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THE SESQUITERPENES OF *LACTARIUS DELICIOSUS* AND *LACTARIUS DETERRIMUS*

OLA BERGENDORFF and OLOV STERNER*

Organic Chemistry 2, Lund Institute of Technology P.O.B. 124, S-221 00 Lund, Sweden

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Key Word Index—*Lactarius deliciosus*; *L. deterrimus*; Agaricaceae; mushrooms; milc caps; guaiane sesquiterpenes.

Abstract—The sesquiterpenoid contents of the fruit-bodies of *Lactarius deliciosus* Fr. and *L. deterrimus* Gröger have been reinvestigated. Undamaged fruit-bodies of both species were found to contain only a single sesquiterpene, as two fatty acid esters. If the fruit-bodies are injured (e.g. by cutting), the esters are in minutes converted to five free sesquiterpenes. Three of these have previously been isolated from *L. deliciosus* while two are new, and the structures of the new compounds have been elucidated by spectral and chemical methods. The conversions of sesquiterpenes in the injured fruit-bodies appear to be enzymatic, and the possibility that they are part of a chemical defence system is discussed.

INTRODUCTION

A general characteristic of the genus *Lactarius* (family Russulaceae of the Basidiomycotina subdivision of Fungi), is that the fruit-bodies contain a latex which can be observed if the fruit-bodies are cut or broken. The colour and taste of this latex varies between different species, a fact that has great taxonomical significance, and the chemical backgrounds for such distinctions have been subjected to several investigations. The latex of the fruit-bodies of *Lactarius deliciosus* Fr. and *L. deterrimus* Gröger is first carrot-coloured, but slowly (minutes) darkens and eventually turns green, and these colours have in *L. deliciosus* previously been shown to be due to guaiane sesquiterpenes. Besides lactaroviolin (**1**) [1], the free dihydroazulene alcohol **2a** [2] as well as its stearic acid ester **2b** [2], and lactarazulene (**3**) [3] have been isolated from European specimens of *L. deliciosus*. In addition, lactarofulvene (**4**) has been isolated from Californian specimens of *L. deliciosus* [4], while the aldehyde **5** was isolated from Indian specimens of *L. deterrimus* [5]. However, most *Lactarius* species that have been investigated are devoid of guaiane sesquiterpenes. Many have a colourless latex with an intense pungent taste, owing to the presence of unsaturated dialdehyde sesquiterpenes with marasmane, lactarane and seco-lactarane skeletons [6]. The pungent unsaturated dialdehydes, which are formed enzymatically from a precursor sesquiterpenoid (a fatty acid ester of a marasmane sesquiterpene) as a response to injury to the fruit-bodies, possess potent antibiotic and antifeedant activities [6].

The formation and transformation of sesquiterpenes in the pungent *Lactarius* species (e.g. *L. vellereus* and *L. torminosus*) obviously protects the fruit-bodies from parasites, and thus appear to constitute a chemical defence system. The possibility that a similar system has evolved also in *L. deliciosus* and *L. deterrimus*, motivated a reinvestigation of these species by the techniques developed to study the sesquiterpenes of the pungent *Lactarius* species [7].

RESULTS AND DISCUSSION

The initial sesquiterpenoid contents of young and seemingly parasite unaffected specimens of *L. deliciosus* and *L. deterrimus*, were investigated by immersing the specimens in liquid nitrogen at their sites of growth, and extracting them still frozen with hexane. Only a single sesquiterpene, as two fatty acid esters, was found in significant amounts (i.e. detectable with our TLC and HPLC analytical systems) when such extracts were analysed immediately after preparation. The two esters of the same alcohol were revealed by HPLC analysis, and the two esters could be separated by HPLC chromatography. The major component (85% in both species) was found to be the previously isolated stearic acid ester **2b**. Comparison of the ^{1}H NMR data (chemical shift, multiplicity and integral) of the minor component (15% in both species) with those of various free fatty acids, suggest that the minor component is the linolic acid ester **2c**. No traces of the sesquiterpenes previously isolated from the pungent *Lactarius* species (with marasmane, lactarane or seco-lactarane skeletons [6]) were detected in this investigation.

To investigate if these original sesquiterpenoid esters are transformed when the fruit-bodies of *L. deliciosus* and *L. deterrimus* are injured, similar to the situation in the pungent *Lactarius* species, specimens were brought directly from their place of growth to the laboratory where they were ground in a meatgrinder (without the addition of a solvent). At various times after grinding, portions of the mush were extracted with hexane and analysed from sesquiterpenoid contents. This was done both on a preparative scale in order to isolate and characterize the products formed in the ground mushrooms, and on an analytical scale to facilitate the quantitative analysis of the sesquiterpenes as a function of the time between grinding

*Author to whom correspondence should be addressed.

