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The Chemical Basis of Hot-tasting and Yellowing of the Mushrooms *Lactarius chrysorrheus and L. scrobiculatus*+

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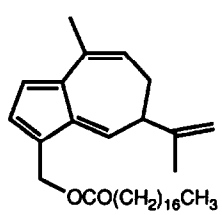
Abstract The chemical background behind yellowing and pungent taste of *Lactarius chrysorrheus* and *L scrobiculatus* has been investigated The intact fruit bodies originally contain a fatty acid ester of velutinal (*i e* compound **2b** and **2a**, respectively) as the only sesquiterpenoid When the fruit bodies are injured the esters are enzymatically converted into sesquiterpene furans, mono- and di-aldehydes, and lactones, which have been isolated and their structures elucidated These compounds have been submitted to conformational analysis by molecular mechanics and ^{1}H NMR in order to make correct stereochemical assignments The pungent taste of the fruit bodies of both species is due to a new dialdehyde, chrysorrhedial (**9**), while a new triene-enolactone (**8**) is involved in the change of the colour

INTRODUCTION

The mushrooms belonging to the genus *Lactarius* (family Russulaceae, Basidiomycotina) contain a milky-juice which can be observed when the fruit-bodies are cut or broken The colour and taste of this latex, as well as those of the flesh, can be different from species to species, a fact of great taxonomical relevance [1] The chemical background for such impressive diversities have been subjected to several investigations in the last two decades [2,3] It is now possible to give a general picture of the biochemical origin and fate of many sesquiterpenes and other secondary metabolites found in these mushrooms. A single compound, which can be specific for each species, for example the guaiane sesquiterpenoid **1** in *L deliciosus* Fr ,[4] velutinal ester **2a** in *L vellereus*,[5,6] phenol stearate **3** in *L fuliginosus* Fr ,[7] is originally present in intact fruit-bodies and can be isolated when mushrooms are worked up in carefully controlled conditions



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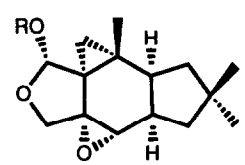
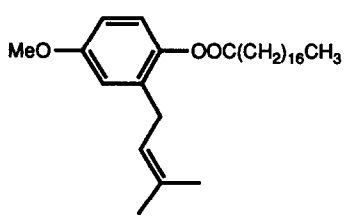


2a R = $CO(CH_2)_{16}CH_3$
2b R = $CO(CH_2)_4CO(CH_2)_{11}CH_3$



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These compounds are probably stored as fatty acid esters in the lipid layers of the cell membranes. In this way they are protected against the action of lipases and other enzymes[7,8] which would promote the cascade of chemical transformations of the original molecules when the mushrooms cells are disrupted A few mushroom enzymes display their activity very rapidly (from a few seconds to minutes), others probably remain active in injured samples for long periods of time and even in contact with organic solvents [7] This can explain the apparent discrepancies in the results reported from different laboratories, where different extraction and isolation processes were followed For example, some sesquiterpene furans and lactones (e.g. **7** and **5**), isolated in the past from *Lactarius* fruit bodies[2,3] and lately regarded with some skepticism as true metabolites,[9] have again been reconsidered as enzymatically formed.[10,11]

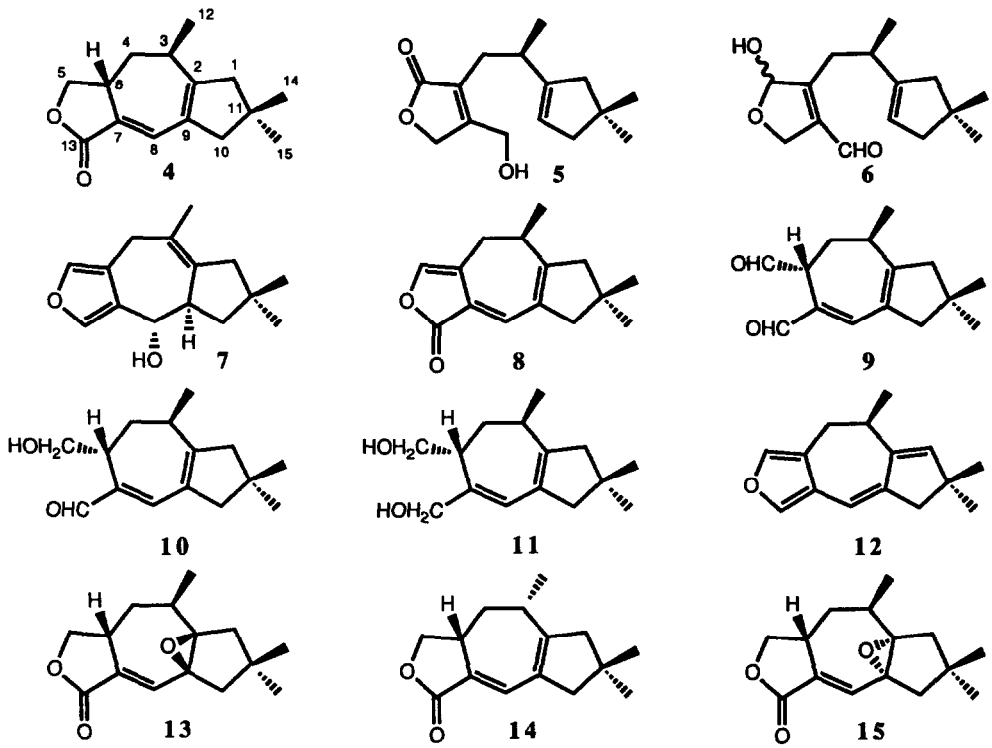
In this context we examined the secondary metabolites of intact and injured fruit bodies of *L chrysorrheus* Fr. and *L scrobiculatus* (Scop ex Fr) Fr. They belong to a relatively small group of inedible *Lactarius* species that have a latex and flesh which, as soon as the fruit bodies are broken, are white and tasteless, but turn yellow and bitter-acrid after a while.[12] The time for observing these variations of colour and taste depends on several factors, mainly on the species and the age and state of the fruit bodies For example, in young, fresh samples of *L scrobiculatus* the changes occur almost immediately while they take 1-5 minutes in *L chrysorrheus* [13] In old specimens yellowing and pungency are much less marked Moreover, the colour fades considerably while the latex drops dry up on the surface of injured specimens Nothing is known about the nature of the molecules involved in these transformations

We isolated in the past a great number of lactarane, secolactarane and norlactarane sesquiterpenes from *L scrobiculatus*,[14-18] however the labile acrid and coloured compounds escaped our investigations In this paper we report our findings on the sesquiterpenes of *L chrysorrheus*, a common mushroom of many Italian forests, and then extend our results to *L scrobiculatus*

RESULTS AND DISCUSSION

In order to compare our results with those reported for similar studies on other *Lactarius* species,[4,7,9-11] we adapted the procedure already followed for *L vellereus* [9] Only young specimens of *L chrysorrheus* that appeared undamaged by parasites were collected To simulate injury, the mushrooms were minced without the addition of solvent and extracted with hexane at room temperature A complete series of extractions was made at different times after injury and the extracts were analysed by TLC and UV spectroscopy In addition, at the same times, few drops of the milky-juice were collected with a capillary tube, suspended in CH_2Cl_2 and rapidly analyzed by TLC This chemical analysis was performed while simultaneously tasting the mushroom's flesh and latex

