/nmeth.1701.

Pierre L, Woods R, Earl S, Masci PP, Lavin MF. 2005. Identification and analysis of venom gland-specific genes from the coastal taipan (Oxyuranus scutellatus) and related species. Cellular and Molecular Life Science 62:2679–2693 DOI 10.1007/s00018-005-5384-9.

Pišlar A, Sabotič J, Šlenc J, Brzin J, Kos J. 2016. Cytotoxic L-amino-acid oxidases from Amanita phalloides and Clitocybe geotropa induce caspase-dependent apoptosis. Cell Death Discovery 2:Article 16021.

Ponnudurai G, Chung MC, Tan NH. 1994. Purification and properties of the L-amino acid oxidase from Malay pit viper (Calloselasma rhodostoma) venom. Archives of Biochemistry and Biophysics 313:373–378 DOI 10.1006/abbi.1994.1401.

Puiffe ML, Lachaise I, Molinier-Frenkel V, Castellano F. 2013. Antibacterial properties of the mammalian L-amino acid oxidase IL4I1. PLOS ONE 8:e54589 DOI 10.1371/journal.pone.0054589.

Rambaut A. 2009. FigTree, a graphical viewer of phylogenetic trees. Available at http://tree.bio.ed.ac.uk/software/figtree/.

Rambaut A, Drummond AJ. 2013. Tracer v16. Available at http://tree.bio.ed.ac.uk/software/tracer/.

Rey-Suárez P, Floriano RS, Rostelato-Ferreira S, Saldarriaga-Córdoba M, Núñez V, Rodrigues-Simioni L, Lomonte B. 2012. Mipartoxin-I, a novel three-finger toxin,
is the major neurotoxic component in the venom of the redtail coral snake *Micrurus mipartitus* (Elapidae). *Toxicon* 60:851–863 DOI 10.1016/j.toxicon.2012.05.023.

Rey-Suárez P, Núñez V, Gutiérrez JM, Lomonte B. 2011. Proteomic and biological characterization of the venom of the redtail coral snake, *Micrurus mipartitus* (Elapidae), from Colombia and Costa Rica. *Journal of Proteomics* 75:655–667 DOI 10.1016/j.jprot.2011.09.003.

Rodrigues RS, Da Silva JF, Boldrini França J, Fonseca FP, Otaviano AR, Henrique Silva F, Hamaguchi A, Magro AJ, Braz AS, Dos Santos JJ, Homsi-Brandeburgo MI, Fontes MR, Fuly AL, Soares AM, Rodrigues VM. 2009. Structural and functional properties of Bp-LAAO, a new L-amino acid oxidase isolated from *Bothrops pauloensis* snake venom. *Biochimie* 91:490–501 DOI 10.1016/j.biochi.2008.12.004.

Rokyta DR, Marges MJ, Calvin K. 2015. Post-transcriptional mechanisms contribute little to phenotypic variation in snake venoms. *G3* 5:2375–2382 DOI 10.1534/g3.115.020578.

Ronquist F, Huelsenbeck JP. 2003. Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19:1572–1574 DOI 10.1093/bioinformatics/btg180.

Sakurai Y, Takatsuka H, Yoshioka A, Matsu T, Suzuki M, Titani K, Fujimura Y. 2001. Inhibition of human platelet aggregation by L-amino acid oxidase purified from *Naja naja kaouthia* venom. *Toxicon* 39:1827–1833 DOI 10.1016/S0041-0101(01)00133-7.

Samel M, Tönismägi K, Rönholm G, Vija H, Siigur J, Kalkkinen N, Siigur E. 2008. L-Amino acid oxidase from *Naja naja oxiana* venom. *Comparative Biochemistry and Physiology. B: Comparative Biochemistry* 149:572–580 DOI 10.1016/j.cbpb.2007.11.008.

Sanz L, Pla D, Pérez A, Rodríguez Y, Zavaleta A, Salas M, Lomonte B, Calvete JJ. 2016. Venomic Analysis of the Poorly Studied Desert Coral Snake, *Micrurus tschudii tschudii*, Supports the 3FTx/PLA<sub>2</sub> Dichotomy across *Micrurus* Venoms. *Toxins (Basel)* 8:E178 DOI 10.3390/toxins8060178.

Skarnes RC. 1970. L-amino-acid oxidase, a bactericidal system. *Nature* 225:1072–1073 DOI 10.1038/2251072a0.

Souza DH, Eugenio LM, Fletcher JE, Jiang MS, Garratt RC, Oliva G, Selistre-de Araujo HS. 1999. Isolation and structural characterization of a cytotoxic L-amino acid oxidase from *Agristodon contortrix laticinctus* snake venom: preliminary crystallographic data. *Archives of Biochemistry and Biophysics* 368:285–290 DOI 10.1006/abbi.1999.1287.

Stábeli RG, Sant’Ana CD, Ribeiro PH, Costa TR, Ticli FK, Pires MG, Nomizo A, Albuquerque S, Malta-Neto NR, Marins M, Sampaio SV, Soares AM. 2007. Cytotoxic L-amino acid oxidase from *Bothrops moojeni*: biochemical and functional characterization. *International Journal of Biological Macromolecules* 41:132–140 DOI 10.1016/j.ijbiomac.2007.01.006.

Stiles BG, Sexton FW, Weinstein SA. 1991. Antibacterial effects of different snake venoms: purification and characterization of antibacterial proteins from *Pseudechis*...
australis (Australian king brown or mulga snake) venom. *Toxicon* **29**:1129–1141 DOI 10.1016/0041-0101(91)90210-I.

