Introduction

Snake venoms are complex mixtures of proteins with or without enzymatic activity, including metalloproteinases, serine proteinases, phospholipases A₂, and L-amino acid oxidase. According to World Health Organization (WHO), snake-envenoming represents a public health problem worldwide and is considered a neglected disease.¹,²

In Brazil, the venomous pit vipers Bothrops are responsible for the majority of snakebites (90%), and B. jararaca and B. jararacussu are amongst the highest venomous species. B. jararaca is found in southern of Brazil, Paraguay, and northern of Argentina; while B. jararacussu is found in Brazil (from Bahia to Santa Catarina), Paraguay, southeastern Bolivia, and northeastern Argentina. Envenoming by these snakes shares some common features, as bleeding, renal or cardiac failure, hemorrhage, edema or massive tissue necrosis.³ In fact, B. jararaca or B. jararacussu bite induces severe symptoms, and usually leads victims to death or amputation of the affected limb. The treatment of snake bites is performed by injecting polyvalent or monovalent antivenom, that despite neutralizing systemic effects and preventing the death of victims, it does not block efficiently local effects, as tissue necrosis.⁴ Besides, as consequences, morbidities and amputation may occur as well as it may induce side
effects into victims, as early anaphylactic reactions and fever. Thus, seeking for strategies to block toxic effects of snake venom is a considerable challenge, and natural products have been investigated, elsewhere. On the other hand, molecules derived from organic synthesis have not been widely investigated. In this context, heterocycles play an essential role in drug design, since they comprise a class of substances of great synthetic interest due to their occurrence in natural products and pharmacologically active compounds. In particular, 1,3-benzoxathiol-2-one and its derivatives have been found to possess diverse biological activities, such as antibacterial, antifungal, antioxidant, anticancer, anti-inflammatory and carbonic anhydrase and monoamine oxidase (MAO) enzymes inhibitors. To the best of our knowledge, the antivenom effect of such class of molecules has not been studied. On the other hand, sulfonamide derivatives have already been reported for their ability to neutralize the hemolytic activity of the venom of Lachesis muta, as well as to inhibit some in vitro and in vivo toxic effects of the venoms of B. jararaca or L. muta. Besides, drugs derived from sulfa have been utilized in some pharmaceutical applications due to their antibacterial, antiviral, antimalarial, antifungal, anticancer, antidepressant and other properties.

In continuation of our efforts on the synthesis of new biologically active 1,3-benzoxathiol-2-one-based compounds, we herein report the synthesis of new 1,3-benzoxathiol-2-one sulfonamides and evaluation of their ability to inhibit some toxic in vitro and in vivo activities of B. jararaca and B. jararacussu venoms.

Experimental

Chemistry

Reactions were routinely monitored by thin-layer chromatography (TLC) on silica-gel precoated F254 Merck plates (Darmstadt, Germany) visualized under UV light (254-366 nm). Melting points were determined on a Fisatom 430 apparatus (São Paulo, Brazil) and are uncorrected. Catalytic hydrogenation reaction was performed on a Paar 4540 reactor (Moline, USA). Infrared (IR) spectra were recorded on a PerkinElmer 1420 spectrometer (Waltham, USA) using KBr pellets and frequencies are expressed in cm⁻¹. Electrospay ionization mass spectra (ESI-MS) in the negative ion mode were recorded on a Waters ZQ-4000 single quadrupole mass spectrometer (Milford, USA). Nuclear magnetic resonance (NMR) spectra were recorded on a Varian Unity Plus 300 and 500 (Palo Alto, USA) spectrometers in dimethyl sulfoxide (DMSO-d6), which was used as the deuterated solvent (Cambridge Isotope Laboratories Inc., Tewksbury, USA). Chemical shifts (δ) are reported in parts per million (ppm) relative to tetramethylsilane (TMS), which was used as an internal standard. All reagents and solvents were used as obtained from commercial suppliers without further purification. In addition, pyridine, ethyl acetate, hydrochloric acid, diethyl ether and magnesium sulfate were purchased from Vetec Química Fina LTDA (Duque de Caxias, Brazil). Finally, 6-hydroxy-benzo[d][1,3]oxathiol-2-one, 4-substituted benzenesulfonyl chlorides and 4-(dimethylamino)pyridine (DMAP) were obtained from Sigma-Aldrich (São Paulo, Brazil).

Procedures for preparing 1,3-benzoxathiol-2-one derivatives 2, 3 and 4

Protocols for the preparation, physical and spectroscopic data of compounds 2, 3 and 4 have already been reported in our previous papers.