Only a single compound, the lipophilic velutinal lactarinic acid ester (**2b**)[6] was found in a specimen of tasteless and colourless latex immediately collected after breakage of the fruit bodies [19] The compound was identical with an authentic sample Surprisingly, neither stearoylvelutinal (**2a**)[5,6] nor other velutinal fatty acid esters[20] could be detected About 5-10 min after injury the taste became extremely acrid and pungent, while the latex turned a nice brilliant yellow colour At the same time a hexane extract showed an intense absorption at 360 nm, well estending over the visible region TLC analysis revealed no significant amount of ester **2b**, instead the major components were found to be a non polar yellow compound and other colourless spots detectable only after spraying with vanillin-H_2SO_4 solution [17] We identified lactaroscrobiculide A (**4**),[15,17] blennin C (**5**),[15,21] lactardial (**6**),[10,11,22] furanol **7**[3] (identified by their spectral data and comparison with authentic samples) and three new lactarane sequiterpenes chysorrhelactone (**8**), chrysorrhedial (**9**) and chrysorrheal (**10**) [23] The thermal and photochemical lability of the last three compounds required them to be handled in the dark and their spectral data recorded as soon as possible after chromatographic separation Triene **8** resulted particularly unstable as a neat liquid and had to be always kept in a solution of aprotic solvent, however, even in a benzene solution at -22 °C in the dark, it decomposes within a few days to a white insoluble material



The structures **8-10** were assigned mainly on the basis of ^1H and ^{13}C NMR measurements, including NOE and selective decoupling experiments and two-dimensional techniques (homo- and hetero-nuclear COSY)

A comparison of the NMR data of **9** and **10** with those of **4** (Tables 1 and 2) suggested that the three compounds are very similar and proved the existence of the following molecular fragments in all three a 4,4-dimethylcyclopentene ring, a pentasubstituted diene system containing the only olefinic proton (at δ 6 81 in **9** and at δ 6 61 in **10**) in β position to a C=O group, one -$CH(CH_3)CH_2CH$- fragment attached to an olefinic quaternary carbon. By using these fragments we established the lactarane skeleton for both **9** and **10** The mass spectrum of **9** displayed a molecular peak at m/z 232 which, along with the NMR data, is consistent with the formula $C_{15}H_{20}O_2$ In the IR spectrum strong bands at 1717, 1670 and 1588 cm^{-1} indicated different kinds of carbonyl functions, which were identified as two formyl groups by the characteristic signals in the ^1H (Table 1 and Fig. 1) and ^{13}C NMR spectra Therefore, dialdehyde **9** must have one CHO group attached to C-6 and the other linked to C-7 UV absorption at 313 nm fully supported the dienal system.

The mass spectrum of **10** showed a molecular peak at m/z 234, two units more than **9**, while IR, UV and NMR spectra confirmed the presence of the dienal function In addition, COSY cross peaks and decoupling experiments proved the existence of a CH_2OH group attached to the allylic methine carbon C-6 Thus, compound **10** is the 5-H derivative of **9**

Although we expected that 1,4-dialdehyde **9** and γ-hydroxyaldehyde **10** could be in equilibrium with the corresponding hemiacetal forms (**16** and **17**, respectively) no significant amount of a cyclic product is observed in the NMR spectra However, cat *p*-TsOH in EtOH completely converted compound **10** into O-ethyl acetals **18**, confirming the relative position of the OH and CHO groups in **10** Furthermore, the ^1H NMR spectrum of **9** shows small signals at δ 9 62 (d, J 1 2 Hz) and 9 41 (s), which were attributed to the C-6 epimeric aldehyde 6-*epi*-chrysorrhedial (*ca* 12%)

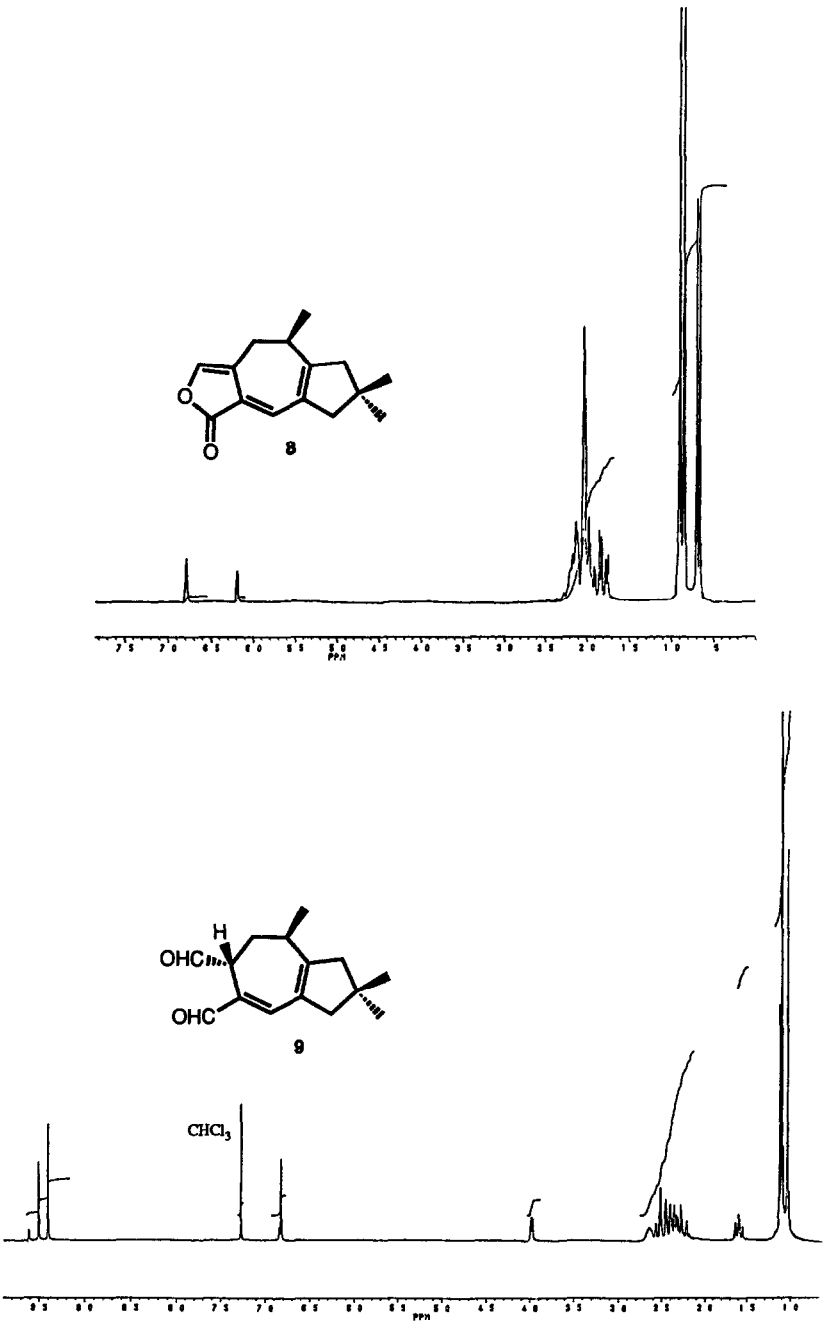


Fig 1 - ^1H NMR spectra of chrysorrhelactone (**8**) and chrysorrhedial (**9**)

Table 1 ^{1}H NMR Spectral Data for Compounds **4**,°+ **8**,§* **9**,°+ **10**,§+ and **11**°+ (δ_H values in ppm from TMS)

