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Original Article

Amino acids L-phenylalanine and L-lysine involvement in *trans* and *cis* piperamides biosynthesis in two *Piper* species

Participação dos aminoácidos L-fenilalanina e L-lisina na biossíntese de piperamidas *trans* e *cis* em duas espécies de *Piper*

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Abstract

Several *Piper* species accumulate piperamides as secondary metabolites, and although they have relevant biological importance, many details of their biosynthetic pathways have not yet been described experimentally. Experiments involving enzymatic reactions and labeled precursor feeding were performed using the species *Piper tuberculatum* and *Piper arboreum*. The activities of the phenylalanine ammonia lyase (PAL) enzymes, which are involved in the general phenylpropanoid pathway, were monitored by the conversion of the amino acid *L*-phenylalanine to cinnamic acid. The activity of the 4-hydroxylase (C4H) enzyme was also observed in *P. tuberculatum* by converting cinnamic acid to *p*-coumaric acid. *L*-[UL-^{14}C]-phenylalanine was fed into the leaves of *P. tuberculatum* and incorporated into piperine (**1**), 4,5-dihydropiperine (**2**), fagaramide (**4**), *trans*-piplartine (**7**), and dihydropiplartine (**9**). In *P. arboreum*, it was only incorporated into the piperamide 4,5-dihydropiperiline (**3**). *L*-[UL-^{14}C]-lysine was successfully incorporated into the 4,5-dihydropiperine piperidine group (**2**), dihydropyridinone, and *trans*- (**7**) and *cis*-piplartine (**8**). These data corroborate the proposal of mixed biosynthetic origin of piperamides with the aromatic moiety originating from cinnamic acid (shikimic acid pathway) and key amide construction with amino acids as precursors.

Keywords: *Piper*, Piperaceae, biosynthesis of natural products, piperamides, piperine.

Resumo

Diversas espécies do gênero *Piper* acumulam piperamidas como metabólitos secundários e, embora tenham relevante importância biológica, detalhes de suas vias biossintéticas ainda não foram descritos experimentalmente. Experimentos envolvendo reações enzimáticas e incorporação de precursores marcados foram realizados utilizando as espécies *Piper tuberculatum* e *Piper arboreum*. As atividades das enzimas fenilalanina amônia liase (PAL), que estão envolvidas na via geral dos fenilpropanoides, foram monitoradas pela conversão do aminoácido L-fenilalanina em ácido cinâmico. A atividade da enzima 4-hidroxilase (C4H) também foi observada em *P. tuberculatum* pela conversão do ácido cinâmico em ácido p-cumárico. L-[UL-^{14}C]-fenilalanina foi administrada em folhas de *P. tuberculatum* e incorporada em piperina (**1**), 4,5-di-hidropiperina (**2**), fagaramida (**4**), *trans*-piplartina (**7**) e diidropiplartina (**9**). Em *P. arboreum*, foi incorporado apenas à piperamida 4,5-dihidropiperilina (**3**). L-[UL-^{14}C]-lisina foi incorporada com sucesso em 4,5-diidropiperina (**2**), *trans*-piplartina (**7**) e *cis*-piplartina (**8**). Esses dados corroboram a proposta de origem biossintética mista de piperamidas com a porção aromática originada do ácido cinâmico (via do ácido chiquímico) e construção de amida chave com aminoácidos como precursores.

Palavras-chave: *Piper*, Piperaceae, biossíntese de produtos naturais, piperamidas, piperina.

