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Synthesis and pharmacological evaluation of prenylated and benzylated pterocarpans against snake venom

Alcides J. M. da Silva,[a] Antonio L. Coelho,[a] Alessandro B. C. Simas,[a] Raphael A. M. Moraes,[b] Diogo A. Pinheiro,[b] Fabrício F. A. Fernandes,[b] Emerson Z. Arruda,[b] Paulo R. R. Costa[a,*] and Paulo A. Melo[b,*]

[a]*Laboratório de Química Bioorgânica (LQB), Núcleo de Pesquisas de Produtos Naturais, Centro de Ciências da Saúde, Bloco H, Universidade Federal do Rio de Janeiro, RJ 21941-590, Brazil*
[b]*Departamento de Farmacologia Básica e Clínica, Centro de Ciências da Saúde, Bloco H, Universidade Federal do Rio de Janeiro, RJ 21941-590, Brazil*

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Abstract—Edunol (**3**), a pterocarpan isolated from *Harpalyce brasiliana*, a plant used in the northeast of Brazil against snakebites, was obtained by synthesis and showed antimyotoxic, antiproteolytic and PLA2 inhibitor properties. These proprieties could be improved through the synthesis of a bioisoster (**5**), where the prenyl group was replaced by the benzyl group.

Viperidae snakebites are an important health problem in Brazil and other South American countries.[1] Among these snakebites, that of the genus *Bothrops* induces local swelling hemorrhage and myonecrosis, which have been attributed to the venoms proteolytic and phospholipase activity.[2] The first aid treatment and management of this kind of accident are among the most neglected and poorly studied areas of medicine. The only specific therapy known so far is the specific antivenom, which has low and limited effectiveness.[2,3]

During the last decades, several extracts of plants with activity against snake venoms have been reported in the literature.[4] The naturally occurring coumestan wedelolactone, isolated from *Eclipta prostrata* and some of its synthetic derivatives, such as PLA2 inhibitors, are among the most active products.[5] On the other hand, pterocarpans prenylated in the A-ring are also described as very active products. For example, the plant popularly called '*Cabeça-de-Negro*' is main ingredient in '*Específico Pessoa*',[6] a northeast Brazilian folk medicine used against snakebites. Two active pterocarpans were isolated from this popular medicine, cabenegrina A-I (**1**) and cabenegrina A-II (**2**) (Fig. 1). More recently a new pterocarpan denominated edunol (**3**) was also isolated from the root of the *Harpalyce brasiliana*, a plant found in the northeast of Brazil and used against snakebites.[7]

While active compounds **1**–**3** have a C5 side chain on the A-ring, maackian (**4**), which is not active against snake venoms, lacks the A-ring side chain.

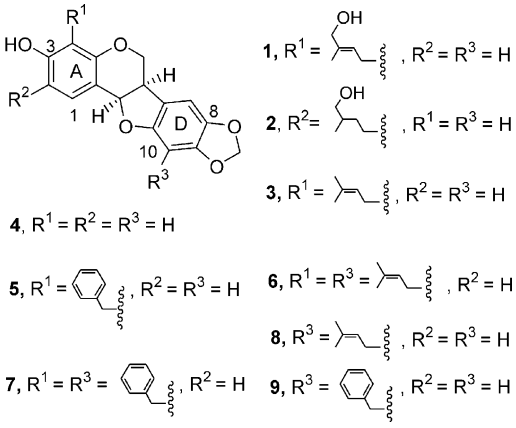

Figure 1. Prenylated and benzylated pterocarpans.

* Corresponding authors. Tel.: +55-212-5626791; fax: +55-212-2702683; e-mail: lqb@nppn.ufrj.br

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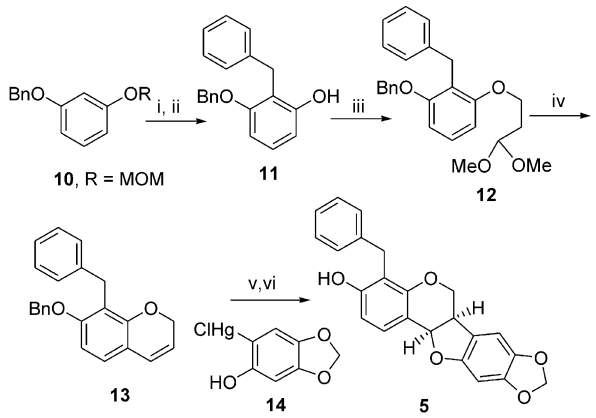

Scheme 1. (i) BuLi (1.2 equiv), THF, 0 °C, 40 min, benzyl bromide; (ii) PPTS (0.2 equiv), ^{i}PrOH, 80 °C; (iii) iodo dimethylacetal, KOH, THF; (iv) HCl (20%); (v) $PdCl_2$, LiCl, acetone; (vi) H_2-Pd/C acetone.

