In Vitro Evaluation of Caffeoyl and Cinnamoyl Derivatives as Potential Prolyl Oligopeptidase Inhibitors

Luciana O. Adolpho[1], **Daniele Marin**[1], **Albert Puigpinos**[2], **Laura Mendieta**[2], **Teresa Tarragó**[2], **Ademir F. Morel**[1], **Ernest Giralt**[2], **Ionara I. Dalcol**[1]

[1] Núcleo de Pesquisa de Produtos Naturais (NPPN), Chemistry Department, Universidade Federal de Santa Maria, Santa Maria – RS, Brazil

[2] Institute for Research in Biomedicine, Barcelona Science Park, Barcelona, Spain

Abstract

A screening of the natural product chlorogenic acid, isolated from the Brazilian medicinal plant *Hypericum brasiliense*, caffeic acid, cinnamic acid, and *p*-methoxycinnamic acid, and derivatives of caffeoylquinic, caffeoyl, and cinnamoyl against the enzymes prolyl oligopeptidase and dipeptidyl peptidase IV was carried out. Caffeoylquinic, caffeoyl, and cinnamoyl derivatives were prepared using simple derivatization procedures and through coupling reactions with the amino acid proline. The dipeptidyl peptidase IV assay showed inhibitory activity of the tested compounds at a high concentration (500 µM) in the range of 81.5–7.2%. In contrast, the derivatives methyl ester and 1,7-acetonide obtained from chlorogenic acid, and caffeic acid and its methyl ester derivative showed selectivity and activity as prolyl oligopeptidase inhibitors, with IC_{50} values of 3 to 14 mM.

Key words

prolyl oligopeptidase · dipeptidyl peptidase IV · chlorogenic acid · caffeoyl and cinnamoyl derivatives · *Hypericum brasiliense* · Hypericaceae

Supporting information available online at http://www.thieme-connect.de/ejournals/toc/plantamedica

Prolyl oligopeptidase (POP, prolyl endopeptidase or post-proline cleaving enzyme, EC 3.4.21.26) is a large intracellular serine protease (80 kDa) found in over 20 human tissue types. This enzyme cleaves short peptides (up to 30 amino acid residues) at the carboxyl side of an internal proline (-Pro-Xaa-; where Xaa is different from Pro) [1,2]. Previous studies indicate that POP activity is involved in key physiological functions, such as learning and memory, cell division and differentiation, signal transduction, as well as in some psychiatric disorders [3–5]. This activity is directly related to the fact that several neuropeptides, such as angiotensin I and II, vasopressin, bradykinin, thyroid-releasing hormone, and substance P, are potential substrates for POP [6,7]. Thus, the activity of this enzyme in the central nervous system (CNS) can decrease the concentrations of neuropeptides related to psychiatric and neurodegenerative disorders, such as schizophrenia and bipolar disorder [8].

The specificity of an inhibitor against POP can be examined by assaying, in parallel, the inhibitory capacity of candidate molecules against the enzyme dipeptidyl peptidase IV (DPP IV; EC 3.4.14.5) [9,10]. This serine protease cleaves peptides and proteins that hold a proline or alanine in the penultimate position of the N-terminus, these being relevant targets in the treatment of type 2 diabetes mellitus (T2DM) [11]. Although POP and DPP IV are related to a number of pathologies and present low amino acid sequence homology, the similarity of their three-dimensional structures causes many compounds to indiscriminately inhibit both enzymes.

We identified the natural 3-caffeoylquinic acid (chlorogenic acid, **1**), isolated from the Brazilian medicinal plant *Hypericum brasiliense* (Hypericaceae) and used as an excitant and antispasmodic, as a new POP inhibitor [12]. Using the commercial chlorogenic acid **1**, caffeic acid **6**, *trans*-cinnamic acid 12, and p-methoxycinnamic acid **13** as starting materials, we synthesized the derivatives series **2**–**5**, **7**–**11**, and **16**–**19** as depicted in ◉ **Figs. 1**–**3**.

