Biosynthesis of Phenylpropanoid Amides by an Endophytic *Penicillium brasiliannum* Found in Root Bark of *Melia azedarach*

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Biosynthetic studies on brasiliamides, potently convulsive and bacteriostatic compounds from an endophytic *Penicillium brasiliannum* isolated from *Melia azedarach* (Meliaceae), confirms their phenylpropanoid origin, which is very uncommon in fungi. Feeding experiments with [2-13C]-phenylalanine indicated the incorporation of two units of this amino acid on brasiliamide structures. The first step in the phenylpropanoid pathway to those compounds was evaluated through enzymatic bioassays and confirmed the phenylalanine ammonia-lyase (PAL) participation. The metabolism of phenylalanine in this fungus is discussed.

**Keywords:** Biosynthesis, *Penicillium*, endophytic, bis-phenylpropanoyl amides, phenylpropanoid

Cumarins and lignans are typical phenylpropanoid compounds, formed respectively of one and two phenylalanine units [20]. The enzyme phenylalanine ammonia-lyase (PAL) converts the amino acid phenylalanine to cinnamic acid, the basic C₆–C₃ carbon skeleton present in these compounds, in a key enzymatic process in the phenylpropanoid pathway [20, 44]. The cinnamic acid may incorporate a triketide (C₃) by the action of chalcone synthase (CHS), forming the flavonoids, one of the most abundant and diverse class of natural products [9, 37]. Phenylpropanoids are considered to be plant-specific secondary metabolites [41]. The expression of the enzyme PAL, the entry point of the phenylpropanoid pathway, has often been reported as a plant response against invading microorganisms, indicating that phenylpropanoid may act as phytoalexins [18, 27].

Although heterologous gene expressions lead some bacteria to produce flavonoids [40, 42], the phenylpropanoid biosynthetic pathway has not been found to be naturally expressed in microorganisms. Recently, the presence of genes encoding the core enzymes of the phenylpropanoid pathway in the fungus *Aspergillus oryzae* was detected [14, 34]. However, these genes are silenced and no phenylpropanoid compounds are produced by this *Aspergillus*. A bacterial route to benzoate via PAL was shown to occur in *Streptomyces*, but leading to flavonin and its analogs [26] instead of phenylpropanoids. There are some reports of cinnamates in filamentous fungi, but their origin (whether they are truly fungal metabolites) appears to be doubtful. One of the first fungi bis-phenylpropanoid compounds reported was the bis-lactone of dihydrocaffeic acid isolated from *Inonotus* sp. [16]. Flavonoids were obtained from extracts of *Aspergillus* species [22, 23, 39], but these compounds may have originated from the soy-based cultivation medium used to grow the fungus. The biosynthetic route leading to bis-phenylbenzoquinone and related pigments and cyclopeptide compounds appears to be an important way for the accumulation of amino acids by fungi secondary metabolism [38], but apparently without the involvement of PAL producing cinnamates as intermediates.

The biosynthesis of phenylpropanoid compounds has received widespread interest, mainly because they have various important biological activities [17], are useful as pigments [35], and are involved in many ecological contexts since they are often related to biotic (e.g., microorganism infection) and abiotic (e.g., temperature, pH) stress induction in plants [6, 21]. Moreover, PAL is one of the few nonhydrolytic enzymes that have important commercial applications [21], being useful for the production of L-phenylalanine (L-Phe) from trans-cinnamic acid through the reverse physiological reaction [47]. In addition, PAL is effective in the treatment of certain mouse tumors [12] and useful in quantitative analysis of serum L-Phe in monitoring patients with phenylketonuria [43].

During our continued studies on the biochemistry of plant–microbe consortiums, we have investigated the compounds produced by an endophytic *Penicillium brasiliannum* isolated from *Melia azedarach* (Meliaceae) [10, 11, 33]. The known
brasiliamides A (1) and B (2), and a new brasiliamide N-oxide, named brasiliamide F (6) [11] (Fig. 1), were isolated and structurally characterized. Compounds 1 and 2 were also previously isolated along with brasiliamides C–E (3–5) by Fujita et al. [13, 36] from a soil–collected P. brasilianum strain. Analyses of the brasiliamides molecular structures indicated that they are apparently biosynthesized from two phenyalanine units, which is uncommon in fungi. In the present work, the biosyntheses of these brasiliamides were investigated through the addition of labeled L-phenylalanine in the culture medium. Extraction and bioassays with PAL were also conducted in order to investigate the possibility of phenylpropanoid pathway occurrence in the fungus metabolism.