General procedure for the synthesis of sulfonamides (5a-5h)

In a round-bottom flask, pyridine (10 mL), 5-amino-6-methoxybenzo[d][1,3]oxathiol-2-one (4) (1 mmol), the appropriate 4-substituted benzenesulfonyl chloride (1.5 mmol) and DMAP (1 mmol) were added. The system was heated at reflux for 8 h. Then, the solution was neutralized with concentrated HCl. The reaction mixture was extracted with ethyl acetate (4 × 20 mL), the combined organic layers were dried over MgSO4 and evaporated under reduced pressure. The oil obtained was triturated with diethyl ether to afford a solid product. After completing the crystallization, the solid was filtered and was washed with hot water followed by diethyl ether.

\[ N-(6-Methoxy-2-oxobenzo[d][1,3]oxathiol-5-yl)-4-methylbenzenesulfonylamide (5a) \]

Yield: 48%; mp 171-173 °C; IR (KBr) ν / cm⁻¹ 3258 (N-H), 1768 (C=O), 1336 (S=O), 1161 (S=O); 1H NMR (500.00 MHz, DMSO-d6) δ 9.52 (s, 1H, NH), 7.64 (s, 1H, H2, J = 8.0 Hz, H2'/H6'), 7.32 (d, 2H, J = 8.0 Hz, H3'/H5'), 7.16 (s, 1H, H4), 3.46 (s, 3H, OCH3), 2.35 (s, 3H, CH3); 13C NMR (125.0 MHz, DMSO-d6) δ 169.7 (C=O), 153.0 (C6), 146.1 (C7a), 142.8 (C4'), 137.4 (C1'), 129.1 (C3'/C5'), 126.7 (C2'/C6'), 123.1 (C5), 120.4 (C7), 112.5 (C3a), 97.2 (C4), 56.1 (OCH3), 20.9 (CH3); ESI-MS m/z, [M – H]⁻ : 350.1 ([M – H]⁻ 100%).

\[ N-(6-Methoxy-2-oxobenzo[d][1,3]oxathiol-5-yl)-4-nitrobenzenesulfonylamide (5b) \]

Yield: 40%; mp 215-217 °C; IR (KBr) ν / cm⁻¹ 3257 (N-H), 1737 (C=O), 1532 (N-O), 1348 (S=O), 1291 (N-O),
1169 (S=O); 1H NMR (500.00 MHz, DMSO-d$_6$) δ 8.36 (d, 2H, J 8.9 Hz, H3'/H5'), 7.92 (d, 2H, J 8.9 Hz, H2'/H6'), 7.65 (s, 1H, H7), 7.17 (s, 1H, H4), 3.41 (s, 3H, OCH$_3$); 13C NMR (75.0 MHz, DMSO-d$_6$) δ 169.7 (C=O), 153.8 (C6), 149.6 (C7a or C1' or C4'), 146.9 (C7a or C1' or C4'), 146.0 (C7a or C1' or C4'), 128.2 (C2'/C6'), 124.0 (C3'/C5'), 122.3 (C7), 121.9 (C5), 112.7 (C3a), 97.3 (C4), 56.0 (OCH$_3$); ESI-MS m/z [M – H$^-$]: 381.1 ([M – H$^-$] 100%).

4-Fluoro-N-(6-methoxy-2-oxobenzo[d][1,3]oxathiol-5-yl) benzenesulfonamide (5e)

Yield: 25%; mp 123-125 °C; IR (KBr) ν / cm$^{-1}$ 3247 (N-H), 1760 (C=O), 1340 (S=O), 1168 (S=O), 1157 (C-F); 1H NMR (500.00 MHz, DMSO-d$_6$) δ 7.73 (dd, 1H, J 9.0, 5.2 Hz, H2'/H6'), 7.64 (s, 1H, H7), 7.36 (d, 2H, J 8.9 Hz, H3'/H5'), 7.16 (s, 1H, H4), 3.43 (s, 3H, OCH$_3$); 13C NMR (75.0 MHz, DMSO-d$_6$) δ 170.2 (C=O), 164.7 (d, 3J$^C$=F 251.1 Hz, C4'), 153.9 (C6), 147.0 (C7a), 132.7 (d, 3J$^C$=F 3.3 Hz, C1'), 130.2 (d, 3J$^C$ 9.6 Hz, C2'/C6'), 123.1 (C5), 122.0 (C7), 116.4 (d, 3J$^C$ 22.7 Hz, C3'/C5'), 113.0 (C3a), 97.7 (C4), 56.6 (OCH$_3$); ESI-MS m/z [M – H$^-$]: 354.1 ([M – H$^-$] 100%).