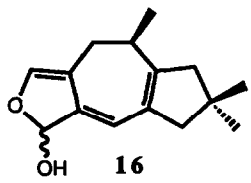
Proton	4	8	9	10	11	J (Hz)	4	8	9	10	11
H-1	2 22 d[a]	z	2 25 d[a,b]	2 25 d[a,b]	2 07 d[a]	1,1'	17 2	u	17.5[b]	u	16 5
H-1'	2 56 d[a]	z	2 46 d[a,c]	2 56 d[a,c]	2 38[r]	10,10'	15 2	u	14 5[b]	u	14 0
H-3	2 62 m	2 15 m[r]	2 63 m	2 58 m	2 38[r]	4α,4β	13 5	14 9	14 2	14 0	14 0
H-4α	1 67 ddd	1 80 dd	2 35 ddd	2 07 ddd	1 95 ddd	4β,6	3 0	-	3 0	2 0	1 8
H-4β	1 78 dt	2 10 m[r]	1 59 ddd	1 49 ddd	1 62 ddd	4β,3	3 0	u	10 5	11 5	11 7
H-5	4 56 t	6 18 bs	9 50 s	3 56 m	3 67 dd	4α,6	11 5	-	6 5	6 0	6 5
H-5'	3 81 t	-	-	3 56 m	3 73 dd	4α,3	3 6	5 1	4 5	4 3	5 0
H-6	3 31 m	-	3 98 dd	3 29 tdd	2 68 dddd	5,5'	8 8	-	-	u	10 0
H-8	6 86 d	6 80 bs	6 81 s	6 61 s	5 76 s	5,6	8 8	-	0	7 2	5 2
H-10	2 36 d[a]	z	2 37 d[a,b]	2 31 d[a,b]	2 15 d[a]	5',6	8 8	-	-	7 2	8 7
H-10'	2 45 d[a]	z	2 53 d[a,c]	2 49 d[a,c]	2 38[r]	6,8	3 2	-	-	-	-
H-12	1 14 d	0 69 d	1 12 d	1 10 d	1 02 d	3,12	7 0	7 0	7 0	7 0	7 0
H-13	-	-	9 40 s	9 30 s	4 03 bd	13,13'	-	-	-	-	12 0
H-13'	-	-	-	-	4 12 d						
H-14	1 07 s[b]	0 86 s[b]	1 04 s[e]	1 04 s[e]	1 01 s						
H-15	1 08 s[b]	0 91 s[b]	1 11 s[e]	1 11 s[e]	1 07 s						

° 300 MHz, § 250 MHz, + $CDCl_3$ solution, * C_6D_6 solution,
[a] each line further splitted by long range couplings, [b, c, e] assignments in the same vertical column may be interchanged,
[u] undetermined, [r] overlapping signals, [z] overlapping ABq at *ca* 2 05

Table 2 ^{13}C NMR Spectral Data[#] for Compounds **4**,°+ **8**,°* **9**,§+ and **10** °*

Carbon	4	8	9	10
C-1	54 2 (2)[a]	54 6 (2)[a]	53 3 (2)[a]	53 3 (2)[a]
C-2	154 2 (0)	156 7 (0)	157 3 (0)	155 7 (0)
C-3	34 8 (1)	33 7 (1)	34 0 (1)	33 1 (1)
C-4	32 5 (2)	29 4 (2)	32 1 (2)	34 1 (2)
C-5	70 5 (2)	140 8 (1)	199 9 (1)	62 6 (2)
C-6	35 6 (1)	119 1 (0)	47 5 (1)	37 4 (1)
C-7	128 9 (0)[b]	130 3 (0)	137.8 (0)	143 3 (0)
C-8	131 5 (1)	132 2 (1)	147 8 (1)	145 9 (1)
C-9	128 5 (0)[b]	126 8 (0)	129 2 (0)	129 1 (0)
C-10	52 6 (2)[a]	51 6 (2)[a]	52 4 (2)[a]	52 8 (2)[a]
C-11	36 9 (0)	37 0 (0)	36 5 (0)	36 3 (0)
C-12	21 7 (3)	16 4 (3)	19 6 (3)	15 4 (3)
C-13	172 1 (0)	168 8 (0)	192 6 (1)	193.5 (1)
C-14	29 1 (3)	29 0 (3)[b]	29 0 (3)[b]	29 2 (3)[b]
C-15	29 1 (3)	29 1 (3)[b]	28 8 (3)[b]	28 9 (3)[b]

[#] The number (in parentheses) of protons attached to each carbon was determined by DEPT experiments, ° 62.5 MHz, § 75 5 MHz, + $CDCl_3$ solution, δ_c values in ppm relative to $CDCl_3$ at 77 0, * C_6D_6 solution, δ_c values in ppm relative to C_6D_6 at 128 0,
[a, b] assignments in the same vertical column may be interchanged.



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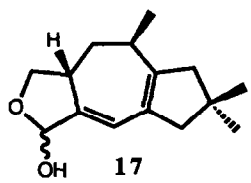

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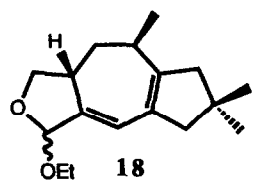

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Compound **4**, **9** and **10** have the same absolute configuration, as shown in the formulae Irradiation of H-6 in lactone **4** gave a NOE effect at H_3-12 (1.5%) and H-5 (5.6%) and irradiation of H_3-12 gave NOE effects at H-6 (8%) and H-1β (5.5%). Thus, H-6 and CH_3-C(3) are *cis* positioned on the cycloheptadiene ring The same relationship between these hydrogens is maintained in compounds **9** and **10**, notwithstanding the different molecular conformations (*vide infra*). In fact, hydride reduction of **4**, **9** and **10** afforded the same diol **11** with identical optical rotation. A CD measurement of **11** gave a positive Cotton effect ($\Delta\varepsilon$ +1.12) for the $\pi\rightarrow\pi^*$ transition of the diene system. According to the diene helicity rule[24] this indicates a positive skewness of the chromophore in **11** and allowed to establish the absolute configuration of the diol (and indirectly of **4**, **9**, **10**) by inspecting Dreiding models of the preferred conformation **11A** (Fig. 2), established by molecular mechanics.

The mass spectrum of compound **8** showed a strong molecular peak at m/z 230 which is consistent with the molecular formula $C_{15}H_{18}O_2$ The latter was confirmed by hydrogens and carbon counting from the NMR spectra IR bands at 1760 (s), 1675 (m), 1615 (m) and 1565 indicated the presence of an unsaturated γ-lactone and conjugated double bonds, identified by NMR signals as one tetrasubstituted and two trisubstituted olefins Comparison of the NMR data of **8** with those of **4** (Tables 1 and 2) proved that the former contains a further unsaturation in the furanone ring, while the remaining part of the two molecules are identical. The strong absorption of sesquiterpene **8** at 370 4 nm was that expected for the cross-conjugated dienone-triene chromophore and is responsible for the yellow colour and photolability of this compound Although only biosynthetic reasons (*vide infra*) support the absolute stereochemistry of the C-3 stereocenter, this should be the same as in sesquiterpenes **4**, **9** and **10**

The constant finding of furanol (**7**), blennin C (**5**) and lactardial (**6**) in different batches of *L chrysorrheus* confirmed that they are true metabolites of this mushroom and are formed enzymatically.

