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Sesquiterpenes of *Lactarius* and *Russula* (Mushrooms): An Update[1]

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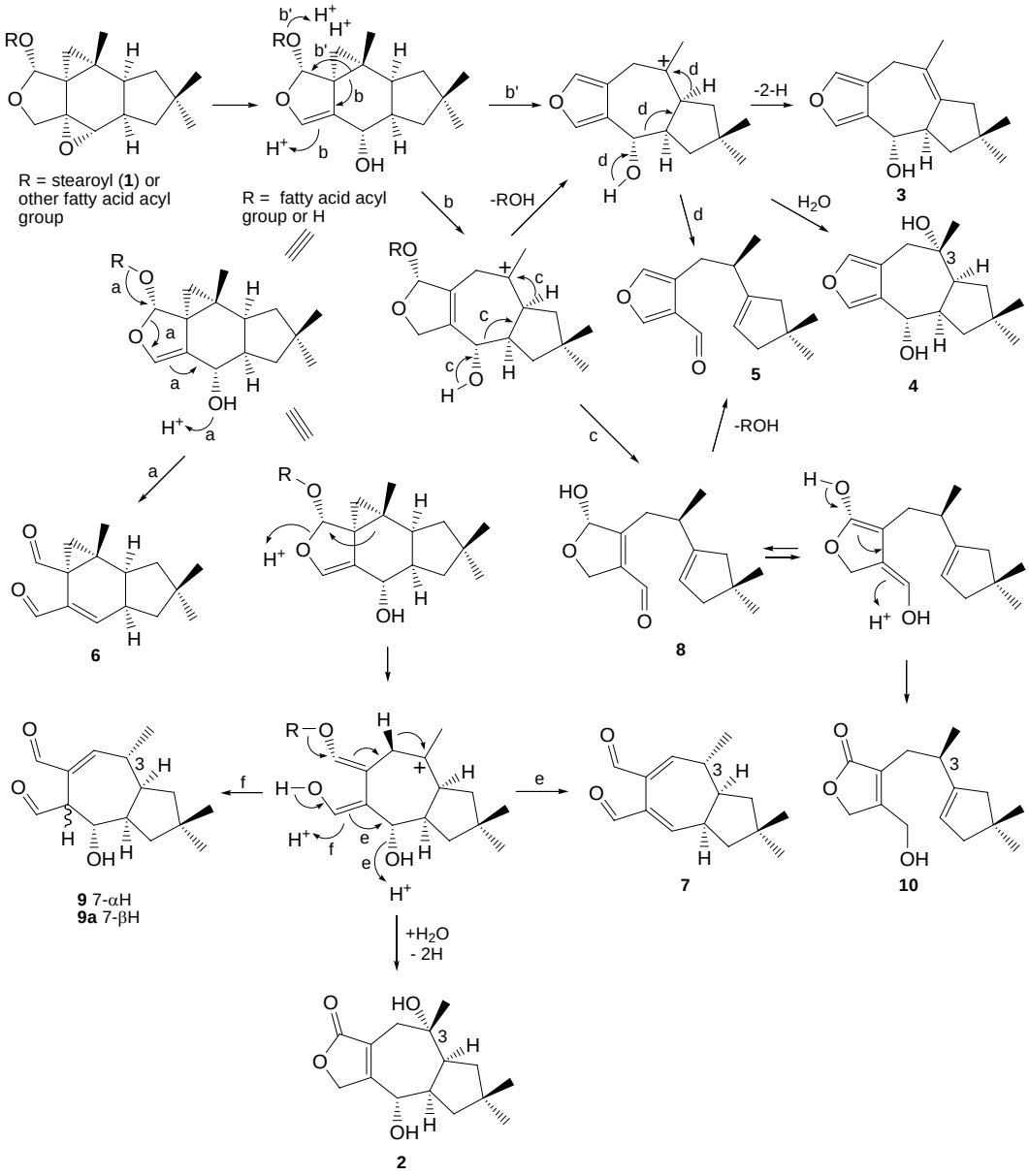
In this review the biogenesis, structures, and bioactivities of all sesquiterpenoids isolated from *Russula* and *Lactarius* species in the last decade are critically discussed, and divided into sections according to their skeletons. A brief chemotaxonomic overview of the family Russulaceae is reported in the final part of the review.

Keywords: *Lactarius*, *Russula*, Basidiomycetes, chemical defense system, lactarane, marasmane, protoilludane, guaiane, aristolane, nardosinane sesquiterpenoids.

The vast kingdom of Fungi comprises both microscopic species like molds, as well as species forming large fruiting bodies (mushrooms and toadstools), which are mainly found in the Basidiomycota and Ascomycota divisions. Fungi of the genera *Lactarius* and *Russula* belong to the division Basidiomycota, order Russulales, family Russulaceae [2]. They are distributed worldwide, forming different mycorrhiza with higher plants. More than 150 species of *Lactarius* [3] and 300 of *Russula* [4] are reported to grow in Europe, usually in woods. Although different alkaloids, phenols, triterpenoids, sterols, etc., often with new structures, have been isolated from Russulaceae, sesquiterpenes of several types are the most characteristic secondary metabolites of these mushrooms for the reasons outlined below.

Two reviews published by us have covered the literature on different aspects of *Lactarius* constituents until the end of 1997 [5]. In this paper, after a brief introduction on some important general aspects of the sesquiterpenoids of Russulaceae, we will discuss the results collected on *Lactarius* sesquiterpenes in the last decade. In addition, we will report a comprehensive account of the sesquiterpenoids isolated from *Russula* species, which have been almost neglected in the previous two reviews.

Lactarius and *Russula* species share several morphological features, in particular some micromorphological characters related to the spore ornamentation and to the presence of specialized hyphae (cystidia); however, they are easily distinguishable by a simple test. In fact, broken fruiting bodies of *Lactarius* exude a juice (latex) that is more or less milky or aqueous; by contrast *Russula* species produce no latex. The flesh of a few species is mild (e.g. *L. deliciosus*, *L. volemus*, *R. cyanoxantha*, *R. aurata*), whilst the taste of many *Lactarius* and *Russula* species, even if initially mild, becomes acrid or bitter with time. The pungent-burning sensation develops in one's mouth from within a few seconds up to a few minutes. This empirical observation is routinely employed by mushroom hunters to distinguish inedible and sometimes toxic-irritating species from edible ones. Moreover, this separation between "mild" and "acrid" species is also used as a diagnostic criterion in morphological taxonomy [6].



Scheme 1. Proposed enzymatic conversions of velutinal esters in fruitbodies of Russulaceae.

The latex exuded from carpophores of *Lactarius* is white or colored, or it changes from white to different colors with time; similarly, the taste of the milky juice may remain mild or becomes hot, slowly or very rapidly [3]. These facts have a significant taxonomic relevance and arise from changes in the chemical contents of the mushrooms. In these chemical transformations sesquiterpenes of different skeletons play a major role (*vide infra*).

Several evidences indicate that usually one, more rarely few biologically inactive and tasteless fatty acid esters of a precursor sesquiterpene alcohol are contained in intact fruiting bodies of most Russulaceae [5]. These compounds, in consequence of an injury to the fruiting bodies, are converted into several derivatives with the same skeletons of the precursors or different ones, and often possessing various bioactivities [5]. They are responsible for the characteristic pungent and/or bitter tastes of the flesh, and for the changes of the color of the latex of some *Lactarius*.

It has been suggested that these conversions of sesquiterpenes constitute chemical defense systems that protect fruiting bodies of Russulaceae against parasites and predators [7]. Although other apolar sesquiterpene esters can be involved [5], depending on the mushroom species, the most widespread precursor in these conversions is the marasmane

sesquiterpenoid stearoylvelutinal (**1**) [8], which has been found in intact fruiting bodies of a number of pungent species [5]. As a response to injury, ester **1** is converted to dozens of derivatives, the majority of which have the marasmane, lactarane, and secolactarane skeletons (Scheme 2) and usually contain a characteristic γ-lactone ring, like the bitter lactarorufin A (**2**) and the seco-derivative blennin C (**10**), or a furan ring, like furandiol (**3**), furanol (**4**), lactaral (**5**), or a 1,4-dialdehyde group, like the hot-tasting isovelleral (**6**), velleral (**7**), lactardial (**8**), and piperdial (**9**). These dialdehydes, as well as other less distributed ones [5], in general show antimicrobial, cytotoxic, antifeedant, and mutagenic activities, and are therefore considered to be the active components of the chemical defense system occurring in Russulaceae pungent species [9].

Rearrangements of the marasmane skeleton of **1** into the lactarane and secolactarane ones are believed to be controlled by enzymes and initiated by hydrolysis of the ester group and opening of the oxirane ring. A cascade of reactions then ensues (Scheme 1), involving carbocation-like species or, most likely, intermediates linked to nucleophilic groups of enzymes [10]. In particular, the configurations at the C(3) stereocentres of formed compounds reflect the different ways by which the cation at this carbon is annihilated by an internal or an external nucleophile. Moreover, although most conversions do not involve a change in the oxidative state of the products, formation of γ-lactones like lactarorufin A (**2**) requires an additional oxidative step.

