Co-Production of Bisphenylpropanoid Amides and Meroterpenes by an Endophytic *Penicillium brasilianum* Found in the Root Bark of *Melia azedarach*

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A fungus, isolated from the root bark of *Melia azedarach* (Meliaceae), from which a series of meroterpenes have been reported, was identified as *Penicillium brasilianum* based on analysis of the ITS region of ribosomal DNA. From a rice culture of this fungus, the known phenylpropanoid amides brasiliamide A and B were obtained together with a new, slightly modified congener, along with the meroterpenoids preaustinoid A1, preaustinoid B2 and austinolide. The compounds were isolated by the use of combined chromatographic procedures and identified by physical methods, mainly 1D and 2D NMR experiments, with distinction for ¹H{¹⁵N} HMBC applied to brasiliamide A. The amides were tested for their antimicrobial activity and showed only weak inhibitory effects, against a set of pathogenic bacteria.

Key words: *Melia azedarach*, *Penicillium brasilianum*, Bisphenylpropanoid, Brasiliamide

Introduction

During our continuous studies on endophytic microorganisms (Santos *et al.*, 2003), a *Penicillium* sp. was found associated with *Melia azedarach* (Meliaceae). In previous works, we reported that this *Penicillium* sp. is a rich source of interesting secondary metabolites such as insecticidal meroterpenoids, a class of complex metabolites derived from a mixed terpenoid-polyketide biosynthetic pathway, and tremorgenic alkaloids (verruculogen) (Santos *et al.*, 2003; Santos and Rodrigues-Filho, 2002, 2003a, b, 2004; Fill *et al.*, 2007). Analysis of the ITS region of the ribosomal DNA allowed its identification as *P. brasilianum*. It has been shown now that this fungus produces bisphenylpropanoid amides (brasiliamides **1**–**5**), as already reported in the literature (Fujita *et al.*, 2002; Fujita and Hayashi, 2004). Brasiliamides A (**1**) and B (**2**) (Fig. 1) were first identified as secondary metabolites from *P. brasilianum* isolated from soil (Fujita *et al.*, 2002), and, when evalu-

ated, these compounds showed convulsive activities against silkworms (*Bombyx mori*).

From *P. brasilianum* found in *M. azedarach* we obtained two known bisphenylpropanoid *N*-acetyl amides, **1** and **2**, and one new congener from the methanolic extract obtained when the fungus was cultivated on sterilized rice for three weeks. We report here the production, isolation and identification of these amides.

Material and Methods

General experimental procedures

Optical rotations were measured on a Perkin Elmer 241 polarimeter. UV spectra were obtained in CH₂Cl₂ on a Hewlett Packard 8452-A spectrophotometer, and IR spectra were measured with a Bomen MB-102 spectrophotometer using KBr pellets. Low-resolution ESIMS data were acquired in the negative ion mode, using a QUATTRO-LC instrument (Micromass, Manchester, UK) equipped with an ESI/APCI ion

Fig. 1. Chemical structures of the brasiliamides produced by *P. brasilianum*.

source. ¹H, ¹³C and ¹⁵N NMR experiments were conducted on a BRUKER DRX-400 spectrometer with CDCl₃ as the solvent and TMS as the internal standard.

Isolation of the microorganism

Details on the plant material collection and fungus isolation procedure are given elsewhere (Santos and Rodrigues-Filho, 2002, 2003a). The general procedures adopted followed the methodology described by Petrini *et al.* (1992). The *P. brasilianum* used in the present work (LaBioMi024) is deposited at the Laboratório de Bioquímica Micromolecular de Microorganismos (LaBioMMi) of the Departamento de Química, Universidade Federal de São Carlos, São Carlos, Brazil.

Rice culture of *Penicillium* sp. and isolation of the amides

Fifty Erlenmeyer flasks (500 ml) containing about 90 g of rice ("Uncle Ben's", Mars, Canada) and 75 ml of distilled water per flask were autoclaved twice at 121 °C for 40 min. A small disc of the PDA medium from the Petri dish containing mycelium of *Penicillium* sp. was transferred under sterile conditions to 47 of the 50 Erlenmeyer flasks containing sterilized rice. Three flasks were kept

for control purposes. After 20 d of growth, the water that had accumulated in the flasks was separated by filtration from the fungal biomass. About 150 ml of methanol were added to each flask; the mixture was allowed to stand for 5 h and filtrated by gravity. The methanol was evaporated under reduced pressure, producing a yellowish residue (6 g), which was subjected to low-pressure silica gel CC eluted with a hexane to methanol gradient (hexane; hexane/dichloromethane 90:10, 70:30, 50:50, 30:70; dichloromethane; dichloromethane/methanol 99:1; methanol 100%). The medium polar fractions eluted with dichloromethane/methanol 95:5 were reiteratively chromatographed on silica gel columns, and the meroterpenoids preaustinoid A1, preaustinoid B2 and austinolide were finally purified by preparative TLC (hexane/ethyl acetate 1:1). The fraction eluted with dichloromethane/methanol 90:10 gave the brasiliamides **1**, **2** and **6** after further purification by preparative TLC (dichloromethane/ethyl acetate/methanol 7:2:1).

