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Production of a benzylated flavonoid from 5,7,3′,4′,5′-pentamethoxyflavanone by *Penicillium griseoroseum*

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ABSTRACT

Penicillium griseoroseum, isolated as an endophytic microorganism from *Coffeea arabica* seeds, was grown in Czapeck's medium containing 5,7,3′,4′,5′-pentamethoxyflavanone. The fungus incorporated a dimethylated tetraketide, clavatol, a typical fungal secondary metabolite, into the flavanone structure at C-6, resulting in a novel benzylated flavonoid. Clavatol was also found free in the fungus extract. The compounds were isolated by chromatographic procedures and identified by extensive spectroscopic studies, including MS/MS and 1D and 2D NMR. The process probably involves enzymes catalyzing C–C bound formation, which is uncommon in fungi. The possibility of fungi participation in the biosynthesis of plant benzylated flavonoids is discussed.

1. Introduction

Flavonoids are naturally occurring compounds whose occurrence appears to be almost exclusive to plants [1,2]. These natural products are produced by the phenylpropanoid biosynthetic pathway which is initiated by the enzyme phenylalanine ammonia-lyase (PAL). Since this enzyme is frequently stimulated after plants have being exposed to pathogenic microorganisms, flavonoids and other phenylpropanoid are frequently isolated as antimicrobial compounds [3,4]. Actually flavonoids may accumulate in plants after fungi inoculation and also shows in vitro antifungal activity [5]. By the other hand, although many microorganisms have genetic ability to produced PAL, phenylpropanoid compounds are rarely found in fungi [6].

These chemical-ecologies facts presumes that those microorganisms successfully colonizing plant tissues without causing disease are potentially good biotransformers of chemical constituents produced by the host plant [7], and are good sources for new valuable enzymes. We have recently established a program to verify these hypothesis using endophytic microorganisms associated with plants living in tropical areas [8,9]. Besides the gain in comprehending host-microbe biochemical interactions, the biotransformation process can result in biologically active substances useful to human medicine.

During our previous studies we obtained a large collection of endophytic filamentous fungi from coffee tree (*Coffeea arabica*) [10]. One *Penicillium* isolate, later identified as *P. griseoroseum*, was reiterated obtained from fruits and grew very well in any cultivation medium containing certain amount of powdered coffee seeds. When coffee seeds constituents were individually added to the cultivation medium, we found that this *Penicillium* was able to selectively mono-N-demethylate caffeine producing theophiline [11]. Therefore the purpose of the present work was to test this demethylation ability shown by *P. griseoroseum* using other methylated substrates, containing O-methyl instead of N-methyl groups. Flavanone 5,7,3′,4′,5′-pentamethoxyl ether (**1**) was chosen due the many possibilities for demethylation. We report here that the fungus surprisingly incorporated a small polyketide into the exogenous flavanone structure forming a new benzylated flavonoid, through a uncommon C–C bound formation.

2. Experimental

2.1. Microorganisms

The fungus *P. griseoroseum* was isolated from coffee seeds during two collections in March 2003 and November 2005 from a small farm in "Aguas da Prata" city, state of São Paulo, Brazil. The general procedures adopted for endophytic fungi isolation followed the methodology described by Petrini et al. [12]. Immediately after collection, the coffee fruits were washed, first with water and then with ethanol, after which it was sterilised with 11% aqueous sodium hypochloride for 1 min. The sterilised fruits

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Table 1
NMR spectroscopic data of **1** and **2** ($CDCl_3$, 400 for ^1H and 100 MHz for ^{13}C)[a].