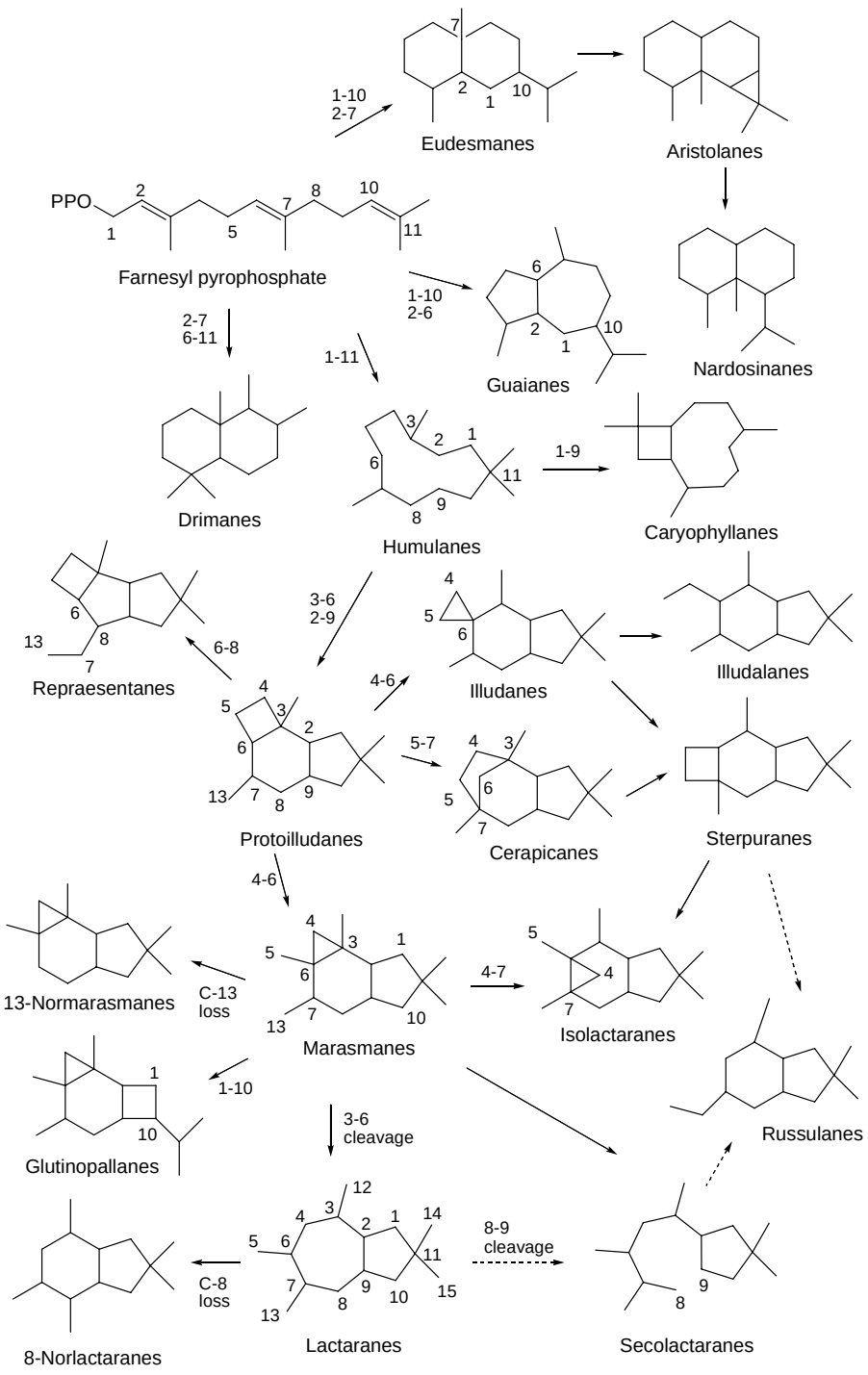
The precursor sesquiterpene esters, in particular velutinal esters (**1**), are chemically labile, so that they are isolatable only under careful procedures, i.e. by using an inert solvent at a relatively low temperature for extraction, and non-acidic chromatographic phases for separations of extracts [5]. On the contrary, alcoholic solvents, or solvents containing traces of acids, as well as untreated silica gel, rapidly transform velutinal esters into different lactarane derivatives, among which furans, like furandiol (**3**), are the most abundant [11]. Since these same compounds may also be formed enzymatically, sometimes it is difficult to recognize whether an isolated compound is a true fungal metabolite or a chemical artifact.

In the authors' experience, the best procedure to extract the original apolar compounds present in intact fruiting bodies is to freeze freshly collected undamaged mushrooms at -20 °C and then soak them in either *n*-hexane or CH_2Cl_2 at -20°C for a few minutes. In contrast, to monitor the complex cascade of reactions occurring in injured fruiting bodies, and to isolate the compounds thus formed, fruiting bodies are minced without adding any solvent and different samples of the mush are extracted with CH_2Cl_2 or THF or EtOAc at room temperature, at different times after injury. Separations of the extracts are then made on silica gel, pre-washed with mixtures of *n*-hexane and Et_3N or, preferably, on a RP-18 phase.

It must be stressed that extraction of fruiting bodies of Russulaceae with either acetone or alcohols almost inevitably produces artifacts, as the presence of alkoxy groups in the structures of several isolated compounds clearly indicates. Even the use of EtOAc as solvent of extraction is not always safe, since significant amounts of harmful AcOH may be formed by hydrolysis of the solvent. Notwithstanding that such precautions have been clearly indicated in the literature [5], it is, therefore, rather surprising that, even recently, some authors use alcohols as favorite solvents for the extraction of *Lactarius* and *Russula*.

Families of sesquiterpenes isolated from Russulaceae. The presumed biogenetic pathways to the main *Lactarius* and *Russula* sesquiterpenoids is presented in Scheme 2 which, for completeness, also includes the related sterpuranes, not yet isolated from Russulaceae, but found in other mushrooms [12]. They have been divided into different families according to the biosynthetic origin from farnesyl pyrophosphate (FPP).

Farnesane sesquiterpenes possess the skeleton of the acyclic precursor farnesol, while drimanes, humulanes, guaianes, and eudesmanes arise from different FPP cyclizations, the mode of cyclizations being indicated by the numbers above the arrows. The eudesmanes are precursors of aristolanes and nardosinanes. Two different cyclizations of the humulane precursor give rise to the families of caryophyllanes and protoilludanes, with the sesquiterpenes formally derived from a protoilludane precursor constituting the largest group of Russulaceae sesquiterpenes [5]. Two different cyclobutane ring contraction in the protoilludane skeleton give rise to the marasmane and the very rare (in Russulaceae) illudane skeletons, whereas different rearrangements of marasmanes lead to the glutinopallane, lactarane, isolactarane, and secolactarane skeletons. In principle, the



Scheme 2. .Proposed biogenetic pathways to sesquiterpenoid skeletons of Russulaceae

secolactarane backbone may be formed by C(8)-C(9) bond cleavage of a lactarane precursor; however, the results of some biomimetic-like reactions *in vitro* seem to indicate their direct origin from marasmanes [5]. As an alternative to the protoilludane-marasmane pathway, isolactaranes may originate from rearrangement of a suitable sterpurane intermediate [12], although no sterpurane sesquiterpene has yet been isolated from Russulaceae. A different Meerwein-like rearrangement of the cyclobutane ring of protoilludanes may afford the cerapicane skeleton, whereas contraction of the cyclohexane ring may lead to the repraesentane structure. The rare nor-sesquiterpenoid russulane skeleton has been proposed to derive from the secolactarane one; however, its derivation from either the sterpurane or the

cerapicane skeleton cannot be excluded. Contraction of the seven-membered ring of lactaranes with loss of the C(8) carbon atom may produce the 8-norlactarane skeleton, whereas loss of the C(13) carbon of marasmanes leads to the 13-normarasmane backbone.

Most of these transformations are hypothetical since systematic biosynthetic studies on sesquiterpenes of Russulaceae are still lacking; however, the absolute configurations assigned to most sesquiterpenes isolated from *Lactarius* and *Russula*, and a few results of incorporation of labeled precursors are consistent with this general scheme. Moreover, the occurrence of sesquiterpenes with different skeletons in the same species, for instance marasmane, normarasmane, isolactarane, lactarane, and secolactarane sesquiterpenes in *L. vellereus*, points to their common biogenesis [5].

Sesquiterpenes with farnesane, drimane, glutinopallane, isolactarane, humulane, aristolane caryophyllane, eudesmane, nardosinane, cerapicane, and russulane skeletons have been isolated so far from very few Russulaceae species; therefore, they may be considered as chemotaxonomic markers. Representative sesquiterpenoids [5] belonging to the farnesane, drimane, glutinopallane, and isolactarane families are shown in Figure 1, since no other examples will be encountered in this review. By contrast, sesquiterpenes formed by the protoilludane-marasmane cascade are much more common in the fruiting bodies of Russulaceae.

Drimane, guaiane, farnesane, humulane, caryophyllane, eudesmane, and aristolane sesquiterpenoids are typical products of plant metabolism. Instead, sesquiterpenes having skeletons derived from a protoilludane precursor have been isolated so far only from Baidiomycetes; however, they are not unique to Russulaceae species, as it will discussed in the final part of this review (*vide infra*).

In the following paragraphs we will discuss the sesquiterpenoids isolated from *Lactarius* after 1997 and all sesquiterpenoids isolated from *Russula* so far. The compounds have been grouped in the different families represented in Scheme 2, following the same criteria of our previous reviews [5].

It is interesting to note that before 1997 only sesquiterpenoids of European Russulaceae species were investigated, with the exception of very few North American and Indian *Lactarius* species. From

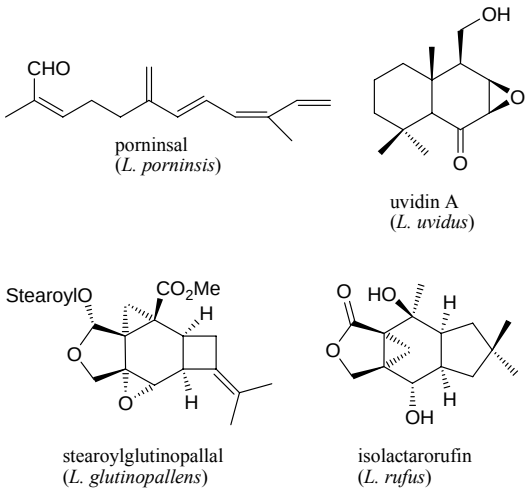
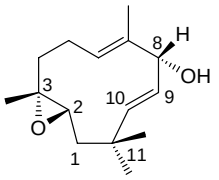


Figure 1: Reprsesentative sesquiterpenoids isolated from *Lactarius* having the farnesane, drimane, glutinopallane, and isolactarane skeletons.

1997 on, sesquiterpenoids contained in Japanese and Chinese *Lactarius* and *Russula* mushrooms have also attracted a growing interest from research groups of Europe and Asia.

The structures of the new compounds reported in the following paragraphs were established mainly on the basis of routine 2D-NMR spectroscopic data and, therefore, they will not be discussed in detail, except when some important stereochemical assignations were made.

Humulane sesquiterpenes. Humulane precursors have always been considered to have a central role in the biogenetic pathways leading to the majority of Russulaceae sesquiterpenoids (Scheme 2). However, until a few years ago, no representative of this family had ever been isolated from either a *Lactarius* or *Russula* species. Therefore, it is of great interest the very recent isolation from *Lactarius hirtipes* J. Z. Ying, collected in China, of a new oxygenated humulane sesquiterpenoid, which was assigned structure **11** [13]. The relative stereostructure was established by ROESY experiments that indicated the presence of a *trans*-epoxide at C(2)-C(3) thanks to the correlations of 2-αH with 1-αH, 4-αH, 5-αH, 15-H_3, and 1-βH. This assignation was confirmed by comparing the data of **11** with those of other 2,3-epoxy-humulenes. The signal for C-2 at 61.3 ppm was assigned to a carbon bearing a β-configured epoxide bond, while the signal for C-3 at 61.4 ppm was assigned to a carbon linked to an α-configured epoxide bond. Other ROESY experiments suggested the α-configuration of the hydroxyl at C-8 and the *E*-configuration of the C(9)-C(10) double bond.



