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Short Report

Lanostane Triterpenes from the Fungus *Pisolithus tinctorius*

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Um novo triterpeno, 3β,22ξ,23ξ-triidróxi-22ξ-acetóxi-24-metil-lanosta-8,24(28)-dieno-31-al, denominado pisosteral, e quatro triterpenos lanostanos conhecidos, 3β,22ξ,23ξ-triidróxi-22ξ-acetóxi-24-metil-lanosta-8,24(28)-dieno (pisosterol), 3α,22ξ,23ξ-triidróxi-22ξ-acetóxi-24-metil-lanosta-8,24(28)-dieno (3-*epi*-pisosterol), 3β,22ξ,23ξ-triidróxi-23ξ-acetóxi-24-metil-lanosta-8,24(28)-dieno e 3β,22ξ,23ξ-triidróxi-22ξ-acetóxi-24-etil-lanosta-8,24(28)-dieno, foram isolados do corpo de frutificação do fungo *Pisolithus tinctorius*, coletado em uma plantação de *Eucalyptus*. De uma linhagem de *P. tinctorius* isolada de *Pinus taeda*, foram isolados quatro triterpenos conhecidos derivados do lanostano, 3β,22ξ-diidróxi-24-metil-lanosta-8,24(28)-dieno, 3β,22ξ-diidróxi-24-etil-lanosta-8,24(28)-dieno, 3β-hidróxi-lanosta-8,24-dieno (lanosterol) and 3β-hidróxi-lanosta-7(8),9,24-trieno (agnosterol). Essas substâncias foram identificadas a partir de métodos químicos e físicos, principalmente espectrometria de massas e ressonância magnética nuclear 1D e 2D.

A new triterpene, 3β,22ξ,23ξ-trihydroxy-24-methyllanosta-8,24(28)-diene-31-al 22-acetate, named pisosteral, and four known lanostane triterpenes derivatives, 3β,22ξ,23ξ-trihydroxy-24-methyllanosta-8,24(28)-diene 22-acetate (pisosterol), 3α,22ξ,23ξ-trihydroxy-24-methyllanosta-8,24(28)-diene 22-acetate (3-*epi*-pisosterol), 3β,22ξ,23ξ-trihydroxy-24-methyllanosta-8,24(28)-diene 23-acetate and 3β,22ξ,23ξ-trihydroxy-24-ethyllanosta-8,24(28)-diene 22-acetate, were isolated from fruiting bodies of *Pisolithus tinctorius*, collected in an *Eucalyptus* plantation. From cultivation of a strain of *P. tinctorius* isolated from *Pinus taeda*, four known triterpenes lanostane derivatives, 3β,22ξ-dihydroxy-24-methyllanosta-8,24(28)-diene, 3β,22ξ-dihydroxy-24-ethyllanosta-8,24(28)-diene, 3β-hydroxylanosta-8,24-diene (lanosterol) and 3β-hydroxylanosta-7(8),9,24-triene (agnosterol), were isolated. These compounds were identified on the basis of chemical and physical methods, mainly mass spectrometry and 1D and 2D nuclear magnetic resonance.

Keywords*:* triterpene, lanostane, *Pisolithus tinctorius*, fungus, basidiomycete

Introduction

Pisolithus tinctorius (Basidiomycete) is commonly found in nature forming ectomycorrhizas, mainly with *Pinus* and *Eucalyptus* trees, in tropical and sub-tropical countries.[1,2] This ectomycorrhizal fungus is commercially important since its basidiospore inoculum may be used to facilitate creation of artificial forest.[3] The mycorrizal formation in the host root depends strongly of the *P. tinctorius* strains used. In this kind of symbiotic association, colonization is effective only among those high compatible plant-fungus interactions, resulting in benefits for the development of both organisms.[4,5] The fungus collected for the present study was found colonizing *Eucalyptus grandis* growing next to a *Pinus* plantation (*Pinus taeda*). It was not found any *P. tinctorius* colonizing the *Pinus* plants in this area. A chemical study was conducted to compare the secondary metabolites production by *Eucalyptus* associated *P. tinctorius*, collected in field, and a fungus strain specialized on colonizing of *Pinus taeda*, cultivated *in vitro*.

