Pergamon

PII: S0031-9422(97)00007-1

Phytochemistry, Vol. 45, No. 3, pp. 499–504, 1997

0031-9422/97 $17.00+0.00

TRITERPENOIDS FROM *PISOLITHUS TINCTORIUS* ISOLATES AND ECTOMYCORRHIZAS

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(*Received in revised form* 28 *October* 1996)

Key Word Index—*Pisolithus tinctorius*; ectomycorrhiza; triterpenoid; lanostane derivatives.

Abstract—Two new triterpenoids have been identified by spectroscopic methods from mycelia of *Pisolithus tinctorius* as 24-ethyllanosta-8,24(24^1)-diene-3β,22ξ-diol and (22*S*)-24,25-dimethyllanosta-8-en-22,24^1-epoxy-3β-ol-24^1-one (25-methylpisolactone) along with the two known triterpenoids 24-methyllanosta-8,24(24^1)-diene-3β,22ξ-diol and (22*S*)-24-methyllanosta-8-en-22,24^1-epoxy-3β-ol-24^1-one (pisolactone). Quantification of these compounds in fungal isolates (surface and suspension cultures) and *Pinus sylvestris* ectomycorrhizas showed that the amount of the new triterpenoids was markedly higher in the mycorrhizas as in the isolates.

INTRODUCTION

The formation of ectomycorrhizas involves the development of specialized fungal tissues, the short root-covering sheath and the Hartig net within the root cortex [1]. These morphological changes are most likely accompanied by significant biochemical changes, such as differential accumulation of fungal polypeptides assumed to be a result of reorganization of structural proteins [2]. Likewise, one would expect dramatic changes in the pattern of fungal secondary compounds, that might play a role in the interactions occurring between tree roots and soilborne ectomycorrhizal fungi. Such changes are unknown, as are the possible functions of these compounds.

In our current biochemical studies on the establishment and functional maintenance of ectomycorrhizas, we became interested in the secondary metabolism of *Pisolithus tinctorius* (Pers.) Cocker & Couch, a commercially important ectomycorrhizal fungus that is considered to have a broad host range [3, 4]. It has been proposed that ectomycorrhizal fungi stimulate the plant growth in low fertility soils by the additional fungal supply of minerals to the host [5]. The main secondary metabolites in fruit bodies of some varieties of *P. tinctorius* were known to be naphthalenoid pulvinic acid derivatives [6] and various triterpenoids [7–9]. We report here on a chemical study concerning triterpenoids from *P. tinctorius* mycelia, comparing isolates (surface and suspension cultures) with the fungus from ectomycorrhizas of *Pinus sylvestris-P. tinctorius*.

RESULTS AND DISCUSSIONS

Four-week-old suspension cultured *Pisolithus tinctorius* mycelia were freeze-dried and extracted with diethyl ether, resulting in a pale yellow solid material corresponding to *ca* 10% of the dry weight of the fungal biomass. GC-mass spectrometry analysis showed four triterpenoids as main components. These compounds were purified by preparative reversed-phase (C_8) HPLC. Complete assignments of ^{1}H NMR and ^{13}C NMR signals on the basis of ^{1}H,^{1}H COSY, HMQC and HMBC experiments were established (Tables 1 and 2). Two compounds were identified as pisolactone [(22*S*)-24-methyl-lanosta-8-en-22,24^1-epoxy-3β-ol-24^1-one] (**3**) [7] and 24-methyllanosta-8,24(24^1)-diene-3β,22ξ-diol (**1**) [8] previously isolated from the fruit bodies of *P. tinctorius*. The other two compounds are newly described methyl derivatives of **1** and **3**.

The molecular formula of compound **2** was assigned to be $C_{32}H_{54}O_2$ by HR-EIMS. The mass spectral fragmentation of **2** is shown in Fig. 1. Ions at *m/z* 372 (**a**) and 344 (**b**) are considered to have been due to the fission of the C-22/C-23 and the C-20/C-22 bond, respectively. Furthermore, this fragmentation is evident for a hydroxyl function at C-22. A fragment ion due to the elimination of the side chain accompanied by the loss of one hydrogen atom was observed at *m/z* 314 (**c**) to give the basic lanosterol nucleus [10]. Two