4-Bromo-N-(6-methoxy-2-oxobenzo[d][1,3]oxathiol-5-yl) benzenesulfonamide (5d)

Yield: 33%; mp 164-166 °C; IR (KBr) ν / cm$^{-1}$ 3261 (N-H), 1768 (C=O), 1339 (S=O), 1161 (S=O); 1H NMR (500.00 MHz, DMSO-d$_6$) δ 9.78 (s, 1H, NH), 7.75 (d, 2H, J 8.6 Hz, H2'/H6'), 7.65 (s, 1H, H7), 7.59 (d, 2H, J 8.6 Hz, H3'/H5'), 7.18 (s, 1H, H4), 3.45 (s, 3H, OCH$_3$); 13C NMR (125.0 MHz, DMSO-d$_6$) δ 170.2 (C=O), 153.9 (C6), 147.1 (C7a), 140.1 (C1'), 132.3 (C2'/C6'), 129.1 (C3'/C5'), 126.9 (C4'), 122.9 (C5), 122.0 (C7), 111.3 (C3a), 97.7 (C4), 56.6 (OCH$_3$); ESI-MS m/z [M – H$^-$]: 415.9 ([M – H$^-$] 100%).

4-Chloro-N-(6-methoxy-2-oxobenzo[d][1,3]oxathiol-5-yl) benzenesulfonamide (5e)

Yield: 40%; mp 98-100 °C; IR (KBr) ν / cm$^{-1}$ 3244 (N-H), 1761 (C=O), 1340 (S=O), 1165 (S=O); 1H NMR (500.00 MHz, DMSO-d$_6$) δ 7.67 (d, 2H, J 8.6 Hz, H2'/H6'), 7.64 (s, 1H, H7), 7.60 (d, 2H, J 8.6 Hz, H3'/H5'), 7.17 (s, 1H, H4), 3.46 (s, 3H, OCH$_3$); 13C NMR (75.0 MHz, DMSO-d$_6$) δ 170.2 (C=O), 153.9 (C6), 147.1 (C7a), 139.7 (C1' or C4'), 138.0 (C1' or C4'), 129.4 (C2'/C6'), 129.1 (C3'/C5'), 123.0 (C5), 121.9 (C7), 113.1 (C3a), 97.8 (C4), 56.6 (OCH$_3$); ESI-MS m/z [M – H$^-$]: 370.0 ([M – H$^-$] 100%).

N-(6-Methoxy-2-oxobenzo[d][1,3]oxathiol-5-yl) benzenesulfonamide (5f)

Yield: 46%; mp 184-186 °C; IR (KBr) ν / cm$^{-1}$ 3247 (N-H), 1762 (C=O), 1344 (S=O), 1161 (S=O); 1H NMR (500.00 MHz, DMSO-d$_6$) δ 9.54 (s, 1H, NH), 7.69 (d, 2H, J 7.4 Hz, H2'/H6'), 7.64 (s, 1H, H7), 7.60 (tt, 1H, J 7.4, 1.2 Hz, H4'), 7.52 (t, 2H, J 7.4 Hz, H3'/H5'), 7.14 (s, 1H, H4), 3.44 (s, 3H, OCH$_3$); 13C NMR (75.0 MHz, DMSO-d$_6$) δ 169.7 (C=O), 153.2 (C6), 146.3 (C7a), 140.3 (C1'), 132.5 (C4'), 128.7 (C3'/C5'), 126.6 (C2'/C6'), 122.9 (C5), 121.0 (C7), 112.5 (C3a), 97.2 (C4), 56.1 (OCH$_3$); ESI-MS m/z [M – H$^-$]: 336.1 ([M – H$^-$] 100%).

4-Methoxy-N-(6-methoxy-2-oxobenzo[d][1,3]oxathiol-5-yl) benzenesulfonamide (5g)

Yield: 41%; mp 171-173 °C; IR (KBr) ν / cm$^{-1}$ 3265 (N-H), 1765 (C=O), 1334 (S=O), 1161 (S=O); 1H NMR (500.00 MHz, DMSO-d$_6$) δ 7.61-7.63 (m, 3H, H7/H2'/H6'), 7.16 (s, 1H, H4), 7.03 (d, 2H, J 9.0 Hz, H3'/H5'), 3.81 (s, 3H, OCH$_3$), 3.50 (s, 3H, OCH$_3$); 13C NMR (125.0 MHz, DMSO-d$_6$) δ 169.8 (C=O), 162.3 (C6 or C4'), 153.0 (C6 or C4'), 146.1 (C7a), 131.9 (C1'), 128.9 (C2'/C6'), 123.3 (C5), 120.4 (C7), 113.9 (C3'/C5'), 112.5 (C3a), 97.2 (C4), 56.2 (OCH$_3$), 55.6 (OCH$_3$); ESI-MS m/z [M – H$^-$]: 366.1 ([M – H$^-$] 100%).