Besides its relevance as an ectomycorrhizal fungus,[2,3] *P. tinctorius* forms an abundant biomass, which has not yet been explored for any other uses. The main secondary metabolites in fruiting bodies of some varieties of *P. tinctorius* are lanostane triterpene[6-10] and naphthalenoid

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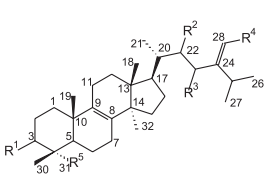
pulvinic acid derivatives.[11] The present work was focused on triterpenes, since they could play important role in the mechanism of plant colonization[12] and may have antiviral[13] and immunosuppressive[8] activities. We report nine lanostane triterpenes occurring in fruiting bodies of the fungus collected in the field and mycelia from strain 185 (*P. taeda*) grown *in vitro*. The present paper represents the first description of the co-production of the triterpenes **1**-**3** with different side chains.

Results and Discussion

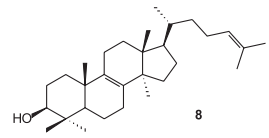
P. tinctorius was collected in a small artificial *Eucalyptus* forest within the campus of the Universidade Federal de São Carlos, next to the Chemistry Department. Fresh fruiting bodies were cut in small pieces and extracted with organic solvents. Triterpenes **1**- **5** were isolated from this collection by the use of successive column and preparative thin-layer chromatography techniques. The triterpenoid structures of these colorless metabolites were deduced from the ^{1}H and ^{13}C NMR data, and in particular from the application of two-dimensional ^{1}H-^{13}C correlation experiments and by comparison with literature data.[9,10,14,15] Triterpenes **1** and **2** differs only by the hydroxyl configuration at C-3. The H-3 signal appears in the ^{1}H NMR spectra of both as a double doublet (δ 3.24, J 11.7 and 4.6 Hz) and triplet (δ 3.64, J 3.6 Hz) respectively. The lanostane **3** is an isomer of **1** and **2** and shows a slight different side chain at C-17. The positioning and stereochemistry of the hydroxyl and acetyl groups in the side chain was solved by the use of single crystal X-ray analysis and nOe techniques applied to pisosterol derivatives.[14,15]

Electron ionization mass spectrometry (EIMS) of these lanostane triterpenes as silyl ether derivatives was also recognized as a good tool to distinguish C-22 and C-23 regioisomerism.[9] Thus, bond cleavage between C-22 and C-23 produce abundant ions at *m/z* 171 (**A**) for **1** (100%) and **2** (71%) (22-acetyl, 23-hydroxy) and 517 ([M-$C_8H_{13}O_2$]$^+$, 8%) for **3** (22-hydroxy, 23-acetyl). The triterpene **1** was isolated in good yields from the mycorrhizal *P. tinctorius* and was used as a reference compound for the identification of other triterpenes, since it is well characterized.

The electron ionization mass spectrum of the bistrimethylsilyl ether of triterpene **4** showed peaks at *m/z* 672 ($M^{+\bullet}$), 657 ([M-CH_3]$^+$), 612 ([M-AcOH]$^{+\bullet}$), 597 ([M-AcOH-CH_3]$^+$) and 507 ([597-TMSOH]$^+$). When the mass spectrum of **4** was obtained by atmosphere pressure chemical ionization (APCI), an abundant peak at *m/z* 551 ([M+Na]$^+$, 100%) was detected. These information's, besides the ^{1}H and ^{13}C NMR spectra analyses, allowed to deduce the molecular formula $C_{33}H_{52}O_5$ (528 Da). The MS and NMR data of **4** (Table 1) are almost like those obtained for **1**. The ^{13}C NMR chemical shifts at δ 76.5 (C-22), 73.0 (C-23), 156.8 (C-24) and 109.8 (C-28) and the peak at *m/z* 171 (100%, **A**) detected in the MS were used to indicate that the side chain of **4** is the same of **1**. The EIMS of the trimethylsilyl ethers of **1** and **4** shows the same profile, but most of the ion fragments in these spectra differs by 14 mass unit, including the $M^{+\bullet}$ (*m/z* 658 for **1** and 672 for **4**), suggesting the presence of an additional oxidation in triterpene **4**. The IR spectrum indicated the presence of a second carbonyl group (ν_{max}: 1710 cm^{-1}) in **4**, in addition to the acetate ester (ν_{max}: 1738 cm^{-1}). The cross peak of δ 206.9 with the ^{1}H singlet at δ 9.40 in the HSQC spectrum of **4**, indicated that the carbonyl group belongs to an aldehyde function which may have arisen from oxidation of one of the methyl group of triterpene **1**. The analysis of the HMBC spectrum and comparison with the NMR data of **1**,[10,14] confirmed the presence of the methyl groups CH_3-18 (δ 0.72, s), CH_3-19 (δ 1.03, s, correlation with C-9 at δ 134.0), and CH_3-32 (δ 0.87, s, correlation with C-8 at δ 134.9), in **4**. Thus, by exclusion, the oxidized CH_3 should be one of the 4,4-dimethyl groups. This assignment was confirmed by HMBC correlations (Table 1, summarized in