1. Introduction

Piperamides are an important class of bioactive metabolites found in *Piper* species. The first isolation of a piperamide took place over 200 years ago, in 1819, when piperine (**1**), a spicy flavored compound, was isolated by

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the Danish chemist Hans Christian Ørsted from the fruits of black pepper (*Piper nigrum*) (Drobnik and Drobnik, 2016). The Piperaceae family is considered a primitive angiosperm, comprising more than 4000 species in tropical and subtropical regions and includes five genera: *Peperomia* (approximately 1700 species), *Piper* L. (2000 species), *Manekia* ret. (formerly called *Sarcorhachis*), *Verhuellia,* and *Zippelia* (Wanke et al., 2007). The chemical diversity of Piperaceae is commonly known, and phytochemical studies have led to the isolation of amides involved in important biological activities, including several examples that highlight the potential for the development of new antifungal, antitumor, and trypanocidal agents (Bosc et al., 2018; Silva et al., 2002; Valli et al., 2017; Vieira et al., 2018). In addition to aliphatic and aromatic piperamides (Alécio et al., 1998; Cotinguiba et al., 2009; Navickiene et al., 2000), several classes of compounds have been isolated from *Piper* species, including lignans (Felippe et al., 2008), polyketides (Cheng et al., 2003), piperolides (Lago et al., 2005), benzoic acid derivatives (Baldoqui et al., 1999; Morandim et al., 2005), kavalactones (Mazzeu et al., 2018), chromenes, and prenylated chromenes (Batista Júnior et al., 2008; Mota et al., 2009).

Biosynthetic studies involving piperamides are scarce. One of the first studies described the incorporation of *L*-[U-^{14}C]-phenylalanine and *L*-[U-^{14}C]-lysine into *trans*-piplartine (also called piperlongumine) (**7**) in *Piper longum*, which demonstrated that these amino acids are the precursors of the phenylpropanoid and dihydropyridinone moieties (Prabhu and Mulchandani, 1985). The main step in amide formation in piperine (**1**), which is the condensation of piperoyl-CoA with piperidine by an acyltransferase obtained from shoots of *P. nigrum,* is the reaction of precursors with the semipurified enzyme piperidine piperoyltransferase (Geisler and Gross, 1990). Recently, Schnabel et al. (2021a) identified and characterized this enzyme as piperine synthase in *Piper nigrum* (Schnabel et al., 2021a). Schnabel et al. (2020) also identified the activity of a CoA ligase, an enzyme capable of converting the substrate piperic acid in piperoyl-CoA, which is required for the biosynthesis of piperine in black pepper fruits (Schnabel et al., 2020). Jin et al. (2020) also identified CoA thioester synthases, which are fundamental in the later stages of amide formation (Jin et al., 2020). Another enzyme identified by Schnabel et al. (2021b) was the cytochrome P450 enzyme CYP719A37, which catalyzes the conversion of the guaiacyl (4-hydroxy-3-methoxy) group to the methylenedioxy group. The conversion of feruperic acid (5-(4-hydroxy-3-methoxyphenyl)-2,4-pentadienoic acid) to piperic acid was observed (Schnabel et al., 2021b).

In our study, questions regarding piperamide formation were experimentally investigated, including determination of the amino acids that could give rise to the phenylpropanoid pathway and the formation of piperidine and dihydropyridinone rings. Two species that accumulate piperamides were used: *Piper tuberculatum*, which accumulates piperine (**1**), 4,5-dihydropiperine (**2**), fagaramide (**4**), piperlonguminine (**5**), 4,5-dihydropiperlonguminine (**6**), *trans*-piplartine/piperlongumine (**7**), *cis*-piplartine (**8**), and dihydropiplartine (**9**) amides; and *Piper arboreum,* which accumulates piperamide dihydropiperiline (**3**) (Figure 1).

