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Cadinane sesquiterpenoids of *Phomopsis cassiae*, an endophytic fungus associated with *Cassia spectabilis (Leguminosae)*

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Abstract

Five cadinane sesquiterpenes derivatives were isolated by bioassay-guided fractionation from *Phomopis cassiae*, an endophytic fungus isolated from *Cassia spectabilis*. The structures of the two diastereoisomeric 3,9,12-trihydroxycalamenenes (**1**, **2**); 3,12-dihydroxycalamenene (**3**); 3,12-dihydroxycadalene (**4**) and 3,11,12-trihydroxycadalene (**5**) were established on the basis of analyses of 1D and 2D NMR and HRTOFMS experiments. Antifungal activity of the isolates was evaluated against *Cladosporium sphaerospermum* and *Cladosporium cladosporioides*, revealing **5** as the most active compound.

Keywords: *Phomopsis*; Endophytic fungi; Cadinane Sesquiterpenes; Antifungal

1. Introduction

Endophytic fungi are organisms living within the tissues of hosts plants (Andrews and Hirano, 1991). These organisms are known as potential sources of biologically active secondary metabolites (Dreyfuss and Chapela, 1994; Tan and Zou, 2001; Strobel et al., 2004) and relatively little is known about the potential of these microorganisms from Brazilian endemic plant species (Azevedo et al., 2000, 2004).

As part of our studies on bioactive agents from metabolites produced by endophytic fungi associated with species of native plants from the Brazilian Cerrado, we initially selected 16 species of plants, which had already been phytochemically profiled by our group. Those species were submitted for isolation of endophytic fungi and 82 pure isolates were obtained, preserved, cultivated in liquid media and extracted with ethyl acetate to obtain crude extracts. Some metabolites produced by fungi were already published by our group (Teles et al., 2005; Cafêu et al., 2005; Silva et al., 2005).

The crude EtOAc extract of *Phomopsis cassiae* isolated from *Cassia spectabilis* showed moderate antifungal activity against *Cladosporium sphaerospermum* and *Cladosporium cladosporioides*, stimulating further studies. Bioassay guided fractionation of this extract afforded five new sesquiterpenes (**1**–**5**).

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2. Results and discussion

Compound **1** was isolated as a white amorphous powder and its HRESI mass data suggested the molecular formula $C_{15}H_{22}O_3$ ($[M + Na]^+$ at *m*/*z* 273.1486). Their IR spectra revealed the presence of hydroxyl (3410 cm^{-1}) and phenolic groups (1466, 1690 cm^{-1}). The ^1H NMR spectrum of **1** (Table 1) showed two aromatic signals, one at δ 6.87 (1H, *s*) and the other at δ 6.43 (1H, *s*), suggesting a tetra-substituted aromatic spin-system with a *para* configuration. A series of complex signals at δ 3.76 (1H, *m*) 3.35 (4H, *m*); δ 3.04 (1H, *m*) and δ 2.30 (1H, *m*) were indicative of two carbinolic and two benzyl hydrogens, respectively. Further signals at δ 0.51 (3H, *d*, $J = 7.0$ Hz), δ 1.03 (3H, *d*, $J = 7.0$ Hz), δ 2.05 (3H, *s*) revealed the presence of two aliphatic and one aromatic methyl groups in the molecule.

The ^{13}C NMR chemical shifts for **1** along with the information obtained from a DEPT-135 experiment (Table 1), indicated a total of 15 signals, suggesting a sesquiterpene: six aromatic carbons at δ 152.9 (*s*), 141.1 (*s*), 128.1 (*d*), 127.4 (*s*), 121.9 (*s*) and 114.7 (*d*), confirming the tetra-substitution for the aromatic moiety and the *para* hydrogen system; two carbinolic signals: a methine at δ 67.8 and a methylene at δ 64.4; three signals for methines at δ 40.1, 38.4 and 36.5; one aliphatic methylene at δ 24.6 and three signals for methyl groups: δ 16.7, 15.9 and 10.8. The above data suggested a cadinane skeleton (Zhang et al., 1998; Nagashima et al., 1996). The total attribution for the observed signals was accomplished based on *g*HMQC correlations.