Since the benzyl group is a bioisoster of the prenyl group, compound **5** is designed as an analogue of **3**, as well as compounds **6** and **7**, which have an additional prenyl or benzyl group on ring D. Compounds **8** and **9** were also designed as analogous of **3**. In fact, the compounds **1**–**4**, **8** and **9** get the prenyl or benzyl group positioned between two alkoxy groups.

The natural product Edunol (**3**) and the analogues (**6**, **7**, **8** and **9**) were prepared using *ortho*-metalation chemistry (DMG) on derivatives of **4**, as previously described.[8] Herein, we report the synthesis of the new analogue **5** using an alternative synthetic pathway. First, resorcinol derivative **10** was regioselectively lithiated[9] and the resulting intermediate was trapped with benzyl bromide followed by in situ hydrolysis of the MOM protecting group, leading to compound **11**. This compound was transformed in to chromen **13** in a two step sequence involving *O*-alkylation of 3-iodopropionaldehyde dimethylacetal, followed by cyclization in acid medium. Compound **13** was allowed to react with organomercurial (**14**) in the presence of $PdCl_2$/LiCl, leading to the *O*-benzylated pterocarpan. The protecting benzyl group was removed by hydrogenolysis leading the target, compound **5** (Scheme 1).

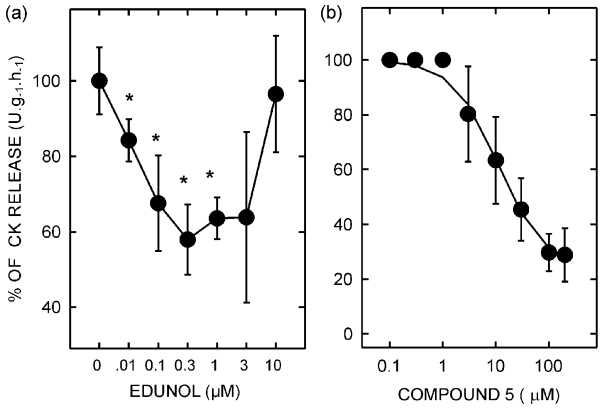

Figure 2. Effect of Edunol (**3**) or its derivative compound **5** on the myotoxic effect of *B. jararacussu* crude venom in vitro. Data in panel A and B ($p<0.05$) show the percent of inhibition of the increased rate of CK release induced by *B. jararacussu* crude venom (25 μg/mL) on isolated EDL mouse muscles. Each point represents the mean ± standard error ($n=4$–6), after 60 min of exposure to the crude venom alone or associated to **3** or **5**, respectively. The basal rate of CK release in physiologic saline solution was 0.35 ± 0.05 $U.g^{-1}.h^{-1}$($n=83$). The curve was determined by non-linear fitting to the Hill equation. Each point represents the mean ± standard error ($n=4$–6; $IC_{50}=9.97$ μM).

The activity of edunol derivatives against muscle damage induced by *B. jararacussu* crude venom was tested. This crude venom is myotoxic, inducing tissue hemorrhage and myonecrosis mainly due to the presence of proteolytic and phospholipase A2 enzyme activities.[10] Sarcolemmal damage and myotoxicity induced by *B. jararacussu* crude venom were assessed in vitro by the rate of loss of creatine kinase (CK) from isolated extensor digitorum longus (EDL) muscles mouse.[11] At 0.1–30 μM, many edunol derivatives (compounds **4**, **6**–**9**) did not antagonize the increased rate of CK release induced by the venom in vitro (Table 1). Meanwhile, Edunol (**3**) inhibited the myotoxic activity in vitro, only at concentrations of 0.1–3 μM (Fig. 2A), whereas compound 5 showed a potent antivenom activity (Fig. 2B).

The compound **5** was able to fully inhibit the venom's myotoxic activity in vitro with an IC_{50} of 9.97 micromolar (Fig. 2B). Interestingly, at 100 μM, this pterocarpan also inhibited 65% the venom phospholipase activity, as well as more than 80% of its proteolytic activity.