2. Materials and Methods

2.1. Plant material and isolation of piperamides

The plant material used was collected from specimens grown in greenhouse conditions at the Institute of Chemistry, UNESP, Araraquara-SP, Brazil (21°48'26.62″ S, 48°11'33.51″ W). *P. tuberculatum* was identified by Dr. Guillermo E. D. Paredes (Universidad Pedro Ruiz Gallo, Lambayeque, Peru), and *Piper arboreum* by Dr. Inês Cordeiro (Instituto de Botânica, Secretaria do Meio Ambiente, São Paulo-SP, Brazil). Both vouchers (Kato-163 and Cordeiro-1936, respectively) were deposited at the Herbarium of the Institute of Biosciences, USP, São Paulo-SP, Brazil (Navickiene et al., 2000; Silva et al., 2002). Seeds from this collection were cultivated and plantlets were utilized for feeding experiments with labeled precursors (Navickiene et al., 2003). Amides **1**–**2** and **4**–**9** were previously isolated from *P. tuberculatum* (Cotinguiba et al., 2009; Navickiene et al., 2000), whereas amide **3** was retrieved from *P. arboreum* (Silva et al., 2002). These piperamides were used as standards for monitoring retention time and obtaining spectra for comparison with biosynthesized substances after incorporation of labeled precursors.

2.2. Protocol for obtaining enzyme source for monitoring of the activity of the PAL (Phenylalanine Ammonia Lyase) enzyme

Protocol for PAL activity: For this experiment, the protocols described by Alokam et al. (2002) and Rösler et al. (1997) were adopted (Rösler et al., 1997) with modifications. *P. tuberculatum* and *P. arboreum* leaves (25 g each) were separately ground in liquid N_2 using a mortar and pestle. Thereafter, 100 mL of 0.1 M borate buffer (pH 8.8) was added. The buffer suspension contained PVPP (10% w/w),

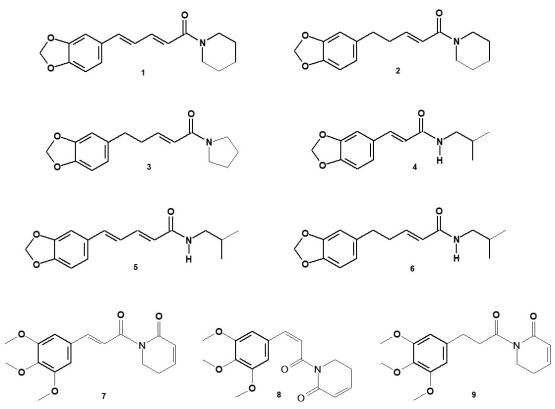

Figure 1. Piperine (1), 4,5-dihydropiperine (2), fagaramide (4), piperlonguminine (5), 4,5-dihydropiperlonguminine (6), *trans*-piplartine (7), *cis*-piplartine (8), and dihydropiplartine (9) are piperamides biosynthesized by *P. tuberculatum*; 4,5-dihydropiperiline (3) is biosynthesized by *P. arboreum*.

sucrose (0.25 M), ascorbic acid (40 mM), EDTA (1 mM), and DTT (5 mM). The suspension was stirred for 15 min in an ice bath prior to the experiment. The solution was homogenized with the buffer, and the extract was filtered through cheesecloth, stored at 4 °C, and centrifuged at 11 950 × g for 20 min. The supernatant was stored on ice and the pellet was discarded. **Enzyme assay:** The enzyme assay was initiated by adding 250 µL of 0.1 M borate buffer (pH 8.8) to a microtube with 125 µL of enzyme solution. This mixture was kept in a bath at 40 °C for 5 min. The reaction was initiated by adding 150 µL of L-phenylalanine (10 mM) and incubating at 40 °C with stirring. After 60 min, the reaction was stopped by adding 25 µL of HCl (6 M). The blank for comparison contained the precursor L-phenylalanine, an enzymatic extract containing PAL and was previously treated with 6M hydrochloric acid for enzyme inactivation. The solution was stirred and centrifuged at 14 000 rpm for a few seconds. All reactions were performed in triplicate with reproducible results. The products of the enzymatic reaction were analyzed by high-performance liquid chromatography (HPLC) using the following: sample volume injected = 10 µL, flow = 1.0 mL/min, λ= 275 nm, column = Supelcosil LC-18 (25 cm× 4.6 mm, 5 mm), mobile phase = MeOH: H_2O (with 0.1% of acetic acid) of 60:40.