1: R^1 = β-OH; R^2 = OAc; R^3 = OH; R^4 = H; R^5 = CH_3
2: R^1 = α-OH; R^2 = OAc; R^3 = OH; R^4 = H; R^5 = CH_3
3: R^1 = β-OH; R^2 = OH; R^3 = OAc; R^4 = H; R^5 = CH_3
4: R^1 = β-OH; R^2 = OAc; R^3 = OH; R^4 = H; R^5 = CHO
5: R^1 = β-OH; R^2 = OAc; R^3 = OH; R^4 = CH_3; R^5 = CH_3
6: R^1 = β-OH; R^2 = OH; R^3 = H; R^4 = CH_3; R^5 = CH_3
7: R^1 = β-OH; R^2 = OH; R^3 = H; R^4 = H; R^5 = CH_3



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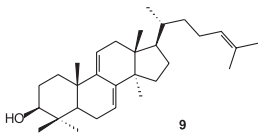

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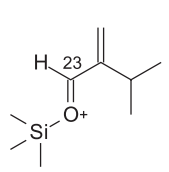
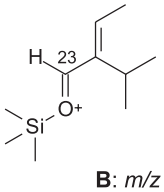
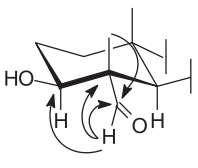


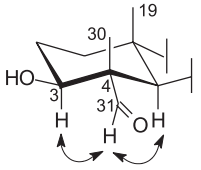
A: *m/z* 171



B: *m/z* 185



C



D

Tablel 1. ^{13}C (100 MHz) NMR Spectra data of the triterpenes **4** and **5** ($CDCl_3$)

	4		**5**
	δ ^{13}C	$^{13}C \rightarrow {}^{1}H$ Correlation (gHMBC)	δ ^{13}C
1	35.2	H-19	35.0
2	25.6	H-2; H-30	26.7
3	71.7	H-5; H-30	78.3
4	55.3	H-5; H-30; H-31	38.0
5	43.6	H-5; H-30; H-31	50.0
6	19.8	H-5	20.8
7	26.5	nd	27.9
8	134.9	H-32	134.3
9	134.0	H-19	134.3
10	35.9	H-19	36.0
11	18.9	nd	18.3
12	26.8	H-18	25.8
13	44.5	H-18; H-32	46.0
14	50.1	H-18; H-32	50.0
15	31.0	H-32	30.0
16	30.9	H-16	30.6
17	47.1	H-18; H-21	47.0
18	15.5	H-18	14.7
19	19.4	H-19	19.3
20	36.8	H-20	35.8
21	13.0	H-21; H-22	12.0
22	76.5	H-21	75.0
23	73.0	H-28[a]; H-28b	68.3
24	156.8	H-23; H-25; H-26; H-27	145.0
25	31.4	H-26; H-27; H-28a; H-28b	27.5
26	22.6	H-25; H-26; H-27	22.0
27	22.6	H-25; H-26; H-27	20.3
28a	109.8	H23; H-25; H-28a; H-28b	121.3
28b	-	H23; H-25; H-28a; H-28b	-
29	-	-	12.0
30	8.7	H-5; H-30; H-31	14.7
31	206.9	H-5; H-30; H-31	27.1
32	24.3	H-15; H-32	23.3
$\underline{C}H_3CO_2$	20.9	$C\underline{H}_3CO_2$	20.3
$CH_3\underline{C}O_2$	169.9	$C\underline{H}_3CO_2$; H-22	170.0

nd = not detected.

C) of the aldehyde hydrogen (δ 9.40) with C-4 (δ 55.3) and the CH_3-30 (δ 1.00) with the aldehyde carbonyl C-31 (δ 206.9). The aldehyde hydrogen H-31 (δ 9.40) showed NOESY with H-3α (δ 3.79) and with H-5 (δ 1.51) confirming the CH_3-31 as the oxidized methyl group. These nOe are summarized in **D**. Due to structural similarity with pisosterol (**1**), the new lanostane triterpene **4**, which appears to be a new compound, was named pisosteral.