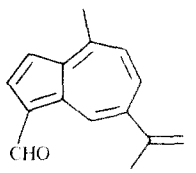
and extraction. As in the pungent *Lactarius* species [7], although less rapidly, the original esters are converted to other compounds when the tissue of the fruit-body is disrupted. No differences, neither qualitative nor quantitative, could be detected between *L. deliciosus* and *L. deterrimus* in this respect. The following products could be identified in the fresh extracts: the aldehydes lactaroviolin (**1**) and compound **6** (for compound **6** we propose the name delicial), the alcohols **2a** and **7a** (for compound **7a** we propose the name deterrol), and lactaroazulene (**3**). Delicial (**6**) and deterrol (**7a**) are new compounds, and the elucidations of their structures are discussed below. The green colour that the latex assumes with time is due to the formation of violet and blue compounds [lactaroviolin (**1**) and deterrol (**7a**)], and their mixing with the yellow compounds [the alcohol **2a**, the fatty acid esters of **2a**, and delicial (**6**)] already present or also formed. Unfortunately, a complete quantitative analysis of these sesquiterpenes as a function of time between grinding and extraction, similar to that made for *L. vellereus* [7], was impossible to perform. The instability of the alcohol **2a** and delicial (**6**), as well as the formation of small amounts of a large number of new compounds (believed to be artifacts) during the handling and storage of the extracts, made the HPLC analyses unreliable. One of these new compounds could be isolated, and ^{1}H NMR and mass spectral data suggest that it is a dimer of two guaiane sesquiterpenes. The formation of such dimers has previously been observed during work-up of similar extracts [8]. However, for lactaroviolin (**1**) and deterrol (**7a**), which both are reasonably stable products, the HPLC analyses were reproducible for extracts made up to two hr after grinding, and the results are shown in Table 1. Both these sesquiterpenes are slowly formed and accumulate in the mushroom tissue during the first hours after injury.

TLC analysis of the extracts made 10 min after grinding at room temperature indicated the presence of large amounts of delicial (**6**). However, when attempts to isolate delicial (**6**) were made, it proved to be very unstable. Chromatography on alumina, for example, was not possible, and when exposed to light it rapidly polymerized. Repeated flash chromatography, in the dark and with cold solvents, on silica gel prewashed with cold ethyl ether, finally gave small amounts of the pure compound and spectral data could be obtained. The elucidation of its structure is largely based on the analysis of the ^{1}H NMR spectrum, which shows close similarities with those of the previously reported 7,8-dihydroguaianes (**2a** and **2b**) [2]. A major difference is the down-field shift of the ring protons H-3 and H-6 (see Scheme 1 for the numbering of the guaiane skeleton), which is in accordance with the genesis of a carbonyl function at C-15. In addition, the signal for H-15 of the alcohol **2a** (2H, δ 4.52) has been replaced by a singlet (1H) at 9.78, indicating that the hydroxymethyl group has been oxidized to an aldehyde. The mass spectrum shows the characteristic losses of a formyl, a methyl, and an isopropenyl group, and the UV and IR data also support the suggested structure **6**. However, an attempt to correlate delicial (**6**) chemically to the alcohol **2a** by potassium borohydride reduction failed, no isolatable product was obtained.

Table 1. The amounts (w w %) of lactarviolin (**1**) and deterrol (**7a**) in the hexane extracts of ground fruit-bodies of *L. deliciosus*, at various times after grinding

Time (min)	Lactaroviolin (1)	Deterrol (7a)
0.5	0.14	0.00
3	0.52	0.15
10	1.02	0.21
30	1.35	1.08
120	2.00	1.33

The same experiment with *L. deterrimus* gave similar results.



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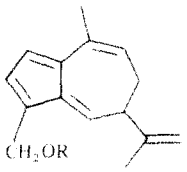
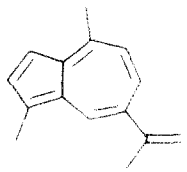


2a R = H
2b R = $CO(CH_2)_{10}Me$
2c R = $CO(CH_2)_3(CH{=}CHCH_2)_2(CH_2)_4Me$



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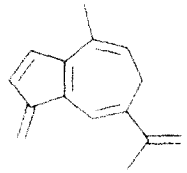

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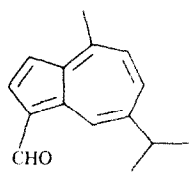

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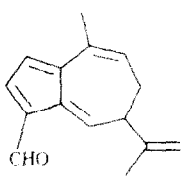

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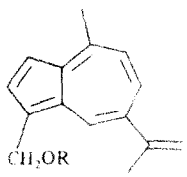
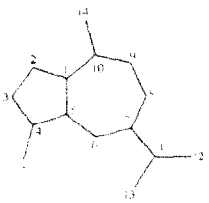


7a R = H
7b R = $CO(CH_2)_{10}Me$



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Scheme 1.