Materials

Venoms, animals, and reagents

Bothrops jararaca and B. jararacussu venoms were kindly supplied by Fundação Ezequiel Dias (FUNED), Belo Horizonte, Minas Gerais State, Brazil, and stored at −20 °C until use. The collection of snake venom was conducted under the authorization of the Brazilian System for Management Genetic of Heritage and Associated Traditional Knowledge (SisGen) (process number A39CD4E). BALB/c mice (18-20 g) were obtained from the Laboratory Animal Care of the Federal Fluminense University (UFF) and were housed under constant temperature (24 ± 1 °C) and light conditions. Experiments were approved by the UFF Institutional Committee for Ethics in Animal Experimentation (protocol number 508),...
that are in accordance with the guidelines of the Brazilian Committee for Animal Experimentation (COBEA). All solvents or reagents were of the best grade available.

**Biological assays**

**Coagulant activity of venoms**

Different concentrations of *B. jararaca* or *B. jararacussu* venom (5-70 μg mL\(^{-1}\)) were added to plasma and coagulation time was monitored using a digital Amelung coagulometer (model KC4A, Labcon, Germany). The amount of venom (μg mL\(^{-1}\)) able to clot plasma around 60 s was called minimum coagulation dose (MCD), and such venom concentration (*B. jararaca*, 35 μg mL\(^{-1}\) or *B. jararacussu*, 50 μg mL\(^{-1}\)) was incubated for 30 min at 25 °C with compounds, at 1:10 venom:compound ratio (m/m). After incubation, mixture was added to the medium reaction and coagulation was monitored, as described. Control experiments were performed by incubating compounds, dimethyl sulfoxide (DMSO) (0.8%, final concentration) or saline with plasma in the absence of venoms.

**Proteolytic activity of venoms**

Proteolytic activity of *B. jararaca* and *B. jararacussu* venom was determined\(^ {23}\) using azocasein as a substrate (0.2% m/v, in 20 mM Tris-HCl, 8 mM CaCl\(_2\), pH 8.8), with modifications. Different concentrations of each venom (2-40 μg mL\(^{-1}\)) were incubated with azocasein, and the amount of each venom that achieved supramaximal activity was considered as 100% of proteolytic activity, and called effective concentration (EC). The effect of compounds was evaluated by incubating one EC of *B. jararaca* or *B. jararacussu* (10 μg mL\(^{-1}\)) venom with compounds (100 μg mL\(^{-1}\)) for 30 min at 25 °C. Then, proteolytic activity was determined, as described. Control experiments were performed by incubating compounds, DMSO (0.9%, final concentration) or saline, instead of venoms.

**Hemolytic activity of venoms**

Hemolytic activity of the venom of *B. jararaca* or *B. jararacussu* was performed, because of the low hemolytic activity of this venom. On the other hand, the degree of hemolysis caused by the venom of *B. jararaca* was determined by the indirect hemolytic test using human erythrocytes and hen’s egg yolk emulsion, as substrate.\(^ {24}\) After performing a concentration-response curve (5-50 μg mL\(^{-1}\)), the amount of venom (μg mL\(^{-1}\)) able to induce 100% of hemolysis was called minimum indirect hemolytic concentration (MIHC). Then, one MIHC of *B. jararaca* (24 μg mL\(^{-1}\)) venom was incubated with compounds (240 μg mL\(^{-1}\)) or solvents (DMSO or saline) for 30 min at 25 °C, followed by the hemolytic test. Control experiments were performed by incubating venom with solvents in the absence of compounds or by adding solely compounds or solvents to reaction medium.