Compound **5**, was also co-produced with **1** by the mushroom collected in field. Compared to **1**, **5** shows structural differences only with regard to the side chain. The ^{1}H NMR spectrum of **5** showed an additional signal of methyl group at δ 1.65 (d, 7.0 Hz, H-29) and an olefinic proton at δ 5.62 (q, 7.0 Hz, H-28) corresponding to an ethylidene group. The E-geometry of this double bond was deduced by a 1D nOe experiment, which showed nOe effect at δ 1.65 (CH_3-29) when H-25 (δ 2.83) was irradiated. The ^{1}H NMR chemical shifts of **5** were compared with those lanostane triterpene isolated from a *Pisolithus*[10]. However its ^{13}C NMR data is being reported for the first time (Table 1). The EIMS spectrum of the trimethylsilyl ether of **5** showed a peak at *m/z* 185 (**B**, 100%) as result of C-22 – C-23 bond cleavage, that confirmed the presence of an extra methyl group at C-28 and a hydroxyl group attached at C-23.

P. tinctorius, strain 185 isolated from *Pinus taeda*, was cultivated *in vitro*. The filtrate and the mycelial biomass obtained in this cultivation were extracted with organic solvents and submitted to the same chromatographic procedures used above yielding the triterpenes **6**-**9**. Triterpenes **6** and **7** show less complex side chains. The positioning of the hydroxyl group at C-22 of both triterpenes (**6** and **7**) was achieved by interpretation of ^{1}H-^{1}H COSY spectrum, which showed correlations between CH_3-21 with H-20 and H-20 with H-22. The tetracyclic rings system of **6** and **7** were identical to that of **1** and showed almost the same ^{13}C NMR data allowing their identification in mixture by comparison with literature data.[12] Compounds **8** and **9** were identified as the well-known lanosterol and agnosterol triterpenes respectively, by comparison with literature.[16,17]

Triterpenes with highest oxidation level, like **1**-**5**, were not produced in isolable amounts when the fungus (strain 185) was cultivated *in vitro*. The wild mushroom studied was found associated with *Eucalyptus grandis* and the strain used for the laboratory cultivation was collected from *Pinus taeda*. We are currently investigating whether this difference in secondary metabolites is due to influence of the substrate composition, different origins of the organisms, or absence of biotic induction conducted by any factor in field.

Experimental

General procedures

Optical rotations were measured on a PERKIN ELMER 241 polarimeter. IR spectra were measured with a BOMEN MB-102 spectrophotometer in KBr pellets. GC-EIMS experiments were carried out on a CARLO ERBA GC 8000 gas chromatography coupled with a MICROMASS PLATFORM II mass spectrometer. The capillary GC column used was a SUPELCO DB-1MS (30 m lenght, 0.25 mm I.D. and 0.25 μm film thickness). The temperature program was as follows: stand at 80 °C for 4 min, then increased at 9.0 °C/min to 250 °C and at 3.0 °C/min to 325 °C. Trimethylsilyl ethers were produced by adding excess of trimethylchlorosilane (TMSCl) to a pyridine

solution of tritepenes **1**-**9**. After 30 min. of reaction, the reactional mixture was partitioned between water and cyclohexane. The organic phase was dried with Na_2SO_4 and injected (3 μL) into GC-MS system. Low-resolution APCIMS data were acquired in positive ion mode, using a MICROMASS QUATTRO-LC instrument equipped with an API "Z-spray" ion source. ^1H and ^{13}C NMR spectroscopic experiments were recorded on a BRUKER DRX-400 spectrometer with $CDCl_3$ as solvent and TMS as internal standard.

Fungi material

Fruiting bodies of *Pisolithus tinctorius* were collected during the summer season (late January) of 1992, in an *Eucalyptus* plantation within the campus of Universidade Federal de São Carlos, in São Carlos, São Paulo State, Brazil. After collection, the fungi material was freeze-dried until the extraction procedures. The *P. tinctorius* isolate 185 (*Pinus taeda*) used in the present work was kindly provided by Dr. Sérgio F. Pascholati and Dr. Mírian J. Baptista from Escola Superior de Agricultura Luís de Queiroz (ESALQ), Universidade de São Paulo - at Piracicaba, São Paulo.