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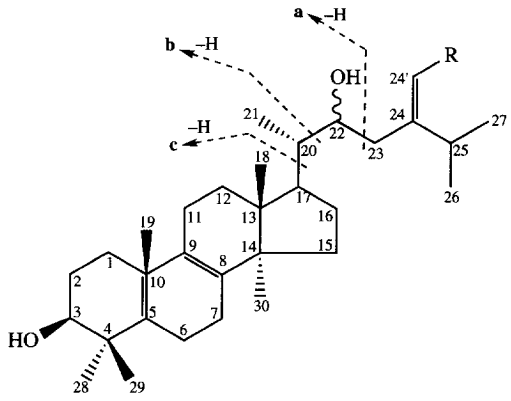
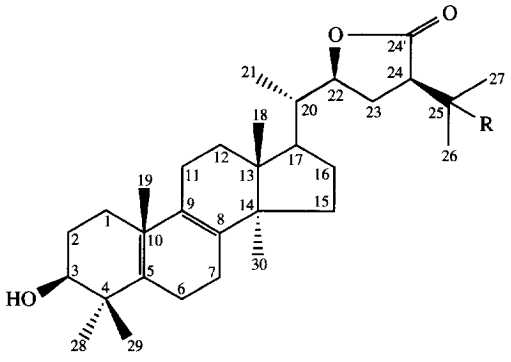


R

1 H

2 CH3



R

3 H

4 CH3

Fig. 1. Structures of the triterpenoids **1–4** and mass spectral fragmentation of the triterpenoids **1** (R = H) and **2** (R = CH_3) from *Pisolithus tinctorius* mycelia.

peaks at δ 79.0 and 71.0 (Table 1) in the ^{13}C NMR spectrum confirmed the presence of two oxygenated carbons corresponding to C-3 and C-22 [8]. The ^{13}C NMR spectrum of **2** exhibited two sp^2 carbon resonances at δ 134.4 and 134.6 corresponding to the lanosterol $\Delta^{8,9}$-double bond. The ^1H NMR ($CDCl_3$) revealed resonances at δ 0.70 (3H, *s*, H-18), 0.81 (3H, *s*, H-29), 0.92 (3H, *s* H-30), 0.99 (3H, *s*, H-19), 1.00 (3H, *s*, H-28) and 3.24 (1H, *dd*, J = 11.4; 4.6 Hz, H-3) which were superimposable with **1**. This indicates that **2** has an identical ring structure to that of **1**. The ^1H NMR spectrum of **2** showed an additional methyl signal (δ 1.65, 3H, *d*, J = 6.8 Hz) and an olefinic proton (δ 5.25, 1H, *q*, J = 6.8 Hz) corresponding to an ethylidene group instead of two olefinic protons (δ 4.89, 1H, *dd*, J = 1.3; 1.3 Hz and δ 4.79, 1H, *dd*, J = 2.4; 1.3 Hz) in **1**. NOESY crosspeaks between H-25 and R(-CH_3) on the one hand and H-22 and H-24^1 on the other hand confirmed the *E*-configuration at the C-24/C-24^1 double bond. Comparison of the ^{13}C NMR spectra of **2** with **1** suggested that **2** was a methyl derivative of **1** indicating that one H of the =CH_2 group at position 24^1 in **1** (δ 109.2) was replaced by a methyl group in **2** to δ 120.6 (C-24^1). Based on the NMR and mass spectral data we suggest that **2** is 24-ethyllanosta-8,24(24^1)-diene-3β,22ξ-diol.

The molecular formula of **4** was assigned to be $C_{32}H_{52}O_3$ by HR-EIMS which showed the molecular ion peak at m/z 484.3905 ([M]$^+$, calcd for $C_{32}H_{52}O_3$: 484.3917) and significant peaks at m/z 469 [M − Me]$^+$ and 451 ([M − Me − H_2O]$^+$. The chemical shift values of C-1 to C-23 and C-24^1 to C-30 in the ^1H NMR and ^{13}C NMR spectra of **4** were almost superimposable with those of corresponding data of **3** (Table 2). This was further supported by the presence of a characteristic resonance at δ 177.2 (C-24^1) indicating the presence of a lactone ring [7]. The ^1H NMR spectrum of **4** also showed five tertiary and one secondary methyl signals as required by the basic lanostane skeleton. Three additional tertiary methyl signals were observed at δ 1.07 which were identified according to their HMBC cross peaks with C-24, C-25 and the CH_3 signals at δ 29.6 (3 × CH_3) as a tertiary butyl group attached to C-24. Thus the structure of **4** was determined to be 25-methylpisolactone.