Investigation on *L scrobiculatus* was carried out by the same procedure followed with *L chrysorrheus*, but in this case fruit bodies had to be extracted *ca* one minute after the injury, as yellowing is much more rapid. We confirmed earlier findings[20] that intact fruit bodies contain only stearoyl velutinal (**2a**), accompanied by traces of other unsaturated fatty acid esters of velutinal. From the extracts of injured mushrooms we isolated, in addition to compounds **4-6**, **8-10**,[23] small amounts of furanodiene **12** This has already been obtained by synthesis,[22,25] but has never been described before as a natural product. Although furanolactarane sesquiterpenes must always be considered with some caution as true metabolites of *Lactarius* mushrooms,[22] it is likely that, like furanol (**7**) and secolactaranes **5** and **6**, diene **12** is formed directly from the velutinal esters by a secondary route (*vide infra*)

Besides **12**, the presence of other furanoid sesquiterpenes in *L scrobiculatus* must be regarded as highly uncertain, even if traces [for example of furanol (**7**)] could have escaped our present investigation This contrasts dramatically with the large amounts of furans isolated from previous extracts of the same mushroom.[15,17] Therefore, incorrect extraction and isolation methods possibly caused the "unnatural" formation of these compounds

Epoxide **13**, isolated in the past from *L scrobiculatus*,[14] was not found during this investigation, even in extracts made one hour after injury Therefore, because of the easy oxidizability in air of lactone **4** to compound **13**, the latter is strongly suspected of being an artefact Interestingly, MCPBA oxidation of **4** to **13** is completely diastereoselective

Conformational Analysis of Lactarane Sesquiterpenes 4, 9-11, 13

A number of stereochemical problems associated with sesquiterpenes **4**, **9-11**, and **13** required a careful conformational analysis of these compounds. In fact, the presence of a cycloheptadiene ring confers them a certain degree of conformational flexibility which cannot be quantitatively evaluated by simple inspection of Dreiding models. Therefore, in order to make correct stereochemical assignments, we related the observed ^{1}H NMR data with the results of molecular modeling performed using the MM2 program.[26]

To confirm the relative configuration of lactaroscobiculide A (**4**), inferred by the NOE experiments (see above), the conformational space of the stereostructure **4** and of its diastereoisomer **14** was explored. Besides a certain degree of conformational mobility due to the cycloheptadiene ring (ring B), the cyclopentene ring (ring A) introduces additional flexibility limited, however, only to the interconversion of the ^{11}E and E_{11} forms due to the sp^2 hybridization of the carbon atoms at the ring fusion On the contrary, the lactone ring (ring C) does not deserve special attention, as its conformation is dictated by the conformation of the adjacent ring B

The conformational search of compounds **4** and **14** made large use of the single and double driver option of the MM2 program extensively applied to the torsional angles of ring B After location of all the conformers deriving from the puckering of this ring, single driving of a proper torsional angle inside the cyclopentene ring inverted its envelope conformation and doubled the number of the local minima. Table 3 reports the relative energies, the equilibrium percentages and selected torsional angles of the conformers of **4** and **14** found in a range of 5 kcal/mol above the global minimum Only conformers **4A,B** and **14A,B** contribute to the overall populations, all the other conformers are high energy practically unpopulated local minima. It is worthy pointing out that the four populated conformers present the same geometry in the rings B and C as can be seen by the values almost identical of the torsional angles describing the geometries; each **A,B** couple of conformers differ in the conformation of ring A as can be seen by the opposite value of the torsional angle (C-1–C-11–C-10–C-9)

Table 3 Relative Energies (kcal/mol), Equilibrium Percentages and Selected Torsional Angles (degrees) for the Conformers of Lactaroscrobiculide A (**4**), its Epimer **14**, and the Epoxides **13** and **15**

Conf	E_{rel}	%	C-2–C-9–C-8–C-7	C-2–C-3–C-4–C-6	C-3–C-4–C-6–C-7	C-1–C-11–C-10–C-9
4A	0 00	53 2	-5	66	-75	-28
4B	0 08	46 8	-6	66	-74	27
4C	4 53	<0 1	-40	-36	-39	-27
14A	0 00	80 1	-10	62	-79	-28
14B	0 83	19 8	-8	67	-77	26
14C	4 24	<0 1	-39	-21	-50	-27
14D	4 78	<0 1	-29	-68	19	-26
14E	4 81	<0 1	-40	-18	-52	24
13A	0 00 [a]	77 8	-13	60	-79	-24
13B	1 02	13 9	-10	59	-80	33
13C	1 66	4 7	-45	-46	-25	-25
13D	1 90	3 1	-39	-63	0	-25
13E	3 35	0 3	-49	-47	-26	35
13F	3 77	0 1	-43	-64	-2	35
15A	0 00	98 2	-48	-40	-39	24
15B	2 53	1 4	-5	68	-73	24
15C	3 41	0 3	-6	69	-72	-31
15D	4 05	0 1	-39	-69	6	-26

[a] 2 70 kcal/mol relative to **15A**

Table 4 Calculated ^{1}H NMR Vicinal Coupling Constants J (Hz) of Compounds **4**, **9-11**, **13-15**

Compound	4β,3	4α,3	4β,6	4α,6	5,6	5',6
4	3 5	2 7	1 7	11 9	8 7	9 8
14	2 7	12 3	1 4	11 5	9 1	9 2
13[a]	3 2	3 9	2 0	11 1	8 6	9 3
15	11 3	5 7	6 8	10 7	3 6	8 9
9	11 1	4 5	1 4	6 8		
10	12 0	3 4	1 3	6 3		
11	12 0	4 3	1 2	6 5		

[a] Observed vicinal coupling constants of compound **13** [14] 4β,3 5 0, 4α,3 3 0; 4β,6 3 5, 4α,6 11 5, 5,6 8 5, 5',6 9 0

The ^{1}H NMR vicinal coupling constants for the hydrogen atoms at C-3, C-4, C-6, and C-5 were then calculated[27] for each diastereoisomeric lactone as weighted averages of the values of each conformer and are reported in Table 4. The close agreement of the experimental data (Table 1) with the values calculated for **4** ensures that the tricyclic lactone has the relative configuration indicated by the formula **4**. Fig 2 reports the three-dimensional plots of conformers **4A** and **4B**; it can be easily seen that H-6 is close to H_3-12 [the distance between H-6 and C-12 is 2.72 Å (2 73 Å) in **4A** (**4B**)] as was suggested by NOE experiments.

The choice between configurations **13** and **15** for the 2,9-epoxide of lactaroscrobiculide A (**4**),[14] was made in a similar way. Owing to the strict similarity of **13**,**15** to **4**,**14** the strategy for the conformational search was the same as above described for the latter compounds. an extensive conformational search in ring B followed by envelope inversions of ring A The conformational behavior of **13** was shown to be quite different from the behavior of **15**, so ensuring the possibility to distinguish them by spectroscopy While the most populated conformations **13A**,**B** of epoxide **13** (which account for about 92% of the overall population) have a geometry of ring B closely resembling that of **4A**,**B**, the global minimum **15A** of epoxide **15** (which accounts for 98% of the overall population) was found completely different from them (see Table 3 and Fig 2) Very different patterns of ^{1}H NMR vicinal coupling constants could, therefore, be calculated for **13** and **15** (Table 4), only the data of **13** well fit the experimental data ensuring that structure **13** correctly describes the product of oxidation of **4** It is worthy pointing out that, as the energy of conformer **15A** is calculated 2.70 kcal/mol lower than that of **13A**, the epoxidation process is confirmed to be under kinetic control Quite surprisingly, the apparently more hindered face of the C-2–C-9 double bond is shown more easily accessible by the oxidizing agent.