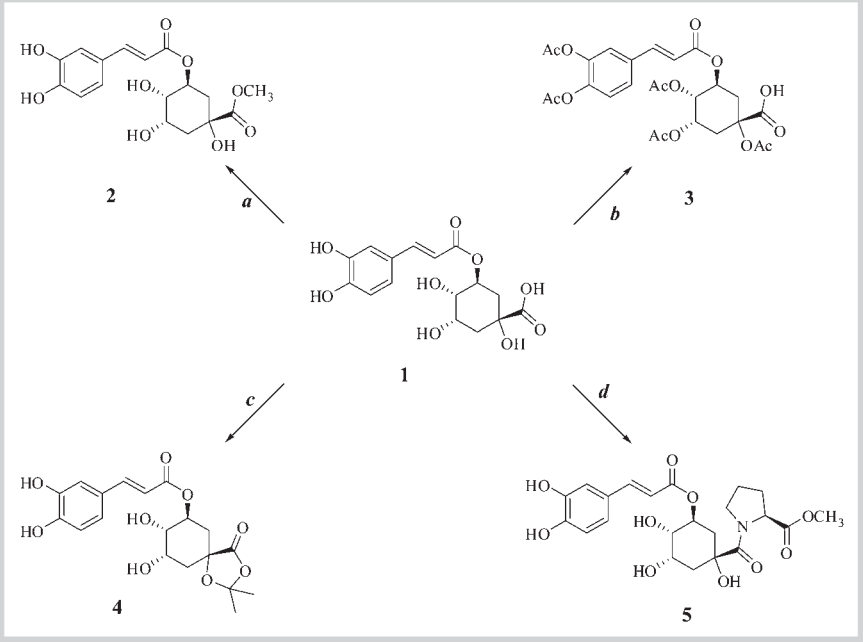


Fig. 1 Synthesis of the caffeoylquinic derivatives. Reagents and conditions: (*a*) MeOH, Amberlite IRA 120 H; (*b*) Ac_2O, DMAP, pyridine; (*c*) acetone, H_2SO_4; (*d*) Pro-OMe. HCl, HATU, DIEA in DMF.

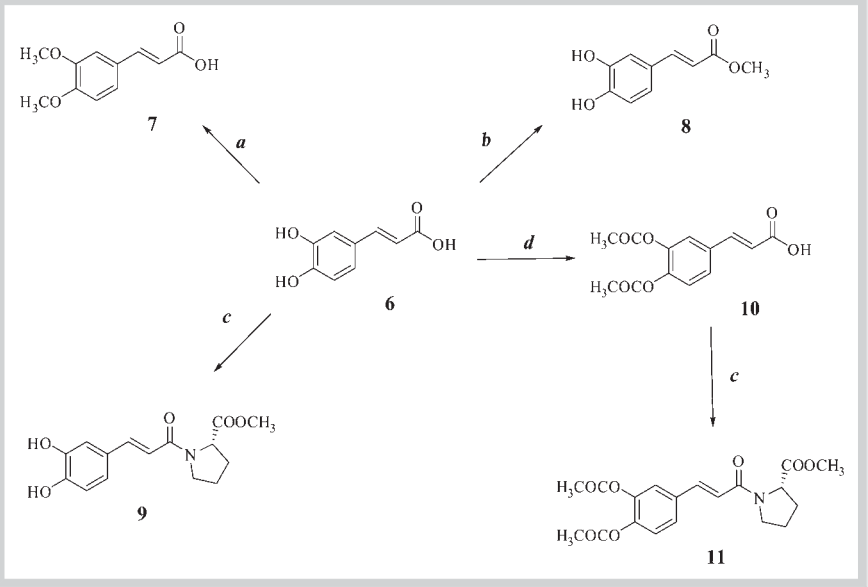
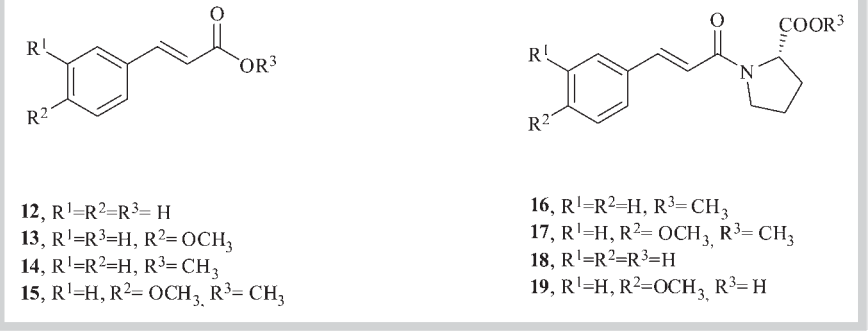


Fig. 2 Synthesis of the caffeoyl derivatives. Reagents and conditions: (*a*) Me_2SO_4, NaOH; (*b*) MeOH, H_2SO_4; (*c*) Pro-OMe. HCl, HATU, DIEA in DMF; (*d*) Ac_2O, DMAP, pyridine.