To reveal possible changes in the pattern of compounds **1–4** during establishment of the ectomycorrhiza, a comparative quantitative study was carried out with isolates (surface and suspension cultures) and mycelia from ectomycorrhizas of *Pinus sylvestris-P. tinctorius*. The proportion of fungal biomass in the mycorrhizas was estimated from the fungal ergosterol content in relation to the ergosterol content of the isolates according to Martin *et al.* [11]. GC-MS of the mycorrhizal diethyl ether extracts revealed the same triterpenoid profile as from surface- and suspension-cultured mycelia. The results from quantitative GC studies are shown in Fig. 2. It is obvious that the amount of the triterpenoids **2** and **4** was markedly higher in the mycorrhizas compared with the isolates. Unfortunately, we cannot distinguish between mycelia from the sheath and the Hartig net in order to localize this differential accumulation. Nevertheless, it seems to be associated with the development of the specialized fungal tissues in the *Pinus sylvestris-Pisolithus tinctorius* ectomycorrhiza. Further studies will have to show the role of these triterpenoids in the fungus–plant symbiotic relationship, possibly part of fungal membrane reorganization.

EXPERIMENTAL

Fungal isolate. The mycelia of *Pisolithus tinctorius* (Pers.) Coker et Couch, strain Lelly/Marx 298, kindly provided by Dr V. Wiemken (Institute of Botany, Basel, Switzerland), were kept at 25° on MMN medium [12]. Plugs of inoculum from this culture were transferred to agar plates with reduced glucose (2 g l^{-1}) and malt extract (0.3 g l^{-1}) concns [13]. After growth for 7–9 days the plugs were used for ecto-

Table 1. ^{13}C and ^{1}H NMR data of compounds **1** and **2** [chemical shifts in $CDCl_3$, coupling constants (Hz) in parentheses]

	1		2	
Position	^{13}C NMR	^{1}H NMR	^{13}C NMR	^{1}H NMR
1	35.6	1.72, 1.23	35.6	1.72, 1.23
2	27.9	1.66, 1.58	27.9	1.67, 1.58
3	79.0	3.239 *dd* (11.7, 4.5)	79.0	3.24 *dd* (11.6, 4.6)
4	38.9	—	38.9	—
5	50.4	1.04	50.4	1.05
6	18.3	1.68, 1.49	18.3	1.68, 1.50
7	26.5	2.03, 2.03	26.5	2.04, 2.04
8	134.4	—	134.4	—
9	134.6	—	134.5	—
10	37.0	—	37.0	—
11	21.0	2.03, 2.01	21.0	2.01, 2.01
12	31.0	1.79, 1.68	21.0	1.78, 1.67
13	44.5	—	44.5	—
14	49.9	—	49.9	—
15	30.8	1.61, 1.21	30.8	1.61, 1.20
16	27.7	2.00, 1.36	27.7	2.00, 1.35
17	47.1	1.91	47.2	1.92
18	15.6	0.70 (3H, *s*)	15.6	0.70 (3H, *s*)
19	19.1	0.99 (3H, *s*)	19.1	0.99 (3H, *s*)
20	41.1	1.43	41.3	1.42
21	12.0	0.92 *d* (6.6)	12.0	0.91 *d* (6.6)
22	70.8	3.81 *ddd* (9.4, 3.9, 1.4)	71.0	3.77 *ddd* (8.6, 4.6, 1.5)
23	41.2	2.26 *dd* (14.3, 9.4) 2.10 *dd* (14.3, 3.9)	38.7	2.05, 2.05
24	153.4	—	142.4	—
25	33.5	2.24 *sept* (6.8)	28.6	2.85 *sept* (7.0)
26*	21.7	1.06 *d* (6.8)	21.2	1.06 *d* (7.0)
27*	22.0	1.04 *d* (6.9)	21.0	1.00 *d* (6.9)
24[1]	109.2	4.89 *dd* (1.3, 1.3) 4.79 *dd* (2.4, 1.3)	120.6	5.25 *q* (6.8)
28	28.0	1.00 (3H *s*)	28.0	1.00 (3H *s*)
29	15.4	0.81 (3H *s*)	15.4	0.81 (3H *s*)
30	24.3	0.92 (3H *s*)	24.4	0.91 (3H *s*)
R (CH_3-)	—	—	12.9	1.65 *d* (6.8)

* Assignments are interchangeable.

mycorrhiza synthesis. For obtaining submerged cultures, mycelia of 3–4-week-old agar plates (MMN-medium) were homogenized and transferred to 50 ml MMN medium in 250 ml-Erlenmeyer flasks. Cultures were kept in the dark at 25° on a rotary shaker at 80 rpm. The cultures were harvested after 4 weeks and freeze dried.