The spectral data of deterrol (**7a**), including ^{13}C NMR data and elemental analysis, were all in agreement with the suggested structure **7a**, and the reduction of lactaroviolin (**1**) with potassium borohydride in ethanol yielded an alcohol identical in all respects to deterrol (**7a**). The stearic acid ester **7b** has previously been isolated from fruit-bodies of *L. indigo* [8], and the ^1H NMR data reported for **7b** are very similar to those of deterrol (**7a**). The ester **7b** appears to be the major sesquiterpenoid originally present in fruit-bodies of this species, and it is responsible for the deep blue colour of their latex. All data of the lactaroviolin (**1**) isolated in this investigation were in agreement with those previously reported, and this also goes for the alcohol **2a** and lactarazulene (**3**). No traces of lactarofulvene (**4**) or the aldehyde **5** could be detected in this investigation, although very small amounts may have escaped our attention. An explanation of this apparent difference between specimens from different continents may be the existence of sub-species of *L. deliciosus* and *L. deterrimus*.

It is very interesting indeed to note both the similarities as well as the differences between the pungent *Lactarius* species and *L. deliciosus* and *L. deterrimus*. The fruit-bodies of each group originally contain fatty acid esters of a single sesquiterpene, and these esters are transformed to sesquiterpene aldehydes and alcohols as a response to injury. In both groups these conversions are assumed to be enzymatic, as they never have been observed *in vitro*. However, the sesquiterpenes of *L. deliciosus* and *L. deterrimus* have a guaiane skeleton, which is not formed by the same pathway as the types of sesquiterpenes found in the pungent *Lactarius* species. The formation and transformation of sesquiterpenes in the pungent *Lactarius* species is relatively easy to follow, and the biological activities (or lack of biological activities) of the precursor, the primary, and the secondary products strongly suggest that they constitute a chemical defense system. However, at this stage it is impossible to support or discard a similar hypothesis for the guaiane sesquiterpenes in the species investigated here. The instability of the compounds make it difficult to study the kinetics of their formation (and transformation?) in the injured mushroom tissue. In addition, it is essential to obtain more information about their biological activities (e.g. antimicrobial and antifeedant activities). Apart from the antibiotic activity of lactaroviolin (**1**) [9], little is known in this respect. This is surprising, not the least as *L. deliciosus* and *L. deterrimus* are very palatable and common mushrooms, and are consumed in large quantities every year.

EXPERIMENTAL

The fruit bodies were collected in the south of Sweden in September 1984 and 1985. Only young specimens that appeared unaffected by parasites were chosen. The investigation of the original sesquiterpene contents of the mushrooms was performed by freezing approximately 100 g of young, unaffected specimens in 1 l liquid N_2, which was brought to the collection site in a Dewar flask. Back in the laboratory, the frozen mushrooms were put in 500 ml hexane at $-20°$ in a turbomixer and thoroughly macerated. The hexane phase was rapidly filtered through Na_2SO_4 and immediately evaporated, and the extracts were stored at $-70°$. To simulate injury, 0.5–1 kg of fresh mushrooms were ground in a meatgrinder at room temperature (without the addition of an extraction solvent), whereupon, at intervals, 50g portions of the ground mushroom were extracted with 100 ml of freshly distilled hexane. The time elapsed between grinding and extraction was recorded for each portion, and the extracts were immediately evapd (protected from light) to dryness below room temperature and stored at $-70°$.

TLC was performed on 'Merck Kieselgel 60 F_{254}' silica gel plates (prewashed with Et_2O), developed with EtOAc–hexane or Et_2O–hexane mixtures. The HPLC analytical system was composed of a 20 × 0.5 cm column packed with 10μ LiChrosorb Si 60, and a Waters UV-detector (380 nm) connected to a Hewlett Packard HP 3390 integrator. The eluent in the HPLC experiments was 1–10% EtOAc in hexane and the flow rate 2 ml per min. Semipreparative HPLC separations were made with a 50 × 1.0 cm column packed with 10 μ LiChrosorb Si 60. Preparative low pressure separations were performed on 'Merck Kieselgel 60, (0.063–0.200 mm, water content 5%), 'Merck Lobar prepacked columns' silica gel columns and, for flash chromatography, 'Merck Kieselgel 60' (0.049–0.063 mm, water content 5%), all eluted with EtOAc–hexane mixtures. All preparative columns were prewashed with Et_2O and hexane.

^1H NMR: 300 MHz J are given in Hz. ^{13}C NMR spectra, proton noise-decoupled and coupled, 76 MHz. Chemical shifts: tetramethylsilane as internal standard. $CDCl_3$ [filtered through 'Merck Aluminiumoxid 90' (activity I) and Na_2CO_3] was used as solvent. Mps: uncorr.