Additional *g*HMBC correlations of H-5 (δ 6.87) with C-15 and C-7 (δ 15.9 and δ 36.5, respectively) and H-2 (δ 6.43) with C-10 and C-4 (δ 40.1 and δ 121.9) allowed us to fully determine the aromatic moiety. The phenolic function linked at C-3, was supported by the correlations between H-15 (δ 2.05) and C-3/C-5 (δ 152.9 and δ 128.1, respectively). The linkage of the *p*-menthane moiety was determined through the correlations shown by C-1 (δ 141.1) with H-14/H-9 (δ 1.03/δ 3.76) and C-6 (δ 127.4) with, H-11/H-8β (δ 2.70/δ 1.52). Observed correlations between C-12 → H-7 (δ 64.4/3.04) and H-13 → C-7 (δ 0.51/36.5) allowed us to fully determine the calamenene skeleton (Nagashima et al., 1996).

*g*NOESY was useful to establish the relative configuration of the stereocenters for **1**. A correlations between Me-14 and Me-13 confirmed a *cis*-calamenene, according to reported literature (Croft et al., 1978; Anderson et al., 1972; Nabeta et al., 1993). Further observed correlations between H-9α and H-7 allowed us to confirm a (*cis*)-1,3-*pseudo*-diaxial relationship between these centers (Nabeta et al., 1993). We were not able to determine the configuration of the stereocenter at C-11, due to free rotation of this center with C-7–C-11 bond. Compound **1** was thus identified as the rel. (−)-(7*S*,9*S*,10*S*)-3,9,12-trihydroxycalamenene.

Compound **2** was isolated as a white amorphous powder and its HRESI mass data suggested the molecular formula $C_{15}H_{22}O_3$ ($[M + H{-}H_2O]^+$) at *m*/*z* 233.1542. The obtained mass and similarities observed in the ^1H and ^{13}C NMR spectra (Table 1) indicated that **2** was a diastereoisomer of **1**. The main spectroscopic differences observed for this molecule were elucidated by using *g*NOESY. A correlation between Me-14 and Me-13 indicated that both groups remained in a *cis* conformation. The appearance of a strong correlation of Me-14 and H-9 indicated that H-7 and H-9 were in a *trans* conformation. Further comparisons with reported data (Croft et al., 1978; Anderson

Table 1
^1H and ^{13}C NMR spectroscopic data for compounds **1**–**5** (500 MHz)[a]

