Three Highly Oxygenated Caryophyllene Sesquiterpenes from *Pestalotiopsis* sp., a Fungus Isolated from Bark of *Pinus taeda*[#]

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A *Pestalotiopis* sp. was isolated from the trunk bark of *Pinus taeda.* The fungus was cultivated in liquid medium and produced three highly oxygenated caryophyllene sequiterpene derivatives, named pestalotiopsolide A, taedolidol and 6-epitaedolidol, respectively. The sesquiterpenes were isolated by silica gel based chromatographic procedures and their structures were elucidated by NMR spectroscopic data.

Key words: Pestalotiopsis, *Pinus taeda*, Caryophyllene

Introduction

The research activities in the field of secondary metabolism of endophytic microorganisms were increased after the finding of paclitaxel (Taxol™) production by *Taxomyces andrenae* (Stierle *et al.*, 1993; Stierle and Strobel, 1995), a fungus found living in association with *Taxus brevifolia*, the plant from which paclitaxel was first isolated (Wani *et al.*, 1971). Paclitaxel is a highly functionalized diterpene with strong anticancer activity mainly against breast and ovarian cancer (Holmes *et al.*, 1995). Many other fungi species associated with *Taxus* have shown paclitaxel production (Rohr, 1997; Li *et al.*, 1996; Li, 1998a; Bashyal *et al.*, 1999). Surprisingly, reports of paclitaxel production by fungi isolated from plants which are not taxol producers (Li *et al.*, 1998b), and not even related to *Taxus* (Strobel *et al.*, 1997), have been published recently. Thus, the taxol-producing fungus *Pestalotiopsis guepinii* was isolated as an endophyte from *Wollemia nobilis* (Wollemi pine), an araucareaceous plant occurring in the Wollemi National Park in Australia (Strobel *et al.*, 1997).

Besides taxol, *Pestalotiopis* species also produce acetogenins (Pulici *et al.*, 1997) and sesquiterpenes (Pulici *et al.*, 1996a; Pulici *et al.*, 1996b). Caryophyllene type sesquiterpenes with immunosuppressive activity were produced by strains of *Pestalotiopsis* sp. obtained from *Taxus brevifolia* (Pulici *et al.*, 1996c). Species of *Pestalotiopsis* are therefore interesting source of natural substances and is being subject of investigation in our research program of secondary metabolism of endophytic microorganism. Recently, we obtained a collection of microorganisms from *Pinus taeda*, a Pinaceae plant successfully introduced into the south-east area of Brazil. We report here the isolation of a *Pestalotiopsis* sp., the predominant fungus in this collection, and the production, isolation and identification of three new high oxidized caryophyllene sesquiterpenes named pestalotiopsolide A (**1**), taedolidol (**2**) and 6-epitaedolidol (**3**).

Materials and Methods

General experimental procedures

UV spectra were obtained in CH_2Cl_2 solution on a Hewlett Packard 8452-A spectrophotometer, and IR spectra were measured with a Bomen MB-102 spectrophotometer in KBr pellets. The ^1H and ^{13}C NMR experiments were recorded using a BRUKER DRX spectrometer, which was operated at 400 MHz for ^1H and 100 MHz for ^{13}C, respectively, using deutero chloroform ($CDCl_3$) as solvent, with TMS as the internal standard. MS data were measured using a low-resolution ESIMS in the positive ion mode in a MICROMASS QUATTRO-LC instrument equipped with an ESI/APCI "Z-spray" ion source. Molecular modeling

[#] This is part of the MS thesis of RFM.

of the sesquiterpenes was conducted following the MM+ minimum energy optimization routines using the HyperChem (Froimowitz, 1993) for Windows (Release 3) program from Autodesk, Inc (Sausalito, CA).

Plant material

Fresh trunk bark of *Pinus taeda* was collected in the Campus of the Universidade Federal de São Carlos, São Paulo State, Brazil early June of 1999. A voucher specimen was deposited in the Herbarium of the university's Department of Botany.

Isolation of the microorganism

The general procedures adopted followed the methodology described by Petrini *et al.* (1992). Immediately after collection, the trunk bark was separated and washed with water followed by ethanol and then sterilized with 11% aqueous sodium hypochloride for 1 min. The material was then deposited on a Petri dish containing PDA medium (potato-dextrose-agar) and incubated in the dark at 25 °C for one week. *Pestalotiopsis* sp. was isolated by replication and grew as a salmon colored culture. The fungus was identified and deposited (number LaBioMi-102) at the Laboratório de Bioquímica Micromolecular – LaBioMi – of the Departamento de Química at Universidade Federal de São Carlos, São Carlos, Brazil.