Isolation of triterpenes from the mushroom collected in field

Fruiting bodies of *P. tinctorius* (1.4 Kg) was cut in small pieces and extracted by percolation during three days with CH_2Cl_2 (3 L), CH_2Cl_2-MeOH (1:1) (3 L) and MeOH (3 L) (three extractions with each solvent). The extracts were combined and evaporated to dryness under reduced pressure. The residue was re-suspended in a mixture of MeOH and water (1:4) (2 L) and extracted with CH_2Cl_2 (3 x 1 L). The CH_2Cl_2 phase was evaporated to dryness and partitioned between *n*-C_6H_{14} (1 L) and MeOH (1 L). The MeOH extract (5.0 g) was subjected to a low-pressure silica gel CC eluted with CH_2Cl_2- MeOH gradient. Four main fractions (M1 to M4) were collected. The triterpene diols rich fraction (M2) was subjected to silica gel (230-400 mesh) CC eluted with *n*- *n*-C_6H_{14} - CH_2Cl_2-Me_2CO gradient resulting in 58 sub-fractions. Fractions 15-21 and 28-32 were chromatographed in preparative TLC [*n*-C_6H_{14} - CH_2Cl_2- Me_2CO (50:45:5)], yielding **1** (130.1 mg), **2** (3.0 mg), **3** (3.5mg), **4** (2.8 mg) and **5** (4.3mg).

Cultivation of strain 185 and Isolation of triterpenes

Mycelia of strain 185 were grown at 28 °C for 30 days in Petri dishes containing Melin-Norkrans medium,[18] modified according to WONG & FORTIN (MMN).[19] Five disks of medium containing mycelium were transferred to 100 mL MMN medium in 250 mL Erlenmeyer flasks (30 flasks). After 30 days of growth in the dark at room temperature, the mycelial suspension was separated by vacuum filtration and the mycelia was dried in a stove at 50 °C, ground and extracted with ethyl acetate to obtain the extract (AM185). The AM185 extract was chromatographed on a silica gel open column eluted with *n*-C_6H_{14}, *n*-C_6H_{14} - CH_2Cl_2 (1:1), CH_2Cl_2, and CH_2Cl_2-ethyl acetate (98:2, 95:5, 4:1 and 1:1). The fractions collected were analyzed by TLC (silica gel 60 F_{254}) using *n*-C_6H_{14} - CH_2Cl_2-Me_2CO (50:48:2) as eluent and revealed with vanillin-sulphuric acid. Initial fractions 13-17 (18.0 mg) were combined and purified by preparative TLC using *n*-C_6H_{14} - CH_2Cl_2- Me_2CO (50:48:2) to give a mixture of **8** and **9** (5.8 mg). Fractions 23-29 were re-crystallized from MeOH to yield a mixture of **6** and **7** (8.3 mg).

*Pisosterol (**1**)*

White amorphous powder; $[\alpha]_D^{25}$ + 33.2° (CH_2Cl_2, *c* 0.001,); IR (KBr) ν_{max} /cm^{-1}: 3443, 2927, 2877, 1739, 1630; EIMS (bistrimethylsilyl ether): *m/z* (rel. int.): 658 $[M]^{+\cdot}$ (6), 598 (23), 493 (8), 399 (9), 309 (15), 281 (4), 211 (39), 171 (100), 129 (25), 73 (99).

*Pisosteral (**4**)*

White amorphous powder; $[\alpha]_D^{25}$ + 30.6° (CH_2Cl_2, *c* 0.001); IR (KBr) ν_{max} /cm^{-1}: 3463, 2924, 2853, 1738, 1710, 1636; ^1H NMR (400 MHz, $CDCl_3$): δ 9.40 (s, H-31), 5.12 (brs, H-28a), 5.04 (d, *J* 8.0, H-22), 5.00 (brs, H-28b), 4.16 (d, *J* 8.0, H-23), 3.79 (dd, *J* 12.0 and 3.5, H-3), 2.31 (septet. *J* 6.9, H-25), 2.02-2.05 (m, H-20), 1.99 (s, CH_3CO_2), 1.07 (d, *J* 6.8, CH_3-27), 1.05 (d, *J* 6.8, CH_3-26), 1.00 (d, *J* 6.8, CH_3-21), 1.00 (s, CH_3-30), 1.03 (s, CH_3-19), 0.87 (s, CH_3-32), 0.72 (s, CH_3-18); ^{13}C NMR (100 MHz,$CDCl_3$): see Table 1; EIMS (trimethylsilyl ether): *m/z* (rel. int.): 672 $[M]^{+\cdot}$ (1), 657 (3), 612 (11), 597 (12), 507 (3), 484 (2), 413 (3), 211 (23), 171 (100), 129 (13), 73 (99); APCIMS: *m/z* (rel. int.): 551 $[M+Na]^+$ (100).

*Triterpene (**5**)*

White amorphous powder; ^{13}C NMR (100 MHz, $CDCl_3$): see Table1; EIMS (bistrimethylsilyl ether): *m/z* (rel. int.): 672 $[M]^{+\bullet}$ (1), 613 (4), 470 (1), 309 (3), 281 (1), 225 (4), 185 (100), 129 (8), 73 (82).

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