12, R^1=R^2=R^3= H
13, R^1=R^3=H, R^2= OCH_3
14, R^1=R^2=H, R^3= CH_3
15, R^1=H, R^2= OCH_3, R^3= CH_3

16, R^1=R^2=H, R^3= CH_3
17, R^1=H, R^2= OCH_3, R^3= CH_3
18, R^1=R^2=R^3=H
19, R^1=H, R^2=OCH_3, R^3= H

Fig. 3 Structures of the cinnamoyl derivatives.

Briefly, compound **1** was esterified following the procedure described by Lopez Giraldo et al. [13] to yield the methyl chlorogenate **2**, while the methyl esters **8, 14**, and **15** were prepared by the Fischer method. Acetylation of **1** and **6** with acetic anhydride and pyridine/4-(dimethylamino)pyridine [14,15] yielded the derivatives pentaacetylchlorogenic acid **3** and diacetylcaffeic acid **10**, respectively. To obtain 1,7-acetonide **4**, chlorogenic acid **1** was allowed to react with anhydrous acetone and concentrated H_2SO_4 [16]. Dimethylcaffeic acid **7** was prepared from **6** in reaction to Me_2SO_4 in NaOH [17]. The structures of these derivatives were confirmed by NMR and mass spectroscopy and compared with spectral data in the literature [14–19]. Compounds **5, 9, 11, 16**, and **17** were synthesized by reacting the appropriate natural acid with proline methyl ester hydrochloride, using either *O*-(7-azabenzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate (HATU)/diisopropylethylamine (DIEA) in dimethylformamide or isobutyl chloroformate/N-methylmorpholine in tetrahydrofuran [20,21]. Alkaline hydrolysis of **16** and **17** yielded the corresponding derivatives **18** and **19**, respectively. The structures of the synthesized derivatives were confirmed by MS, optical rotation, and NMR spectra (see Supporting Information).

Fluorimetric assays were used to estimate POP and DPP IV inhibition [4,10,22]. POP was obtained by expression in *E. coli* following a procedure described in the literature [22]. Porcine DPP IV was from a commercial source. Initially, we screened 500 µM of all the derivatives obtained and the natural acids **1, 6, 12**, and **13** as inhibitors of POP and DPP IV (**Table 1**). From these results, we selected the active compounds to be tested in an IC_{50} assay. At 500 µM, all the derivatives of the cinnamoyl and *p*-methoxycinnamoyl series (compounds **12–19**) showed inhibitory activity below 50% against POP and DPP IV. Also, the DPP IV inhibition preliminary assay at 500 µM displayed only moderate inhibitory activity for caffeoylquinic derivatives **1–5**, caffeic acid **6**, and its methyl ester **8**, in the range of 57–73%, while **7, 9, 10**, and **11** registered low activity (< 50%), so the IC_{50} for these compounds was not determined. In contrast, all derivatives from the caffeoylquinic and caffeoyl series, except **5** and **7**, showed interesting results regarding POP inhibition, so we proceeded to determine their IC_{50} values against this enzyme (**Table 1**). Among the caffeoylquinic series, **2** and **4** showed POP inhibition with IC_{50} values of 3.0 and 14.3 µM, respectively, while from the caffeoyl series, ester **8** (IC_{50} 5 µM) and caffeic acid **6** (IC_{50} 12.5 µM) showed the highest inhibitory capacity. Liposoluble compounds are of interest for biological applications because they can easily pass through biological barriers such as the blood-brain barrier. Unfortunately, the results obtained for derivatives **3** (IC_{50} 99 µM) and **10** (IC_{50} 28 µM) indicated that, in comparison with the starting natural products, derivatization with acetyl groups did not improve inhibitory capacity against the enzymes.