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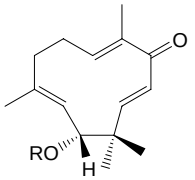
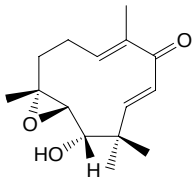


12a R = H
12b R = CO-$(CH_2)_7CH{=}CH(CH_2)_7CH_3$
12c R = CO-$(CH_2)_7CH{=}CHCH_2CH{=}CH(CH_2)_4CH_3$



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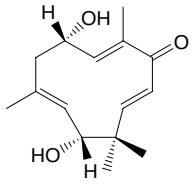

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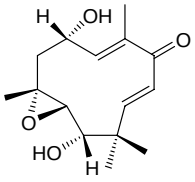

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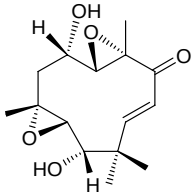

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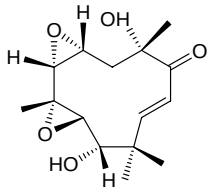

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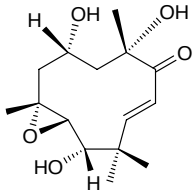

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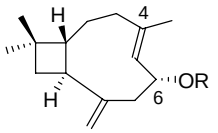
Notably, the free alcohol **11** was isolated along with lactarinic acid (6-oxostearic acid). Since this acid was never isolated before from Russulaceae in a free form, but always esterified with different sesquiterpenoid alcohols [5], one wonders whether the original compound present in fruiting bodies of *L. hirtipes* is, actually, the lactarinate ester of alcohol **11**.

Seven new humulane derivatives, strongly related to epoxide **11**, were isolated by Liu's group from *Lactarius mitissimus*, collected in China [14-16]. They were named mitissimols A-G (**12-18**) and the structure of free mitissimol A (**12a**) was confirmed by single-crystal X-ray diffraction analysis [14], whereas the absolute configuration of mitissimol F (**17**) was established by ^{1}H-NMR analysis of the corresponding diastereomeric MTPA esters [16]. Notably, two esters of mitissimol A, the oleate **12b** and the linoleate **12c**, could also be isolated.

These results stand in high contrast with the structures of the compounds isolated from fruiting bodies of *L. mitissimus* Fr. collected in Europe. In fact, from specimens of this mushroom collected in Sweden [5], Poland [5], and by us (*vide infra*), no humulane derivative has been isolated; instead, sesquiterpenes belonging to the marasmane-lactarane cascade have been found. Therefore, we wonder if the Chinese fungal material belongs to a species different from the European *L. mitissimus* Fr.

Caryophyllane sesquiterpenes. Caryophyllane sesquiterpenoids are quite common in plants, but quite rare in Basidiomycetes. Until 1999 the only example found in Russulaceae was 12-hydroxycaryophyllene-4,5-oxide isolated by Daniewski and collaborators from fruiting bodies of *Lactarius camphoratus* [17]. A new caryophyllane allylic alcohol (**19b**) was later isolated by our group [18] from *L. subumbonatus* Lindgr. (synonym *L. serifluus* DC), belonging to the same Section Olentes as *L. camphoratus* [3]. By different extractions of the fruiting bodies with CH_2Cl_2 at -20°C and at room temperature, we demonstrated that this caryophyllene derivative is present in undamaged mushrooms only as **19a**, i.e. esterified by the new fatty acid (*S*)-6-hydroxystearic acid, while the free alcohol **19b** was formed in injured fruiting bodies by slow hydrolysis of the ester **19a**. In addition, a preliminary toxicity test against *Artemia salina* showed that **19b** and free (*S*)-6-hydroxystearic acid had an LD_{50} of 11 ppm and 28 ppm, respectively, whereas the ester did not show any activity. It was, therefore, supposed that conversion of **19a** into **19b** may constitute a new variant of the general chemical defense system found in most Russulaceae, which is based on the hydrolysis, in injured fruiting bodies, of inactive esters to afford bioactive derivatives used by the mushrooms to protect themselves against predators. The relative stereochemistry of **19a-b** was established through molecular modeling studies of the macrocyclic ring of **19b** and of the epimeric 6β-alcohol. AM1 calculations showed that inversion of the configuration at C-6 has a dramatic effect on the preferred conformation of the nine-membered ring. In fact, while **19b** mainly exists in the ββ conformation, the corresponding 6-*epi*-stereoisomer exists, almost

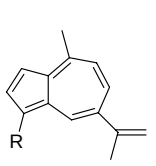


19a R = $CH_3(CH_2)_{11}CHOH(CH_2)_4CO$
19b R = H
19c R = PhCO

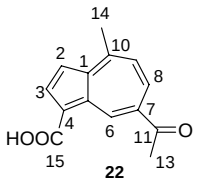
completely, as the βα conformer. Since NOESY correlations better agreed with through space protons interactions expected for the ββ conformation, the 6-OH-caryophylladiene should have the **19b** relative configuration. The absolute configuration of the alcohol **19b** was determined through theoretical calculations of the CD curve of the corresponding benzoate **19c**, by using the De Voe coupled oscillators theory [19].

The structure of 6-hydroxystearic acid, obtained by hydrolysis of ester **19a**, was confirmed by the identity with the product obtained by $NaBH_4$ reduction of the very well-known 6-oxostearic acid (lactarinic acid). The (6*S*)-configuration was assigned by using (*R*)-2-methoxy-2-naphthylacetic acid (2-NMA) as NMR chiral shift reagent [20]. Thus comparing the resonances of the protons belonging to the C_1-C_5 and the C_7-C_{18} parts of the (*R*)-2-NMA derivative of 6-hydroxystearic acid methyl ester with the corresponding signals of the free alcohol, and employing the semiempirical model originally proposed by the authors for the (*R*)-2-NMA esters [20], the (*S*)-configuration was assigned to 6-hydroxystearic acid isolated from *L. subumbonatus* [18].

Guaiane sesquiterpenes. Among Russulaceae, guiane sesquiterpenoids have been isolated so far only from *Lactarius* and they are considered chemotaxonomic markers of species of the Section Dapetes. These mushrooms are characterized by the secretion of a strongly colored milky juice and are usually edible and tasty. About a dozen different guaiane sesquiterpenoids are responsible for the orange, red, green, or even blue color of the latex of each species. These compounds are extraordinarily chemically sensitive and could be isolated only by employing very mild extraction and purification conditions [5]. Thus, extraction of young, undamaged fruiting bodies, frozen in liquid nitrogen at their growing site, and subsequently soaked in *n*-hexane at -20°C, allowed the isolation of fatty acid esters of guaiane alcohols. However, in aged or injured fruiting bodies, the chemical contents change, as often indicated by a change of the color of the latex.



20 R = CHO
21 R = CH_2OH



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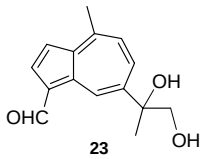

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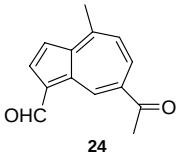

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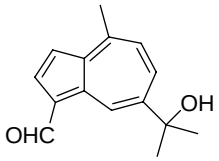

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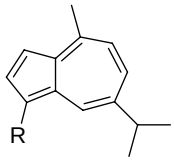


26 R = COOH
27 R = CHO

For instance, the color of the latex of *L. deterrimus* and *L. deliciosus*, which originally is carrot-orange, becomes dark green, and guaiane sequiterpenes having a formyl or a free hydroxymethyl group at C-4, or an azulene structure, are formed [5].

Serious difficulties in distinguishing true fungal metabolites from artifacts formed by incorrect isolation procedures, have caused some confusion in the literature and may explain, at least in part, why sometimes different guaiane sesquiterpenes have been isolated from mushrooms of the same species growing in different places of the world.

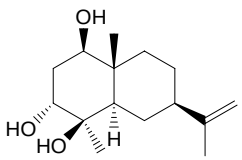
In this context, the recent results on two species of Dapetes collected in the Yunnan Province of China, are of a certain interest. The former species was *Lactarius deliciosus* [21,22]. This mushroom is distributed worldwide and European specimens have been thoroughly studied in the past [5]. Four guaiane sesquiterpenoids were isolated from the Chinese species, among which compounds **22-24** were new, while lactaroviolin (**20**) occurs in several other Dapetes, namely, *L. deliciosus*, *L. deterrimus*, *L. indigo*, *L. sanguifluus*, and *L. semisanguifluus* [5].

Indeed, the green color assumed by the latex of *L. deliciosus* and *L. deterrimus* on aging is due to the formation of the violet-colored aldehyde **20** and the blue-colored alcohol deterrol (**21**). The purple compound **22**, the purple-red **23**, and the brown-red **24** appear to be oxidation products of lactaroviolin **20**, and the oxygenation patterns suggest the possible oxidative sequence **20**→ **23**→ **24**→ **22**.

Quite surprisingly, several characteristic guaiane sesquiterpenoids isolated from European specimens of *L. deliciosus* [23], in particular a few fatty acid esters like the stearate of deterrol (**21**), were not reported by the Chinese authors.

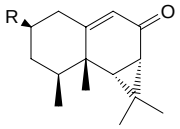
The same group recently described the isolation of two new azulene pigments during the first study of the sesquiterpenoids of *L. hatsudake* [24]. This mushroom is slightly bitter and it is widely distributed in Asia, mainly in China and Japan, where it is consumed. Similarly to *L. deliciosus*, the orange milky juice of *L. hatsudake* turns green-blue when fruiting bodies are injured. Extraction of fruiting bodies with acetone, followed by repeated chromatography on silica gel, led to the isolation of the new red-purple aldehyde **25** and the new purple acid **26**, along with aldehyde **27**, previously isolated from *L. deterrimus* and *L. sanguifluus* [5]. To demonstrate that acid **26** was a true natural metabolite and not an artifact formed by air oxidation of aldehyde **27**, extraction and separation of compounds were repeated under anaerobic conditions, leading to re-isolation of the acid.

Eudesmane sesquiterpenes. Only one sesquiterpenoid with the eudesmane skeleton has been isolated so far from a Russulaceae species, namely the triol **28** from *Lactarius laeticolorus* [25]. Only 5.7 mg of compound **28** was isolated from 2.7 kg of fruiting bodies, which were soaked in EtOH at room temperature for six weeks. Indeed, these are considered quite harsh conditions for the extraction of *Lactarius* fruiting bodies, so that a reinvestigation of this mushroom following a milder procedure of extraction, is highly recommendable. Triol **28** showed a LD_{50} of 4.5 μg/mL in a cytotoxicity test against KB cells.

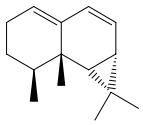


28

Aristolane and nardosinane sesquiterpenes. *Russula lepida* is a beautiful red-cap mushroom, commonly found in Italy in late summer – early autumn in broad-leaved woods, in particular of oaks and chestnuts. The cystidia of *R. lepida* do not respond to the so-called "sulfoaldehyde test". This microscopical test, used by mycologists for *Russula* identification [4, 6, 8b], is considered positive when



29 R = H
29a R = OH



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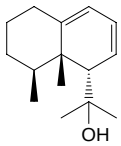

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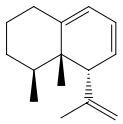


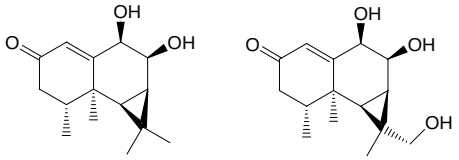
32

the contents of cystidia, treated with a solution of vanillin (or other aromatic aldehydes) in H_2SO_4, turn black or brownish black. This color indicates, indeed, the presence of velutinal esters that are immediately transformed by the acid into lactarane sesquiterpenes, like the furanols **3** and **4** which, by reacting with the sulfoaldehyde reagent produce a blackish spot. The mushroom is sometimes eaten in Italy, but it is considered of poor quality, owing to its hard texture.