Plant material. Seeds of *Pinus sylvestris* L., obtained from the Conrad Appel GmbH-Forst-und Gehölzsaaten (Darmstadt), were germinated and the seedlings grown in a greenhouse at 20–25° with a 16-hr-day period in plastic dishes containing perlite. One-month-old plantlets were transferred into plastic pots containing a mixture of peat/perlite (1:1, v/v). After further growth of 8 weeks, the plantlets had developed sufficient amounts of fine roots and were transferred in plates for initiating ectomycorrhiza formation.

Ectomycorrhiza synthesis. The synthesis and cultivation of ectomycorrhizas were carried out with *P. sylvstris* seedlings in Petri dishes (150 mm i.d.) containing peat [14]. The Petri dishes were placed vertically in plastic boxes in a greenhouse for a 16 hr 8 hr 20°/15° day/night cycle, light intensity of 200 μmol m^{-2} s^{-1}, and 70% rel. humidity. After 2 weeks the plantlets were inoculated with mycelial plugs from the agar medium. Well-developed mycorrhizas were harvested after 5 weeks of incubation. They were collected using a stereomicroscope and immediately freeze-dried.

Extraction and isolation. Freeze-dried mycelia (5 g) from submerged cultures of *Pisolithus tinctorius* were powdered in liquid N_2 and extracted with Et_2O (3 ×). The combined extracts were evaporated to dryness yielding 500 mg of a pale yellow solid containing mainly a mixture of triterpeonids. The solid was redissolved in 2-PrOH and centrifuged. Sepn and purification of the triterpenoids was carried out by repeated chromatography on a reverse-phase (C_8) HPLC column (PrepPack Bondapak 25 × 200 mm) using 80% aq. MeOH (adjusted to pH 3 with formic

Table 2. ^{13}C and ^{1}H NMR data for compounds **3** and **4** (chemical shifts in $CDCl_3$, coupling constants (Hz) in parenthesis)

	3		4	
Position	^{13}C NMR	^{1}H NMR	^{13}C NMR	^{1}H NMR
1	35.6	1.72, 1.23	35.6	1.72, 1.23
2	27.8	1.67, 1.58	27.9	1.68, 1.58
3	78.9	3.24 *dd* (11.3, 4.5)	78.9	3.24 *dd* (11.4, 4.6)
4	38.9	—	38.9	—
5	50.4	1.05	50.4	1.05
6	18.2	1.68, 1.50	18.2	1.68, 1.50
7	26.5	2.03, 2.03	26.5	2.03, 2.03
8	134.2	—	134.2	—
9	134.7	—	134.7	—
10	37.0	—	37.0	—
11	21.0	2.03, 2.03	21.0	2.03, 2.02
12	30.9	1.78, 1.65	30.9	1.77, 1.65
13	44.6	—	44.6	—
14	49.8	—	49.8	—
15	30.8	1.61, 1.23	30.8	1.61, 1.23
16	27.8	2.00, 1.35	27.9	2.01, 1.34
17	47.4	1.96	47.4	1.96
18	15.6	0.71 (3H, *s*)	15.6	0.70 (3H, *s*)
19	19.1	0.98 (3H, *s*)	19.1	0.98 (3H, *s*)
20	39.8	1.60	39.8	1.58
21	12.2	0.94 *d* (6.7)	12.2	0.94 *d* (6.7)
22	80.5	4.48 *ddd* (10.6, 6.2, 1.4)	79.4	4.39 *ddd* (10.7, 6.1, 1.5)
23	26.9	1.98, 1.88	28.5	2.01, 1.92
24	46.8	2.59 *ddd* (12.3, 9.0, 4.9)	50.6	2.46 *dd* (12.6, 8.9)
25	27.5	2.22 *d sept* (5.0, 6.8)	31.8	—
26*	20.7	1.04 *d* (6.9)	29.6	1.07 (3H, *s*)
27*	18.2	0.93 *d* (6.8)	29.6	1.07 (3H, *s*)
24[1]	178.4	—	177.2	—
28	27.9	1.00 (3H, *s*)	28.0	1.00 (3H, *s*)
29	15.4	0.81 (3H, *s*)	15.4	0.81 (3H, *s*)
30	24.2	0.89 (3H, *s*)	24.2	0.89 (3H, *s*)
R (CH_3-)	—	—	29.6	1.07 (3H, *s*)

* Assignments are interchangeable.

acid) as solvent at a flow rate of 10 ml min^{-1} (liquid chromatograph from Beckman Instruments, U.S.A). The triterpenoids were detected at 210 nm.