Given that POP and DPP IV have a high affinity for substrates containing proline, it was expected that the presence of a proline residue in the derivatives (compounds **5, 9, 11**, and **16–19**) would

Compound	DPP IV Inhibition (%)*	POP Inhibition (%)**	POP IC_{50} (μM)
Caffeoylquinic derivatives	**(500 μM)**	**(500 μM)**	
1	81.5 ± 3.9	88.0 ± 2.9	23.0 ± 1
2	64.3 ± 2.6	92.6 ± 2.6	3.0 ± 1
3	53.8 ± 6.0	81.9 ± 0.8	98.9 ± 1
4	62.7 ± 4.1	88.3 ± 1.7	14.3 ± 1
5	66.5 ± 3.3	75.5 ± 1.6	210.0 ± 4
Caffeoyl derivatives			
6	50.1 ± 1.2	93.8 ± 1.1	12.5 ± 5
7	45.0 ± 1.2	29.4 ± 1.2	198.0 ± 2
8	69.4 ± 3.8	90.5 ± 1.2	5.0 ± 2
9	48.8 ± 5.6	96.3 ± 2.9	105.7 ± 1
10	28.6 ± 5.0	72.9 ± 0.4	28.0 ± 1
11	43.9 ± 1.4	98.3 ± 1.1	120.0 ± 1
Cinnamoyl derivatives			
12	4.4 ± 1.6	16.1 ± 2.8	–
13	11.5 ± 4.3	< 10.0	–
14	7.2 ± 4.2	20.0 ± 3.6	–
15	20.5 ± 3.4	22.0 ± 4.0	–
16	< 10.0	< 10.0	–
17	17.0 ± 1.4	42.8 ± 4.0	–
18	35.5 ± 5.5	14.6 ± 3.9	–
19	14.5 ± 1.3	< 10.0	–
Control			
Verbascoside	–	–	3.4 ± 0.2
Isoleucine thiazolide	0.52§		
Z-prolyl-prolinal	–	–	4.9×10^{-3}

Table 1 POP and DPP IV inhibition of caffeoylquinic, caffeoyl, and cinnamoyl derivatives.

Data are represented as mean ± SEM. * Percentages of DPP IV inhibition calculated using GP-AMC as the substrate. ** Percentages of POP inhibition calculated using ZGP-AMC as the substrate. § DPP IV IC_{50} (μM)

enhance their performance as inhibitors. However, none of these derivatives displayed a relevant inhibitory capacity. Compounds **5** and **11** showed IC_{50} values of 210 and 120 μM, respectively. The DPP IV inhibitory capacity of all derivatives and natural acids **1**–**11** at a high concentration (500 μM) was not exceptional (81.5–7.2%). We therefore determined the IC_{50} values of only natural compounds **1** and **6**, which were found to be 128 μM and > 200 μM, respectively.

These general results are significant and demonstrate simple structural modifications of natural products improving their performance as enzymatic inhibitors, making them potential candidates for the development of new drugs. None of the compounds tested were DPP IV inhibitors; however, methyl ester **2** and 1,7-acetonide **4** derived from natural products chlorogenic acid, methyl ester **8**, and caffeic acid **6** proved to be interesting and selective POP inhibitors. These observations indicate the influence of the free 4,5-dihydroxyl groups in the caffeoyl moiety for the activity of these compounds.

Many natural products are known POP inhibitors in the micromolar range, such as the flavonoids oroxylin [23] and baicalin [9], the alkaloid berberin [24], and 6-(8′Z-pentadecenyl)salicylic acid [25]. In addition, some naturally occurring compounds containing one or more caffeoyl groups have also been reported to exert POP inhibitory activity: the phenylpropanoid verbascoside [10], isolated as the main constituent of the crude extract of *Buddleja brasiliensis* Jacq.; rosmarinic acid [26], a polyphenol found in various species of the Boraginaceae; the pentacyclic triterpenoid 3a-(3″,4″-dehydroxy-*trans*-cinnamoyloxy)-D-friedo-olean-14-en-28-oic acid present in leaves *Tamarix hispida* Willd [27]; and also chlorogenic acid, isolated by our group from *Hypericum brasiliense* [12]. From the three series of caffeoyl and cinnamoyl derivatives obtained in this study, we observed that the derivatives presenting the 4,5-dihydroxyl groups in the free caffeoyl moiety, such as the methyl ester **2** and 1,7-acetonide **4** derived from chlorogenic acid, the methyl ester **8**, and the caffeic acid **6**, were the good POP inhibitors, showing selective inhibition in a dose-dependent manner, with IC_{50} values in the low micromolar range (3.0–14.3 μM). Although the mechanism of the POP inhibition by these derivatives has not been elucidated, the findings of this study indicate the potential of the molecules as POP inhibitors. Thus, these compounds are of interest since they can be used as leads in the development of new potent therapeutic drugs to treat neuropsychiatric disorders.