2.3. Protocol for obtaining microsomes for monitoring of the activity of the enzyme C4H (Cinnamate 4-Hydroxylase)

Microsome extraction: The microsome fraction of *P. tuberculatum* leaves was obtained as described by Durst et al. (1996) with a few modifications (Durst et al., 1996). The tissues (20 g) were ground to a fine powder in liquid nitrogen, and homogenized in 100 mL of extraction buffer (42.75 mL of 0.1 M sodium phosphate, pH 7.4, containing 250 mM sucrose, 1 mM EDTA, 1 mM DTT, and 40 mM sodium ascorbate), by shaking at 4 °C. After the dissolution of the reactants (stirring), 10% glycerol (v/v) and 4 g PVPP were added. The suspension was shaken for 10 min at 4 °C and the phases were separated by centrifugation (10 000 × g for 15 min. at 4 ºC). The pellet was discarded, and 1 M $MgCl_2$ was added to the supernatant to achieve a concentration of 30 mM. The solution was then re-stirred for 20 min at 4 °C and centrifuged at 40 000 × g. The pellet was dried, resuspended in 1 mL phosphate buffer, and used as an enzyme source. The protein concentration was measured using the Bradford method. **Enzyme assay:** This enzyme assay used parts of the microsome fraction that contained at least 0.1 mg of protein. Cinnamic acid (10 mM) was used as the precursor, and NADPH (10 mM) as the hydrogen donor. These reagents were incubated for 30 minutes at 30 ºC and the samples were analyzed by HPLC (volume injected = 10 µL, flow = 1.0 mL / min, λ = 278 nm, column = Supelcosil LC-18 (25 cm × 4.6 mm, 5 mm), mobile phase = H_2O (0.01% acetic acid), and MeOH/9:1. The experiment was performed in triplicate with reproducible results. LC-MS/MS analysis were performed on an UltrOTOFQ ESI-TOF apparatus (Bruker-Daltronics, USA) at University of São Paulo, Ribeirão Preto, Brazil.

2.4. Administration of L-[UL-^{14}C]-phenylalanine in plantlets of P. tuberculatum and P. arboreum and L-[UL-^{14}C]-lysine in plantlets of P. tuberculatum and HPLC-Radiomatic analysis of the labeled extracts

Plantlets of *P. tuberculatum* and *P. arboreum* were fed for 1, 4, and 24 h with 100 µL of a solution containing 1 µCi *L*-[UL-14C]-phenylalanine, and with *P. tuberculatum* only, using *L*-[UL-14C]-lysine (precursors labeled with carbon-14 isotope. Each experiment was performed using nine plantlets, in addition to the positive control (without labeled precursor). After feeding, the specimens (separated into leaves, stems, and roots) were frozen with liquid nitrogen, triturated, homogenized, and extracted (3 × 10 mL) with dichloromethane-methanol (2:1). The solvent was evaporated, the residues resuspended in methanol (1 mg mL^{-1}), and radioactivity measurements were conducted using a liquid scintillation analyzer. In sequence, the isotopic labeled extracts (20 µL each sample) were analyzed by HPLC-UV (λ= 275 nm) coupled with a complementary Radiomatic 150 TR 3.0 detector (β-particle detection), as described by Sartorelli et al. (2001).

3. Results

3.1. Phenylalanine Ammonia Lyase (PAL) activity in P. tuberculatum and Piper arboreum

The tetrameric enzyme PAL (phenylalanine ammonia lyase/ EC 4.3.1.24) is the first enzyme involved in phenylpropanoid biosynthesis and is present in higher plants but rarely in prokaryotes (Moore and Hertweck, 2002). In eudicot plants, this enzyme is highly selective for *L*-phenylalanine as a substrate, whereas in monocots, *L*-phenylalanine and *L*-tyrosine could both be catalyzed by enzymes with PAL/TAL activity (EC 4.3.1.25) (Hsieh et al., 2011; Jun et al., 2018). Enzymes that recognize only *L*-tyrosine as a substrate are called TAL (tyrosine ammonia lyase/EC 4.3.1.23) and their activity was observed in monocot species of Gramineae/Poaceae (Achyuthan et al., 2010; Hossain et al., 2007).