Cultivation of Pestalotiopsis sp., and isolation of the sesquiterpenes

The recent isolated fungus was seeded in a Petri dish containing PDA (potato-dextrose-agar) and allowed to grow for 6 days. Fifty 1-liter Erlenmeyer flasks, each containing 300 ml of liquid medium (80 g glucose, 0.48 g NH_4NO_3, 5.0 g KH_2PO_4, 1.0 g $MgSO_4$, 0.1 g $FeSO_4$, 0.015 g $CuSO_4$, 0.161 g $ZnSO_4$, 0.01 g $MnSO_4$, and 0.1 g $(NH_4)_2MoO_4$ dissolved in 1.5 l of distilled water) were inoculated with pieces of the PDA (potato-dextrose-agar) culture containing mycelium and were allowed to grow at 25 °C standing in the dark during 52 days. The mycelium was separated by gravity filtration and the liquid phase were partitioned with ethyl acetate. The solvent was dried under sodium sulfate and removed in vacuum to give a yellowish residue (8.3 g), which was subjected to low-pressure Si gel CC eluted with a hexane-CH_2Cl_2 (1:1 v/v) to methanol gradient. The medium polarity fractions obtained with hexane-CH_2Cl_2-MeOH (25:25:2 v/v) were subjected to Si gel column chromatography using CH_2Cl_2-MeOH (99:1 – 9:1 v/v); the sesquiterpenes pestalotiopsolide A (28 mg), taedolidol (4 mg), and 6-epitaedolidol (16 mg) were finally purified by preparative thin layer Si gel chromatography [hexane-CH_2Cl_2-MeOH (25:25:5 v/v)] .

Pestalotiopsolide A (**1**):

White dense oil; IR ν_{max} KBr cm^{-1}: 3113, 2993, 2956, 1645, 1610, 1399, 1283, 878 and 721 (KBr); ESIMS: *m/z* (%) 333 ($[M+K]^+$, 8), 317 ($[M+Na]^+$, 78), 295 ($[M+H]^+$, 63) and 263 ($[M+H\text{-}CH_3OH]^+$, 100); ^{1}H-NMR ($CDCl_3$, 400 MHz) and ^{13}C-NMR ($CDCl_3$, 100 MHz): Table I.

Taedolidol (**2**):

White dense oil; IR ν_{max} KBr cm^{-1}: 3345, 3059, 2983, 2939, 1607, 1431, 1269, 891 and 701 (KBr); ESIMS: *m/z* (%) 319 ($[M+K]^+$, 2), 303 ($[M+Na]^+$, 8), 281 ($[M+H]^+$, 32), 263 ($[M+H\text{-}H_2O]^+$, 100) and 231 ($[M+H\text{-}H_2O\text{-}CH_3OH]^+$, 11); ESIMS, daughter ions of *m/z* 281, 20 eV: *m/z* (%) 281 ($[M+H]^+$, 0), 263 ($[M+H\text{-}H_2O]^+$, 4), 231 ($[M+H\text{-}H_2O\text{-}CH_3OH]^+$, 9), 213 (22), 205 (51), 189 (58), 171 (77), 145 (65), 143 (100), 119 (62) and 105 (48); ^{1}H-NMR ($CDCl_3$, 400 MHz) and ^{13}C-NMR ($CDCl_3$, 100 MHz): Table I.

6-Epitaedolidol (**3**):

White dense oil; IR ν_{max} KBr cm^{-1}: 3348, 3054, 2985, 2931, 1603, 1422, 1265, 895 and 705 (KBr); ESIMS: *m/z* (%) 319 ($[M+K]^+$, 1), 303 ($[M+Na]^+$, 6), 281 ($[M+H]^+$, 61), 263 ($[M+H\text{-}H_2O]^+$, 100) and 231 ($[M+H\text{-}H_2O\text{-}CH_3OH]^+$, 28); ESIMS, daughter ions of *m/z* 281, 20 eV: *m/z* (%) 281 ($[M+H]^+$, 8), 263 ($[M+H\text{-}H_2O]^+$, 12), 249 ($[M+H\text{-}CH_3OH]^+$, 245 ($[M+H\text{-}2\times H_2O]^+$, 231 ($[M+H\text{-}H_2O\text{-}CH_3OH]^+$, 32), 213 (31), 203 (37), 189 (31), 171 (68), 147 (81), 143 (69), 119 (100) and 105 (81); ^{1}H-NMR ($CDCl_3$, 400 MHz) and ^{13}C-NMR ($CDCl_3$, 100 MHz): Table I.