	1		**2**		
	$^{13}C(\delta)$	^1H [δ mult. (J Hz)]	$^{13}C(\delta)$	^1H [δ mult. (J Hz)]	$^1H\{^{13}C\}$ HMBC
2	79.3	5.34 dd (13.3/2.8)	79.0	5.39 dd (12.8.3/2.0)	–
3a	45.6	3.03 dd (15.5/13.3)	46.0	3.10 dd (16.5/12.8)	C-2, C-4
3b		2.79 dd (15.5/2.8)		2.82 dd (16.5/2.0)	C-2, C-4
4	189.0	–	189.1	–	–
5	164.8	–	160.1	–	–
6	93.5	6.18 d (2.3)	111.9	–	–
7	165.9	–	162.2	–	–
8	93.1	6.11 d (2.3)	104.1	6.14 s	C-6, C-7, C-9, C-10
9	162.2	–	162.0	–	–
10	105.9	–	n.d.	–	–
1′	134.2	–	132.0	–	–
2′	103.2	6.68 s	103.0	6.69 s	C-2, C-1′, C-3′, C-4′
3′	153.4	–	153.1	–	–
4′	138.1	–	137.3	–	–
5′	153.4	–	153.1	–	–
6′	103.2	6.68 s	103.0	6.69 s	C-2, C-1′, C-3′, C-4′
1″	–	–	26.0	2.52 s	C-2″, C-3″
2″	–	–	202.1	–	–
3″	–	–	111.0	–	–
4″	–	–	159.0	–	–
5″	–	–	107.0	–	–
6″	–	–	160.2	–	–
7″	–	–	113.9	–	–
8″	–	–	132.1	7.33 brs	C-2″, C-4″, C-6″, C-7″, C-10″
9″a			n.d.	3.99 d (15.0)	C-6, C-5″
9″b	–	–		3.85 d (15.0)	C-6, C-5″
10″	–	–	16.1	2.10 d (0.7)	C-6″, C-7″, C-8″
OCH_3	60.7	3.99 s	60.5	3.93	
	56.1	2× 3.90 s	56.0	3.92	
	56.1	3.86 s	56.0	3.89	
	55.5	3.84 s	55.2	2× 3.88	
OH-4″	–	–	–	13.04 s	C-3″, C-4″
OH-6″	–	–	–	7.44 brs	n.d.

n.d.: not detected.
[a] ^{13}C Chemical shifts were obtained from HMBC spectrum;

were depulped and the seeds deposited on a Petri dish containing PDA (potato–dextrose–agar) and incubated in the dark at 25 °C for 1 week. *P. griseoroseum* was isolated by replication and grew as a bluish colony. The fungus was identified and deposited at the Laboratório de Biotecnologia at EMBRAPA (Empresa Brasileira de Pesquisa Agropecuária), city of Jaguariuna, São Paulo state, in Brazil.

2.2. Substrate for biotransformation

The 5,7,3′,4′,5′-pentamethoxyflavanone and its flavone congener were obtained from leaves of *Murraya paniculata* following a procedure adapted from Kinoshita and Firman [13].

2.3. Preparation of cultivation medium

After isolation the fungus was maintained on potato agar slants and mineral oil. The fungus was seeded in a Petri dish containing PDA (potato–dextrose–agar) and allowed to grow for 6 days. Fifteen 1-l Erlenmeyer flasks, each containing 300 ml of Czapeck based liquid medium (30 g glucose, 3.0 g $NaNO_3$, 1.0 g KH_2PO_4, 0.5 g $MgSO_4$, 0.01 g $FeSO_4{\cdot}7H_2O$, 0.5 g $MgSO_4$, 0.5 g KCl dissolved in 1.5 l of distilled water and 20 g yeast extract) were used for the biotransformation experiment.

2.4. Biotransformation of 5,7,3′,4′,5′-pentamethoxyflavanone using growing cells

A solution of **1** (20 mg in 2 ml of acetone) was equally distributed into 10 of the 15 flasks containing culture medium previously autoclaved. The 15 flasks were inoculated with pieces of the PDA (potato–dextrose–agar) culture containing mycelium and allowed to growth at 25 °C in the dark for 20-day. Five flasks were kept for control purpose. After this cultivation time the mycelium was harvest by gravity filtration and extracted three times with ethanol, which was evaporated to dryness under vacuum. The filtrate was partitioned three times with 100 ml ethyl acetate. The ethyl acetate phases were combined and dried over anhydrous sodium sulfate and distilled under vacuum. The ethanolic and ethyl acetate extracts were combined and subjected to silica gel (0.05–0.2, Merck) column chromatography using hexane:ethyl acetate (from 95:05 to 1:9 in steps of 5%) as eluent. Final purification of flavonoid **2** (3.8 mg) was achieved by preparative TLC on silica gel 60 (F_{254}, 0.2 mm thick, home made) eluted with hexane:ethyl acetate (4:1).

The benzylflavanone obtained by biotransformation is a white amorphous powder; ^1H NMR (400 MHz, $CDCl_3$), ^{13}C NMR (100 MHz, $CDCl_3$) and HMBC data, obtained in Bruker DRX 400 spectrometer: see Table 1.; ESI-MS/MS (product ion scan, 20 eV) data obtained in a QuattroLC Micromass mass spectrometer: 553 ($[M+H]^+$, 100), 387 (48), 375 (82), 221 (25), 193 (35), 181 (22).