**Hemorrhagic activity of venoms**

Hemorrhagic lesions produced by *B. jararaca* and *B. jararacussu* venoms were quantified by the procedure described by Kondo et al.,\(^ {25}\) with modifications. *B. jararaca* or *B. jararacussu* venom was injected intradermally (i.d.) into the abdominal skin of mice, and 2 h later, animals were euthanized by decapitation, abdominal skin removed, stretched, and inspected for visual changes in the internal aspect in order to localize hemorrhagic spots. One minimum hemorrhagic dose (MHD) was defined as the amount of venom (μg per mouse) able to produce a hemorrhagic halo of 10 millimeters that was 12 μg per mouse. The effect of compounds on venom-induced hemorrhage was investigated by incubating compounds with two MHD of *B. jararaca* or *B. jararacussu* venom (24 μg per mouse) for 30 min at 25 °C. After incubation, mixture was injected i.d. into mice, and hemorrhagic activity was performed, as described. Negative controls were performed by injecting solely saline, DMSO or compounds, instead of venom. The volume of injection of samples was 100 μL.

**Edematogenic activity of venoms**

Edema-inducing activity of venom was determined in accordance to Yamakawa et al.,\(^ {26}\) with modifications. Groups of five mice received a subcutaneous (s.c.) subplantar single injection of *B. jararaca* or *B. jararacussu* venom into the right paw; while the left one received a single injection of saline or DMSO. Then, 1 h after injection, the paws were amputated, weighed and edema was evaluated and expressed as the percentage of increase in the weight of the right foot paw compared to the left one. The effect of compounds was evaluated by incubating each venom (7 μg per mouse) with compounds (20 μg per mouse) for 30 min at 25 °C, and then, an aliquot of this mixture was injected s.c. into mice. Control experiments were performed by injecting compounds or solvents in the absence of venom. The volume of injection of samples was 50 μL.

**Lethality activity of venoms**

*B. jararaca* or *B. jararacussu* venom (50 μg per mouse) was incubated with solvents for 30 min at 25 °C, and an aliquot was injected intraperitoneally (i.p.) into mice, and deaths were observed and registered. Antilethality effect was performed by incubating each venom with compounds (150 μg per mouse) at the same conditions. After incubation,
mixture was injected i.p. into mice, and time of death was observed, and compared to the group that received injection of venoms alone. Negative control group received injection solely of compounds or solvents, instead of venom. After the end of injections, number of deaths of mice was observed over a period of 24 h. The volume of injection of samples was 100 μL.

Statistical analysis

Results are expressed as means ± standard deviation (SD). The statistical significance of differences amongst experimental groups was evaluated using the Student’s t-test. p values < 0.05 were considered significant.

Results and Discussion

Synthesis

The 1,3-benzoxathioli-2-one sulfonamides 5a-5h were synthesized as shown in Scheme 1. First, the nitro derivative 2 was prepared from selective nitration at 5-position of the commercially available 6-hydroxybenzo[d][1,3]oxathiol-2-one 1, using HNO₃ 65% in CH₂Cl₂ at room temperature for 2 h, in 75% yield. Subsequently, methylation of phenolic hydroxyl group of 2 using methyl iodide and K₂CO₃ in N,N-dimethylformamide (DMF) at room temperature for 24 h, leading to the methoxy derivative 3 in 74% yield. Key intermediate 5-amino-6-methoxybenzo[d][1,3]oxathiol-2-one (4) was obtained in excellent yield (93%) by catalytic hydrogenation of 3 with 10% Pd/C in ethanol under 20 bar H₂ pressure at 50 °C for 6-8 h. Melting points and spectral data of the intermediates 2-4 were consistent with literature.¹³⁻¹⁵ The new 1,3-benzoxathioli-2-one sulfonamides 5a-5h were obtained from the coupling of 4 with 4-substituted benzenesulfonyl chlorides in the presence of pyridine and DMAP in 1 equivalent amount for 8 h at reflux in 25-51% yields.

Sulfonylation reaction between the amine 4 and different 4-substituted benzenesulfonyl chlorides was attempted with other methodologies by varying solvent, base, catalyst and temperature, as shown in Table 1. The protocols presented in entries 1 to 9 were unsuccessful. It is noteworthy the comparison between entries 9 and 10, which corroborates with a proposal that, in this reaction, the DMAP plays a dual role as a base and nucleophilic catalyst, and therefore can activate both reactants in this reaction (Figure 1).²⁷

The structures of the newly synthesized compounds were characterized by spectral data (¹H NMR, ¹³C NMR, IR and ESI-MS). 2D-NMR techniques (correlated spectroscopy (COSY), heteronuclear single quantum coherence spectroscopy (HSQC) and heteronuclear multiple bond correlation (HMBC)) helped us to assign the correct signals of the compounds. The results of the spectral analysis were in accordance with the proposed structures.