Ergosterol and triterpenoid assay. Lyophilized material (10–20 mg) of mycorrhizal short roots of *Pinus sylvestris* or mycelia from agar plates was exhaustively extracted with Et_2O (3 × 1 ml) in Eppendorf-tubes (2 ml safe-lock) using zirconia beads (1 mm diameter) and a Bead Beater (Bio Spec Products, Inc., Bartlesville, OK, U.S.A.). After centrifugation at 10000*g* (5 min) using a bench centrifuge at 4° the combined supernatants were evaporated and the residue was dissolved in 1 ml 2-PrOH. The extracts were analysed for free ergosterol content by analytical HPLC. The liquid chromatograph (Waters 600-MS system controller) was equipped with a 5-μm Nucleosil C_8 column (250 × 4 mm i.d.). A linear gradient elution system was applied at a flow rate of 1 ml min^{-1} within 15 min from 70% solvent A (1.5% *ortho*-phosphoric acid in water) to solvent B (100% MeCN) and 20 min at 100% B. Injections of 10 μl were carried out by an automatic sampler. Compounds were detected at 278 nm (ergosterol) and 210 nm (triterpenoids) by photodiode array detector. The ergosterol peak was eluted at approx. 21.4 min., triterpenoids were eluted at 18.12 (**4**), 18.54 (**3**), 18.57 (**1**) and 19.08 (**2**) min. Quantitative ergosterol values were calculated from external standardization with ergosterol, recrystallised from 2-PrOH. The fungal biomass in the ectomycorrhizas was calcd from the ergosterol content of surface cultures of *Pisolithus tinctorius* [11].

Capillary gas chromatography. Column HP-5 (30 m × 0.32 mm; 0.25 μm film thickness); inj. temp. 225°; column temp. 280° (isothermal); FID (temp. 290°); carrier gas N_2, flow rate 2 ml min^{-1}, split injection (split ratio 1:10). The content of the triterpenoids was calcd from their peak area in comparison with lupeol as int. standard.

GC-MS. EI (70 eV); column DB-5MS (15 m × 0.25 mm; 0.25 μm film thickness), inj. temp. 250°, temp. Programme: 170° for 1 min then elevated to 270° within 25° min^{-1}, then raised to 290° at a rate of 2 grd min^{-1}, carrier gas He, flow rate 0.8 ml min^{-1}.

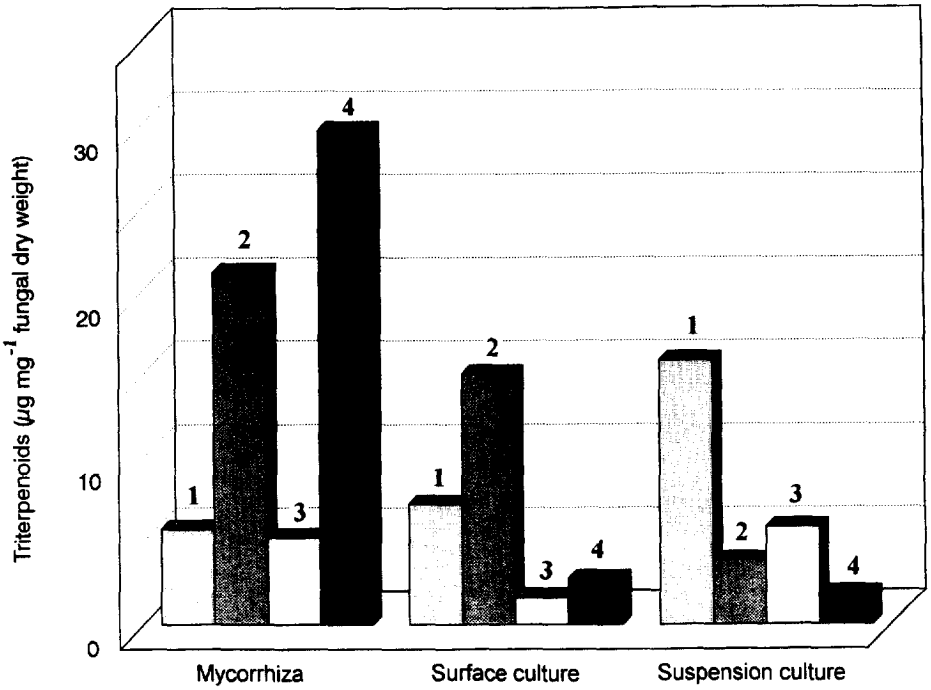


Fig. 2. Amounts of the triterpenoids **1**–**4** (lupeol equivalents) from *Pisolithus tinctorius* isolates (surface and suspension cultures) and *P. tinctorius-Pinus sylvestris* ectomycorrhiza.