2.5. Isolation of clavatol (**3**)

The fungus grew in the absence of flavonoid **1**, in the same medium used for biotransformation. The extract obtained from this cultivation was chromatographed on silica gel columns the same way as above for purification of **2** and finally the substance clavatol (**3**) was obtained in pure form (2.1 mg) along with ergosterol and ergosterol endo-peroxide, and other non-identified secondary metabolites.

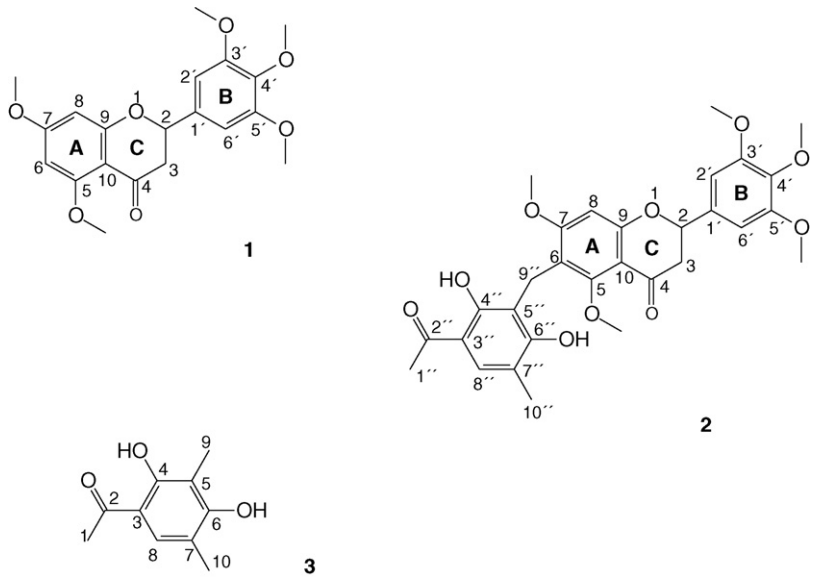


Fig. 1. . Molecular structures of the flavanone (**1**) used as substrate for *P. griseoroseum*, the bezylated flavanone (**2**) produced and the clavatol (**3**), isolated from fermentation extract.

The spectral data obtained for **3** is in accordance with the literature. ^{1}H NMR (400 MHz, $CDCl_3$): δ 12.66 (s, 4-OH), 7.37 (s, H-8), 5.34 (brs, 6-OH), 2.55 (s, 3H-1), 2.21 (s, 3H-10), and 2.14 (s, 3H-9); ESI-MS (full scan, negative ion mode): 179 ([M−H]$^-$, 100).

3. Results and discussion

The fungus was grown in modified Czapeck's medium, with the sugar used as carbon source (dextrose) reduced from 80 to 30 g/L. The polymethoxyl flavanone (**1**) was added to the medium prior to fungus inoculation. After 20 days of growing the fungus was harvest and the ethanol extract of mycelium and ethyl acetate from aqueous liquid phase (partition) were analyzed. The residual flavonoid starting material was detected by TLC only at trace amount. Only one product keeping flavonoidal characteristics was recovered (ca. 3.8 mg of the 20 mg starting material). This compound (**2**) was purified by preparative TLC and analyzed by spectroscopic methods, in comparison with the polymethoxyl flavanone **1**. Attempts were made to identify minor products by LCMS but no conclusive structural elucidation was achieved.