Results and Discussion

For practical reasons, the Pinus trees chosen for fungi isolation were localized in a plantation within the campus of the Universidade Federal de São Carlos, closed to the Chemistry Department. The trunk bark of eight individuals were sampled for fungi isolation. Leaves (needles) were not collected. The fungus *Pestalotiopsis* sp. predominates in many collections. It grew as a salmon colored and dense colony in PDA plate and contains spores (conidia) with four to five cells. The two or three central cells are dark brown and the spores contains at least two apical appendages or hairs (Fig. 1A and 1B). The species in this genera shows very similar morphological aspects (Raj, 1985). The identification of the fungus at specie level is therefore a hard task.

Pulici *et al.* (1996) have reported the production of pestalotiopsins A, B and C, which are highly functionalized caryophyllene sesquiterpenes, by a *Pestalotiopsis* sp. obtained as an endophyte from *Taxus brevifolia.* The oxatricyclic pestalotiopsin C was characterized by single-crystal X-ray diffraction. Its NMR data (Pulici *et al.*, 1997) were very useful as reference for the identification of the caryophyllenes **1**, **2**, and **3** herein described. These sesquiterpenes were obtained as white dense oil, soluble in chloroform, dichloromethane and ethyl acetate; and showed no significant UV-light absorption.

The analysis of the NMR data obtained for **1** in comparison with those reported for pestalotiopsin C (Pulici *et al.*, 1997) suggested that they must have an identical carbon skeleton and with almost the same functions. In the HMBC spectrum of **1**, the two geminal methyl groups attached to C-11 (δ 1.02 and 1.18) and the hydrogen at δ 5.15 (*d*, 2.6 Hz, H-14) shows ^{3}J with the high deshielded quaternary carbon at δ 99.5 (C-1). In compound **1** the carbon C-14 is part of an acetal. This was confirmed by the HMBC correlation of the methoxyl hydrogens at δ 3.40 (*s*) with the signal at δ 115.7 (C-14). Thus the cyclobutane-tetrahydropyran oxabicyclic system present in compound **1** is similar to that present in pestalotiopsin C.

The presence of a trisubstituted double bound in compound **1** could be identified through analysis of the ^{13}C (δ 122.5, CH; 139.1, C; and 16.9, CH_3) and ^{1}H (4.93, *dd*; and 1.78, *br s*) NMR spectra. Along with the methyl group, the other two substituente at this double bond are a methylenic carbon (CH_2), which is geminal to the methyl group; and a CH carbon attached to a methoxyl group. Thus, a partial structure containing a C_5 unit is established by the analysis of the COSY and HMBC NMR data.

Following the ^{1}H-^{1}H couplings in the COSY spectrum it was deduced that two oxymethine carbons (O-CH) connects this C_5 unit to the oxabicyclic system identified above in a similar way found in pestalotiopsin C. The analysis of the molecular formula ($C_{17}H_{26}O_4$, 5 double bond equivalent) and IR (no absorption for OH group), and considering that two of the four oxygen atoms are part of the acetal function and one forms the methoxyl group at C-6, it was deduced that an ether bridge between C-2 and C-7 is present in the molecule.

A study using NOE experiments and geometry optimization by computational software (Froimowitz, 1993) allowed us to suggest the stereochemistry presented for compound **1** which agrees mostly with