Materials and Methods

Provenance, purity of substances, and equipment used is given in Supporting Information.

(E)-methyl 1-(3-(3-(3,4-dihydroxyphenyl)acryloyloxy)-1,4,5-trihydroxycyclohexanecarbonyl)pyrrolidine-2-carboxylate (**5**): This derivative was synthesized using the coupling method described by Salmi et al. [20] with slight modifications. Chlorogenic acid **1** (1.10 mmol) and L-proline methyl ester hydrochloride (1.10 mmol) were dissolved in DMF (3 mL). Next, DIEA (1.10 mmol) was added to the mixed solution at 0 °C. After 15 min, the coupling reagent HATU (1.10 mmol) was added at 0 °C and the resulting mixture was stirred for 72 h at room temperature. The reaction mixture was then poured into chloroform and extracted with HCl 1 N, a saturated solution of $NaHCO_3$, and water. The organic phase was dried with $MgSO_4$ and evaporated to dryness, resulting in an oil, which was purified by recrystallization with $CHCl_3$. Yield 40%. $[\alpha]_D$ − 34 (*c* 0.05, CH_3OH). ^1H NMR (CD_3COD, δ [ppm], *J* [Hz]): 7.64 (d, *J* = 15.9, H-7), 7.41 (s, H-2), 7.21 (d, *J* = 8.3, H-5), 7.01 (d, *J* = 8.3, H-6), 6.35 (d, *J* = 15.9, H-8),

5.44 (s, OHs), 4.52 (m, H-8′), 4.37 (m, H-2′), 4.12 (m, H-1′), 3.83 (s, OC$\underline{H}_3$), 3.44 (m, H-11′), 3.12 (m, H-3′), 2.3–1.9 (m, H-9′, H-6′, H-4′, and H-10′). ^{13}C NMR (CD_3COD, δ [ppm]): 169.7, 169.0, and 168.9 (C-7′, C-9, and C-12′), 147.0 (C-4), 146.8 (C-3), 141.0 (C-7), 129.9 (C-1), 122.9 (C-6), 122.1 (C-8), 116.5 (C-5), 115.2 (C-2), 86.5 (C-5′), 74.0 (C-2′), 71.0 (C-1′), 70.5 (C-3′), 58.8 (C-8′), 49.5 (-O$\underline{C}H_3$), 47.0 (C-11′), 37.3 (C-4′), 37.0 (C-6′), 30.4 (C-9′), 23.2 (C-10′).

(E)-methyl 1-(3-(3,4-dihydroxyphenyl)acryloyl)pyrrolidine-2-carboxylate (**9**): Compound **9** was prepared as described for **5** using caffeic acid **6** and L-proline methyl ester hydrochloride. The oily product obtained was purified by chromatography on silica gel, eluting with dichloromethane. Yield: 40%. $[\alpha]_D$ −64 (*c* 0.05, CH_3OH). ^{1}H NMR (DMSO-d_6, δ [ppm], *J* [Hz]): 7.31 (d, *J* = 15.4, H-7), 7.06 (s, H-2), 6.97 (d, *J* = 8.6, H-5), 6.76 (d, *J* = 8.6, H-6), 6.66 (d, *J* = 15.4, H-8), 4.40 (m, H-1′), 3.72 (m, H-4a'), 3.68 (s, -OC$\underline{H}_3$), 3.50 (m, H-4b'), 2.19–1.86 (m, H-2′, and H-3′). ^{13}C NMR (DMSO-d_6, δ [ppm]): 172.4 and 164.1 (C-5′ and C-9), 147.4 (C-4), 145.3 (C-3), 141.7 (C-7), 126.3 (C-1), 120.6, 115.5, and 115.2 (C-6, C-8, and C-5), 114.6 (C-2), 58.4 (C-1′), 51.4 (-O$\underline{C}H_3$), 46.3 (C-4′), 28.5 (C-2′), 24.2 (C-3′).