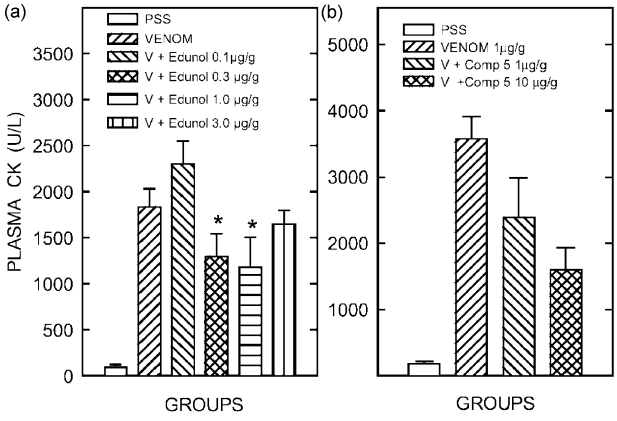

Figure 3. Effect of the treatment with edunol (**3**) and its derivative compound **5** on the myotoxic effect of *B. jararacussu* crude venom in vivo. Panel A shows the effect of intramuscular injection of *B. jararacussu* crude venom (V; 1.0 μg/g) on plasma creatine kinase (CK) activity and the effect of the treatment with edunol. Edunol (0.1–3.0 μg/g) was administered after preincubation in vitro with the venom. Plasma CK activity of the physiological saline solution (PSS) control group was 181.16 ± 26.44 U/l ($n=5$). The values reported are means ± SEM ($n=5$). $p<0.05$ for the difference between the control (venom alone) and the values for each of the edunol concentration plus venom (ANOVA). Panel B data shows the inhibition of the increase of plasma CK activity induced by *B. jararacussu* crude venom injection by compound **5**, in dose of 1.0 and 10 μg/g, respectively. The compound was pre-incubated with the venom and administered by i.m. injection. The mice received the venom injection at a dose of 1 μg/g and the value of plasma CK activity of PSS control group was 184.34 ± 31.10 U/L ($n=5$). The values were expressed as mean ± standard error; $p<0.05$ (ANOVA) for the difference between the control (venom alone) and the values from the two different doses of the compound.

Table 1. Effect of Edunol and different pterocarpan derivatives on the myotoxic effect of *B. jararacussu* crude venom

Compds	Concentration μM	Effect	% of Inhibition[a]
Edunol (**3**)	0.1–1.0	+	>40%
	10.0–30.0	–	0%
4	0.1–30.0	–	0%
5	0.1–10.0	++	>70%
6	0.1–30.0	–	0%
7	0.1–30.0	–	0%
8	0.1–30.0	–	0%
9	0.1–30.0	–	0%

Each compound, Edunol and pterocarpan derivatives, was tested 4–6 times.

[a] The Data represents the % of the inhibition of the increase of the rate of CK release induced by 25 μg/mL of *B. jararacussu* venom on isolated EDL mouse muscles after 60 min of exposure.

For in vivo experiments (Fig. 3), the increase of plasma CK activity was measured after *i.m.* injection of the crude venom.[11] The compounds **3** and **5** protected the mice in a dose dependent response. In these experiments, *B. jararacussu* crude venom (1.0 mg/kg), alone or preincubated together with **3** or **5** for 15 min at 37 °C (in vitro), was injected *i.m.* into each mouse. The final volume of venom injected, alone or plus **3** or **5**, was 0.1 mL. These venom doses were selected based on previous studies.[12] The animals were lightly anesthetized with diethyl ether immediately before and 2 h after venom injection for blood collection according to the guidelines for care and use of laboratory animals. Plasma was separated by centrifugation and stored at 4 °C for subsequent determination of CK activity. The procedure for the measurement of CK activity has been described previously (**1**, **5**–**8**). Three weeks after venom injection more than 50% of mice that received *i.m.* venom injection did not survive. Meanwhile, the animals that received treatment with edunol (**3**) or compound **5** survived the test.[4b]

These results support our hypothesis that natural compound **3** and synthetic derivative can inhibit snake venoms and, the new compound **5** also can be improved and deserves further study as a potential new antivenom.

The protective effect of compound **5** is due to inhibition of phospholipase and proteolytic activity of *B. jararacussu,* as shown in Figure 4. Compound **3** did not present protection against proteolytic or phospholipase activity.