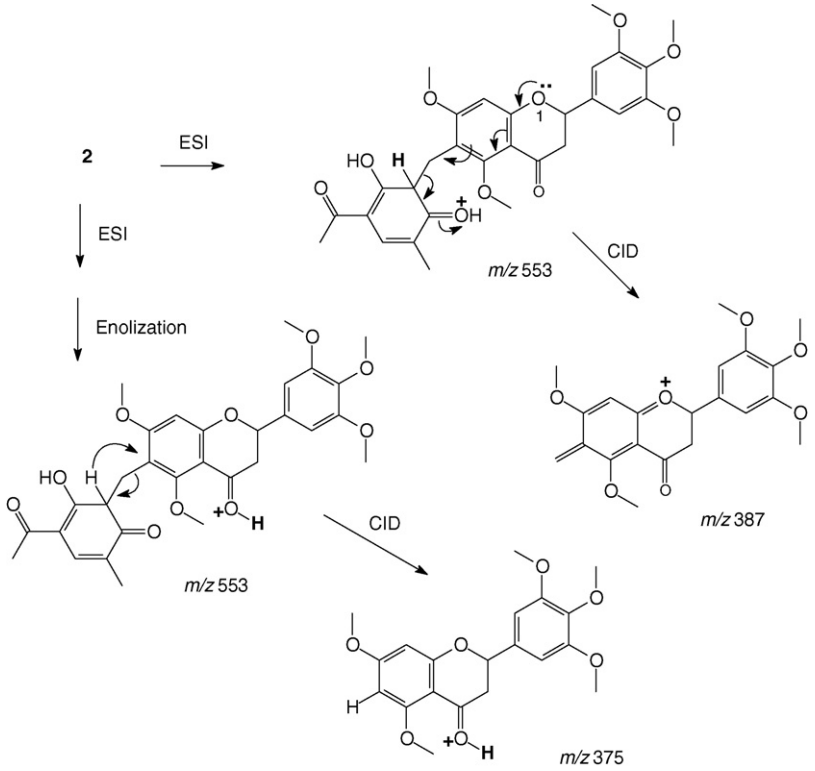
3.1. Compounds identification

The ESI mass spectrum of **1** shows a protonated molecule at *m*/*z* 375 ([M+H]$^+$) in agreement with the molecular formula $C_{20}H_{22}O_7$ (374 Da). The spectrum of **2** (Fig. 1S), obtained in the same condition used for **1**, revealed a peak at *m*/*z* 553 ([M+H]$^+$) which is associated with a compound whose molecular weight is 552 Da. The product ion spectrum obtained from *m*/*z* 553 (compound **2**) shows ions detected at *m*/*z* 375, which correspond to the loss of 178 Da, probably due the fragment added into the molecule of flavonoid **1** during the biotransformation experiment.

The comparison of both flavonoids **1** and **2** through 1D ^{1}H NMR spectra (Table 1, Fig. 2S) was very elucidative to locate the modification done by the fungus. The ^{1}H NMR spectrum for compound **1** shows a pair of doublet for H-6 (δ 6.18, 2.3 Hz) and H-8 (δ 6.11, 2.3 Hz) at ring A; and a 2H singlet (δ 6.68) for H-2′ and H-6′ at ring B. The flavanone flavonoidal subclass is prompt identified in the ^{1}H NMR spectrum by the presence of the ABX spin system formed of H-2 (δ 5.34, dd, 13.3 and 2.8 Hz), H-3a (δ 3.03, dd, 15.5 and 13.3 Hz) and H-3b (δ 2.79, dd, 15.5 and 2.8 Hz) at ring C. The fourth set of signals in the ^{1}H NMR spectrum of compound **1** (Fig. 2S) is formed of four singlets for the five methoxyl groups (δ 3.99, 3H; δ 3.90, 6H; δ 3.86, 3H; and δ 3.84, 3H). The ^{1}H NMR spectrum of the product **2** is clear to indicate that rings B (2H singlet at δ 6.69) and C [ABX system at δ 5.39, dd, 12.8 and 2.0 Hz (H-2); δ 3.10, dd, 16.5 and 12.8 Hz (H-3a); and δ 2.82, dd, 16.5 and 2.0 Hz (H-3b)] were not modified during the biotransformation process. By the other hand the pair of doublet for H-6 and H-8 at ring A is absent in the ^{1}H NMR spectrum of **2** and was substituted by a 1H singlet at δ 6.14 (H-8). Therefore the 178 Da substituent indicated by the MS data is attached to ring A of flavonoid **1**.

In comparison with the ^{1}H NMR spectrum of **1**, compound **2** shows extra signals ascribed to two phenolic hydroxyl groups at δ 13.04 (quelated; Fig. 3S) and 7.44 (free); an aromatic 1H singlet at δ 7.33 (H-8″); an AB –CH_2– multiplete at δ 3.99 (d, 15 Hz) and 3.85 (d, 15 Hz) (H-9″a and H-9″b), and two methyl groups at δ 2.52 (H-1″) and 2.10 (H-10″). These signals and the MS data are compatible with a penta-substituted aromatic ring as the partial structure bounded to the flavanone **1** forming compound **2**. The methylic hydrogens at δ 2.52 correlates with a deshielded ^{13}C (δ 202.0, C-2″) in the HMBC spectrum (Table 1, Fig. 4S), which indicates that this methyl groups is part of methylketone substituent. The HMBC correlations detected for H-8″ (see Table 1) were decisive to establish the relative position of these substituentes in the additional aromatic ring in **1**, with the benzilic methyl group and the methylketone *meta* orientated each other and with the two hydroxyls also *meta* to H-8″, as shown in Fig. 1. These relative positioning was also confirmed by 1D NOE spectra. Thus, Irradiation at the frequency of 3H-10″ (δ 2.10, Fig. 5S) and 3H-1″ (δ 2.52, Fig. 6S) produced enhancements of the signals of H-8″ (δ 7.33).