The three bicyclic compounds **9**, **10**, and **11** will be discussed together as their conformational behavior is very similar In these cases the flexibility of rings A and B is expected to be higher than in the previously studied compounds due to the absence of ring C, moreover, hydroxymethyl and formyl groups produce other conformational freedom owing to rotation around single bonds Therefore, the strategy of conformational search applied above for **4**,**13-15** was completed by a full analysis of the rotamers deriving, in the case of the hydroxymethyl groups, from the rotation of the oxygen atom around the (O–)C–C bond and the rotation of the hydrogen atom around the (H–)O–C bond (3x3 rotamers), analogously, when formyl groups are present, rotation around the (O=)C–C bond has to be considered giving rise to two rotamers when the formyl group is linked to the C-7 sp^2 carbon atom or to three rotamers when it is linked to the C-6 sp^3 carbon atom

Each conformer of diol **11** presents, therefore, a cluster of 81 (3x3x3x3) conformations deriving from different orientations of the two hydroxymethyl groups, this figure becomes 18 (3x3x2) for hydroxyaldehyde **10** and 6 (3x2) for dialdehyde **9**. The energy of all the conformers in each cluster have been calculated, however, as rotation of the side groups does not significantly affect the geometry of the ring moieties, only the conformers with the lowest energy in each cluster have been reported in Table 5

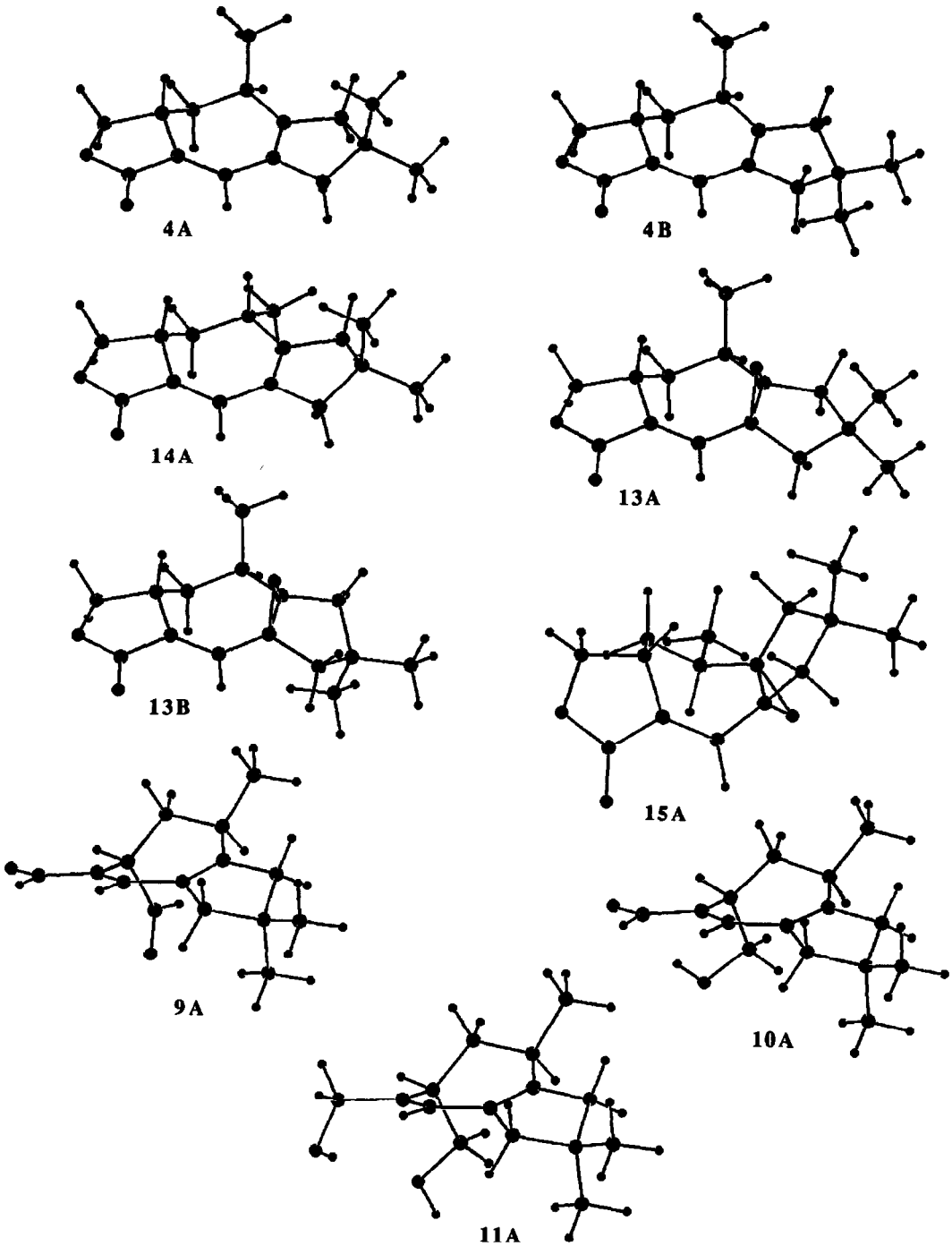


Fig 2 Three-dimensional plots of the most populated conformations of compounds **4**, **9-11**, **13-15**

Due to the absence of ring C in **9-11** the flexibility of ring B was found higher than in **4,13-15**, several local minima were located in a range of 5 kcal/mol above the global minimum. However, the global minimum, and the related second minimum (which differs from it only in the conformation of ring A) account for a very high percentage of the overall population. 87%, 96%, and 98% for **9**, **10**, and **11**, respectively. The geometry of the global minima (Fig. 2) is completely different from that in **4** and **13**, in fact, the orientation of the 3-methyl group is, in **9-11**, pseudo-equatorial, in contrast to the pseudo-axial orientation found in **4** and **13** The weighted averages of the vicinal coupling constants well fit the experimental data ensuring the calculated conformations be a good representation of the solution conformations, moreover, the NOE observed in **11** on irradiation of H-6, i.e the enhancements of H-5 (3 5%) and H-13 (2 6%), are explained by the calculated r^{-6} averaged distances H-6–H-5 (2 62 Å) and H-6–H-13 (2.55 Å).

Analysis of the preferred orientations of the formyl groups in dialdehyde **9** showed a marked preference (by about 3 kcal/mol) of the 7-formyl group for an orientation of the carbonyl oxygen anti to C-8. Though rotation around the C-5–C-6 bond is easier than around the bond C-7–C-13, the two orientations showing H–C-6–C-5–H angles of 108° and -113° were preferred by about 1 kcal/mol over the third one showing a

Table 5 Relative Energies (kcal/mol), Equilibrium Percentages and Selected Torsional Angles (degrees) for the Conformers of Chrysorrhedial (**9**), Chrysorrheal (**10**), and Chrysorrhediol (**11**)

Conf	E_{rel}	%	C-2–C-9–C-8–C-7	C-2–C-3–C-4–C-6	C-3–C-4–C-6–C-7	C-1–C-11–C-10–C-9	C-5–C-6–C-7–C-13
9A	0 00	73 4	21	-51	87	30	-105
9B	0 99	13 8	29	-40	84	-26	-107
9C	1 74	3 9	14	81	-61	26	-47
9D	1 75	3 8	15	80	-60	-27	-48
9E	2 28	1 6	39	18	55	24	-118
9F	2 51	1 1	-23	54	-90	27	7
9G	2 51	1 1	-16	59	-88	-28	-1
9H	2 70	0 8	42	20	54	-25	-119
9I	2 99	0 5	-43	-36	-45	-27	22
9J	3 78	0 1	-45	-37	-43	22	23
10A	0 00	81 0	16	-56	83	29	-95
10B	1 02	14 5	16	-58	83	-27	-95
10C	2 16	2 1	20	81	-50	27	-56
10D	2 25	1 8	21	81	-51	-26	-57
10E	3 55	0 2	-24	52	-92	27	9
10F	3 65	0 2	-20	57	-92	-28	4
10G	3 91	0 1	-43	-36	-45	-27	20
10H	4 34	<0 1	38	33	39	24	-108
10I	4 72	<0 1	-45	-37	-43	22	21
10J	4 79	<0 1	40	30	43	-25	-113
11A	0 00	81 4	21	-51	85	29	-107
11B	0 95	16 4	25	-49	85	-26	-109
11C	2 69	0 9	16	82	-57	26	-51
11D	2 77	0 8	15	80	-60	-27	-49
11E	3 63	0 2	38	31	41	23	-115
11F	3 73	0 1	-10	66	-84	-28	-17
11G	3 81	0 1	-15	62	-87	27	-12
11H	4 04	0 1	41	29	43	-23	-115
11I	4 31	<0 1	-43	-37	-44	-27	24

value of 6° for the same torsional angle. On these basis the absence of a detectable coupling between H-5 and H-6 in the ^{1}H NMR spectrum can be explained by the almost perpendicular orientation of the two C–H bonds