No.	**1**		**2**		**3**		**4**		**5**	
	δH	δC	δH	δC	δH	δC	δH	δC	δH	δC
1		141.1		139.0		142.1		134.7		130.6
2	6.43 *s*	114.7	6.50 *s*	114.3	6.60 *s*	114.6	7.15 *s*	106.9	7.2 *s*	105.5
3		152.9		152.9		151.6		155.1		153.3
4		121.9		120.9		121.1		127.7		124.6
5	6.87 *s*	128.1	6.83 *s*	129.0	6.98 *s*	130.2	7.79 *s*	125.9	8.6 *s*	129.1
6		127.4		128.4		125.8		131.3		133.7
7	3.04 *m*	36.5	2.90 *m*	34.1	2.86 *m*	38.1		138.6		139.3
8α	1.42 *m*	24.6	1.51 *m*	27.5	1.53 *m*	19.9	6.93 *d* (7.5)	120.7	7.21 *d* (7.5)	120.7
8β	1.52 *m*		1.68 *m*		1.75 *m*					
9α	3.76 *m*	67.8	3.65 *m*	69.1	1.52 *m*	28.5	7.03 *d* (7.5)	126.7	7.08 *d* (7.5)	124.8
9β					1.66 *m*					
10	2.30 *m*	40.1	2.10 *m*	40.6	2.81 *m*	32.4		128.1		125.9
11	2.70 *m*	38.4	2.50 *m*	38.8	2.25 *m*	39.3	3.59 *m*	37.7		75.3
12α	3.35 *m*	64.4	3.30 *m*	64.5	3.62 *dd* (10.5; 6.5)	66.8	3.74 *dd* (10.5; 5.0)	69.0	3.7 *m*	69.4
12β	3.30		3.30		3.72 *dd* (10.5; 6.5)		3.47 *dd* (10.5; 7.5)			
13	0.51 *d* (7.0)	10.8	0.66 *d* (7.0)	12.5	0.82 *d* (7.0)	12.5	1.30 *d* (6.5)	18.4	1.61 *s*	26.6
14	1.03 *d* (7.0)	16.7	1.14 *d* (7.0)	21.5	1.26 *d* (7.0)	23.2	2.43 *s*	19.7	2.47 *s*	19.4
15	2.05 *s*	15.9	2.05 *s*	15.8	2.22 *s*	15.5	2.30 *s*	17.2	2.30 *s*	17.2

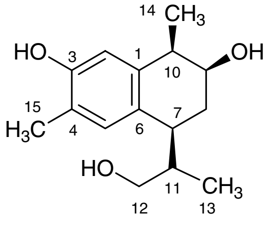
[a] Chemical shifts (relative to TMS) are in (δ) ppm, multiplicities and coupling constants in Hz in parentheses. Assignments were aided by ^1H–^1H *g*COSY and *g*HMQC.

et al., 1972; Nabeta et al., 1993) allowed us to provisionally deduce identify **2** as rel. (+)-(7*S*, 9*R*, 10*S*) the C-9 diastereoisomer of **1.**

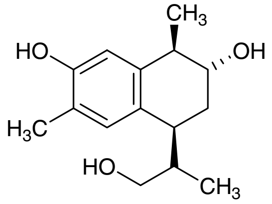
Compound **3** was isolated as a white amorphous powder. HRESI mass data allowed us to propose the molecular formula $C_{15}H_{22}O_2$ ($[M+H]^+$ at *m*/*z* 235.1777). The absence of an oxygen atom in this molecule allowed us to provisionally deduce that a reduction occurred at position C-9. This reduction was confirmed by ^{13}C NMR and DEPT-135 (Table 1) due to the presence of the signal at δ 28.5 and the lack of the carboxymethine signal at δ 67.8/69.1 present in **1** and **2**, respectively.

A correlation between Me-14 and Me-13 was observed in the *g*NOESY experiment thus concluding that **3** has a *cis* calamenene relative stereochemistry. Observed correlations between H-9α and H-8α and H-7 defined their relative configuration also as *cis*. Further observed correlations between H-8β and H-9β, confirmed the relative configuration established for the *p*-menthane moiety. Compound **3** was hence identified as the rel. (+)-(7*S*, 10*S*) 3,12-dihydroxycalamenene.

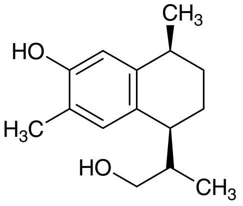
Compound **4** was isolated as a white amorphous powder. A molecular formula $C_{15}H_{18}O_2$ ($[M+H]^+$ at *m*/*z* 231.1405) was obtained after HRESI mass data analysis. The ^1H NMR data (Table 1) showed a new aromatic hydrogen spin system at δ 7.03 (1H, *d*, $J = 7.5$ Hz) and δ 6.93 (1H, *d*, $J = 7.5$ Hz) with an *ortho* configuration. The downfield shift observed for Me-14 ($\approx\Delta\delta$ +1.3) was indicative of an aromatization of the *p*-menthane moiety (Nabeta et al., 1994). ^{13}C NMR and DEPT-135 (Table 1) data showed 10 aromatic carbons, three methyls: two benzylic and one aliphatic; one carboxymethylene and one benzylic methine, indicative of the presence of a naphthalene skeleton. All correlations were determined using *g*COSY, *g*HMQC, *g*HMBC, and compared with reported data for cadalene analogues (Kubo et al., 1996; Nabeta et al., 1994; Stipanovic et al., 1975).