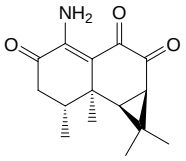
A few years ago, during our studies on non-pungent Russulaceae species, we investigated the chemical contents of undamaged fruiting bodies of Italian specimens of *R. lepida* [26]. They were frozen at – 20°C and extracted with CH_2Cl_2 in the cold. Free acids were removed by CC on activity III Al_2O_3 and the neutral fraction was further separated by CC on RP18 and by centrifugal circular chromatography on silica gel plates to afford sesquiterpenes **29-32**. This finding was well in accordance with the negative "sulfoaldehyde test" signalling the absence of velutinal esters and derivatives thereof. Compound **29** was identical to (+)-aristolone (α-ferulone). The CD spectrum of rulepidanol (**31**) showed a positive CE for the π→π* transition of the skewed conjugated diene; this indicated a P helicity for the chromophore and hence the absolute configuration 4*S*, 5*S*, 6*S* for compound **31**.

The structures of rulepidadienes A (**30**) and B (**32**), were established by standard NMR techniques. Nardosinane sesquiterpenes, as **31** and **32**, are believed to derive in nature from aristolane precursors. The co-occurrence of **31** and **32** with aristolanes **29** and **30** in the same mushroom reinforces this hypothesis and suggests that the absolute configurations of compounds **30** and **32** correspond to those of **29** and **31**, respectively.

Aristolane and nardosinane sesquiterpenes are of types rather rare in nature; they have been isolated



33 **34**

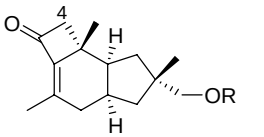


35

from terrestrial plants and marine organisms and only from *R. lepida* among fungal species. It may be of evolutionary significance that fungal aristolane and nardosinane sesquiterpenes **29-32** are antipodal to those usually found in higher plants and belong, instead, to the enantiomeric series typical of several liverworts and Octocorallia.

R. lepida is used as a food and medicinal agent in China. Moreover, an extract of the fruiting bodies showed an interesting antitumor activity [27]. A few years ago, Chinese specimens of *R. lepida* were collected at the Ailao Mountain in the Yunnan Province, which is one of the areas with the richest fungal sources in the world. Extraction was made with EtOH and $CHCl_3$/MeOH 1:1. Repeated chromatography on silica gel gave four aristolane sesquiterpenoids, rulepidol (**29a**), rulepidadiol (**33**), rulepidatriol (**34**), and lepidamine (**35**) [27,28]. Their absolute configurations have not been determined. Compound **35** is particularly important, because it is the first aristolane-type alkaloid isolated from nature. It has a bright-yellow colour, due to a broad absorption maximum at 409 nm. The amino group was revealed by the signals in the ^{1}H NMR spectrum at 9.84 ppm (br *s*, 1H) and 6.34 ppm (br *s*, 1H), which were assigned to two N-bound protons.

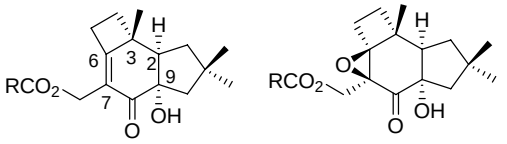
As already observed for studies on *Lactarius deliciosus*, although the skeletons are the same, the structures of sesquiterpenoids isolated from apparently identical species collected in Europe and Asia, are often different. Although these discrepancies may depend on different factors, the possibility exists that such highly oxidized derivatives, for instance **33** and **34**, are produced from less oxidized precursors upon exposure to fungal oxidases that are not completely deactivated even in 30% EtOH at 25°C [5].



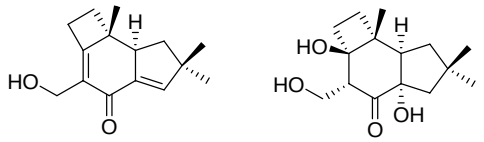
36 R = $CO(CH_2)_4CO(CH_2)_{11}CH_3$
37 R = H

Protoilludane and related sesquiterpenes. Although many different families of Russulaceae sesquiterpenoids have always been considered to originate from a protoilludane precursor [5] (Scheme 2), it was only in 1998 that the first two protoilludane sesquiterpenes, i.e. violascensol (**37**) and its lactarinate ester (**36**), were isolated by us from a *Lactarius* species, namely from *L. violascens* Fr. [29]. The absorption at 264 nm in the UV spectra of **36** and **37** was indicative of the conjugated ketone carbonyl group, while the isolated methylene group at C-4 gave rise to a diagnostic AB quartet at δ 2.68 in **36** and at δ 2.62 in **37**.

Four years after this discovery, a chemical investigation of the fruiting bodies of *Lactarius atlanticus* allowed us to isolate four new protoilludane sesquiterpenes, named atlanticones A-D (**38-41**) [30]. Extraction of undamaged fruiting bodies afforded enone **38** and the corresponding β-epoxide **39**, both as a mixture of oleate and linoleate esters, in a ratio of 9:1. On the other hand, no esters were extracted from injured fruiting bodies; instead, the free alcohols **40** and **41** were isolated in significant amounts when the extract was chromatographically separated on silica gel. By contrast, only triol **41**, but no dienone **40**, was isolated when RP-18 was used as the chromatographic phase. Both compounds appeared to be formed from atlanticone A (**38**), but dienone **40** was likely to have been derived from acidic silica gel promoted dehydration of the fragile tertiary alcohol. Contrary to the absolute configuration of the violascenol ester **36**, which could not be inferred with confidence from the CD curve [29], that of atlanticone A (**38**) was firmly established by chiroptical studies along with extensive molecular modelling [30]. The enone system of **38** was skewed, both from PM3 calculations (calculated angle ω of about 150°) and the relatively low intensity of the UV absorption band at 250 nm (ε = 5000). Accordingly, the CD spectrum of atlanticone A showed two Cotton effects above 220 nm: a weak, negative band at 335 nm (n→π* transition), with Δε = -2, and a positive band at 246 nm (π→π* transition), with Δε = +18.5. According to the semiempirical helicity rules derived for interpreting the signs of CD bands of skewed



38 **39**

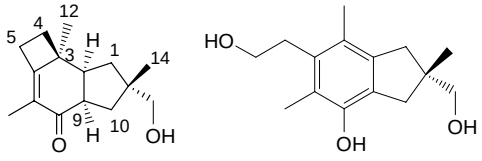


40 **41**

s-trans 2-cyclohexenones, the dihedral angle of the enone system in compound **38** was thus positive, corresponding to the absolute configuration 2*R*,3*R*,9*S*.

The finding of compounds **36**, **38**, and **39** gives further support to the hypothesis that when sesquiterpenoids of different families are contained in intact fruiting bodies of Russulaceae, they occur in the form of esters of long chain fatty acids [31].

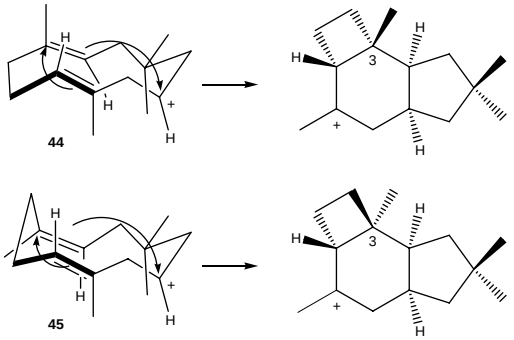
For different reasons the results of an investigation on *Lactarius piperatus* collected in the Yunnan province, China have been quite surprising [32]. This mushroom has been one of the first *Lactarius* to be extensively studied in Europe by the Swedish school in Lund [5]. These authors nicely demonstrated that undamaged fruiting bodies of *L. piperatus* contained stearoyl velutinal (**1**) from which isovelleral, velleral, and furanolactarane sesquiterpenes were formed upon injury to the mushroom tissues [5].



42 **43**

From an ethanolic extract of Chinese specimens of *L. piperatus*, the new isoprotoilludanol **42** was isolated in a mixture with marasmane, isolactarane, and lactarane sesquiterpenes (see the following paragraph) [32]. This has represented the first finding of protoilludane, marasmane, and lactarane sesquiterpenes in the same mushroom, and thus it corroborates the hypothesis of a common biogenesis (Scheme 2):

Compound **42** was named isoplorantinone, since the configuration at C-3 was inverted relative to that of



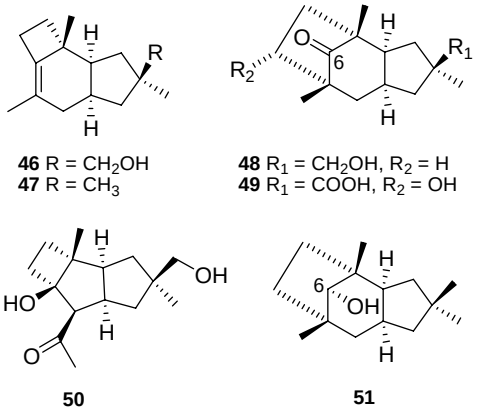
44 **45**

Scheme 3: Cyclizations of *E*,*E*- and *Z*,*E*-humulyl cations to give the protoilludane and the isoprotoilludane skeletons, respectively.

plorantinone A (**60**) [33], whose stereostructure typifies the stereochemistry of all protoilludanes isolated from Russulaceae. Thus, **42** represents the first and only known exception having the methyl at C(3) *cis* oriented to the bridgehead hydrogens. Since the configuration at C(3) of the protoilludane skeleton is created during the cyclization of a folded humulyl cation precursor (Scheme 2), most protoilludanes derive from cyclization of the *E*,*E*-cation **44**, while **42** may originate from the *Z*,*E*-cation **45** (Scheme 3).

The stereostructure of compound **42** was deduced by the strong correlations in the NOESY spectrum between 2-αH and 9-αH, between 12-H_3 and 9-αH, 4-αH, and 5-αH, and by the weak correlation between 12-H_3 and 2-αH. Similar correlations between 14-H_3 and 1-βH and 10-βH indicated that the methyl group linked to C(11) was β-oriented [34]. The results of a MM2 force-field molecular-modelling were consistent with the NOESY spectrum of **42** and indicated a boat conformation for the cyclohexane ring and a slightly twisted envelope conformation for the cyclopentane ring. Along with **42**, the Chinese authors isolated the new illudalane sesquiterpene **43**, whose structure was confirmed by a single-crystal X-ray diffraction determination [32]. We believe that triol **43** is, indeed, a degradation product of **42**, owing to the presence of acids during the isolation step, as it will be discussed below in connection with an ester of 43 isolated from *Russula pseudodelica*.