Brasiliamide A (1): Colourless amorphous powder; m.p. 132–134 °C. – [α]_D²⁵ –0.45° (c 2.7, CHCl₃). – UV: λ_{max} (CH₃OH) = 211, 233, 255(sh) nm. – IR (KBr): ν_{max} = 3455, 3288, 2932, 2850, 1700, 1672, 1636 cm^{–1}. – ¹H NMR (400 MHz, CDCl₃, standard

SiMe₄): see Table I. – ¹³C NMR (100 MHz, CDCl₃, standard SiMe₄), HSQC and HMBC: see Table I. – ESIMS (daughter ions, 10 eV): *m/z* = 439 (40) [M+H]⁺, 380 (100), 338 (30). – ESIMS (daughter ions, 20 eV): *m/z* = 437 (5) [M–H][–], 419 (15), 395 (5), 378 (30), 336 (50), 58 (100).

Brasiliamide F (6): Colourless amorphous powder; m.p. 119–124 °C. – UV (CH₃OH): λ_{max} = 213, 276 (sh) nm. – IR (KBr): ν_{max} = 3436, 2920, 2845, 1733, 1673, 1644, 1497 cm^{–1}. – ¹H NMR (400 MHz, CDCl₃, standard SiMe₄): see Table I. – ¹³C NMR (100 MHz, CDCl₃, standard SiMe₄), HSQC and HMBC: see Table I. – ESIMS (daughter ions, 20 eV): *m/z* = 409 (60) [M–H][–], 394 (30), 379 (5), 367 (100), 352 (35).

Results and Discussion

Among the numerous fungi collected from organs of *M. azedarach* (Santos *et al.*, 2003), only one *Penicillium* sp. was found to be a producer of meroterpenes (Santos *et al.*, 2003; Santos and Rodrigues-Filho, 2002, 2003a, 2003b, 2004), evaluated by liquid chromatography coupled with mass spectrometry (LC/MS) (Santos and Rodrigues-Filho, 2004). These meroterpenoid compounds show close structure similarities with limonoids, which are endogenous compounds produced by the host plant. Other characteristic secondary metabolites produced by this *Penicillium* sp. were verruculogen and methyl penicillate (Santos *et al.*, 2003). In the present work, ribosomal DNA analysis was conducted to identify this fungus at species level. A total of 507 base pairs (ITS region) were decoded and compared with the GeneBank database. Two good matches were found with *P. brasilianum* strains CBS 253.55 and RMF 8007, showing an error value of 0.0 (100% reliability).

A literature survey of secondary metabolites produced by this fungus species showed that it is a producer of bisphenylpropanoid amides, named brasiliamides (Fujita *et al.*, 2002; Fujita and Hayashi, 2004). These amides cause convulsion to silkworms. The phenylpropanoid units in each of these compounds are a methoxypiperonyl cinnamic acid derivative and a modified phenylalanine. Although genes encoding the phenylpropanoid biosynthetic pathway have been found in fungi (Seshine *et al.*, 2005a, 2005b), there is no report on the expression of these genes to produce fungal phenylpropanoid compounds. Thus

we were motivated to look for this type of compounds in our strain of *P. brasilianum*.

The fungus was cultivated over sterilized rice, extracted and submitted to a combination of chromatographic procedures which led to the purification of the previously reported meroterpenoids preaustinoid A1, preaustinoid B2 and austinolide (Santos and Rodrigues-Filho, 2003b), and of three compounds with brasiliamide profile, when analyzed by ¹H NMR. Only five members of this class of secondary metabolites are known until now (Fujita *et al.*, 2002; Fujita and Hayashi, 2004). All of these compounds contain a phenyl group, recognized in the NMR spectrum by the presence of a 5H multiplet at ca. δ 7.15–7.35, and a methoxypiperonyl group which shows two signals for *meta* coupled hydrogen signals at δ 6.28 (*d*, *J* = 1.5 Hz, H-5) and 6.23 (*d*, *J* = 1.5 Hz, H-9), two AB oxymethylenic hydrogen atoms at δ 5.96/5.94, and a methoxy group at δ 3.84 (*s*, 3H-3''), like observed for brasiliamide A (Fujita *et al.*, 2002) (Table I). Both the methoxypiperonyl and the phenyl groups may be present as benzyl partial structures (**I** and **II**, Fig. 2), and this is seen by the two pairs of diastereotopic methylenic hydrogen atoms at ca. δ 2.30–4.30. The atom numbering system adopted so far for brasiliamides is ambiguous (Fujita *et al.*, 2002; Fujita and Hayashi, 2004). In the present work we numbered the atoms considering that the amino acid phenylalanine is a precursor of these brasiliamides.