1-*Formyl*-6,7-*dihydro*-4-*methyl*-7-*isopropenylazulene* (**6**) (*delicial*), 5 mg, was obtained as a yellow oil after repeated chromatography on silica gel. UV λ_{max}^{hexane} nm(log ε): 229 (4.06), 265 (3.98), 298 (3.71), and 435 (3.39); IR ν_{max}^{neat} cm^{-1}: 2940, 1670, 1480, 1395, 1365, 1300, 1220, 1030, 905, 830, 760; ^1H NMR: δ 9.78 (1H, *s*, H-15), 8.00 (1H, *dd*, $J_{3,6}=1$, $J_{6,7}=4.1$, H-6), 7.25 (1H, *dd*, $J_{2,3}=2.9$, $J_{3,6}=1$, H-3), 6.50 (1H, *d*, $J_{2,3}=2.9$, H-2), 5.82 (1H, *ddd*, $J_{8a,9}=7.3$, $J_{8b,9}=6.0$, $J_{9,14}=1$, H-9) 4.85 (1H, *m*, H-12a) 4.78 (1H, *m*, H-12b) 3.31 (1H, *ddd*, $J_{6,7}=4.1$, $J_{7,8a}=10.2$, $J_{7,8b}=3.2$, H-7), 2.60 (1H, *dddd*, $J_{7,8a}=10.2$, $J_{8a,8b}=16$, $J_{8a,9}=7.3$, $J_{8a,14}=1$, H-8a), 2.35 (1H, *ddd*, $J_{7,8b}=3.2$, $J_{8a,8b}=16$, $J_{8b,9}=6.0$, H-8b), 2.01 (3H, *m*, H-14), 1.81 (3H, *m*, H-13); EIMS (probe) 70 eV *m/z* (rel. int.): 212[M]$^+$ (100), 197[M − Me]$^+$ (25), 183[M − CHO]$^+$ (38), 169[M − C(Me)CH_2]$^+$ (86), 149 (74), 129 (67), 128 (69), 115 (82), 57 (81), 55 (72), and 43 (94); insufficient pure compound was obtained for ^{13}C NMR and elemental analysis.

1-*Hydroxymethyl*-4-*methyl*-7-*isopropenylazulene* (**7a**) (*deterrol*). 140 mg, was obtained as deep blue needles, mp 100–101°, by Al_2O_3 and silica gel chromatography. UV λ_{max}^{EtOH} nm (log ε): 239 (4.34), 290 (4.67), 370 (3.88); IR ν_{max}^{KBr} cm^{-1}: 3280, 2920, 1620, 1420, 1280, 1200, 1070, 1005, 890, 830, 780, 770; ^1H NMR: δ 8.67 (1H, *d*, $J_{6,8}=2.0$, H-6), 7.80 (1H, *d*, $J_{2,3}=3.9$, H-3), 7.68 (1H, *dd*, $J_{6,8}=2.0$, $J_{8,9}=9.0$, H-8), 7.29 (1H, *d*, $J_{2,3}=3.9$, H-2), 7.13 (1H, *d*, $J_{8,9}=9.0$, H-9), 5.35 (1H, *m*, H-12a), 5.21 (1H, *m*, H-12b), 5.14 (2H, *s*, H-15), 2.87 (3H, *m*, H-14), 2.28 (3H, *m*, H-13); ^{13}C NMR: δ 146.9, 146.3, 138.5, 135.7, 135.5, and 129.9 (*s*, C-1, C-4, C-5, C-7, C-10, and C-11), 135.9, 134.6, 132.6, 125.8, and 114.2 (*d*, C-2, C-3, C-6, C-8, and C-9), 114.4 (*t*, C-12), 58.6 (*t*, C-15), 24.3 and 23.2 (*q*, C-13 and C-14); EIMS (probe) 70 eV *m/z* (rel. int.): 212 [M]$^+$ (89), 210 (22), 196 (25), 195 (100), 179 (20), 165 (40), 152 (16), 128 (17), 57 (21), 41 (21), and 40 (66); Elemental analysis, found: C, 84.74; 7.78%. $C_{15}H_{16}O$ requires C, 84.87; H, 7.60%.

Lactaroviolin (**1**) (100 mg) was reduced to deterrol (**7b**) with $NaBH_4$ in EtOH. The reaction was monitored by TLC, and was completed in 10 min at room temp. The reaction mixture was diluted with Et_2O and filtrated through a short Al_2O_3 column, the solvent was evapd and the residue was subjected to silica gel chromatography. Deterrol (**7a**) (84 mg, 83%) was the only product obtained.

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