1



2



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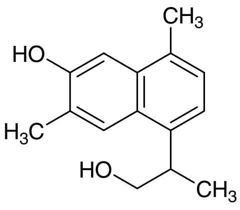

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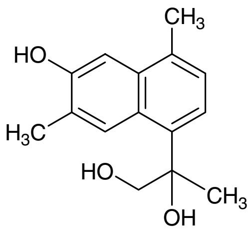

5

Further correlations observed in the *g*HMBC experiment, particularly the ones of H-8 (δ 6.93) with C-6/C-11 and H-9 (δ 7.03) with C-1/C-14 allowed us to fully determine the substitution pattern of the B ring for the naphthalene moiety. Compound **4** was identified as 3,12-dihydroxycadalene.

Compound **5** was isolated as a white amorphous powder and its HRESI mass data suggested the molecular formula $C_{15}H_{18}O_3$ ($[M+Na]^+$ at *m*/*z* 269.1179). The appearance of a hydroxyl group in **5** was corroborated by the loss of the signal assigned to H-11 (δ 3.59) in **4** thus suggesting that oxidation had occurred at this position. ^{13}C NMR and DEPT-135 spectroscopic data corroborated this assumption showing a signal at δ 75.3 (*s*) which exhibited a correlation showed in the *g*HMBC experiment with H-8 (δ 7.21) indicating that the new oxidized position was at C-11. Other correlations were determined using *g*COSY, *g*HMQC, and compared with reported data for analogues (Kubo et al., 1996; Nabeta et al., 1994; Stipanovic et al., 1975). Compound **5** was identified as the new 3,11,12-trihydroxycadalene.

The antifungal activity of compounds **1**–**5** against the phytopathogenic fungi *C. cladosporioides* and *C. sphaerospermum* was evaluated by bioautography (Rahalison et al., 1991). The compound 3,12-dihydroxycadalene (**2**) exhibited potent activity against two fungi. The detection limit for the compound **5** was found to be 1.0 μg, comparable with the same amount of the standard nystatin.

Cytotoxicity of the compounds **1**–**5** against human cervical tumor cell line (HeLa) were tested using the MTT assay (Mosmann, 1983). Compound **4** exhibited cytotoxicity against cell line (IC_{50} 20 μmol L^{-1}), and **3** and **5** a weak cytotoxicity (IC_{50} 100 and 110 μmol L^{-1}, respectively). Cisplatin, a cytotoxic agent, was used as positive control with IC_{50} 5 μmol L^{-1}.

3. Experimental

3.1. General experimental procedure

Optical rotations were determined at room temperature using Perkin–Elmer 241 polarimeter. IR and UV spectra were recorded on Perkin–Elmer 1710 spectrometer and Perkin–Elmer UV–Vis 14P spectrometer. Melting points were measured on Microquimica MQAPF-301. ^{1}H and ^{13}C NMR spectra; *g*HMBC and *g*HMQC, *g*COSY and TOCSY experiments were recorded on a Varian Unity 500 NMR, with TMS as internal standard. Electrospray mass and tandem mass spectra in both positive and negative ion modes were recorded on a Q-Tof (Micromass) mass spectrometer with a quadrupole (Qq) and high-resolution orthogonal time of flight (o-TOF) configuration. The sample introduction was performed using a syringe pump (Harvard Apparatus, Pump 11) set to 10 μl/min pumped through an uncoated fused-silica capillary. All samples were dissolved in MeOH–H_2O (1:1). The HRESI-MS mass spectra were acquired using a ESI capillary voltage of 3 kV and a cone voltage of 10 V. ESI-MS/MS spectra of mass selected ions were acquired using 10–20 eV collisions with argon. For preparative HPLC separations, Varian Star Dynamax model SD-1 pump, and a Phenyl-hexyl Luna Phenomenex column, (250 × 21.20 mm, 5 μm), were used in this study. Peaks were detected using Varian model 320-chromato-integrator connected to a UV detector.