During a search for plant growth regulators in Russulaceae mushrooms, repraesentin A (**46**), a new protoilludane, was isolated from Chinese specimens of inedible, bitter tasting *Lactarius repraesentaneus* Britz., along with three rearranged protoilludanes, named repraesentins B, C, and F (**48**-**50**), and two lactaranes, named repraesentins D and E (*vide infra*) [35,36]. Notably, repraesentins A-C, at a



46 R = CH_2OH
47 R = CH_3

48 R_1 = CH_2OH, R_2 = H
49 R_1 = COOH, R_2 = OH

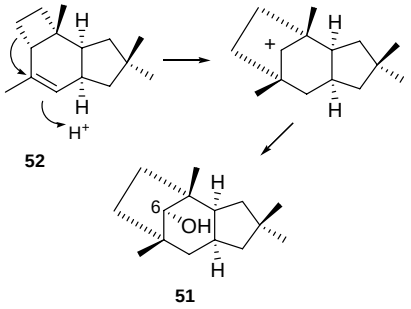
50 **51**

concentration of 67 ppm, promoted radicle elongation of lettuce seedlings by 136%, 118%, and 184%, respectively. Instead, repraesentin F (**50**) is only moderately active, showing a value of 116% at a concentration of 3.6×10^2 μM: The structure of repraesentin A (**46**) was confirmed by comparing its spectroscopic data with those of 6-protoilludene (**47**), previously isolated from two different Basidiomycetes, namely, *Fomitopsis insularis* and *Omphalotus olearius* [37]. The authors suggested that **46** and **47** should then have the same absolute configuration, i.e. 2*S*, 3*R*, 9*S*.

Repraesentins B (**48**) and C (**49**) showed a different tricyclic skeleton, consisting of a five-, a six-, and a five-membered ring assembled as in the structure of cerapicol (**51**), which was isolated from cultures of the ascomycete *Ceratocystis piceae* [38]. We propose the name cerapicane for the skeleton of **48**, **49**, and **51**. This rare sesquiterpene skeleton is clearly derived by enlargement of the cyclobutane ring of a protoilludane precursor; the presence of an oxygenated group at C(6) suggests a rearrangement initiated, for example, by protonation of the double bond of 7-protoilludene (**52**) (Scheme 4).

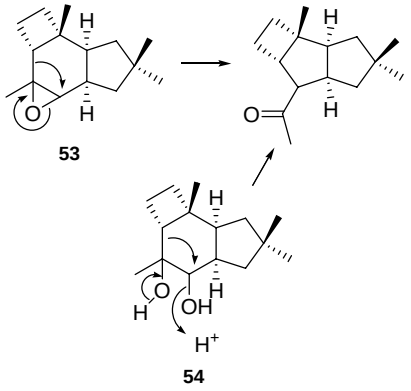
More intriguing appears to be the rearrangement of the protoilludane skeleton to that of ketone **50**, which we propose to name repraesentane. The presence of an acetyl group suggests that it may derive from a pinacol like rearrangement of either 7,8-epoxyprotoilludene (**53**) or of the corresponding diol **54** (Scheme 5):

Protoilludane sesquiterpenoids were also isolated during thorough studies [33, 39, 40] of the contents of fruiting bodies of *Russula pseudodelica* collected in China [41]. The mushroom belongs to a group of Russulae (Compactae, section Plorantinae), which is distinguished by morphological characters indicating



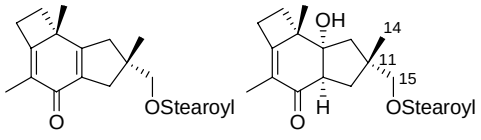
52 **51**

Scheme 4: Proposed mechanism for the rearrangement of the protoilludane to the cerapicane skeleton.



53 **54**

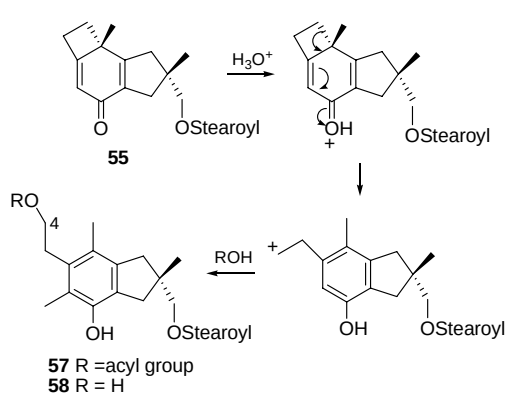
Scheme 5: Proposed mechanisms for the rearrangement of the protoilludane to the repraesentane skeleton.



55 **56**

primitivity. The authors reported a detailed analysis of the sesquiterpenes present in intact fruiting bodies and of the derivatives formed from these precursors by enzymatic conversions initiated by injury to the fruiting bodies. Moreover, artifacts produced by the presence of a trace of acid during extraction and isolation processes, were examined.

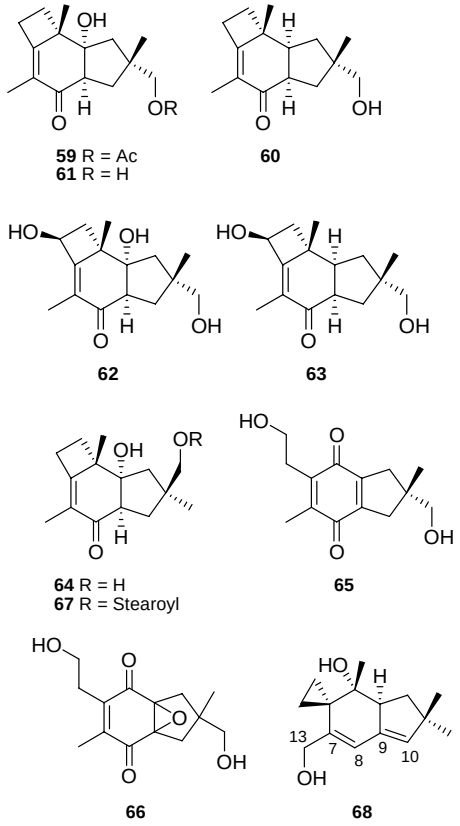
A CH_2Cl_2 extract of undamaged fruiting bodies of *R. pseudodelica* contained two sesquiterpenoids, stearoyldelicone (**55**) and stearoylplorantinone B (**56**) in a ratio of about 3:1. Moreover, mixtures of C(4) fatty acid esters of the illudalane-type **57** were obtained in small amounts, owing to the presence of free fatty acids (stearic, oleic, palmitic acid in Scheme 6) in the CH_2Cl_2 extract, which may react with **55**, and at a much slower rate with **56**, according to the mechanism shown in Scheme 6. Similarly, chomatographic purification of dienone **55** was possible only on neutral alumina, since it decomposed on silica gel, possibly because of traces of acid, to give the triol stearate **58**.



Scheme 6: Mechanism of the degradation of dienone 55 by acids.

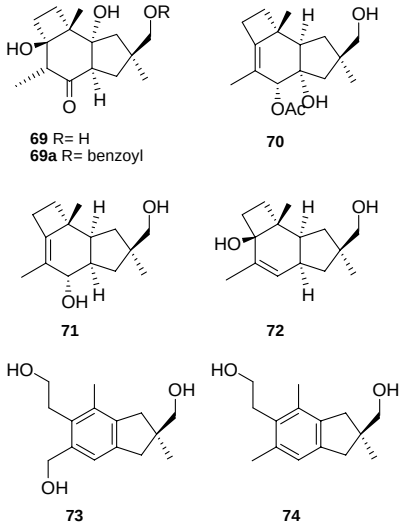
When intact fruit bodies of *R. pseudodelica* were extracted with EtOAc, instead of CH_2Cl_2, stearoyldelicone (**55**) could not be isolated, but the major component was the illudalane acetate **57** (R = Ac). According to the authors, this phenol derived from degradation of **55** caused by the acetic acid formed by hydrolysis of EtOAc. Similarly, injured fruiting bodies of *R. pseudodelica* extracted with EtOAc, yielded acetylplorantinone B (**59**), which was not obtained from the CH_2Cl_2 extract. Again, the acetyl group was likely introduced from the solvent. Apart from **59**, the two extracts showed comparable chemical compositions, both containing plorantinones A (**60**), B (**61**), C (**62**), and small amounts of plorantinone D (**63**). These plorantinones can be considered as products formed by the enzymatic hydrolysis of the stearates **55** and **56**, followed by oxidation and/or reduction.

The C(11)-epimer of plorantinone B, *epi*-plorantinone B (**64**), and two new 12-norsesquiterpenes, deliquinone (**65**) and 2,9-epoxydeliquinone (**66**) were also obtained, although in small amounts, from both extracts of injured fruiting bodies of *R. pseudodelica* [40]. The relative stereochemistry of **66** could not be established on the basis of the data from NOESY experiments. It is, at present, not clear how compounds **64**-**66** are formed. In particular, the formation of epimer **64** as a result of the enzymatic conversions initiated by injury is puzzling because, contrary to **56**, which is the immediate precursor of plorantinone B (**61**), *epi*-stearoylplorantinone B (**67**), a possible precursor of compound **64**, was never detected in the extracts. On the other hand, it is difficult to imagine an enzymatic epimerization of the C(11) stereocentre of **56** or **61**, and a C(15) reduction / C(14) oxidation sequence is also unlikely.



The origin of the illudane diene **68**, present in minor amounts in both extracts of injured specimens, is also unclear, as it was the only sesquiterpenoid obtained from *R. pseudodelica* lacking a hydroxy group at C(14) or C(15), and carrying, instead, a hydroxy group at C(13). Moreover, compound **68** is the only illudane derivative isolated so far from a Russulaceae species and it is identical to a sesquiterpenoid previously isolated from submerged cultures of the Basidiomycete *Agrocybe aegerita* [42]. Geometry optimization of compound **68** by the MO-SCF AM1 method afforded the lowest energy conformer, having a skewed diene moiety, the 7-8-9-10 ω-angle being equal to 163°. Moreover, the CD spectrum of **68** showed a positive Cotton Effect at about 250 nm ($\Delta\varepsilon$ = +9.0) associated with the longest wavelength $\pi\rightarrow\pi^*$ transition of the *s-trans* diene chromophore. According to the skewed dienes helicity rule, and taking into account that this feature gave the predominant contribution to the observed CE, it could be deduced that the diene moiety in **68** was right-handed and, therefore, it indicated the absolute configuration of the compound as shown in the formula.