Spectroscopy. 2D NMR spectra were recorded at 500 MHz (solvent: $CDCl_3$; TMS δ 0 as int. standard), ^{13}C NMR spectra at 75 MHz (solvent: $CDCl_3$; $CDCl_3$ δ 77.0 as int. standard). MS: 70 eV EIMS, HR-EIMS. Mps are uncorr.

24-*Methyllanosta*-8,24(24^1)-*diene*-3β,22ξ-*diol* (**1**). Crystallization from 2-PrOH afforded 190 mg. Mp 177–179°, $[\alpha]_D^{24°}$ +49.7 ($CHCl_3$, *c* 0.2). EIMS, *m/z* (rel. int.): 456 $[M]^+$ (32), 441 $[M-Me]^+$ (5), 423 $[M-Me-H_2O]^+$ (9), 405 (4), 372 (**a**, 8), 357 [**a**−Me] (100), 344 (**b**, 6), 339 [**a**−Me−H_2O] (35), 329 (5), 321 [**a**−Me−2H_2O] (9), 314 (**c**, 6), 311 (13), 299 [**c**−Me], (11), 281 [**c**−Me−H_2O] (10), 215 (6), 187 (7), 161 (6), 135 (6), 95 (8).

24-*Ethyllanosta*-8,24(24^1)-*diene*-3β,22-*diol* (**2**). Yield 70 mg. Mp 170–180°, $[\alpha]_D^{24°}$ +39.27 ($CHCl_3$, *c* 0.45). EIMS, *m/z* (rel. int.): 470.4096 [M^+, calcd for $C_{32}H_{54}O_2$ 470.4124] (16), 455 $[M\text{-}Me]^+$ (6), 437 $[M\text{-}Me\text{-}H_2O]^+$ (8), 419 (3), 372.3037 [**a**, calcd for $C_{25}H_{40}O_2$ 372.3029] (36), 357.2782 [**a**−Me, calcd for $C_{24}H_{37}O_2$ 357.2794] (100), 344 (**b**, 8), 339.2664 [**a**−Me−H_2O, calc for $C_{24}H_{35}O$ 339.2688] (32), 329 (5), 321 [**a**−Me−2H_2O] (6), 314 (**c**, 9), 311 (11), 299 [**c**−Me] (12), 281 [**c**−Me−H_2O] (9), 215 (6), 187 (8), 161 (7), 109 (9).

(22S)-24-*Methyllanosta*-8-*en*-22,24^1-*epoxy*-3β-*ol*-24^1-*one* (pisolactone, **3**). Yield 18 mg. Mp 267–273°, $[\alpha]_D^{24°}$ +48.6 ($CHCl_3$, *c* 0.238). EIMS, *m/z* (rel. int.): 470 $[M]^+$ (20), 455 $[M-Me]^+$ (100), 315 (8), 299 (10), 281 (21), 227 (15), 215 (14), 213 (15), 187 (20), 161 (19) 159 (18), 147 (15), 135 (17), 121 (19), 95 (23), 55 (22).

(22S)-24,25-*Dimethyllanosta*-8-*en*22,24^1-*epoxy*-3β-*ol*-24^1-*one* (25-methylpisolactone, **4**). Yield 8 mg of colourless needles. Mp 313–316°, $[\alpha]_D^{24°}$ +38.7° ($CHCl_3$; *c* 0.23). EIMS *m/z* (rel. int.): 484.3905 [M^+, calcd for $C_{32}H_{52}O_3$ 484.3917] (28), 469 $[M-Me]^+$ (80), 451.3542 $[M-Me-H_2O]^+$ [calcd for $C_{31}H_{47}O_2$ 451.3576] (100), 329 (7), 299 (9) 281 (14), 227 (9), 215 (8), 213 (8), 187 (10), 161 (10), 159 (9), 147 (6), 135 (17), 121 (10), 95 (15), 57 (18).

Acknowledgements—We thank Elke Gebauer and Ulrike Hintsche for dependable technical assistance, as well as Dr M. Weiss for valuable discussions on ectomycorrhiza cultivation. This work was supported by the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

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