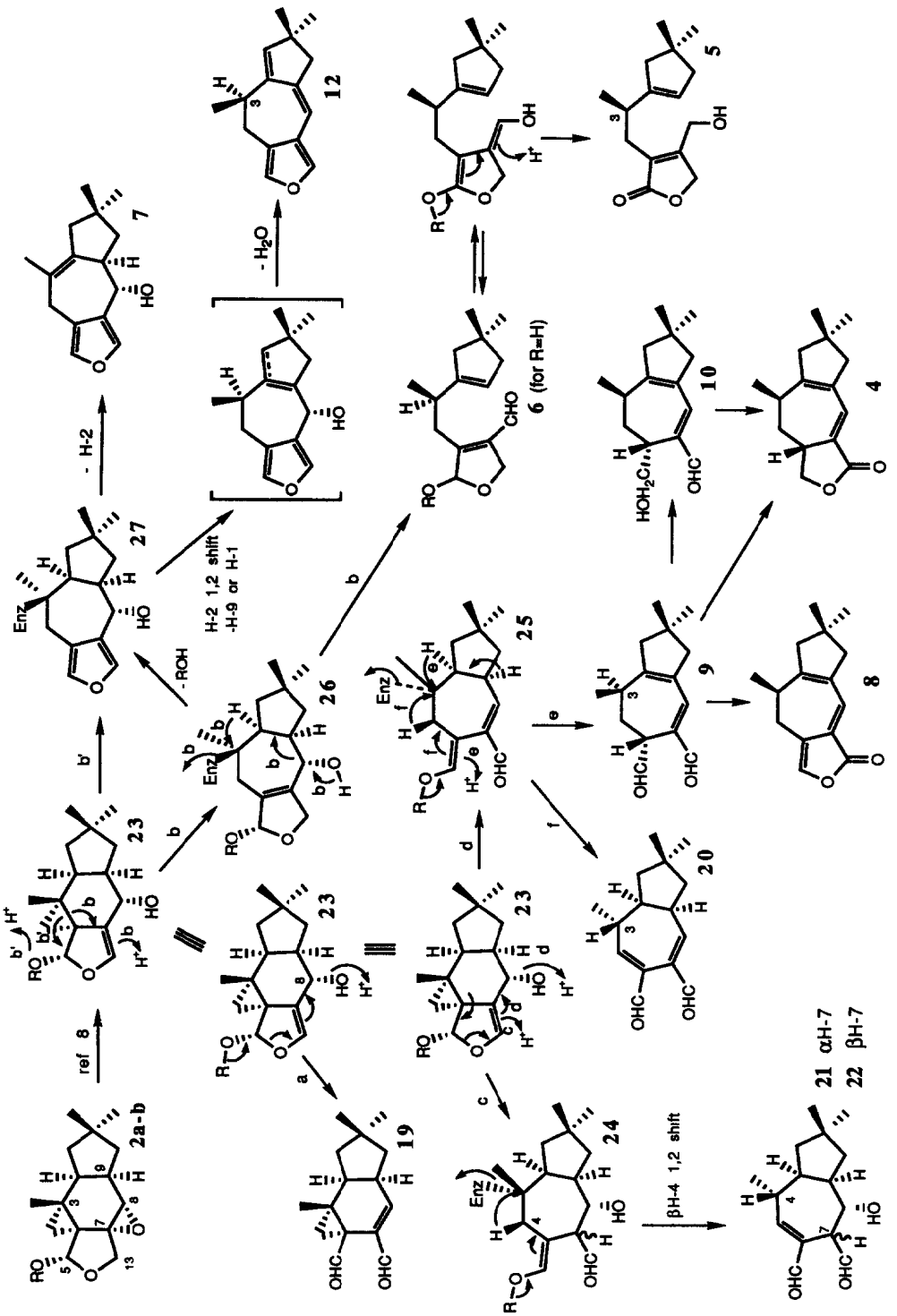
The ^{1}H NMR spectrum of chrysorrheal (**10**) did not indicate the presence of any detectable amount of hemiacetalic tricyclic form (**17**). Inspection of conformer **10A** shows a somewhat divergent orientation of the C-5–C-6 and C-7–C-13 bonds (the torsional angle C-5–C-6–C-7–C-13 is -95°), ring closure to hemiacetal would require a severe conformational rearrangement at a high energetic cost; this fact, added to the cost for the loss of the resonance energy deriving from the conjugation of the carbonyl group to the C-7–C-8 double bond, makes improbable the cyclization to **17** Moreover, the pseudo-axial orientation of the C-5–C-6 bond in **10** and **11** is confirmed by the proximity of H_2-5 to H-3 (see conformers **10A** and **11A** in Fig 2) as demonstrated by the NOE enhancements of H_2-5 on irradiation of H-3 in **10** (2.3%) and **11** (2 4%)

Biological Significance of Sesquiterpenes 4, 6, 8-10

The formation of triene lactone **8** is involved in the yellowing of the latex and flesh of *L scrobiculatus* and *L chrysorrheus* shortly after an injury of the fruit bodies Chrysorrhedial (**9**) is extremely pungent and, along with lactardial (**6**),[10] appears to be responsible for the pungency of these mushrooms Lactaroscrobiculide A (**4**) and chrysorrheal (**10**) are bitter and slightly astringent, but not acrid. Despite these similarities, the compounds are formed by specific enzymatic processes from two different velutinal precursors **2a** in *L scrobiculatus* and **2b** in *L chrysorreheus* Moreover, with respect to triene **8**, the relative amount of dialdehyde **9** is higher in the former than in the latter species, while lactone **4** is more abundant in *L chrysorrheus* This confirms earlier findings[9-11] that the pattern of sesquiterpenes produced by each *Lactarius* species differs from one species to another and can be a chemotaxonomical marker Unfortunately, a complete quantitative analysis of the sesquiterpenes of *L chrysorrheus* and *L scrobiculatus* as a function of time between grinding and extraction, similar to that made for *L vellereus*[9] was impossible to perform. The rapidity of the initial enzymatic reactions in injured mushroom tissues and the instability of many compounds made it difficult to study the kinetics of their formation and to isolate possible intermediates. However, at least in *L chrysorrheus* where changes occur less rapidly, compounds **8**, **9** and **10** appear in the extracts almost simultaneously (within 80-90 sec from the injury), while lactone **4** seems to be formed slightly later Along with small amounts of **5**, **6** and **10** the latter becomes the main compound in the (colourless) extracts made *ca* 1/2 h after grinding By this time the pungent dialdehyde **9** and the yellow trienelactone **8** have practically disappeared Together with these chemical changes, the peppery taste of the mushroom turns bitter and the yellow colour fades away considerably. Compared with *L chrysorrheus*, disappearance of lactone **8** is faster in *L scrobiculatus*, while lactaroscrobiculide A (**4**) increases less rapidly

When the fruit bodies of *L scrobiculatus* were cut in the dark and under an inert (N_2) atmosphere, yellowing of the flesh and latex occurred as promptly as in the air. This experiment demonstrates that atmospheric free oxygen is not directly involved, but specific enzymes in the mushrooms promote the oxidation of substrates **2a-b** to compound **8**