The electrospray (ESI) mass spectra obtained for the major brasiliamide **1** showed prominent

Fig. 2. Partial structures deduced for brasiliamides based on NMR data.

Table I. NMR spectroscopic data of brasiliamides **1** and **6** (CDCl₃, 400 MHz for ¹H and 100 MHz for ¹³C).

Position	1			6		
	¹³ C	¹ H	¹ H{ ¹³ C} and ¹ H{ ¹⁵ N} HMBC	¹³ C	¹ H	¹ H{ ¹³ C} HMBC
1	122.1	6.92 <i>d</i> (10.5)	C-2, C-3, N-10, N-11, N ¹⁰ COCH ₃	113.0	6.15 <i>brs</i>	C-1'', C-2, C-2', C-3
2	122.4	–	–	121.1	–	–
3a	40.1	3.30 <i>d</i> (14.5)	C-1, C-4, C-5, C-9, N-11	38.0	4.27 <i>brm</i>	–
3b	–	3.19 <i>d</i> (14.5)	C-1, C-4, C-5, C-9, N-11	–	3.44 <i>d</i> (15.6)	C-1, C-2, C-4, C-5, C-9
4	131.3	–	–	132.1	–	–
5	102.9	6.28 <i>d</i> (1.5)	C-3, C-6, C-7, C-9	102.3	6.36 <i>brs</i>	C-3, C-4, C-6, C-7, C-9
6	149.9	–	–	148.8	–	–
7	134.4	–	–	137.1	–	–
8	143.8	–	–	143.4	–	–
9	108.2	6.23 <i>d</i> (1.5)	C-3, C-5, C-7, C-8	108.1	6.36 <i>brs</i>	C-3, C-4, C-6, C-7, C-9
1'a	56.6	4.53 <i>d</i> (17.0)	C-2, C-2', N ¹¹ COCH ₃	166.4	–	–
1'b	–	3.02 <i>d</i> (17.0)	C-2, C-2', N ¹¹ COCH ₃	–	–	–
2'	206.9	–	–	52.0	4.93 <i>brm</i>	n.d.
3'a	47.8	3.82 <i>d</i> (16.0)	C-1', C-2', C-4', C-5'/9'	35.0	2.99 <i>dd</i> (11.7, 12.0)	n.d.
3'b	–	3.74 <i>d</i> (16.0)	C-1', C-2', C-4', C-5'/9'	–	2.50 <i>d</i> (12.0)	C-1', C-4', C-5', C-9'
4'	132.6	–	–	133.8	–	–
5'/9'	129.4	7.18 <i>m</i>	C-3', C-5', C-7'	129.2	7.15 <i>m</i>	C-3', C-4', C-5', C-9'
6'/8'	128.8	7.34 <i>m</i>	C-4', C-6'	128.8	7.32 <i>m</i>	–
7'	127.4	7.29 <i>m</i>	C-5'/9'	126.9	7.27 <i>m</i>	C-3', C-4', C-7'
OCH ₃	56.7	3.84 <i>s</i>	C-8	56.7	3.87 <i>s</i>	C-4'
OCH ₂ O	101.4	5.96/5.94 <i>m</i>	C-6, C-7	101.3	5.93 <i>brs</i>	C-5', C-9'
N ¹⁰ COCH ₃	23.1	2.10 <i>s</i>	C-1, N ¹⁰ COCH ₃ , N-10	21.5	2.23 <i>s</i>	C-8
N ¹⁰ COCH ₃	168.2	–	–	169.2	–	C-6, C-7
N ¹¹ COCH ₃	20.0	1.87 <i>s</i>	N ¹¹ COCH ₃ , N-11	–	–	C-1''
N ¹¹ COCH ₃	170.7	–	–	–	–	–
N ¹⁰ -H	–	9.63 <i>d</i> (10.5)	C-2, N ¹⁰ COCH ₃	–	–	–

Coupling constants (Hz) in parentheses.
n.d., Not detected.

peaks at *m/z* 439, when positive ions were scanned, and *m/z* 437 in the negative ion mode, confirming a molecular weight (MW) of 438 amu. By counting the ¹³C NMR signals and using the ¹H NMR peak area, along with the MS data, the molecular formula C₂₄H₂₆N₂O₆ is suggested for this fungal metabolite. This molecular formula is the same as obtained for brasiliamide A (Fujita *et al.*, 2002). All of the physical data reported for this compound are coincident with ours, confirming its identification. Further confirmation of the brasiliamide A structure was given by analysis of ¹⁵N NMR data (Table I). Thus, ¹H{¹⁵N} HSQC showed correlation of the amide hydrogen signal at δ 9.63 (*d*, *J* = 10.5 Hz) with a nitrogen nucleus (N-10); in the ¹H{¹⁵N} HMBC spectrum there were correlations between H-1 with N-10 and N-11, H-3a and H-3b with N-11, and H-1'a and H-1'b with N-11. Brasiliamide B (C₂₄H₂₆N₂O₅, 422 amu) was identified in the same way as for brasiliamide A, but

without the use of ¹⁵N NMR spectroscopy due the small amount of the compound isolated.