Our data shows that compound **5** antagonized some activities of *B. jararacussu* crude venom which contains peptides and proteins that act on the physiological mechanisms of the body. To improve snakebite treatment we need to acquire new molecules from plants as well new synthetic antagonists that can prevent the toxins from reaching the mammalian targets and acting as cytotoxic agents. Polyvalent antivenin are composed of specific antibodies that neutralize individual venom component and they are limited to some venom components.[13] Our previous investigations

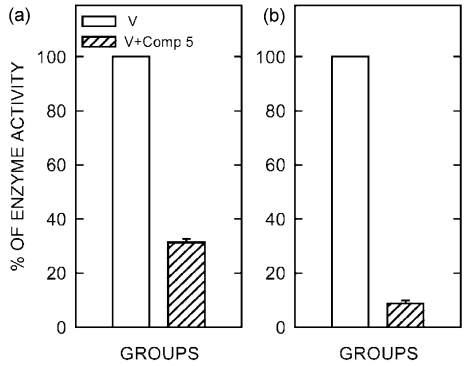

Figure 4. Effect of compound **5** on the phospholipase and proteolytic activity of *B. jararacussu* crude venom. Panel A and B show the inhibition of phospholipase and proteolytic activity of *B. jararacussu* crude venom (V; 10.0 μg/mL) by compound **5** (100 μM) respectively. Panel A shows the phospholipase activity of *B. jararacussu* crude venom incubated with hen's egg-yolk buffered emulsion (pH 7.8), mixed and carried out at 37 °C during 30 min, alone or in presence of premixed different concentrations of compound **5**. The absorbance change between each 30 min was measured spectrophotometrically at 925 nm. Data from four identical experiments was measured at 30 min. $p < 0.05$ for the difference between the control (venom alone) and the values for the compound **5** plus venom (ANOVA). Panel B shows the effect of compound **5** on proteolytic activity of the venom. Aliquots of crude venom alone or venom plus compound **5** (100 μM) were incubated with 0.2% azocasein in 0.2 M Tris–HCl, 20 mM $CaCl_2$, pH 8.8, for 90 min at 37 °C, in a final volume of 0.8 mL. The reaction was stopped with 0.4 mL of 10% trichloroacetic acid and fractions were centrifuged for 10 min. The supernatant (1 mL) was mixed with 2 M NaOH (0.5 mL) and absorbance was measured at 480 nm. The data were expressed in % of control enzyme activity.

and the present work, by using crotalid snake venoms,[5] have allowed us not only to confirm the observations from folk medicine, but also to develop new antisnake venoms that can neutralize many venom activies. Compound **5** is a new synthetic drug with potential therapeutic application, but needs further studies. The development of a drug against snakebites is a relevant issue and a challenge, because snake venoms are not composed of single compounds, being instead a complex mixture causing an even more complex local reaction.[14]

In conclusion, the antivenom activity of the natural product edunol (**3**) was improved by changing the prenyl group at position 4 to a benzyl group. On the other hand, the acyivity was completely removed by the introduction of an additional prenyl or benzyl group in position 10.

1. Experimental

1.1. Chemistry

Melting points were determined with a Thomas-Hoover apparatus and are uncorrected. Column chromatography was performed on silica gel 230–400 mesh (Aldrich). ^{1}H NMR spectra were recorded on a Varian Gemini (200 MHz) instrument using tetramethylsilane (TMS) as standard and $CDCl_3$ as solvent. *J* Values are given in Hz. ^{13}C NMR spectra were obtained at 50 MHz.