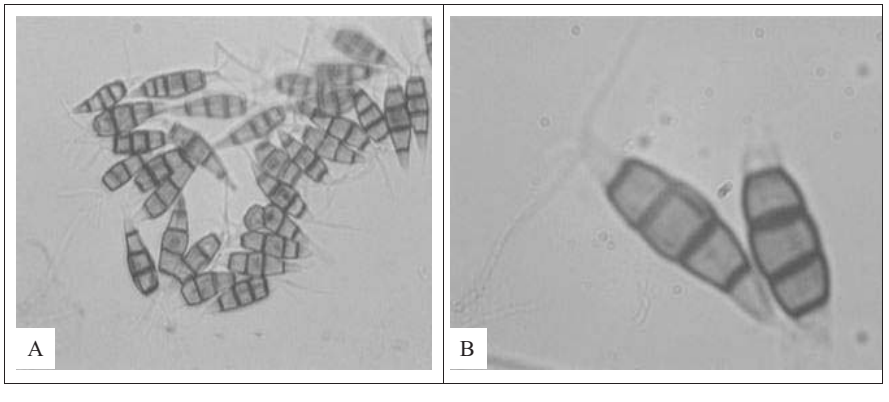
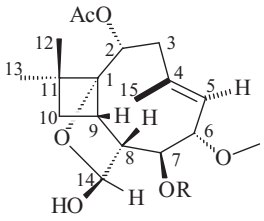
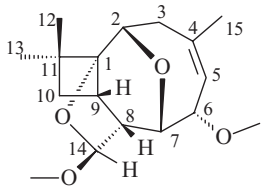


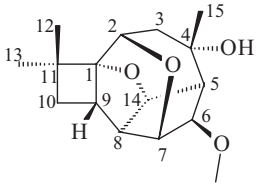
Fig. 1. Morphological aspects of *Pestalotiopsis* sp., strain LaBioMi-102, isolated from *Pinus taeda* collected in São Carlos, S. P. – Brazil. Conide details are shown in **A** and in **B**.



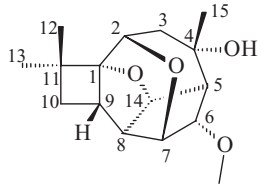
R = H: Pestalotiopsin A
R = CH_3: Pestalotiopsin C



1: Pestalodiopsolide A



2: Taedolidol



3: 6-Epitaedolidol

Fig. 2. Structures of the sesquiterpenes produced by *Pestalotiopsis* sp. isolated from *Pinus taeda.*

Table I. NMR spectroscopic data for sesquiterpenes **1–3**[*] ($CDCl_3$, 400 for ^1H and 100 MHz for ^{13}C).

	1			**2**			**3**		
	^{13}C	^1H	HMBC	^{13}C	^1H	HMBC	^{13}C	^1H	HMBC
1	99.5	–	–	95.6	–	–	95.5	–	–
2	70.1	4.10 *dd* (10.7, 5.7)	n. d.	72.5	4.04 *dd* (8.8, 7.0)	n. d.	71.3	4.21 *m*	4,9
3α	45.2	2.43 *m*	1,2,4,5,15	45.1	1.79 *dd* (14.4, 8.8)	1,2,4,5,15	40.4	1.90 *br d* (15.8)	1,2,4,5
3β	–	2.43 *m*	n. d.	–	2.14 *dd* (14.4, 7.0)	n. d.	–	2.09 *dd* (15.8, 4.1)	n. d.
4	139.1	–	–	78.9	–	–	75.9	–	–
5	122.5	4.93 *d* (11.7)	3,15	50.7	2.19 *dd* (3.9, 2.0)	3,15	56.9	2.29 *dd* (10.0, 6.7)	3,4,6,8,14
6	82.1	3.73 *dd* (11.7, 6.7)	4,6,7	86.9	4.20 *dd* (3.0, 2.0)	4,6,7	89.1	3.27 *t* (6.7)	4,5
7	76.9	3.87 *d* (6.7)	6,7,9,14	75.9	3.90 *dd* (3.0, 2.8)	6,7,9,14	76.7	4.14 *t* (6.7)	6,9
8	63.4	2.41 *m*	1,7,9	48.9	2.50 *dd* (8.2, 2.8)	1,7,9	59.6	2.80 *ddd* (7.0, 7.0, 3.6)	6,10
9	36.2	2.70 *m*	1,7,11,14	37.2	2.59 *t* (8.6)	1,7,11,14	33.7	2.60 *ddd* (9.3, 5.8, 3.6)	n.d.
10α	41.3	1.47 *dd* (12.1, 6.3)	1,8,9,12,13	36.3	1.65 *dd* (10.7, 8.6)	1,8,9,12,13	40.4	1.39 *dd* (11.9, 5.8)	1,8,9,11,13
10β	–	1.86 *dd* (12.1, 9.6)	1,13	–	1.49 *dd* (10.7, 8.6)	1,13	–	1.98 *dd* (11.9, 9.3)	8,9,13
11	43.2	–	–	37.7	–	–	37.3	–	–
12	23.3	1.18 *s*	1,10,11,12,13	24.5	1.29 *s*	1,10,11,12,13	26.4	1.22 *s*	1,10,11,13
13	26.8	1.02 *s*	1,10,11,12,13	22.3	1.01 *s*	1,10,11,12,13	24.4	1.09 *s*	1,10,11,12
14	115.7	5.15 *d* (2.6)	1,7	87.3	5.14 *dd* (8.2, 3.9)	1,7	83.7	4.99 *dd* (10.0, 7.0)	1,9
15	16.9	1.78 *br s*	3,4,5,15	30.3	1.15 *s*	3,4,5,15	30.1	1.22 *s*	3,4,5
OCH_3	55.2	3.24 *s* (at C-6)	6	56.5	3.22 *s* (at C-6)	6	57.6	3.42 *s* (at C-6)	6
OCH_3	55.2	3.40 *s* (at C-14)	14	–	–	–	–	–	–