Analogously, molecular mechanics calculations (utilising Allinger's MM3-92 force-field) and the semi-empirical MO-SCF method AM1 indicated an



angle ω of 153° and 146°, respectively, for the non-planar enone moiety in the calculated lowest energy conformer of plorantinone B (**61**) [33]. Interestingly, experimental coupling constants deduced from the ^{1}H-NMR spectrum of 61 were in better agreement with the AM1 geometry. On the other hand, in the CD spectrum of 61, the Cotton effects associated with the R and K bands showed a negative and a positive sign, respectively. Therefore, according to the helicity rule for the skewed 2-cyclohexenones, plorantinone B (**61**) was assigned the absolute configuration shown in the formula. For biosynthetic reasons the other sesquiterpenoids isolated from *R. pseudodelica* should have the same configuration as compound **61**. This stereochemistry is in agreement with the absolute configuration of naturally occurring marasmane sesquiterpenes, which biogenetically are formed by cyclobutane ring contraction of protoilludanes (Scheme 2).

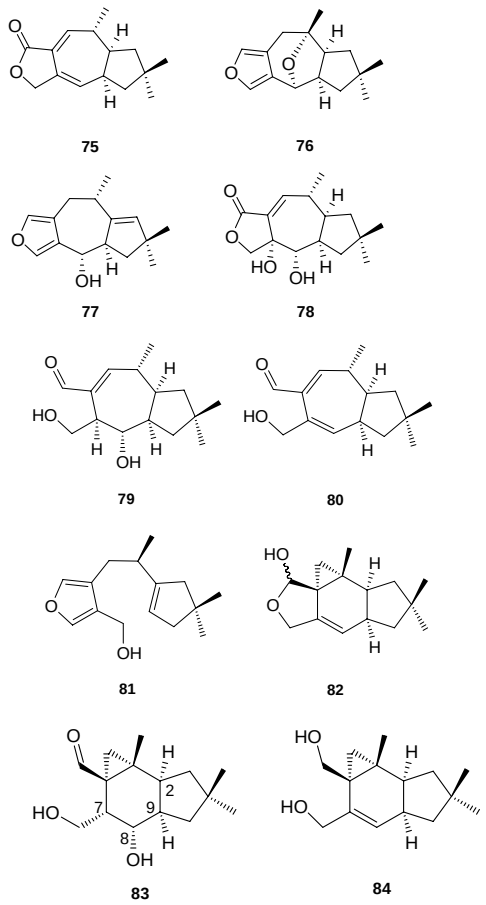
A related group of protoilludane sesquiterpenoids were recently isolated from *Russula japonica* Hongo [43]. *R. japonica* grows in colonies in broad-leaved forests throughout Japan. The fungus is taxonomically very close to *R. pseudodelica*, and according to some authorities, even identical to it. Surprisingly, nothwithstanding the very well-known deleterious effects of alcoholic solvents on the integrity of most Russulaceae sesquiterpenoid precursors, fresh fruiting bodies were extracted with 70% EtOH at room temperature for 4 weeks. The structures of six new illudoid sesquiterpenes, russujaponols A-F (**69**-**74**), four protoilludanes and two illudalanes, were elucidated by extensive NMR data. In addition, the absolute configuration of compound **69** was assigned on the basis of the X-ray analysis of benzoate **69a**. Interestingly, all protoilludanes isolated from *R. japonica* have a β oriented CH_2OH group at C(11), whereas, in the closely related species *R. pseudodelica*, protoilludanes with the opposite stereochemistry at C-11 were predominant. Compounds **69**, **70**, and **72** were not active in a cytotoxicity assay against 39 human cancer cell lines, whereas russujaponol A (**69**) suppressed invasion of human fibrosarcoma (HT 1080) cells into Matrigel in a concentration dependent manner and caused 63% inhibition at 3.73 μM (positive control, doxorubicin 52% at 0.17 μM).

Marasmane, isolactarane, and lactarane sesquiterpenes. These families comprise a large number of sesquiterpenoids that are biogenetically strictly related and have a great importance in the vegetative life of most Russulaceae mushrooms, as discussed in the introductory part of this review. Therefore, they shall be discussed together in this paragraph.

The finding of lactarane sesquiterpenoids in *Russula sardonia* Fr. represents a kind of milestone in the history of the chemistry of Russulaceae metabolites since, for the first time, sesquiterpenes identical or related to those isolated from *Lactarius* species, were also identified in a *Russula* [44]. This allowed us to infer that lactarane sesquiterpenes are widespread in many pungent *Russula* species and these compounds are not only characteristic of the milky juice mushrooms, but of many Russulaceae species.

The sesquiterpenes isolated from *R. sardonia* were lactarorufin A (**2**), furanol (**3**), furandiol (**4**), lactaral (**5**), vellerolactone (**75**), and the new compounds furan ether A (**76**), furosardonin A (**77**), and sardonialactone A (**78**) [44]. The absolute configuration of ether **76** was confirmed by dehydration of furandiol **4** with $MeSO_2Cl$ in hot pyridine, while the stereochemistry of lactone **78** was established by osmilation of **75** with OsO_4, which yielded a diol identical to the natural sardonialactone A. Furthermore, the coupling constant between 8-H and 9-H (J = 10.0 Hz) indicated a *trans* relationship between the two protons, hence the hydroxy groups at C(7) and C(8) were *cis* H-9.

R. sardonia is one of the most common pepper tasting red *Russula* species (Piperinae), which in Italy grow in autumn in pine woods. However, none of the compounds isolated is particularly hot-tasting to the tongue, while lactarorufin A (**2**) is highly bitter. Indeed, it was common practice, at the time of these



studies, to leave minced fruiting bodies in EtOH or Me_2CO for a long time, and this was also the procedure followed by us for the extraction of *R. sardonia* [44]. Therefore, the true pungent metabolites present in injured fruiting bodies of *R. sardonia*, as well as their precursor(s) in intact fruiting bodies, have still to be identified.

Five years later after these investigations, another pungent Russula, *R. queletii*, was analyzed by B. Wickberg and O. Sterner in Lund, during their pioneering studies on the sesquiterpenes constituting the Russulaceae chemical defense system [7b]. They demonstrated that this species originally contained only the chemically very labile marasmane derivative steroylvelutinal (**1**) [8], while the pungent unsaturated dialdehyde piperdial (**9**) was isolated from *n*-hexane and EtOAc extracts made within a few minutes after grinding. Notably, from extracts made more than 10 minutes after grinding, only very small amounts of **9** were obtained; instead, the C-5 reduced derivative, piperalol (**79**) could be isolated in considerable amounts. The antibacterial activities of **9** and **79** were comparable with those of velleral (**7**) and vellerol (**80**), respectively. Piperdial (**9**) is a labile compound; and in the presence of traces of acid (e.g. from silica gel) it rapidly dehydrates to velleral (**7**). Both **9** and **7** immediately degrade upon contact with alumina. Injured fruiting bodies of *R. queletii* were also found to produce the pungent dialdehydes velleral (**7**) and lactardial (**8**), vellerol (**80**), and very small amounts of furandiol (**4**); small amounts of lactarol (**81**) were also found in extracts made more than 30 minutes after grinding [7b].

Patterns of sesquiterpenoids analogous to those found in intact and injured specimens of *R. queletii*, have been found by us in several other pungent *Russula* species collected in Italy [45]. A brief account of the contents of undamaged and injured fruiting bodies extracted after 10 or 15 minutes, respectively, is reported in Table 1. For comparison, the results obtained on some mild *Russula* species and a few *Lactarius* have also been included.

Except *R. badia* and *R. foetens* (Table 1), all the *Russula* species discussed so far, containing velutinal esters (**1**) in undamaged fruiting bodies, afforded lactarane sesquiterpenoids upon injury. In *R. badia*, instead, isovelleral (**6**) and isovellerol (**82**) have the same marasmane skeleton as their precursors.

The same pattern of sesquiterpenoids was also found in *R. cuprea* Krombh. ex Lange [46]. This mushroom belongs to the section Urentinae, which comprises species having an initially mild tasting flesh that becomes strongly acrid after about 1 min. Extraction of intact fruiting bodies with EtOAc at 0°C, followed by chromatographic separation of the extract on flash silica gel pretreated with $HNiPr_2$, afforded a mixture of velutinal esters **1**. These were hydrolysed and the acids were analysed as methyl esters by GC-MS. Palmitic, linoleic, oleic, and stearic acids were identified, the last two being the major components. Injured fruiting bodies were minced at room temperature and left for 30 min before extraction with EtOAc. Column chromatography on flash silica gel, followed by HPLC separation on RP-18, yielded, in addition to isovelleral (**6**) and isovellerol (**82**), cupreal (**83**) and isovellerdiol (**84**), which had never been found before in nature.

The stereostructure of cupreal (**83**) was supported by the theoretical proton coupling constants of the 2-H/9-H/8-H/7-H spin system of the preferred conformer of cupreal obtained by molecular mechanics calculations (MM3(92) force-field) [46]. The formation of cupreal (**83**) from velutinal esters (**1**) was unclear, but it appeared to be an enzymatic

Table 1: Sesquiterpenoids found in intact and injured fruiting bodies of some *Russula* and *Lactarius* species [45].

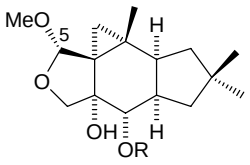
species	Intact fruiting bodies	Damaged fruiting bodies (10 min)	Damaged fruiting bodies (15 min)
R. fellea	velutinal esters of stearic, oleic, palmitic and linoleic acids (**1**)		velleral (**7**), lactaral (**5**), piperalol (**79**) unid. furanols and lactones
R. ochroleuca	velutinal esters of stearic, oleic, palmitic and linoleic acids (**1**)		**7**, **5**, **79**, lactardial (**8**), unid. furanols
R. exalbicans	unid. velutinal esters (**1**)	**7**, **8**, piperdial (**9**), **79**, vellerol (**80**)	
R. torulosa	stearoylvelutinal (**1**)		**5**, **8**, unid. furanols and other lactaranes
R. rutila	stearoylvelutinal (**1**)		pattern similar to *R. torulosa*
R. firmula	stearoylvelutinal (**1**)	**5**, **8**, unid.	**5**, **8**, unid. furanols, and other lactaranes
R. badia	unid. velutinal esters (**1**)	unid. velutinal esters (**1**), isovelleral (**6**), isovellerol (**82**)	
R. foetens	unid. velutinal esters (**1**)		isovelleral (**6**)
R. acrifolia	stearoylvelutinal (**1**)	**5**, **6**, unid. furanols and possibly lactarane lactones	
R. paludosa	unid. velutinal esters (**1**)	**79**	
R. parazurea	unid. velutinal esters (**1**)	**5** and unid. furanols	
R. purpurata	small amounts of unid. velutinal esters (**1**)	Not yet investigated	Not yet investigated
R. xerampelina	small amounts of unid. velutinal esters (**1**)	Not yet investigated	Not yet investigated
R. amara	small amounts of unid. velutinal esters (**1**)	Not yet investigated	Not yet investigated
R. versatilis	small amounts of unid. velutinal esters (**1**)		**79**
R. cyanoxantha	no velutinal esters or other sesquiterpenes could be detected	no velutinal esters or other sesquiterpenes could be detected	no velutinal esters or other sesquiterpenes could be detected
L. rugatus	no velutinal esters or other sesquiterpenes could be detected	no velutinal esters or other sesquiterpenes could be detected	no velutinal esters or other sesquiterpenes could be detected
L. mitissimus	velutinal lactarinate (**1**)		velutinal lactarinate (**1**), **5**, **8**
L. mairei	velutinal lactarinate and stearate (**1**)	Large amounts of **7** after 5 min.	.

process. It might be formed by reduction at C(7) of velutinals or, alternatively, by hydration of isovellerol (**82**).