**Materials and Methods**

**Materials**
The fungus *Penicillium brasilianum* was isolated from root bark of *Melia azedarach* following the procedure described by Santos et al. [33], and is deposited at Laboratório de Bioquímica Micromolecular de Microorganismos in São Carlos, Brazil.

**Instruments and Reagents**
Low-resolution ESI/MS and ESI/MS/MS data were acquired in positive-ion mode, using a Micromass Quattro-LC instrument equipped with an ESI/APCI "Z-spray" ion. This mass spectrometer was coupled with a Waters HPLC (Alliance 2695) equipped with PDA detector (Alliance 2996) for the analysis. *H and C NMR experiments were recorded on a Bruker DRX-400 spectrometer with deuteron chloroform (CDCl₃) as the solvent and TMS as the internal standard. [2-¹³C]-phenylalanine and all other reagents were purchased from Sigma.

**Penicillium brasilianum Cultivation and Feeding Experiments**
The strain was cultivated on dextrose–potato agar (PDA) slants at 25°C. The inoculum was prepared by suspending spores from 7-day cultures in water to a density of 10⁷ spores/ml. The inoculum of *Penicillium brasilianum* was transferred under sterile conditions to 5 of 8 Erlenmeyer flasks containing sterilized chemically defined medium (Czapek) developed in previous studies [12], which contained glucose, 80.0 g; NH₄NO₃, 0.48 g; K₂HPO₄, 5.0 g; MgSO₄, 1.0 g; CuSO₄, 0.015 g; ZnSO₄, 0.161 g; MnSO₄, 0.01 g; FeSO₄·7H₂O, 0.1 g; and (NH₄)₂MoO₄, 0.1 g; per liter of distilled water. The other 3 flasks were kept for control purposes. The initial pH of the medium was adjusted to 6.5. Feeding experiments were performed with use of [2-¹³C]-phenylalanine (99% ¹³C). The precursor was added to the fungal culture before inoculation into the growth media. The final concentration of labeled phenylalanine was 1 mg/ml for each experiment. After fermentation of more than 15 days, the mycelium was separated from the culture medium and extracted with ethanol (150 ml). The ethanol extracts were dried to yield the crude isotopically labeled extract (321.0 mg). Purification of brasiliamide A (1) was performed by column chromatography in a column (diameter, 25 mm) packed with silica gel (70–230 Mesh). Brasiliamide A was eluted with 250 ml of a hexane:ethyl acetate (9:1) mixture. The yield of the vacuum-dried, high-purity brasiliamide A was 1.5 mg.

**Fig. 1.** Chemical structure of brasiliamides produced by the endophytic *P. brasilianum* isolate (1, 2, and 6), and those originated from the soil strain (3, 4, and 5) [13, 36].
Phenylalanine Ammonia-Lyase Activity

The fungus *Penicillium brasilianum* was cultivated for 3 days under static conditions on Czapek medium enriched with 2% of yeast extract, as described previously. The enzymatic activity was measured in two different environments, first using the filtrate (to check if it is an excreted enzyme) and then using the mycelium resuspended in a poor medium (buffer solution) containing Tris-HCl buffer, pH 8.8. The specific substrate for PAL (L-phenylalanine - 2.5 mM) was added to both assays. After 30 min of reaction, an aliquot of 5 ml of the filtrate mixture and the resuspended mycelium were collected and added to 5 ml of HCl (0.1 M) to stop the reaction. The desired product, cinnamic acid, was extracted with 5 ml of alcohol reagent and monitored by HPLC/UV analyses.

**LC/MS/MS Analyses**

A LUNA C18 (Phenomenex) HPLC column (250×4.60 mm, 5 µ particle size) was used for sample separation. The HPLC mobile phase flow rate was set at 0.70 ml/min, using linear gradient elution with acetonitrile and water, for 45 min. In both eluents, 0.1% TFA (trifluoroacetic acid) was added. In the ESI/MS analyses, the capillary voltage was 3.88 kV and the cone voltage was 11 V. In the collision cell, argon was used as the collision gas at a collision energy of 10 eV and a CID pressure of 1.3×10⁻³ mbar. The ion source temperature was held at 50°C.