(E)-4-(3-(2-(methoxycarbonyl)pyrrolidin-1-yl)-3-oxoprop-1-enyl)-1,2-phenylene-diacetate (**11**): Diacetylcaffeic acid **10** and L-proline methyl ester hydrochloride were coupled as described for **5** and **9**. Yield 49%. $[\alpha]_D$ −48 (*c* 0.05, $CHCl_3$). ^{1}H NMR ($CDCl_3$, δ [ppm], *J* [Hz]): 7.64 (d, *J* = 15.4, H-7), 7.38, 7.34, and 7.19 (H-6, H-2, and H-5), 6.67 (d, *J* = 15.4, H-8), 4.59 (m, H-1′), 3.83 (m, H-4′a), 3.74 (s, -OC$\underline{H}_3$), 2.29 (2 -OCOC$\underline{H}_3$), 2.16–1.75 (H-4b', H-2′, and H-3′). ^{13}C NMR ($CDCl_3$, δ [ppm]): 172.6, 168.0, 167.9, 164.4 (C-5′, C-9, and 2 -OCOC$\underline{H}_3$), 143.0 (C-4), 142.3 (C-3), 141.0 (C-7), 134.0 (C-1), 126.3, 123.7, and 122.3 (C-6, C-8, and C-5), 119.1(C-2), 59.0 (C-1′), 52.2 (-O$\underline{C}H_3$), 46.9 (C-4′), 29.1 (C-2′), 22.6 (C-3′), 20.5 (2-OCO$\underline{C}H_3$).

Methyl 1-cinnamoylpyrrolidine-2-carboxylate (**16**): Isobutyl chloroformate (2.7 mmol) was added to a cooled (−15 °C) solution of cinnamic acid **12** (2.7 mmol) and NMM (2.7 mmol) in THF (14 mL) and stirred at −15 °C for 15 min. A solution of L-proline methyl ester hydrochloride (2.7 mmol) and NMM (2.7 mmol) in DMF (5 mL) was then added and the reaction mixture was stirred for 24 h, allowing it to warm up to r.t. slowly [21]. The reaction mixture was evaporated, then poured into chloroform and extracted with a saturated solution of HCl 1 N, $NaHCO_3$, and water. The organic phase was dried with $MgSO_4$ and evaporated to dryness, resulting in an oil which was purified by recrystallization using hexane/ethyl acetate to give **16** as a white solid (m.p. 85.3–88.3 °C). Yield 81%. $[\alpha]_D$ −68 (*c* 0.05, $CHCl_3$). ^{1}H NMR ($CDCl_3$, δ [ppm], *J* [Hz]): 7.61 (d, *J* = 15.5, H-7), 7.39–7.24 (5 Har), 6.63 (d, *J* = 15.5, H-8), 4.49 (m, H1′), 3.75 (m, H-4′a), 3.64 (s, -OC$\underline{H}_3$), 3.59 (m, H-4′b), 1.97 (m, H-2′, and H-3′). ^{13}C NMR ($CDCl_3$, δ [ppm]): 172.7 and 164.8 (C-5′ and C-9), 142.8 (C-7), 142.6, 135.0, 129.6, 128.7, and 127.8, 117.8, (6 C-ar and C-8), 58.9 (C-1′), 52.1(-O$\underline{C}H_3$), 46.8 (C-4′), 29.0 (C-2′), 24.7 (C-3′).