1.2. Synthesis of the Pterocarpan (5)

To a mixture of $PdCl_2$ (0.194 g, 1.1 mmol) and LiCl (0.047 g, 1.1 mmol) in acetone (10 mL) was added chromen **13** (0.360 g, 1.1 mmol) in acetone (10 mL). This mixture was stirred for 15 min at 0 °C and then 2-chloromercurio-4,5-methylenedioxyphenol **13** (0.414 g, 1.1 mmol) in acetone (10 mL) was added to it. The suspension thus obtained was stirred for 12 h at 25 °C. After this time, brine (30 mL) was added to it and the mixture was extracted with acetyl acetate (100 mL), the organic extract dried (Na_2SO_4), and subjected to column chromatography to give the *O*-benzyl pterocarpan as a white solid (0.280 g, 55% yield), mp 78–80 °C. ^{1}H NMR ($CDCl_3$) δ 3.50 (m, 1H), 3.60 (t, $J = 11.1$ Hz, 1H), 4.06 (s, 2H), 4.27 (dd, $J = 11.1$, 4.5 Hz, 1H), 5.08 (s, 2H), 5.50 (d, $J = 6.3$ Hz, 1H), 5.90 (d, $J = 5.5$, 1H), 5.91 (d, $J = 5.5$ Hz, 1H), 6.42 (s, 1H), 6.68 (d, $J = 8.5$ Hz, 1H), 6.71 (s, 1H), 7.26 (m, 11H). Anal. calcd for $C_{30}H_{24}O_5$: C, 77.57; H, 5.21. Found: C, 77.50; H, 5.19. The solution of the *O*-benzyl pterocarpan (0.208 g, 0.40 mmol) in acetone was hydrogenated (3 atm) in the presence of Pd-C (10%). After 3 h the catalyst was filtered and the product purified by chromatography on silica to give (**5**) in 80% yield, mp 60–62 °C. ^{1}H NMR ($CDCl_3$) δ 3.50 (t, $J = 11.0$, 1H), 3.64 (m, 1H), 4.03 (s, 2H), 4.27 (dd, $J = 11.0$, 4.4 Hz, 1H), 5.49 (d, $J = 6.5$ Hz), 5.90 (d, $J = 5.5$ Hz, 1H), 5.91 (d, $J = 5.5$ Hz, 1H), 6.43 (s, 1H), 6.55 (d, $J = 8.4$ Hz, 1H), 6.71 (s, 1H), 7.25 (m, 6H). Anal. calcd for $C_{23}H_{18}O_5$: C, 73.79; H, 4.85. Found: C, 73.82; H, 4.78.

1.3. Data analysis

The concentration–response curves were fitted by the Hill equation, using nonlinear regression. Data from assays of venom-induced CK release was well fitted by a single-site model.

2. Myotoxicity assays

In vitro and in vivo myotoxicity was evaluated at room temperature as previously described in references **4a**,**b**,**c**. Briefly, Extensor digitorum longus muscles were blotted, weighed rapidly and then transferred to sample collecting units of 2.5 mL capacity, where they were superfused continuously at a rate of 3 mL/min with physiological saline solution (PSS) equilibrated with 95% O_2/5% CO*2*. At 30 min intervals, the solutions perfusing the muscles were collected and replaced with fresh media. The CK activity was determined using a diagnostic kit purchased from Sigma Chemical Co. The rate of CK release from the isolated muscles is expressed as international enzyme units released into the medium per gram of muscle per h of collection ($U.g^{-1}.h.^{-1}$). *Bothrops jararacussu* venom alone or associated to the edunol or its derivatives was added to the nutrient solution which superfused the isolated muscles. In vivo the myotoxicity was evaluated by injecting intramuscularly (*i.m.*) the venom dissolved in physiological saline solution (PSS, 0.1 mL) alone or preincubated with edunol or compound **5** (15 min, 37 °C), after which the mixture was *i.m.* injected into the mice. Before and 2 h after the *i.m.* injection the animals were lightly anesthetized with diethyl-ether and the blood was collected by orbital puncture. The plasma was separated by centrifugation and stored at 4 °C for subsequent determination of creatine kinase (CK) activity.

3. Enzymatic assays

Proteolytic and Phospholipase activity were performed according to refs 12a and b respectively. Briefly, Phospholipase activity of *B. jararacussu* crude venom was evaluated by incubating 10 μg of the venom with hen's egg-yolk buffered emulsion (pH 7.8), mixed and carried out at 37 °C during 30 min., alone or in presence of premixed different concentrations of compounds. The absorbance change between each 30 min. was measured spectrophotometrically at 925 nm. Proteolytic activity of the venom was evaluated by added aliquots of crude venom alone or venom plus compound **5** (100 μM) and incubated with 0.2% azocasein in 0.2 M Tris–HCl, 20 mM $CaCl_2$, pH 8.8, for 90 min at 37 °C, in a final volume of 0.8 mL. The reaction was stopped with 0.4 mL of 10% trichloroacetic acid and fractions were centrifuged for 10 min. The supernatant (1 mL) was mixed with 2 M NaOH (0.5 mL) and absorbance was measured at 480 nm. The data were expressed in% of control enzyme activity. Each point represents the data from four identical experiments. $p < 0.05$ for the difference between the control (venom alone) and the values for the compound **5** plus venom (ANOVA).

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