L-phenylalanine were incubated with the enzymatic extracts obtained from *P. tuberculatum* and *P. arboreum*, and the conversion of *L*-phenylalanine to cinnamic acid was clearly observed in the HPLC chromatograms of *P. tuberculatum* (Figure 2).

3.2. Cinnamate 4-Hydroxylase (C4H) activity in P. tuberculatum

The activity of the cinnamate 4-hydroxylase (C4H) enzyme was evaluated in *P. tuberculatum*. Cinnamate 4-hydroxylase is a cytochrome P450-dependent enzyme that catalyzes cinnamic acid hydroxylation and is the second enzyme involved in phenylpropanoids biosynthesis, releasing *p*-coumaric acid, which is a precursor of compounds such as lignans, piperamides and flavonoids (Deng and Lu, 2017). An enzymatic reaction and HPLC analysis were performed as described in the *Materials and Methods* section, and the retention time of the product was compared with that of the standard *p*-coumaric acid. The retention time of the compound was found to be similar

to the standard (Supplementary Material Figure SM1), and its identity was investigated by mass spectrometry, owing to the complexity of the products formed in the enzymatic reaction.

The product was collected and the analyzed by mass spectrometry (ESI) in positive mode (LC-MS). The spectra revealed a peak of the ionized compound at m/z 165.0547 $[M+H]^+$ and a fragment of mass m/z 147.0476 $[M-H_2O+H]^+$, attributed to the loss of one water molecule (Supplementary Material Figures SM2 and SM3) (Ou et al., 2009).

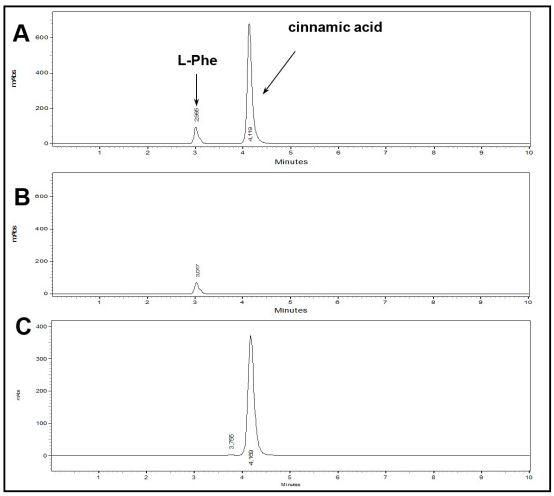

Figure 2. HPLC analysis of the enzymatic reactions (*L*-phenylalanine + enzymatic extract of *P. tuberculatum* leaves). Chromatogram A shows the formation of the cinnamic acid product after incubation of the amino acid *L*-phenylalanine with the enzymatic extract. Chromatogram B show the the blank for comparison (L-phenylalanine + enzymatic extract of *P. tuberculatum* leaves, previously treated with 6M hydrochloric acid for enzyme inactivation). Chromatogram C shows the retention time of the phenylpropanoid cinnamic acid (standard). In addition to the amino acid *L*-phenylalanine, *L*-tyrosine was also used as a possible precursor to phenylpropanoids, but no conversion to *p*-coumaric acid was observed. The same results were observed for *P. arboreum*.