(E)-methyl 1-(3-(4-methoxyphenyl)acryloyl)pyrrolidine-2-carboxylate (**17**): This derivative was prepared as described for **16** using *p*-methoxycinnamic acid **13** and L-proline methyl ester hydrochloride. The oily product obtained was purified by chromatography on silica gel, eluting with chloroform. Yield: 50%. $[\alpha]_D$ −56 (*c* 0.05, $CHCl_3$).^{1}H NMR ($CDCl_3$, δ [ppm], *J* [Hz]): 7.67 (d, *J* = 15.4, H-7), 7.44–6.88 (4 Har), 6.60 (d, *J* = 15.4, H-8), 4.61 (m, H1′), 3.85 (m, H-4′a), 3.82 (s, -OC$\underline{H}_3$), 3.74 (s, -OC$\underline{H}_3$), 3.69 (m, H-4′b), 2.11 (m, H-2′, and H-3′). ^{13}C NMR ($CDCl_3$, δ [ppm]): 172.9 and 165.2 (C-5′ and C-9), 160.9 (C-4), 142.5 (C-7), 129.4 (C-2 and C-6), 127.8 (C-8), 115.4 (C-1′), 114.1 (C-3 and C-5), 58.9 (C-1′), 55.3 and 52.2 (2-O$\underline{C}H_3$), 46.8 (C-4′), 29.1 (C-2′), 24.8 (C-3′).

1-cinnamoylpyrrolidine-2-carboxylic acid (**18**): 1 mL of 1 M NaOH was added to a methanol solution (1 mL) of compound **16** (0.4 mmol). The mixture was stirred at r.t. for 2 h, neutralized with 1 M HCl, and the methanol was evaporated under reduced pressure. The aqueous solution was cooled in an ice bath and acidified at pH 2 with 1 M HCl. The solid obtained was separated by filtration and washed with water. Yield: 87%. RMN ^{1}H (CD_3OD, d [ppm], *J* [Hz]): 7.55 (d, *J* = 15.4, H-7), 7.53–6.93 (5 Har), 6.89 (d, *J* = 15.4, H-8), 4.46 (m, H-1′), 3.76 (m, H-4′), 2.22–1.96 (m, H2′, and H3′). ^{13}C NMR (CD_3OD, d [ppm]): 173.0 and 165.7 (C-5′ and C-9), 142.8 (C-7), 134.8 (C-1), 129.7, 128.5, 127.8, 117.8 (5 C-ar), 117.6 (C-8), 59.2 (C-1′), 30.6 (C-2′), 24.3 (C-3′).

(E)-1-(3-(4-methoxyphenyl)acryloyl)pyrrolidine-2-carboxylic acid (**19**): This derivative was obtained as described for **18**, by alkaline hydrolysis with methanol and 1 M NaOH. Yield: 87%. ^{1}H-NMR (CD_3OD, δ [ppm], *J* [Hz]): 7.65 (d, *J* = 15.6, H-7), 7.68–7.11 (4 Har), 6.95 (d, *J* = 15.6, H-8), 4.72 (m, H-1′) 4,00 (s, -OC$\underline{H}_3$), 3.97 (m, H-4′), 2.50–2.22 (m, H2′, and H3′). ^{13}C NMR (CD_3OD, δ [ppm]): 175.7 and 167.7 (C-5′ and C-9), 162.9 (C-4), 143.9 (C-7), 130.8 (C-2 and C-6), 129.7 (C-8), 116.8 (C-1), 115.5 (C-3 and C-5), 60.7 (C-1′), 55.9 (-O$\underline{C}H_3$), 48.6 (C-4′), 30.3 (C-2′), 25.7 (C-3′).

In vitro measurement of enzyme inhibition: POP and DPP IV activities were determined as described previously [10,,23,28]. The IC_{50} value was defined as the concentration of fraction required to inhibit 50% of enzyme activity. Data were analyzed using GraphPad Prism 6 software. The assays were performed in triplicate and with a blank for each sample without enzyme, and the percentage of inhibition was calculated by comparing the absorbance of the sample to the blank. POP activity was evaluated using verbascoside [10] and Z-prolyl-prolinal as positive controls, while in the DPP IV assay, the standard inhibitor isoleucine thiazolide (P32/98) was used as the control.

Supporting information

Details regarding the synthesis of the known derivatives **2**–**4**, **7**–**8**, **10**, and **14**–**15**, NMR spectra of compounds as well as provenance, purity of substances, and equipment are available as Supporting Information.

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Conflict of Interest

The authors declare no conflict of interest.

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Correspondence
Profa Dra Ionara I. Dalcol
Núcleo de Pesquisa de Produtos Naturais (NPPN)
Chemistry Department
Universidade Federal de Santa Maria
Campus Camobi
97105–900 Santa Maria – RS
Brazil
Phone: + 55 55 32 20 87 98
Fax: + 55 55 32 20 80 31
iidalcol@gmail.com