[*] Coupling constants (Hz) in parentheses.
n. d.: Not detected.

the model compound pestalotiopsin C. In compound **1** the double bound geometry was suggested to be Z since it is part of a seven-member ring, where the presence of an E double bond will produce a molecule with high potential energy. This was confirmed by a NOE observed between the methyl hydrogens at δ 1.78 (H-15) with H-5 (δ 4.93). Therefore the molecular structure of compound **1** was identified as a tetraoxacyclic sesquiterpene that received the trivial name pestalotiopsolide A.

The compound **2** shows a shorter rf in TLC when compared with pestalotiopsolide A. Its IR spectrum contains a strong absorption for a hydroxyl group detected at 3445 cm^{-1}; absorption that could be ascribed to double bonds were not detected. The full scan ESI mass spectrum obtained for compound **2** shows ions at *m/z* 281 (30%) and 263 (100%). The spectrum was also obtained after the addition of a solution containing NaCl and KCl and showed strong peaks at *m/z* 303 ($[M+Na]^+$) and 319 ($[M+K]^+$) confirming the peak at *m/z* 281 as $[M+H]^+$. These data, in conjunction with the ^{13}C and ^1H NMR spectra, indicated the molecular formula to be $C_{16}H_{24}O_4$ (280 a. m. u). The base peak at *m/z* 263 ($[M+H-H_2O]^+$), in agreement with the IR data, confirms the presence of a free hydroxyl group in the molecules of compound **2**.

Based on analysis of the NMR data, a modified caryophyllene skeleton was proposed for the sesquiterpene **2**. These data clearly show that C-4 and C-5, where there was a double bond in pestalotiopsin C and pestalotiopsolide A, are now replaced by an oxygenated quaternary (δ 78.9) and a tertiary (CH) nonoxygenated (δ 50.7) carbons. Therefore, C-5 was probably cyclized with an electrophilic site, resulting in the molecules of **2**.

The two methyl groups (δ 1.29, *s* and 1.01, *s*) attached to the quaternary carbon C-11 shows HMBC with the ^{13}C detected at δ 95.6 (C-1). The deshielding effect over C-1 is due to the presence of the oxygen atom as bridge-head in the oxabicyclic system. This effect was observed in the other caryophyllenes obtained from *Pestalotiopsis* (Pulici *et al.*, 1997), where C-8 and C-14 completes the bicycle system. For compound **2**, H-14 was detected as a doublet of doublets at δ 5.14 (^3J with C-1), and C-14 was found at δ 87.3. Both H-14 and C-14 are shielded when compared with compound **1** (δ 5.15 and 115.7 respectively) indicating that C-14 is not anymore part of an acetal function. The COSY spectrum showed that H-14 is coupled with two CH hydrogens at δ 2.19 and 2.50 which were ascribed to H-5 and H-8, respectively. These data allowed the identification of a cyclobutyl-tetrahydropyranyl-cyclopentyl tricyclic partial structure in the molecules of compound **2**. The analysis of the HMBC and COSY spectra clearly established the sequence of spins couplings H-5 to H-8 and the positioning of the methoxyl group at C-6 (δ 86.9). The methyl group at δ 1.15 shows long range correlation with the ^{13}C atoms at δ 78.9 (C-4), 50.7 (C-5) and 45.1 (C-3) in the HMBC spectrum allowing the positioning of the hydroxyl at C-4.