3.3. Phenylalanine: the first precursor of phenylpropanoids participates of the C6–C3 biosynthesis

To corroborate the involvement of *L*-phenylalanine in phenylpropanoid biosynthesis of moities, a solution containing *L*-[UL-^{14}C]-phenylalanine was incorporated into *P. tuberculatum and P. arboreum* seedlings, where it was metabolized and the crude extracts of leaves, roots, and stems were subjected to HPLC-UV-Radiomatic analysis. *L*-[UL-^{14}C]-phenylalanine was incorporated into several amides from *P. tuberculatum*, as shown in Table 1. The specific incorporation was ≥ 6.55% for piperine (**1**) in stems within 1 h of feeding with *L*-[UL-^{14}C]-phenylalanine and ≥ 7.36% in roots within 4 h; ≥ 3.19% for 4,5-dihydropiperine (**2**) in stems within 1 h; ≥ 3.98% fagaramide (**4**) was obtained in leaves within 1 h and ≥16.70% in stems after 24 h; ≥7.52% 4,5-dihydropiperlonguminine (**6**) in leaves within 24 h; ≥ 8.01% *trans*-piplartine (**7**) in stems within 4 h; and ≥ 4.92% dihydropiplartine (**9**) within 1 h. The experiment with *P. arboreum* seedlings returned specific incorporation of ≥ 4.49% for piperiline (**3**) in roots within 4 h of feeding with *L*-[UL-^{14}C]-phenylalanine (Table 1, Supplementary Material Figure SM4).

3.4. L-Lysine is the precursor of the piperidine moiety in 4,5-dihydropiperine (2) and dihydropyridinone moiety in trans-piplartine (7) and cis-piplartine (8) in P. tuberculatum

An enzymatic study of the biosynthetic origin of the piperidine moiety in the amide piperine (**1**) was published in 1990, showing that an acyltransferase enzyme, (piperidine piperoyltransferase; EC 2.3.1.-), isolated from the shoots of *P. nigrum*, catalyzes the coupling of piperidine

Table 1. Percentage incorporation of activity from radiolabeled *L*-[UL-^{14}C]-phenylalanine into amides 1, 2, 4, 6, 7, and 9 by seedlings (leaves, stems, and roots) of *P. tuberculatum* at 1, 4, and 24 hours. In roots of *P. arboreum* it was only possible to observe the incorporation in amide 3.

Radiolabeled	Tissue	Incubation time (h)	Compound (% of incorporation)
L-[UL-^{14}C]-phenylalanine	*Piper tuberculatum*		
		1	4 (3.98)
	Leaves	4	-
		24	6 (7.52)
		1	1 (6.55); 2 (3.19)
	Stem	4	7 (8.01); 1 (6.20)
		24	4 (16.70)
		1	9 (4.92)
	Roots	4	1 (7.36)
		24	-
		Piper arboreum	
	Roots	4	3 (4.49)

Table 2. Percentage incorporations of activity from radiolabeled L-[UL-^{14}C]-lysine into amides 2, 7, and 8 by plantlets of *Piper tuberculatum*.

Radiolabeled	Tissue	Incubation time (h)	Compound (% of incorporation)
L-[UL-^{14}C]-lysine		*Piper tuberculatum*	
		1	8 (4.58)
	Leaves	4	-
		24	-
		1	-
	Stems	4	-
		24	7 (3.60)
		1	-
	Roots	4	8 (3.40)
		24	2 (2.73)

and the piperoyl-coenzyme A. In addition to amides with a piperidine ring, *P. tuberculatum* also accumulates dihydropyridinone amides such as *trans-piplartine* (**7**) and *cis-piplartine* (**8**). Prabhu and Mulchandani (1985) showed that *L*-[UL-^{14}C]-lysine is the precursor of the piplartine dihydropyridinone ring moiety isolated from *Piper longum*. The piperidine ring therefore undergoes a series of oxidations to form a dihydropyridinone moiety (Prabhu and Mulchandani, 1985).

In our study of *P. tuberculatum*, feeding experiments using *L*-[UL-^{14}C]-lysine performed with seedlings was evaluated by HPLC-UV-Radiomatic analyses, which revealed the incorporation of this isotopically-labeled amino acid in several amide skeletal structures. The data were obtained separately using leaves, stems, and roots, and a co-injection with authentic standards (previously isolated from *P. tuberculatum*) was used for comparison. Radiolabeled precursors were incorporated into amides biosynthesized by *P. tuberculatum*, as shown in Table 2.