The HMBC spectrum also revealed correlations of the AB spin system at δ 3.99 and 3.85 (H-9″a and H-9″b) with the shielded aromatic ^{13}C signals at δ 111.9 (C-6) and 107.0 (C-5″). These two resonances are compatible with aromatic ^{13}C containing two *ortho* hydroxyl or methoxyl groups. Although the 2H-9″ multiplete chemical shift is almost coincident with the methoxylic hydrogens resonances, none of these methylic 3H would correlate with the aromatic ^{13}C at ca. δ 100 (J^4) but will correlate with ^{13}C signals at ca. δ 155–160 (J^3) and δ 55–60 (J^1), as seen in Fig. 4S. Therefore the extra aromatic ring is attached to C-6 of flavanone **1** through the methylenic bridge C-9″. This was further confirmed



Scheme 1. . Pathway of fragmentation suggested for the most abundant ions produced from the benzylated flavanone (**2**) under electrospray ionization and collisional induced decomposition (CID).

by 1D NOE spectrum (Fig. 6S) with irradiation of the resonance at δ 6.14 (H-8), which produced an enhancement of only one methoxyl NMR signal (δ 3.89). In case of C-9″ being bounded to C-8, two methoxyl signals would be enhanced upon irradiation at δ 6.14.

The ions detected in the product ions mass spectrum obtained for compound **2** (Fig. 1S) were also important for the establishment of this substituent positioning. The ion fragment detected at *m*/*z* 387 may have arisen from the C-6–C-9″ cleavage with the help of the lone electron pair at O-1 after ionization at C-5″ by electrospray. The fragment ion at *m*/*z* 375 can be produced by a 1,3 H-rearrangement after the molecule have being ionized at the C-3 carbonyl and suffered enolization in the benzyl group. The interpretation of these fragmentations (Scheme 1) were important for the structural elucidation of compound **2**.

In the course of our biotransformation experiments described here the known pentaketide clavatol (**3**) [14] was obtained along with other common substances (e.g. ergosterol, ergosterol peroxide, etc.) of the fungus secondary metabolism. This compound (**3**) appears to be the acetophenone which have being attached to the exogenous flavanoid added to the culture medium for biotransformation. This reinforces the structural identification of the benzylated flavonoid discussed above.

3.2. Biosynthesis of natural benzylated flavonoids

This is the first demonstration of such benzyl coupling to a flavonoid skeleton by a fungus. Natural benzyl flavonoids have being reported in plants [15–18], although still rarely. The benzyl groups in these natural plant benzylated flavonoids contains only few substituents at the aromatic ring, usually one hydroxyl and a small alkyl group, and are of unknown biosynthetic origin, although they putatively arisen from aromatic amino acids or also from small poliketide. Most of the benzyl groups in these flavonoids are located at C-6 and a few at C-8, and, for the best of our knowledge, they surprisingly occurs almost exclusively in flavanones or flavanonols (no $\Delta^{2(3)}$). Glicosylflavanonols containing benzyl group at ring A were recently reported [19]. In our experiments, when the 5,7,3′,4′,5′-pentamethoxyflavone was used as substrate for biotransformation, the starting material was completely recovered after the experiment work out.

The coupling of flavonoids with benzyl groups is still unknown whether it is mediated by free radicals (e.g. even electron species formation at C-9 in **3** and at C-6 in **1**, with further coupling) or an ionic substitution at C-9 of **3**, after it have being activated by enzymes, by the nucleophylic C-6 (*orto* to two methoxyl). In any of these possible mechanisms, there is certainly the involvement of interesting enzymes. This process represents a C–C bound formation which may find useful application in organic synthesis.

4. Conclusions

Although the expected demethylated reaction did nod occurs for the flavonoid used as substrates, their coupling with a benzyl group which is clearly a polyketide of fungal origin represents an interesting finding and suggest a new form of fungi biochemical adaptation to plant flavonoid contents that is recognized as part of plant defense mechanism. Two of the benzylated flavonoids reported in the literature were isolated from the fruit body of a fungus (*Phellinus igniarius*), but the authors assumed that the flavonoids could be produced by the host plant and translocated to the associated fungus [20]. The literature data and the present work strongly suggest further investigations regard the participation of endophytic fungi on the biosynthesis of those benzylated flavonoids found in plants.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2010.07.022.

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