**RESULTS**

The 13C isotopically labeled phenylalanine (2-13C-Phe) was added to the Czapek’s medium used to grow the fungus *P. brasilianum* in order to investigate the incorporation of this amino acid into brasiliamides. After 15 days of inoculation, the fungus was harvested by filtration under reduced pressure and the amides were extracted from the mycelium using ethanol. Brasiliamide A, the major brasiliamide accumulated in the extract, was monitored for 13C-Phe incorporation using LC/UV/MS/MS and 13C NMR spectroscopy. Brasiliamide A was detected in both experiment (13C-Phe added) and control (only fungus in the basic Czapek’s medium) EtOH extracts as clear chromatographic peaks with identical retention time and UV spectra compared with the brasiliamide A standard.

The positive-ion ESI full-scan mass spectrum of brasiliamide A in the control cultivation contained a prominent peak at m/z 439 ([M+H]+), which corresponds to the molecular formula C₂₄H₂₆N₂O₆. A sodium adduct ([M+Na]⁺) was also detected at m/z 461 (Fig. 2). The MS spectrum obtained from the extract of the experiment (13C-Phe added) and control (only fungus in the basic Czapek’s medium) EtOH extracts as clear chromatographic peaks with identical retention time and UV spectra compared with the brasiliamide A standard.

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labeled compound was used to acquire $^{13}$C NMR data, and two prominent signals were clearly detected at $\delta$ 122.6 and 206.8 (Fig. 4B), which corresponded respectively to C-2 and C-2’ in brasiliamide A (Fig. 4A) [11]. This is in complete agreement with the interpretation of the MS data and confirms that two 2-$^{13}$C-Phe were incorporated into brasiliamide A, the major brasiliamide produced by this *Penicillium*. Minor brasiliamides B (2) and F (6) were also shown to incorporate 2-$^{13}$C-Phe, when analyzed by mass spectrometry (Fig. 5).

As stated before, *bis*-phenylpropanoid compounds are usually formed after phenylalanine molecules have been deaminated by PAL. Then, since the two C$_6$-C$_3$ units are present in brasiliamides, enzymatic experiments were performed in order to verify the possibility of PAL activity in *P. brasili anus*. Enzyme extracts were obtained from growing cells after fungus cultivation in Czapek’s medium. Thus, incubation of $L$-phenylalanine in these enzymatic extracts led to production of cinnamic acid, which was directly identified using LC/MS. It was observed that the enzyme was able to convert the substrate in a short time after incubation, which shows how active this enzyme is during the fungus development.

**DISCUSSION**

The brasiliamides A and B (1 and 2) produced by our endophytic isolate of *P. brasili anus* [11] are identical to those found by Fujita *et al.* in their soil isolate [13, 36]. What these compounds have in common are the presence of two aromatic rings, one of them with no substituent and the other being a methoxypiperonyl partial structure, and at least one N-acetyl group, which characterize them as amides. Although in all of these molecules the aromatic rings are bonded to a C$_3$ side chain, only in brasiliamide F (6) is an intact phenylalanine unit (8) promptly identified (Fig. 1). A retro-synthetic analysis on brasiliamide F (Scheme 1) indicates that the phenylalanine is connected to a more drastically modified C$_6$-C$_3$ (7), which can also originate from another phenylalanine unit, probably with the carboxyl group reduced to an aldehyde (Z corresponding to C=O).

In the present work, it was demonstrated that brasiliamides are biosynthesized by *P. brasili anus* using two Phe units and that cinnamic acid is produced when Phe is incubated with an enzymatic extract obtained from this fungus, indicating that PAL is active in this extract and probably participates in brasiliamides biosynthesis. The six brasiliamides

**Fig. 4.** $^{13}$C NMR spectra obtained for brasiliamide A (A) and $^{13}$C-enriched brasiliamide A (B). Signals labeled with “X” are due to impurities.