As an example, the ¹H NMR spectrum of compound 5a exhibited a broad singlet at 9.82 ppm for N-H. Hydrogens H7 and H4 appeared as singlets at 7.64 and 7.16 ppm, respectively. Hydrogens H2'/H6' and H3'/H5' are shown as duplets at 7.57 ppm (J 8.0 Hz) and 7.32 ppm (J 8.0 Hz), respectively. Two singlets were identified at 3.46 and 2.35 ppm for O⁻CH₃ and CH₃ groups, respectively. The ¹³C NMR spectrum exhibited the C =O signal at 169.7 ppm, OCH₃ at 56.1 ppm and the CH₃ at 20.9 ppm. IR spectrum of 5a showed the N−H and C=O stretching vibrations at 3258 and 1768 cm⁻¹, respectively. The bands at 1336 and 1161 cm⁻¹ represent sulfur-oxygen stretching frequencies in sulfone group (SO₂).

Biological assays

The inhibitory ability of the synthesized compounds (5a-5h) against some activities of the venoms of B. jararaca and B. jararacussu was investigated. The tested activities, coagulant, proteolytic, hemolytic,
Synthesis and Biological Evaluation of Novel 1,3-Benzoxathiol-2-one Sulfonamides

Table 1. Different methodologies tested to obtain the sulfonamide derivatives 5a-5h

| entry | Solvent       | Base     | Catalyst | Temperature | R     | Yield / % |
|-------|---------------|----------|----------|-------------|-------|-----------|
| 1     | acetonitrile  | K₂CO₃    | –        | r.t.        | F     | –         |
| 2     | acetonitrile  | K₂CO₃    | DMAP (cat) | reflux      | F     | –         |
| 3     | THF           | K₂CO₃    | –        | r.t.        | F     | –         |
| 4     | THF           | K₂CO₃    | DMAP (cat) | reflux      | F     | –         |
| 5     | acetone/H₂O   | K₂CO₃    | –        | r.t.        | CH₃   | –         |
| 6     | AcOEt         | Et₃N     | DMF (cat) | reflux      | CH₃   | –         |
| 7     | THF           | Et₃N     | DMAP (cat) | reflux      | CH₃   | –         |
| 8     | pyridine      | pyridine | –        | r.t.        | CH₃   | –         |
| 9     | pyridine      | pyridine | DMAP (cat) | reflux      | CH₃   | –         |
| 10    | pyridine      | DMAP (1 equiv) | reflux | CH₃   | 45     |           |

THF: tetrahydrofuran; DMF: N,N-dimethylformamide; DMAP: 4-(dimethylamino)pyridine; cat: catalyst; r.t.: room temperature.

Figure 1. The role of DMAP as a base and nucleophilic catalyst in the synthesis of compounds 5a-5h.

Hemorrhagic, edematogenic, and lethality are responsible for the most important symptoms after snakebites, and cause into victims many toxic effects, leading to morbidities, amputation or deaths. Thus, it is essential to evaluate such activities of venoms to develop a candidate molecule as antivenom. It is worth mentioning that a similar ratio between B. jararaca or B. jararacussu venom and the compounds was used for most of all the in vitro and in vitro assays, that it was of 1:10 venom:compound (m/m). This strategy was employed to compare better the efficacy of neutralization of all compounds in each assay in this article as well as previous results of our group.¹⁹,²⁰
Anticoagulant activity

Snake venoms are composed of many enzymes, which are responsible for toxic effects observed in victims. The snake venom serine proteases (SVSPs) and snake venom metalloproteases (SVMPs) enzymes are the most important and majority group of enzymes. They act on a variety of tissues and systems. SVSPs alter blood coagulation system, and most of them have thrombin-like activity, leading to the formation of fibrin, and thus, promoting the formation of thrombus. On the other hand, SVSPs may induce hemorrhage of victims because of the high consumption of fibrinogen, impairing blood to clot. In contrast, the SVMPs induce hemorrhage and coagulation of blood. Snakebites caused by the species of Bothrops genus provoke tissue necrosis and intravascular thrombin formation, resulting in coagulopathies and hemorrhage. However, B. jararaca venom is more hemorrhagic than B. jararacussu venom, due to presence of the SVMPs jararhagin28 and bothropasin,29 previously isolated from B. jararaca venom. On the other hand, B. jararacussu venom is more myotoxic due to the action of two myotoxins with phospholipase A2 (PLA2) structure, bothropstoxin I (Bthtx-I, without catalytic activity) and bothropstoxin-II (Bthtx-II, with catalytic activity).30,31 Thus, a molecule able to impair snake venom-induced coagulation, proteolysis or hemorrhage is relevant.