In several aspects the chemical behaviour of *L scrobiculatus* and *L chrysorrheus* is similar to that of other pungent Russulaceae species with permanent white latex.[9-11,20] In fact, in injured tissues of these mushrooms hot tasting sesquiterpenoid unsaturated dialdehydes [*e g* isovelleral (**19**), velleral (**20**), piperdial (**21**) and *epi*-piperdial (**22**)] are rapidly formed by specific enzymatic routes from fatty acid esters of velutinal (**2a-b**) These potent antimicrobial compounds are then reduced to the corresponding less active and non pungent 13-hydroxyaldehydes (*e g* isovellerol, vellerol, piperalol and *epi*-piperalol). It has been suggested that all together these sesquiterpenes constitute a chemical defense system against parasites and predators [9,28] It is likely that in *L chrysorrheus* and *L scrobiculatus* chrysorrhedial (**9**) and, in minor extent, lactardial (**6**) act as the deterrent compounds, like the other unsaturated dialdehydes **19-22**, while blennin C (**5**) and lactaroscrobiculide (**4**), more than chrysorrheal (**10**), seem to be the harmless end products. Interestingly, the deterrent activity decreases in "white juice" *Lactarius* species by reduction of the C-13 carbonyl, instead in "yellow juice" species by reduction of the C-5 formyl group. Lactone **4** can be formed either by oxidation of



Scheme 1 Proposed biosynthetic routes to compounds **4-10, 12, 19-22**

C-13 in chrysorrheal (**10**) or by a Cannizzaro-type reaction in dialdehyde **9**, as the conversion **6** → **5** C-13 oxidation of compound **9** leads directly to chrysorrhelactone (**8**). The rapid formation of a large amount of lactones **4** and **8** shortly after an injury to the fruit bodies indicates that *Lactarius* species secreting a yellowing latex are endowed with an array of oxidizing enzymes much more efficient than species with permanent white latex In the latter mushrooms, indeed, the routes leading to sesquiterpene lactones are still debated.[11]

Lactaroscrobiculide A (**4**) shows no antibacterial activity, while both chrysorrhedial (**9**) and chrysorrheal (**10**) are active against *Bacillus subtilis* and *Staphylococcus aureus* (Kirby-Bauer test). Citotoxicity was tested by bioessaying on *Artemia salina* (brine shrimp essay) [29] The LD_{50} (μg/mL) values (95% confidence) found for **4**, **9**, and **10** were 12.9 (8 4-19.6), 15 7 (9 6-25 7), and 43 6 (24 3-76 8), respectively The instability of chrysorrhelactone **8** made these biological activity tests unreliable, thus its role in mushrooms remains unknown

Biosynthetic Routes to Sesquiterpenes 4-10, 12, 19-22

The enzymatic mechanism of velutinal ester conversion in injured *Lactarius* fruit bodies are poorly studied However, the co-occurrence of sesquiterpenes with related structures in the same mushroom has suggested possible biosynthetic routes.[18,30,31] The finding of specific metabolites patterns indicates that parallel enzymatic pathways must have evolved in each species with small modifications of the individual enzymes pool Stereochemical details of isolated sesquiterpenes are obviously important in searching for possible biosynthetic intermediates The absolute configuration indicated in the formulae **2a-b**, **4-13**, **19-22** were established by CD studies (this paper and ref. 32), enantioselective synthesis of isovelleral (**19**)[33] and chemical correlations [2,8,22,25] It is well established that lactarane and secolactarane sesquiterpenes can have either absolute configuration at C-3 (compare **9** with **20**) As the C-3 stereocenter is a quaternary carbon in velutinal esters **2a-b**, this would depend on which side stereospecific H addition to C-3 occurs Therefore the stereochemistry at this center in each compound, compared to that of other stereocenters in the same molecule, may reveal important clues for the conversion of the marasmane bicyclo[4 1 0]heptane moiety into the lactarane seven membered ring and may suggest further key intermediates

In Scheme 1 we propose a comprehensive picture of the possible pathways leading to sesquiterpenes **4-10**, **12**, and the pungent related dialdehydes isovelleral (**19**), velleral (**20**), piperdial (**21**) and *epi*-piperdial (**22**) As velleral is not formed via isovelleral (**19**),[8] at least four parallel routes stream from the velutinal esters **2a-b** to other *Lactarius* sesquiterpenes one to isovelleral (**19**), another to the velleral (**20**) - piperdial (**21-22**) group, another to chrysorrhedial (**9**) and related compounds and the last one to secolactaranes and furanoid sesquiterpenes The mechanism of the latter enzymatic conversion may well be very similar to that of the non-enzymatic degradation [22,30] It is likely that the epoxide ring is the initial site of reaction, with more or less simultaneous enzymatic ester hydrolysis. This is suggested by the extreme ease of spontaneous hydrolysis of these esters and the findings of a high lipase activity in other *Lactarius* species [4,7] As recently proposed,[8] compound **23** (R=H or a fatty acid acyl) could well be one of the first intermediates in velutinal **2a-b** conversions Notably the hemiacetal ring of **23** already contains two formyl groups at C-5 and C-13 in a masked form. A formal isomerization of **23** (R=H) could lead eventually to **21** or **22**, while elimination of a ROH equivalent could afford **9** or **19** or **20** Two alternative ways of isomerization or ROH expulsion would lead instead to the secolactarane **5** or to furanol (**7**), respectively It is really impressive, indeed, how Nature could manage to create such a large number of different structures by an imaginative variation of these two simple reactions! A deeper scrutiny of the intimate details of these conversions shows that they all fall in the same type of a cooperative electron push-pull mechanism An enzymatic proton source provides the initial electrophile, while an internal (route a) or external (routes b, b', c, d) enzyme nucleophilic group would stabilize the developing positive charge Thus, the fate of the intermediate **23** appears to depend on the site of protonation which may take place on the C-5 oxygen function or on the C-8 hydroxyl group or on the C-7–C-13 double bond The formation of both piperdial (**22**) and *epi*-piperdial (**23**) indicates that the configuration at C-7 is introduced in the course of these conversions, depending on which side protonation occurs Entering of the

external nucleophilic group takes place at C-3 with simultaneous opening of the cyclopropane ring (see intermediates **24-27**) This is followed by a hydride shift from a vicinal carbon with an *anti* expulsion of the group temporarily attached to C-3. A βH-4 shift would thus lead to the configuration at C-3 of velleral (**20**) and piperdials (**21-22**), while a 1,2-H shift from C-2, followed by the loss of H-9 or fragmentation of the C-8–C-9 bond could give rise to chrysorrhedial (**9**), furanodiene (**12**) or lactardial (**6**), respectively Subtle differences in the topology of the enzymes are therefore sufficient for catalyzing the divergent formation of isomeric dialdehydes **9** and **20** from intermediate **25** along the two routes e and f, respectively

EXPERIMENTAL

Melting points were determined on a Fisher-Johns hot plate and are uncorrected. IR spectra were recorded (film or KBr pellets) with a Perkin-Elmer model 881 spectrophotometer NMR spectra were recorded on a Bruker 250 MHz or a Bruker ACE 300 instrument. For the two dimensional homo- and heteronuclear COSY and NOE-difference experiments Bruker standard software was employed. Mass spectra were obtained on a Finnigan MAT 8222 mass spectrometer at 70 eV using a direct inlet system. Specific optical rotations were determined with a Perkin-Elmer model 241 digital polarimeter at 20 °C and CD spectra were obtained with a Jasco J-500A spectropolarimeter UV absorption spectra were determined with a Perkin-Elmer Lambda 5 UV/VIS spectrophotometer Column chromatography was performed on Kieselgel 60 (Merck) 0 040-0.063 mm, slurry packed. TLC analysis was carried out on silica gel plates (GF_{254}, Merck, 0 25 mm) The spots were visualized by spraying the plates with 0 5% vanillin solution in H_2SO_4 (4:1) and then heating at 120 °C for 30 sec All solvents were purified, dried and degassed by standard techniques just before use.