TLC analysis was carried out on precoated silica gel 60 F_{254} plates (Merck). Spots were visualized spraying anisaldehyde 5% in H_2SO_4 followed by heating.

3.2. Plant material

Authenticated *C. spectabilis* plant material was collected in the Chemistry Institute UNESP – Araraquara, SP, Brazil in June 17, 2001 by G.H. Silva. The botanical identification was made by Professor Maria Cláudia Marx Young and a voucher specimen was deposited at the Herbarium of the Botanic Garden of São Paulo, Brazil (Voucher No SILVA – 193).

3.3. Isolation of the endophytic fungus

The fungal strain *P. cassiae* was an endophyte isolated from leaves of *C. spectabilis*. The leaves were washed in running H_2O and surface sterilized by successive soaking in NaOCl 1% for 5 min, 70% EtOH for 1 min and then rinsed twice with water. Aseptically the leaves were cut open and 1 × 1 cm^2 pieces of the leaves were placed on water agar in Petri plates. After incubation at 25 °C for seven days, individual hyphal tips of the emerging fungi were removed and placed on potato dextrose agar (PDA) (Maier et al., 1997). During the first three weeks of incubation, the cultures were periodically checked for purity and successively subcultured by hyphal tipping method until pure culture was obtained. Cultures were maintained on potato dextrose agar. The taxonomic identification as *P. cassiae* was performed based on fungal morphology by Ludwig H. Pfenning and deposited Coleção Micologica de Lavras, Universidade Federal de Lavras fungal herbarium and assigned as CML 292.

3.4. Growth of *P. cassiae* and production of the EtOAc extract

The fungus was cultivated on a small scale in 2 Erlenmeyer flasks (500 mL), each containing 0.8 g of potato extract, 4.0 g dextrose and 200 mL distilled water (PDB) which were autoclaved at 125 °C for 15 min. Approximately 10 small pieces (1 × 1 cm) of PDA medium, from the Petri dish containing biomass of the *P. cassiae* isolated were inoculated into each flask and the flasks were sealed with cotton to permit aerobic growth. After incubation for 28 days at 25 °C on rotary shakers at 150 rpm, the mycelia biomass accumulated in the flasks was separated from the aqueous medium by filtration. The culture broth was extracted with EtOAc (3 × 2,0 L). The EtOAc extracts were combined, filtered, and evaporated *in vacuo* to give a yellow oil (277.12 mg).

3.5. Antifungal assay

The microorganisms *C. cladosporioides* (Fresen) de Vries SPC 140 and *C. sphaerospermum* (Perzig) SPC 491 were used in the antifungal assay. They have been maintained at the Instituto de Botânica, São Paulo, SP, Brazil. Compounds **1**–**5** were applied on a precoated Si-gel TLC plates using a solution (10 μL) containing 100, 50, 25, 10, 5 and 1 μg. After eluting with $CHCl_3$:CH_3OH (9:1) they were sprayed with the fungi (Rahalison et al., 1991). Nystatin was employed as positive control.

3.6. Cytotoxicity bioassays

The human cervical cancer cell line (HeLa) assay was performed as previously described (Mosmann, 1983). Cisplatin was used as positive control (IC_{50} 5.0 μmol L^{-1}).