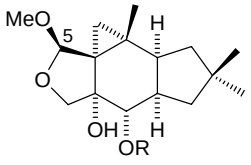
An impressive number of new marasmane and lactarane sesquiterpenoids have been isolated in recent years from *Lactarius* and *Russula* species collected in China and Japan. Most of them apparently correspond to the same species collected in Europe, whose chemical constituents have been reported in several publications, mainly by Swedish and Polish groups [5]. As already observed for other mushrooms, there are contrasting data between the results obtained on European and Asiatic specimens, and we have tried to explain these discrepancies. In addition, the Asiatic authors did not describe the chemical contents of intact and damaged fruiting bodies separately by using proper extraction procedures; instead, mushrooms were immediately ground and alcoholic solvents were largely used for extraction and chromatographic separations. For these reasons, the results have, in our opinion, a limited chemotaxonomic importance; however, they nicely demonstrate that fruiting bodies of Russulaceae are true chemical factories and can be used to generate a large number of different compounds.

From the fruiting bodies of *Lactarius piperatus* (Scop.: Fr.) S. F. Gray, a very hot-taste mushroom collected in Japan, four new marasmane sesquiterpenoids, namely lactapiperanols A-D (**85**-**88**), were isolated [47], together with two known lactarane sesquiterpenes, the quite common lactarorufin A (**2**) [5] and the rare furosardonin A (**77**) [44]. Comparison of the NMR data of the four compounds provided evidence that lactapiperanols A (**85**) and C (**87**), as well as B (**86**) and D (**88**), respectively, only differ from each other by the opposite configuration of the acetal C(5) carbon.

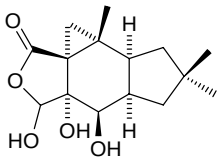
Diversified structural types of sesquiterpenoids, all biosynthetically derived from a protoilludane precursor (Scheme 2), were isolated from specimens of *L. piperatus* (Fr.) S. F. Gray collected from the Kunning area of Yunnan province, China [32] and extracted with EtOH. In addition to the protoilludane isoplorantinone (**42**) and the illudalane **43** discussed above, the new marasmane **89** and secolactaranolide **90** were isolated, together with known marasmanes **91** and **92**, the rare isolactarane isolactarorufin (**93**), and the known 5-lactaranolides lactarorufin A (**2**), blennin A (**94**) and blennin D (**95**) [5]. We believe



85 R = H
86 R = Ac



87 R = H
88 R = Ac



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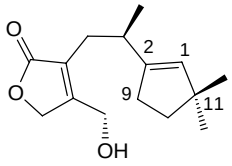

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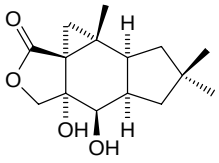

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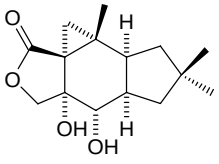

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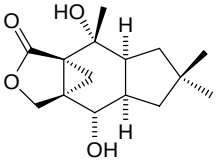

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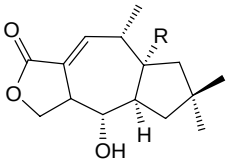
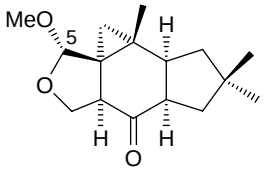


94 R = H
95 R = OH



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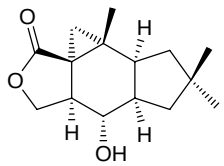

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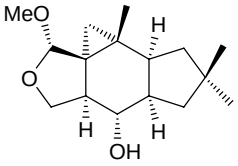

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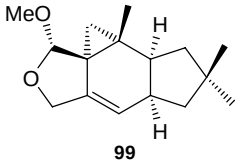

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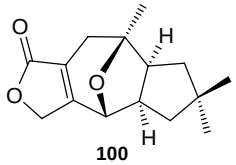


100

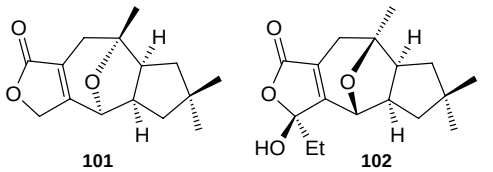
that compound **90**, though claimed to be new, is indeed identical with the very well known blennin C (**10**) [48], which has the trisubstituted double bond located between C(2) and C(9) instead of between C(1) and C(2). A direct comparison of compound **90** with an authentic sample of **10** has not yet been done; however, the key HMBC correlations of compound **90** are fully compatible with structure **10,** and a close inspection of the corresponding NMR data of compounds **10** and **90** shows minor differences which can be attributed to the different solvents in which the spectra have been recorded, i.e. $CDCl_3$ for **10** [48] and acetone-d_6 for lactone **90** [32]. In particular, it is unlikely that the signal of C(11), which resonated at δ 38.3 in the ^{13}C NMR spectrum of **10**, was shifted downfield by only 0.3 ppm in the ^{13}C NMR spectrum of **90**. In comparison, in furosardonin A (**77**) and in 1,2-dehydrolactarolide A (**103**), which have a double bond between C(1) and C(2), the signal of C(11) resonated at δ 42.5 and 41.9, respectively.

Strikingly, nothing has been reported by the Asian authors on the structures of the compounds responsible for the high pungency of *L. piperatus* [32,47]. Moreover, lactarorufin A (**2**) was the only sesquiterpenoid occurring in both the Japanese and Chinese samples and, even more surprisingly, no compound isolated from *L. piperatus* collected in Europe [5] was isolated from the Asiatic specimens.

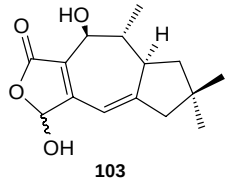
Pubescenone (**96**), a new maramane closely related to lactapiperanols A-D (**85**-**88**), was isolated with the well known 8,9-secofuranolactarane lactaral (**5**) from *L. pubescens*, an inedible mushroom that was collected at Ailao Mountains, in the Yunnan Province of China [49]. The fruiting bodies were exhaustively extracted with $CHCl_3$-MeOH, 1:1, a procedure by which a methoxy group was probably introduced at C(5) of the acetal **96**.

The same procedure was followed by the authors for the extraction of *Russula foetens* (Pers.) Pers. collected in the same habitat as *L. pubescens*. Five marasmanes were isolated and identified as lactapiperanol A (**85**), the lactones **92** and **97**, lactapiperanol E (**98**), and the acetal **99** [50]. The last three compounds were claimed to be new, but **98** and **99**, as well as the other *O*-methyl acetals discussed in this paragraph, were likely to have been derived from reaction of the corresponding free hemiacetals **83** and **82** with MeOH used for extraction.

We conclude this review with a group of 5-lactaranolide sesquiterpenes isolated from different *Lactarius* and *Russula* species. Although marasmane precursors apparently were not present in the extracts, there are no doubts, from our actual Russulaceae, that 5-lactaranolides are derived from



101 **102**



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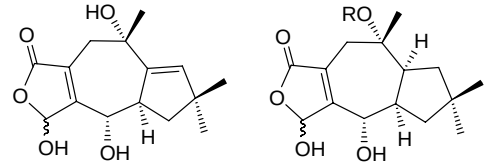
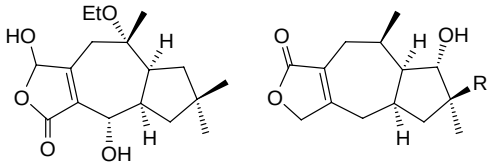


104 **105** R = H, **106** R = Me, **107** R = Et



108 **109** R = CH_2OH, **110** R = CO_2H

knowledge about the biochemistry of the chemical transformations stemming from velutinal esters (**1**) [5]. 5-Lactaranolides, *e.g.* lactarorufin A (**2**), are probably the largest group of sesquiterpenoids isolated from Russulaceae. The γ-lactone ring presumably derives from oxidation of the corresponding 1,4-dialdehyde or γ-hydroxyaldehyde or, alternatively, of a furan ring [5].

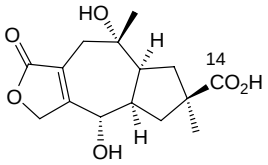
The new 5-lactaranolide rufuslactone (**100**) was recently isolated from a 95% aqueous EtOH extract of the fruiting bodies of *Lactarius rufus*, collected in the Yunnan Province of China [51]. Its structure, included the internal ether between C(3) and C(8) was confirmed by comparison of the NMR data with those of stereomeric γ-lactone **101**, previously isolated from *L. necator*, and subvellerolactone C (**102**) isolated from *L. subvellereus* Peck [5]. The NOE spectra and the small value of the coupling constant (J = 3.3 Hz) between 8-H and 9-H confirmed the β-configuration of the internal ether.

The internal ether **100** was found to inhibit the mycelial growth of some plant pathogenic fungi *in vitro*. *Alternaria brassicae* was the most sensitive to compound **100**, and its mycelium growth inhibition was 68.3% at 100 μg/mL. Interestingly, at 100 μg/mL, the growth of *A. alternata* was almost unaffected by the commercial fungicide carbendazim, while compound **100** inhibited the growth by 38.9% [51].

The mushroom *L. subvellereus* Peck is commonly used in Chinese traditional medicine, and the EtOAc extract has shown cytotoxic and antitumor activities. These effects have been attributed to two 5-lactarolide sesquiterpenes, subvellerolactones A (**103**) and C (**102**) isolated from this mushroom, which were active against different cancer cell lines [52]. Another new lactaranolide, 1,2-dehydrolactarolide A (**104**), together with known lactarorufin A (**2**), 3-*O*-ethyllactarolide A (**107**) and 3-*O*-ethyllactarolide B (**108**) [5], was isolated from fruiting bodies of *L. vellereus* Fr., collected in Japan [53]. The compounds were separated by bioassay-guided purification of the EtOH extract. Two lactarane sesquiterpenes, lactarorufin A (**2**) and lactarolide A (**105**), were isolated with the same procedure from a MeOH extract of *L. subpiperatus* Hongo [53]. Compound **104** exhibited promotional activities of 119%, 152%, and 162% at 3.6 × μM, 3.6×10^1 μM, and 3.6×10^2 μM, respectively, toward radicle elongation of lettuce seedlings, while the other isolated compounds showed no effect up to 3.6×10^2 μM [53].