The stereocenters in the molecule were proposed considering the analysis of the NOE data and geometry optimization studies (Froimowitz, 1993). The methoxyl hydrogens at δ 3.22 shows NOE with the pseudo-axial methyl group at C-4 and with the H-3β (axial) (δ 2.14); H-2 (δ 4.04) shows NOE with H-3 (δ 2.14 and 1.79), H-9β (δ 2.59) and H-12 (δ 1.29); the three hydrogens H-5 (δ 2.19), H-14 (δ 5.14) and H-8 (δ 2.50) are also correlated with each other in the NOESY spectrum, indicating that they are positioned at the same face of the cyclopentane ring. To the best of our knowledge, the substance represented by structure **2**, and trivially named as taedolidol, is a new modified caryophyllene sesquiterpene with an unprecedented oxapentacyclic ring system.

Compound **3** is an isomer of taedolidol (**2**). It has almost the same IR and MS spectral data. Careful analysis of the HSQC and HMBC NMR data indicated that the same cyclobutyl-tetrahydropyranyl-cyclopentyl tricyclic partial structure deduced above for **2** is also present in its isomer **3**. In addition, the ^1H-^1H correlations detected in the COSY spectrum established the same positioning of the functionalities along C-3 to C-7 seen in **2**. The ^1H NMR spectrum of taedolidol (**2**) contains three oxymethine hydrogens at δ 3.80–4.20 which were ascribed to H-6, H-2 and H-7 respectively. In the spectrum of compound **3**, one of these signals (H-6) is high-shielded and was detected at δ 3.27 (Δδ = – 0.93). Also in comparison with the ^1H spectrum of taedolidol (**2**), the signal for H-14 in **3** is shielded (Δδ = – 0.16). These shielding effects were due to the nonpaired electrons at the oxygen atoms in the ether bridge between C-2 and C-7 and in the methoxyl oxygen at C-6, respectively. Therefore, the H-6β hydrogen in **3** is shielded by the β-oxygen at C-7, and H-14 is shielded by the α-methoxy oxygen at C-6. The methyl hydrogens in the methoxyl group is also shielded by the C-7 β-oxygen and are detected at δ 3.22 (Δδ = – 0.20) in compound **2**, where it is β-orientated, and at δ 3.42 for the α-methoxy isomer (**3**). A smaller shielding effect was also observed for the β-methoxyl oxygen over the methyl hydrogens CH_3-15 which were detected at δ 1.15 in **2** and at δ 1.22 (Δδ = –0.07) for compound **3**. The

^{13}C NMR data of compounds **2** and **3** (Table I) is in good agreement with these shielding effects observed in the ^{1}H spectra, with C-5 (δ 50.7) and C-8 (δ 48.9) being shielded by the 6β-methoxyl group in **2**, when compared with **3** (Δδ = – 6.2 and – 10.7, respectively). For compound **3**, which has a methoxyl group at 6α, the signal of ^{13}C-14 is 3.6 ppm upper-field shifted (δ 83.7) compared with **2** (δ 87.3). All of these chemical shift-based deductions were confirmed by NOE experiments. Thus, irradiation at the frequency of H-6 (δ 3.27) in **3** produced a NOE effect in H-3β (δ 2.09), H-5 (δ 2.99), H-7 (δ 4.14), CH_3-15 (δ 1.22), and in the methoxyl hydrogens at C-6 (δ 3.42). Finally, this new natural product is represented by structure **3**, which is an epimer of compound **2**, and was therefore named 6-epitaedolidol, also showing an unprecedented oxapentacyclic ring system.

Although the pestalotiopsins were not isolated in our fermentation experiments, they could be precursors of pestalotiosolide (**1**) and taedolidol (**2**). The ether bridge between C-2 and C-7 present in the compounds discussed here may have been formed by a kind of S_N2 reaction with the oxygen atom at C-7 replacing the acetyl group at C-2. In its turn, the E double bond at C-4(5) in the pestalotiopsins place C-5 close enough to react with the aldehyde or any related electrophilic center at C-14 resulting in the formation of the cyclopentane present in taedolidols **2** and **3**.

Along with the sesquiterpenes described above, the fungus produced huge amounts of fatty acids and glycerides. Steroids, mainly ergosterol, were produced in trace quantities detected only by GCMS. The strain of *Pestalotiopsis* studied here was not yet tested for taxol production.

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