Extraction and isolation of the sesquiterpenes L chrysorrheus was collected in different woods of Appenninines, while *L scrobiculatus* was found in coniferous forests near Champoluc (Aosta Valley). The mushrooms were brought to the laboratory in Pavia and extracted within 24 hours after collecting. Fresh fruit bodies of *L chrysorrheus* (650 g), apparently not damaged by parasites, were minced and left at room temperature for 10 min without adding any solvent They were then extracted three times at 22 °C with degassed hexane and the combined extracts were rapidly dried ($MgSO_4$) and concentrated below 30 °C under reduced pressure in the dark It was very important *not to evaporate the solution completely to dryness* for preventing decomposition of lactone **8** The residual solution (*ca* 2 5 mL) was adsorbed on the top of a silica gel column, eluted with a gradient of AcOEt in hexane (from 1 16 to 1·8). All the operations were performed under dimmed light The sesquiterpenes were eluted in the following order **8** (30 mg), **9** (35 mg), **7** (12 mg), **4** (270 mg), **10** (28 mg), **6** (8 mg), **5** (15 mg). The same procedure was followed for the extraction and isolation of sesquiterpenes **12**, **8**, **9**, **4**, **10**, **6**, and **5** from *L scrobiculatus* However, in this case fruit bodies were minced only one minute prior to extraction Furanol (**7**),[3] furanodiene **12**,[25] lactaroscrobiculide A (**4**),[15] mp 86-88 °C, $[\alpha]_D$ +466 (CH_2Cl_2, *c* 0 8), lactardial (**6**),[22] blennin C (**5**),[15,21] were identical with authentic samples ^1H NMR and ^{13}C NMR spectra of compounds **4**, **8**, **9**, **10**, and **11** are reported in Tables 1 and 2, respectively For obtaining the NMR spectra of lactone **8** chromatographic fractions containing it had to be evaporated to dryness *just before* recording and C_6D_6 was added *immediately*

Chrysorrhelactone (8) Oil, $[\alpha]_D$ -30 4 (CH_2Cl_2, *c* 0 7), UV (CH_2Cl_2) λ_{max} 370 4 nm, ν_{max} 3110, 2920, 1760, 1675, 1615, 1565, 1460, 1365, 1300, 1265, 1155, 1110, 1045, 980, 950, 755, 725 cm^{-1}; EIMS, m/z (rel int) 230 (M^+, 100), 215 (M-Me, 25), 201 (40), 187 (17), 174 (M-isobutene, 22), 159 (15), 146 (35), 91 (20)

Chrysorrhedial (9) Oil, $[\alpha]_D$ +60 2 (CH_2Cl_2, *c* 2 4), UV (CH_2Cl_2) λ_{max} 313 nm (log ε 3 95); ν_{max} 2956, 2720, 1717, 1670, 1588, 1457, 1424, 1363, 1313, 1157, 1048, 737, 702 cm^{-1}, EIMS, m/z (rel int.) 232 (M^+, 50), 204 (28), 203 (40), 189 (21), 185 (30), 175 (45), 169 (25), 159 (24), 147 (43), 133 (27), 131 (26), 129

(24), 128 (24), 119 (65), 117 (24), 115 (30), 105 (75), 95 (23), 91 (85), 83 (30), 81 (28), 79 (34), 77 (47), 69 (50), 57 (45), 55 (70), 43 (49), 41 (100).

Chrysorrheal (10) Oil, $[\alpha]_D$ +29 6 (C_6D_6, *c* 0 15), UV (CH_2Cl_2) λ_{max} 318 nm (log ε 4.17), ν_{max} 3400, 2950, 1675, 1590, 1460, 1425, 1365, 1300, 1245, 1170, 1035 cm^{-1}; EIMS, m/z (rel.int.): 234 (M^+, 100), 216 (M-18, 26), 203 (27), 201 (47), 187 (78), 173 (48), 161 (35), 160 (33), 159 (31), 145 (42), 133 (21), 131 (34), 119 (40), 105 (58), 91 (56), 77 (21), 55 (25), 41 (32)

O-Ethyl acetals 16 of chrysorrheal Compound **10** (8 mg) in EtOH (2 mL) containing *p*-TsOH (0.5 mg) was stirred at room temperature for 2 hours Solid Na_2CO_3 was added and the mixture was filtered and taken to dryness IR no OH bands, ^1H NMR (80 MHz, $CDCl_3$, TMS) δ 1.05 (6H, s, H_3-14 and H_3-15), 1 07 (3H, d, J 7 0 Hz, H_3-12), 1.22 (3H, t, J 7.0 Hz, CH_3CH_2O-), 1.5-2.7 (8H, m, H_2-1, H_2-10, H-3, H_2-4, H-6), 3.35-3 85 (2H, m, H_2-5), 4 05 and 4 27 (totally 2H, 2q, J 7 0 Hz, CH_3CH_2O-), 5.30 and 5 45 (totally 1H, 2s, H-13), 5.90 (1H, bs, H-8); EIMS, m/z (rel int.): 262 (M^+, 62), 217 (M-OEt, 100), 188 (42), 175 (40), 173 (46), 162 (27), 161 (24), 147 (61), 133 (24), 131 (22), 119 (31), 105 (37), 91 (36), 69 (23), 57 (25), 55 (28), 43 (28), 41 (32)

Chrysorrhediol (11) Mp 53-54 °C, $[\alpha]_D$ +59 5 (CH_2Cl_2, *c* 0 2), UV (CH_2Cl_2) λ_{max} 269 4 nm (log ε 4 16), CD (EtOH) λ_{ext} (Δε) 268 nm (+1 12), ν_{max} 3300, 2940, 1620, 1455, 1430, 1360, 1310, 1290, 1230, 1150, 1025, 995, 870 cm^{-1}, EIMS, m/z (rel int.) 236 (M^+, 7), 218 ($M-H_2O$, 67), 187 (100), 159 (21), 145 (31), 131 (28), 119 (25), 105 (40), 91 (37), 55 (20), 41 (24).

a) *From $LiAlH_4$ reduction of lactone 4* Lactaroscrobiculide A (**4**, 21 mg, 0 091 mmol) in THF (1 mL) was added to $LiAlH_4$ (2 6 mg, 0 068 mmol) in THF (1 mL) at -25 °C under argon After stirring for 2 hours, AcOEt (1 mL), then H_2O (0 3 mL) and Celite were added and the mixture was filtered under vacuum, dried ($MgSO_4$) and evaporated The residue was chromatographed on a silica gel column. Elution with hexane-AcOEt (2 1) gave diol **11** (12 mg)

b) *From $LiAlH_4$ reduction of chrysorrheal (10)* Following the above procedure hydroxyaldehyde **10** (6 mg) gave diol **11** (3 5 mg)

c) *From DIBAL-H reduction of chrysorrhedial (9)* 1 0 M DIBAL-H in THF (371 µL, 0 371 mmol) was added to compound **9** (34 4 mg, 0.148 mmol) in THF (1 mL) at -10 °C under argon The reaction mixture was stirred for 90 min at room temperature, then quenched with 10% aq NaOH, diluted with AcOEt and washed with brine The organic layer was dried ($MgSO_4$) and evaporated Chromatographic separation of the residue on a silica gel column, eluted with C_6H_6-AcOEt (7 3), gave diol **11** (10 mg).

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