Incorporation of ≥ 3.60% in dihydropiperine **(2)** was observed in stems within 24 h; ≥ 2.73% for *trans*-piplartine **(7)** in stems within 24 h, ≥ 4.58% in *cis*-piplartine (**8**) in leaves within 1 h, and ≥ 3.40% within 24 h in roots. The results showed the involvement of *L*-lysine in the piperidine and dihydropyridinone rings and the role of PAL in alkamide biosynthesis in *P. tuberculatum* species. This is the first experimental study on the incorporation of the amino acid *L*-lysine in a structure related to piperine (**1**) (Figure 3) (Prabhu and Mulchandani, 1985).

Despite piperamides being important compounds there is still a lack of knowledge of their biosynthesis. This is the first experimental study to demonstrate the role of the amino acid *L*-phenylalanine in the biosynthesis of phenylpropanoid moieties in piperine (**1**) and related structures such as 4,5-dihydropiperine (**2**), 4,5-dihydropiperine (**3**), fagaramide (**4**), and 4,5-dihydropiperlonguminine (**6**). *L*-[UL-^{14}C]-lysine was incorporated into several amides in *P. tuberculatum*, revealing that this amino acid is the precursor of dihydropyridinone and piperidine groups. This is the first experimental study on the incorporation of the amino acid *L*-lysine in a structure related to piperine (4,5-dihydropiperine, **2**).

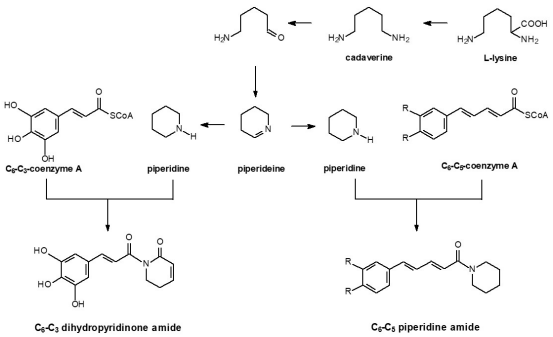

Figure 3. The incorporation of the amino acid *L*-lysine in C_6–C_5 piperidine amide (4,5-dihydropiperine, 2) and two C_6-C_3 dihydropyridinone amides (*trans*-piplartine, 7 and *cis*-piplartine, 8).

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Supplementary Material

Supplementary material accompanies this paper.

Figure SM1. HPLC analysis: A) Chromatogram showing the retention time of cinnamic acid (standard); B) Chromatogram showing the retention time of p-coumaric acid (standard); C) Chromatogram of the enzyme extract without the reagents (blank); D) Chromatogram of the reaction between cinnamic acid and the enzyme extract from P. tuberculatum: several products are formed, including one with a similar retention time to p-coumaric acid. This product was confirmed as p-coumaric acid by mass spectrometry analysis, revealing a peak quasi-molecular ion at m/z 165.0547 [M+H]+ and a fragment of mass m/z 147.0476 [M-H2O+H]+, attributed the loss of one molecule of water (Figure SM1 and SM2).
Figure SM2. MS/MS spectra of the product of the phenylalanine conversion reaction by the enzyme PAL, producing p-coumaric acid. This product was confirmed as p-coumaric acid by mass spectrometry analysis, revealing a iozinded ion at m/z 165.0547 [M+H]+ and a fragment of mass m/z 147.0476 [M-H2O+H]+, attributed the loss of one molecule of water.
Figure SM3. Proposal for the fragmentation of p-coumaric acid.
Figure SM4. Chromatogram of an HPLC-Radiomatic analysis showing incorporation of L-[U-14C]-phenylalanine in 4,5-dihydropiperiline (3) (extract of P. arboreum roots). All L-[UL-14C] -phenylalanine incorporation experiments (in P. arboreum and P. tuberculatum) and L-[UL-14C] -lysine (P. tuberculatum) followed this same analysis protocol.

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