B. jararaca (35 μg mL⁻¹, black columns) or B. jararacussu (50 μg mL⁻¹, white columns) venom was incubated with saline (S), DMSO (0.8% v/v, final concentration), or with compounds 5a-5h (at a 1:10 venom:compound ratio m/m). Then, an aliquot of the mixture was added to plasma, and coagulation time (s) was monitored, as described.

Figure 2. Effect of compounds on coagulation of the venoms of B. jararaca and B. jararacussu. Data are expressed as means ± SD of three individual experiments (n = 9). *p < 0.05 when compared with control.

Antiproteolytic activity

B. jararaca and B. jararacussu venoms are rich sources of proteases, and each venom was able to hydrolyze the chromogenic substrate, azocasein. Then, 100 μg mL⁻¹ of compounds (5a-5h) were incubated with each venom (10 μg mL⁻¹), as described in Experimental section. As shown in Figure 3, compounds 5b (39%) and 5h (27%) were the most efficient to inhibit proteolytic activity of B. jararacussu and B. jararaca venom, respectively. On the other hand, the compounds 5d-5g did not inhibit proteolysis of venoms (Figure 3). None of the compounds solely had proteolytic activity.

Figure 3. Inhibitory effect of compounds on proteolysis of B. jararaca and B. jararacussu venoms. The results express the means ± SD of two individual experiments (n = 6).

B. jararaca or B. jararacussu venom (10 μg mL⁻¹) was incubated with 100 μg mL⁻¹ of compounds (5a-5h) for 30 min at 25 °C, and then proteolytic activity was performed, as described.
Antihemolytic effect

Hemolytic activity was not performed for the venom of *B. jararacussu* because it had low hemolytic activity. The concentration of *B. jararaca* venom (24 μg mL⁻¹) that induced 100% of lysis of red blood cells was called minimum indirect hemolytic concentration (MIHC). This MIHC of venom (24 μg mL⁻¹) was incubated with 240 μg mL⁻¹ of compounds (5a-5h), and then hemolytic activity was performed, as described in Experimental section. As shown in Figure 4, the compounds 5b and 5h inhibited around 55%, while the compounds 5d and 5e inhibited approximately 20%. The compounds 5a, 5c, 5f and 5g did not inhibit hemolysis of *B. jararaca* venom. Hemolytic activity of snake venoms is due to the participation of another group of enzymes, phospholipase A₂ (PLA₂). Beyond hemolysis, PLA₂ enzymes inhibit platelet aggregation, induce edema or myotoxicity, and, thus, participate in the development of tissue necrosis. None of the compounds solely induced hemolysis (data not shown).

240 μg mL⁻¹ of compounds (5a-5h) were incubated with *B. jararaca* venom (24 μg mL⁻¹) for 30 min at 25 ºC. Then, hemolytic test was performed, as described, and inhibition of hemolysis was determined.

It is noteworthy that the most active compound in inhibiting hemolysis of *B. jararaca*, and coagulation and proteolysis induced by both venoms, (N-(6-methoxy-2-oxobenzo[d][1,3]oxathiol-5-yl)-4-nitrobenzenesulfonamide) (5b), bears the nitro group in its structure. The nitro group has a strong electron-withdrawing ability that creates localized or regional electron deficient sites within molecules, allowing interactions with biological nucleophiles present in living systems, such as proteins, amino acids, nucleic acids, and enzymes. The interaction may occur by a nucleophilic addition or displacement, by electron transfer involving oxidation and reduction, or simply by molecular complexation.³²,³³ This fact may suggest the importance of the nitro group for the biological activity. Among the halogenated compounds, the most active to prevent *B. jararaca* venom-induced hemolytic activity was the 4-iodo-N-(6-methoxy-2-oxobenzo[d][1,3]oxathiol-5-yl)benzenesulfonamide (5h), which has an iodine atom on the benzene ring. Interestingly, a decrease in the inhibitory effect was observed with an increasing of halogen atom electronegativity, in the order, compound 5h (iodine), 5d (bromine), 5e (chlorine), 5c (fluorine).