The above identified brasiliamides are di-*N*-acetylated compounds containing a methoxypiperonyl and a benzyl group. A brasiliamide that contains only one 3H singlet for an *N*-acetyl group in the ¹H NMR spectrum was also obtained from *P. brasilianum* extracts. This brasiliamide monoacetate was analyzed by MS and showed [M–H][–] produced by ESI at *m/z* 409, indicating a MW of 410 amu and differing from brasiliamide E (C₂₂H₂₆N₂O₄, 382 amu) (Fujita and Hayashi, 2004), the only brasiliamide monoacetate known so far. The presence of the methoxypiperonyl (δ 6.36, *brs*, H-5 and H-9; δ 5.93, *brs*, 2H-4''; δ 3.87, *s*, 3H-3'') and the benzyl (δ 2.99, *dd*, H-3'a; δ 2.50, *d*, H-3'b; δ 7.15–7.27, *m*, H-5' to 9') groups was evident in the ¹H NMR spectrum of this unknown brasiliamide. The two signals at δ 4.93 (H-2') and δ 4.27 (H-3a) were observed as broad lines, prob-

ably due to a strong influence of vicinal quadrupolar nitrogen atoms, or due to the presence of rotamers like demonstrated for brasiliamide A (Fujita and Hayashi, 2004). The partial structure **III** (Fig. 2) was confirmed for this unknown amide based on the establishment of the connectivities between the methoxypiperonyl (**I**, Fig. 2) and benzyl (**II**, Fig. 2) groups assigned by the 2D HMBC and NOE measurements. Thus, upon irradiation, H-5 and H-9 (both at δ 6.36) showed NOE with H-1 (δ 6.15), 3H-2'' (δ 2.23), the diastereotopic benzylic hydrogen atoms at δ 4.27 (H-3a) and δ 3.44 (H-3b) and the methoxy hydrogen atoms at δ 3.87 (3H-3''). NOE was also observed with H-5/H-9, H-3b and 3H-2'' (*N*-acetyl) when H-1 (δ 6.15) was irradiated. The NMR signal of H-1, which is a doublet in brasiliamide A, was detected as a singlet (δ 6.15), indicating, along with the NOE between H-3/3H-2'', the presence of an *N,N*-disubstituted *N*-acetamide. H-1 is HMBC-correlated with C-2 (δ 121.1), C-3 (δ 38.0), C-2' (δ 52.0) and also with C-1'' (δ 169.2). Thus, the partial structure containing the methoxypiperonyl group (**III**, Fig. 2) seems to be the same as the one present in brasiliamide B. Actually, this unknown amide shows very similar NMR characteristics as reported for brasiliamide B [8], with some broad NMR peaks when the spectrum is measured at ca. 20 °C, probably due to the presence of rotamers. The mass spectral data of this unknown amide (m/z 409 [M-H]⁻, obtained by negative ion ESIMS), along with the above discussed NMR data are compatible with the molecular formula C₂₂H₂₂N₂O₆ (410 amu). The above identified partial structure (**III**, Fig. 2) corresponds to the formula C₂₁H₂₁NO₄, which means that the

fragment CHNO₂ needs to be added to **III** to complete the unknown structure. The ¹³C NMR spectrum contains a shielded carbonyl signal at δ 166.4, which is compatible with the presence of an *N*-hydroxylamide group, when compared with model compounds (Hirsch *et al.*, 1989; Chittari *et al.*, 1998). Thus, this brasiliamide was identified and named brasiliamide F.

The antimicrobial properties of brasiliamide A, the major amide obtained, were tested against a set of pathogenic bacteria, but **1** showed only a weak bacteriostatic effect to *Bacillus subtilis* with a minimal inhibitory concentration (MIC) of 250 µg/ml.

Apparently, these brasiliamides are biosynthesized from two phenylalanine units. In plants, the bisphenylpropanoid pathway produces lignans and other cinnamic acid derivatives (Ferrer *et al.*, 2008). This process begins with the action of the enzyme phenylalanine ammonia lyase (PAL) to produce cinnamic acid and cinnamic acid-derived compounds, and is frequently associated with plant defense mechanisms against invading microorganisms (Essenberg, 2001). This biosynthetic route is not so far found in microorganisms. Thus, *P. brasiliense* deserves a careful study to check the production and expression of the enzyme PAL.

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