**Struck J, Sizer W. 1960.** Oxidation of L-α-amino acids by chicken liver microsomes. *Archives of Biochemistry and Biophysics* **90**:22–30 DOI 10.1016/0003-9861(60)90606-8.

**Suhr SM, Kim DS. 1999.** Comparison of the apoptotic pathways induced by L-amino acid oxidase and hydrogen peroxide. *Journal of Biochemistry* **125**:305–309 DOI 10.1093/oxfordjournals.jbchem.a022287.

**Takatsuka H, Sakurai Y, Yoshioka A, Kokubo T, Usami Y, Suzuki M, Matsui T, Titani K, Yagi H, Matsumoto M, Fujimura Y. 2001.** Molecular characterization of L-amino acid oxidase from *Agkistrodon halys blomhoffii* with special reference to platelet aggregation. *Biochimica et Biophysica Acta* **1544**:267–277 DOI 10.1016/S0167-4838(00)00229-6.

**Tan KK, Ler SG, Gunaratne J, Bay BH, Ponnampalam G. 2017.** In vitro cytotoxicity of L-amino acid oxidase from the venom of *Crotalus mitchellii pyrrhus*. *Toxicon* **139**:20–30 DOI 10.1016/j.toxicon.2017.09.012.

**Teixeira TL, Oliveira Silva VA, Da Cunha DB, Polettini FL, Thomaz CD, Pianca AA, Zambom FL, Da Silva Leitão Mazzi DP, Reis RM, Mazzi MV. 2016.** Isolation, characterization and screening of the in vitro cytotoxic activity of a novel L-amino acid oxidase (LAAOcdt) from *Crotalus durissus terrificus* venom on human cancer cell lines. *Toxicon* **119**:203–217 DOI 10.1016/j.toxicon.2016.06.009.

**Tempone AG, Andrade Jr HF, Spencer PJ, Lourenço CO, Rogero JR, Nascimento N. 2001.** *Bothrops moojeni* venom kills *Leishmania* spp. with hydrogen peroxide generated by its L-amino acid oxidase. *Biochemical and Biophysical Research Communications* **280**:620–624 DOI 10.1006/bbrc.2000.4175.

**Tönismägi K, Samel M, Trummal K, Rönnholm G, Siigur J, Kalkkinen N, Siigur E. 2006.** L-Amino acid oxidase from *Vipera lebetina* venom: isolation, characterization, effects on platelets and bacteria. *Toxicon* **48**:227–237 DOI 10.1016/j.toxicon.2006.05.004.

**Torii S, Naito M, Tsuruo T. 1997.** Apoxin I, a novel apoptosis inducing factor with L-amino acid oxidase activity purified from western diamondback rattlesnake venom. *Journal of Biological Chemistry* **272**:9539–9542 DOI 10.1074/jbc.272.14.9539.

**Toyama MH, Toyama Dde O, Passero LF, Laurenti MD, Corbett CE, Tomokane TY, Fonseca FV, Antunes E, Joazeiro PP, Beriam LO, Martins MA, Monteiro HS, Fonteles MC. 2006.** Isolation of a new L-amino acid oxidase from *Crotalus durissus cascavella* venom. *Toxicon* **47**:47–57 DOI 10.1016/j.toxicon.2005.09.008.

**Vargas LJ, Quintana JC, Pereañez JA, Núñez V, Sanz L, Calvete J. 2013.** Cloning and characterization of an antibacterial L-amino acid oxidase from *Crotalus durissus cumanensis* venom. *Toxicon* **64**:1–11 DOI 10.1016/j.toxicon.2012.11.027.

**Vargas Muñoz LJ, Estrada-Gomez S, Núñez V, Sanz L, Calvete JJ. 2014.** Characterization and cDNA sequence of *Bothriechis schlegelii* L-amino acid oxidase with antibacterial activity. *International Journal of Biological Macromolecules* **69**:200–207 DOI 10.1016/j.ijbiomac.2014.05.039.
Wei JF, Wei Q, Lu QM, Tai H, Jin Y, Wang WY, Xiong YL. 2003. Purification, characterization and biological activity of an L-amino acid oxidase from *Trimeresurus mucrosquamatus* venom. *Sheng Wu Hua Xue Yu Sheng Wu Wu Li Xue Bao* 35:219–224.

Zhang H, Teng M, Niu L, Wang Y, Wang Y, Liu Q, Huang Q, Hao Q, Dong Y, Liu P. 2004. Purification, partial characterization, crystallization and structural determination of AHP-LAAO, a novel L-amino-acid oxidase with cell apoptosis-inducing activity from *Agkistrodon halys pallas* venom. *Acta Crystallographica Section D: Biological Crystallography* 60:974–977 DOI 10.1107/S0907444904000046.

Zhang L, Wei LJ. 2007. ACTX-8, a cytotoxic L-amino acid oxidase isolated from *Agkistrodon acutus* snake venom, induces apoptosis in Hela cervical cancer cells. *Life Sciences* 80:1189–1197 DOI 10.1016/j.lfs.2006.12.024.

Zhong SR, Jin Y, Wu JB, Jia YH, Xu GL, Wang GC, Xiong YL, Lu QM. 2009. Purification and characterization of a new L-amino acid oxidase from *Daboia russellii siamensis* venom. *Toxicon* 54:763–771 DOI 10.1016/j.toxicon.2009.06.004.