Effect of compounds on in vivo activities of *B. jararaca* and *B. jararacussu* venoms

Antihemorrhagic activity

Injection intradermically (i.d.) of *B. jararaca* or *B. jararacussu* (24 μg per mice) venom produced a hemorrhage halo of around 20 mm that was considered as 100% of hemorrhagic activity. This dose of venom, which represents 2 MHD, was incubated for 30 min at 25 ºC with the compounds (240 μg per mice). After incubation, the mixture was injected i.d. into mice, and hemorrhage was analyzed. As seen in Figure 5, all the compounds inhibited the hemorrhagic activity of venoms, but with different inhibitory percentages (from 6 to 62%). Thus, the
compounds interfered with SVMPs, maybe by chelating Zn\(^{2+}\), as they depend on this metal to display hemorrhagic activity. The compound 5f inhibited more efficiently B. jararaca venom-induced hemorrhage, probably due to its ability to bind to the main toxic enzymes of such venom, jararhagin\(^{28}\) or bothropasin.\(^{29}\) The compound 5a did not inhibit B. jararacussu-induced hemorrhage. Thus, one may speculate the inability of the compound to bind to divalent cations (as Zn\(^{2+}\) or Ca\(^{2+}\)), leading to an inefficacy may speculate the inability of the compound to bind to

5a

not inhibit -induced hemorrhage. Thus, one

B. jararacussu

and edematogenic activity was evaluated, as described, and inhibition of edema was determined.

Antilethality activity

A single intraperitoneal (i.p.) injection of 13 µg per mouse of B. jararaca or B. jararacussu venom incubated with saline or DMSO killed mice approximately in 65 min. As seen in Table 2, 120 µg per mouse of the compounds (5a-5h) protected mice of the lethality of B. jararacussu venom. On the other hand, the compounds 5a, 5f and 5g failed to protect mice of death caused by B. jararaca venom. Nevertheless, the other compounds did impair the death of mice induced by such venom. Injection of solely compounds did not cause death of mice, even at higher concentrations (up to 200 µg per mice). The maximal time of observation of the survival of mice was 300 min.

Table 2. Effect of compounds on lethality of B. jararaca and B. jararacussu venoms

| Group                           | Survival time / min |
|---------------------------------|---------------------|
| Venom + saline                  | 65 ± 17.4           |
| Venom + DMSO                    | 60 ± 9.5            |
| Venom + compound 5a             | 68 ± 14.2           |
| Venom + compound 5b             | 130 ± 8.1*          |
| Venom + compound 5c             | 160 ± 18.1*         |
| Venom + compound 5d             | 140 ± 10.4*         |
| Venom + compound 5e             | 160 ± 11.4*         |
| Venom + compound 5f             | 70 ± 17.4           |
| Venom + compound 5g             | 85 ± 14.2           |
| Venom + compound 5h             | 300 ± 22.1*         |

Results are expressed as means ± SD (n = 5), and *p < 0.05 was compared with B. jararaca or B. jararacussu + saline or dimethyl sulfoxide (DMSO).

The compounds 5a-5h (120 µg per mice) were incubated for 30 min at 25 °C with B. jararaca or B. jararacussu venom (13 µg per mouse). Then, the mixture was injected i.p. into mice, and survival time was monitored. Control experiments were performed by incubating B. jararaca or B. jararacussu venom with saline or DMSO. The total time of observation of survival time of mice was 300 min.

Conclusions

In summary, a series of eight novel 1,3-benzoxathiol-2-one sulfonamide derivatives was successfully synthesized and characterized by IR, \(^1\)H and \(^{13}\)C NMR, and ESI-MS
analysis. These compounds were able to inhibit some of the main toxic activities of the venom of *B. jararaca* or *B. jararacussu*, but with different potencies. Thus, being promising molecules to treat envenomation by these snakes as well as to aid the current antivenom serum therapy. However, it is quite difficult to postulate a mechanism of action of compounds based on their chemical structure, because snake venoms are a complex mixture of toxic proteins acting through unknown mechanisms of action.

**Supplementary Information**

Supplementary data are available free of charge at http://jbcs.sbq.org.br as PDF file.

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**Author Contributions**

Eliza de L. Chazin was responsible for the investigation of the synthesis and writing original draft; Leonardo B. Martins for the investigation of the synthesis; Marcus Vinícius N. de Souza and Claudia Regina B. Gomes for the writing review; Ana Cláudia R. da Silva for the investigation of the biological activity and writing original draft; Marcelly C. Branco for the investigation of the biological activity; Eladio F. Sanchez for the project administration; André L. Fuly for the project administration, writing original draft, writing review and editing of the biological activity; Thatyana R. A. Vasconcelos for the project administration, writing original draft, writing review and editing of the synthesis.

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