**Fig. 5.** Chromatograms of product ions generated from precursors of m/z 425 ($^{13}$C-labeled brasiliamide B), 441 ($^{13}$C-labeled brasiliamide A), and 413 ($^{13}$C-labeled brasiliamide F) found in labeled extract.
known so far (Fig. 1) have many structural features in common. With the exception of brasiliamide F (6), in all of the other congeners (1–5), the carboxyl carbon (C-1) of the Phe precursor unit is reduced and aminated. Therefore, a reductive amination in a cinnamaldehyde (11), followed by alternating oxidation and reduction steps, may occur to produce two 1-aryl-3-amino-2-propanone (17) units (Scheme 3), which are putative key intermediates to brasiliamides 1–5. The coupling of these two aminopropanones is very much expected, in the organic chemistry point of view (Scheme 4), to form compounds 1–6. The formation of the enamine function present in brasiliamides A, B, C, and F occurs during the dehydration of the unstable intermediate 20 in this scheme, and this is compatible with the presence of the C–C double bonds at Δ^1(2) in 1, 2, and 6, and at Δ^2(3) in 3. Only two further N-acetylation steps in 21 are necessary to form brasiliamide A (1). Thus, Schemes 3 and 4 are in complete agreement with the ^13C isotopic labeling experiment. Further cyclization of the aminoketone 21 in Scheme 4, followed by reductions and N-acetylation, can lead to the natural products 2–5.

The presence of 1-aryl-3-amino-2-propanone partial structures (18 and 19 in Scheme 4) is rare in natural secondary metabolites. An Aspergillus species has shown to accumulate 1-(4-hydroxyphenyl)-3-amino-2-propanone [1, 15], being one of the very few examples. Another expressive structural feature in these brasiliamides is the presence of a 3-methoxy-4,5-methylenedioxyphenyl (methoxypiperonyl) group. The classes of natural products in which the methoxypiperonyl group is most frequently found are the lignoids (lignans and neolignans) [4, 45] and some flavonoids [3, 5, 31, 32, 46], being typical plant phenylpropanoids, although a huge number of synthetic compounds contains this group. It appears that the only fungal metabolites containing the methoxypiperonyl as a partial structure are the brasiliamides (Fig. 1); the PF118 compounds produced by Chrysosporium, which are three complex antitumor amides [36].

Scheme 1. Retro-synthetic analysis of brasiliamide F (6).

Scheme 2. Fragmentation mechanism suggested for brasiliamide A.
CAN 134:352364; AN 2001:366097; CAPLUS]; and the tremorgenic toxins territrems A and B produced by A. terreus [9], which seem to contain a cinnamic acid derivative, with the aromatic ring being a methoxypteronyl, incorporated in a terpenoyl carbon chain. In plants, many typical enzymes of the phenylpropanoid pathway (e.g., cinnamate 4-hydroxylase, 4-coumarate CoA ligase, caffeic acid/5-hydroxyferulic acid O-methyltransferase, etc.) act on phenylalanine to produce synapate [8], which is further oxidized to form the methoxypteronyl. Thus, brasiliamides have many similarities with plant bis-phenylpropanoids biosynthesis and represent a special fungal metabolism of phenylalanine.

The most frequent ways followed by fungi to incorporate unmodified phenylalanine structures in its metabolites are the production of cyclopeptides and depsipeptides, besides construction of protein [3]. The deamination in phenylalanine/tyrosine leading to diphenylbenzoquinones and related pigments in fungi appears to occur via another enzymatic process producing 2-hydroxycinnamates as first intermediates [24, 25]. In an overall view, it appears that the phenylpropanoid pathway in plants is used to produce larger molecules from phenylalanine, resulting in secondary metabolites that may mediate their biotic (microbes, insects, etc.) and abiotic (mainly UV light) environmental interactions [8, 37, 41, 44]. On the other hand, in fungi, generally this metabolic pathway appears to be shorter and the initial biosynthetic step involves oxidizing enzymes that degrade phenylpropanoid precursors [28] back to smaller molecules such as benzoates and styrene, like some Penicillium species do [2, 29]. Thus, it seems that the phenylpropanoid pathway is an important biochemistry field of battle for survival. A very interesting theory was recently developed, which indicates that the land plants would have acquired the ability to produce phenylpropanoid compounds from microbes via horizontal gene transfer (HGT) encoding the PAL enzyme [7]. During their evolution, many microorganisms appear to have kept these metabolic abilities to defend themselves and be able to associate with plants controlling their chemical responses during colonization. Perhaps the biosynthesis of brasiliamides represents a new strategy developed by the fungus P. brasiliannum to be accepted by...
Melia azedarach as it host plant. Experiments to verify this hypothesis must be designed and tested.

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References