3.7. Extraction and isolation

The extract was subjected to C18 column chromatography employing gradient elution from H_2O to MeOH, to yield 7 fractions (H_2O:MeOH, 85:15, 65:35, 60:40, 55:45, 45:55, 35:65, 00:100 each 150 mL). ^{1}H NMR spectroscopic analyses identified fractions 3, 4 and 5 as promising for further fractionation. The fraction 3 (23.0 mg) was purified by reversed-phase HPLC [Luna Phenyl-hexyl, 10 μm, Phenomenex 21.20 × 250 mm; using $CH_3CN:H_2O$ (19:81) as the solvent, flow rate 10 mL/min; UV detection at 248 nm] to afford sesquiterpene **1** (3.5 mg). Fraction 4 (15.0 mg) was purified by reversed-phase HPLC [Luna Phenyl-hexyl, 10 μm, Phenomenex 21.20 × 250 mm; using $CH_3CN:H_2O$ (22:78) as the solvent, flow rate 10 mL/min; UV detection at 248 nm] to afford sesquiterpenes **2** (2.5 mg) and **4** (4.0 mg). Fraction 5 (30.0 mg) was purified by reversed-phase HPLC [Luna Phenyl-hexyl, 10 μm, Phenomenex 21.20 × 250 mm] using $CH_3CN:H_2O$ (29:71) as the solvent, flow rate 10 mL/min; UV detection at 248 nm] to afford sesquiterpenes **3** (4.0 mg) and **5** (4.8 mg).

*3.7.1. Rel. (7S,9S,10S)-3,9,12-trihydroxycalamenene (**1**)*

Amorphous powder (3.5 mg); $[\alpha]_D^{25}$ −54.8 (*c* 0.30, $CHCl_3$); IR (KBr) cm^{-1}: 3416, 1618, 1466. For ^1H and ^{13}C NMR see Table 1; HRTOF-ESIMS: *m/z* 273.1486 $[M + Na]^+$ Calcd. for $C_{15}H_{23}O_3Na$, 273.1467.

*3.7.2. Rel. (7S,9R,10S)-3,9,12-trihydroxycalamenene (**2**)*

Amorphous powder (2.5 mg); $[\alpha]_D^{25}$ +33 (*c* 0.15, $CHCl_3$); IR (KBr) cm^{-1}: 3419, 1623, 1448. For ^1H and ^{13}C NMR see Table 1; HRTOF-ESIMS: *m/z* 233.1542 $[M + H-H_2O]^+$ Calcd. for $C_{15}H_{21}O_2$, 233.1541.

*3.7.3. Rel. (7S,10S)-3,12-dihydroxycalamenene (**3**)*

Amorphous powder (4.0 mg); $[\alpha]_D^{25}$ +18.1 (*c* 0.46, $CHCl_3$); IR (KBr) cm^{-1}: 3427, 1622, 1454. For ^1H and ^{13}C NMR see Table 1; HRTOF-ESIMS: *m/z* 235.1717 $[M + H]^+$ Calcd. for $C_{15}H_{23}O_2$, 235.1698.

*3.7.4. 3,12-Dihydroxycadalene (**4**)*

Amorphous powder (4.8 mg); $[\alpha]_D^{25}$ −58.2 (*c* 0.14, $CHCl_3$); IR (KBr) cm^{-1}: 3419, 1623, 1448. For ^1H and ^{13}C NMR see Table 1; HRTOF-ESIMS: *m/z* 231.1405 $[M + H]^+$ Calcd. for $C_{15}H_{19}O_2$, 231.1385.

*3.7.5. 3,11,12-Trihydroxycadalene (**5**)*

Amorphous powder (4.0 mg); $[\alpha]_D^{25}$ −18.4 (*c* 0.50, $CHCl_3$); IR (KBr) cm^{-1}: 3420, 1606. For ^1H and ^{13}C NMR see Table 1; HRTOF-ESIMS: *m/z* 269.1179 $[M + Na]^+$ Calcd. for $C_{15}H_{18}O_3Na$, 269.1155.

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