Repraesentins D (**109**) and E (**110**) are two new lactaranes isolated, together with the protoilludane-related sesquiterpenoids repraesentins A (**46**), B, C, and F (**48**-**50**), from *L. repraesentaneus* Britz. Fruiting bodies were collected in Japan and extracted with MeOH at room temperature [36]. The structures of repraesentins D and E are quite interesting for the lack of a hydroxyl group at C(8), which is commonly found in lactaranolides [5], and the rare presence of an oxygenated function at C(1). Compound **109** exhibited a maximum promotional activity of 157% at 3.6×10^2 μM toward radicle elongation of lettuce seedlings, while **110** showed activity of 122%, 143%, and 164% at 3.6×10^{-1} μM, 1.1 μM, and 3.6 μM, respectively, suggesting that the carboxyl group at C(14) was needed for exhibiting strong activity [36]. Compounds **109** and **110** were inactive on the hypocotyl elongation of lettuce seedlings up to 3.6×10^2 μM [36].

Russula brevipes is a short stalked white mushroom growing widely in woods of the north-western Himalayas, during and after the rainy season. It is reported to be edible, although it is not consumed by



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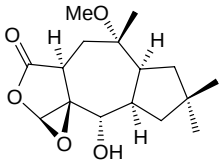

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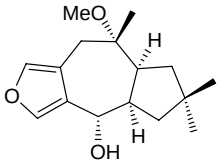

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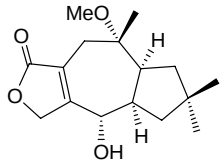

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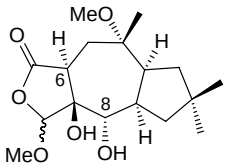
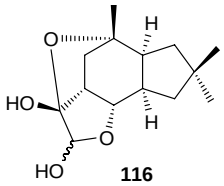


115

local people. Air and oven dried (40°C) finely powdered fruiting bodies were extracted with light petroleum in a Soxhlet and then with MeOH for 40 h to give lactarorufin A (**2**) and the new 14-carboxylactarorufin A, named russulactarorufin (**111**) [54]. To confirm the presence of the carboxylic acid group, russulactarorufin was esterified with CH_2N_2. The up-field shift NMR resonances of quasi-axial 1-H and 10-H in the ester (δ 1.08 and 1.15) compared to the free acid (δ 1.82 and 1.75) could only be accounted for if the protons fall into the shielding cone of the ester carbonyl group.

An interesting new β,γ-epoxy-γ-lactone sesquiterpenoid (**112**) was isolated from the neutral fraction of a MeOH extract of fruiting bodies of *Russula emetica* collected in Japan, together with three known sesquiterpenoids, namely lactarorufin A (**2**), furandiol (**4**), and the corresponding 3-*O*-methyl ether **113** [55]. Compound **112** is the first example of a 5-lactaranolide having an epoxy ring at C(7) and C(13). The IR band at 1805 cm^{-1} was indicative of a β,γ-epoxy-γ-lactone group. Compound **112** was easily transformed into the 3-*O*-methyl ether of lactarolide A (**106**) during purification by silica gel column chromatography. Reduction of **106** with $NaBH_4$ afforded 3-*O*-methyl lactarorufin A (**114**). On the other hand, methanolysis of epoxide **112** gave the dimethoxy derivative **115**. A NOE interaction observed between 6-H and 8-OH indicated the α-configuration for 6-H of **115**. It has been suggested by the authors that a β,γ-epoxy-γ-lactone compound



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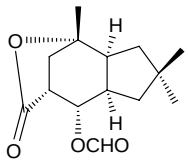

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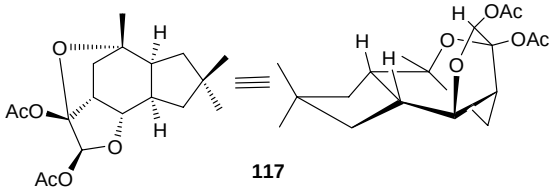

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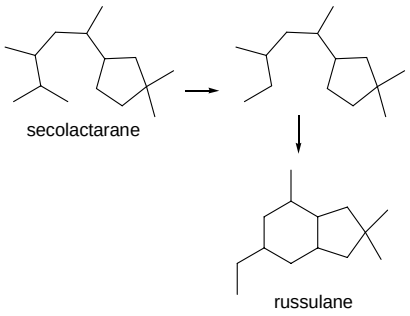
like **112** may be a biogenetic precursor of lactol derivatives of the type of 3-*O*-methyl lactarolide A (**106**) [55]. Actually, we suggest that **112** can be formed by oxidation of the furan ring of furandiol ether **113**, thus being a link between the furanolactarane and the 5-lactarolide groups of sesquiterpenes

We have observed that alkoxy substituted lactaranolides and furanolactaranes, in particular 3-methoxy or ethoxy derivatives, e.g. **106**, **107**, and **113**, have frequently been isolated from alcoholic extracts of different *Russula* or *Lactarius* species. We have serious doubts, indeed, that these compounds are genuine fungal metabolites; instead, we suspect that they are originated from degradation of velutinal precursors (**1**), which are very well known to decompose rapidly upon contact with alcohols [5,11]. On the other hand, γ-hydroxybutenolides, e.g **107** and **108**, can well derive from air oxidation of the corresponding lactarane furans [56].

Fresh fruiting bodies of *R. delica* Fr., purchased in a food market in Japan, were extracted with Et_2O at room temperature for 2 weeks. Russulanorol (**116**), a new norsequiterpenoid with a novel carbon skeleton, was isolated together with known furandiol (4), blennin C (**10**), and lactarolide A (**105**) [57]. Compound **116** exists in solution as a solvent-dependant mixture of two stereoisomers at the C-11 hemiacetal carbon, in a ratio of 2:1 and 10:1 in $CDCl_3$ and CD_3OD, respectively; the β-OH hemiacetal is the major one. The structure of russulanorol (**116**) was based on that of diacetate **117** obtained upon exposure of 116 to Ac_2O-pyridine. NOE experiments indicated the relative stereostructure and the conformation shown in the figure. Treatment of russulanorol (**116**) with PCC-Al_2O_3 in benzene gave the γ-lactone 118, which



Scheme 7: Possible biogenetic pathway for the carbon skeleton of russulanorol (116).

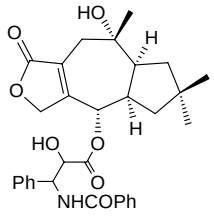
exibited a positive Cotton effect at 217.5 nm ($\Delta\varepsilon$ + 3.28). The application of the lactone CD sector rule suggested the absolute stereostructure shown in figure. The previously unknown norsesquiterpenoid skeleton of **116** was named "russulane" and a possible biosynthetic pathway from the secolactarane skeleton was proposed (Scheme 7) [57]. The co-occurence of russulanorol (**116**) with blennin C (**10**) in the same mushroom seems to reinforce this hypothesis.

In our opinion, however, derivation of the russulane backbone from either a sterpurane or a cerapicane precursor, with loss of C(13), cannot be excluded (Scheme 2).

Biological activities: Various biological activities have been attributed to several sesquiterpenoids isolated from Russulaceae. They include cytotoxic, antitumor, antibacterial, antifungal, algicidal, insecticidal, antifeedant, and mutagenic properties. Earlier data of different bioactivities have been reported in previous reviews [5], whereas those determined for new sesquiterpenoids isolated in the last decade have been outlined in the preceding paragraphs of this update.

Continuing previous studies on the bioactivities of synthetic lactarane derivatives [5], Daniewski and collaborators in Poland prepared a small library of sesquiterpenoid alcohols of *Lactarius* origin esterified with *N*-benzoyl-, *N*-acetyl, and *N*-BOC-phenylisoserinates to mimic the structures of taxol and taxotere [58]. Representative structures are compounds **119** and **120**.

In comparison to original alcohols, the introduction of the various ester moieties moderately enhanced their antifeedant activities, as well as changed their selectivity of activity against the cereal storage pests



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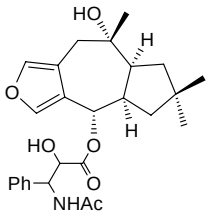

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Tribolium confusum, *Trogoderma granarium*, *Sitophylus granarius*, and *Rhizoperta dominica*. On the other hand, among 16 different *N*-benzoyl-phenylisoserinates tested, six decreased HSV-1 titers. Selectivity indexes ranged from 13.9 to 31.7. On the contrary, no activity against RNA viruses, bacteria, and fungal strains was detected [58].

Chemotaxonomic considerations: To the best of our knowledge, a serious chemotaxonomic study of Russulaceae sesquiterpenoids has never been attempted, apart from the pioneering works of Gluchoff-Fiasson and Kühner [8b]. Only some chemotaxonomic remarks will be reported hereafter since at present a more complete treatment is not possible owing to the almost complete lack of chemical data for species growing in extra-European countries. Only a few data from Eastern Asiatic or North American taxa are available and, as we have already observed, determination of a few of these species may not have been correct. Especially for the genus *Russula*, this is a serious drawback, since most tropical species belong to sections (or subgenera) scarcely or even not represented in the European Flora [59]. Moreover, a detailed chemotaxonomic approach should also take in consideration the important results obtained by Eugster [60] on the constituents of *Russula* pigments (the so called russupteridines).

What appears immediately evident is the widespread presence of velutinal esters (**1**) in fruiting bodies of *Russula* species. Actually, apart from the different esterifying fatty acids (*vide infra*), the marasmane sesquiterpene velutinal has been found in the intact fruiting bodies of all the European *Russula* species investigated so far, with the noteworthy exception of *R. lepida*, which produces aristolane-nardosinane sesquiterpenoids. Other oddities are the eastern Asiatic *R. japonica* and *R. pseudodelica* from which protoilludanes have been isolated. Finally *R. cyanoxantha* represents a singular anomaly, since no sesquiterpenoids at all were isolated. On the other hand, in the genus *Lactarius*, velutinals **1** are present in approximately half of the investigated species.

A remarkable finding is that velutinals, outside Russulaceae, have been isolated from a few genera of the order Aphyllophorales s.l., as *Auriscalpium*, *Gloeocystidiellum, Lentinellus, Artomyces* and others [5]. These genera have some morphological features in common with *Russula* and *Lactarius*, in particular the presence of specialized hyphae, called gloeocystidia, where velutinal esters are stored, and the ornamented spores. Thus, the common presence of velutinal esters confirms the close taxonomic relationship of the above genera to Russulaceae.

Recent results obtained from DNA sequencing [61] have definitely proved that Russulaceae do not belong to Agaricales, but are fully related to Aphyllophorales, and are close to them phylogenetically. Indeed, this represents a remarkable example of a complete consistency of chemical with morphological and genomic data.

A second observation is that the genus *Lactarius* appears to be much more chemically differentiated than the genus *Russula*. In the former, in fact, at least 13 different molecular skeletons have been found, while in the latter, apart from the marasmane backbone and the closely derived ones, like the lactarane and the secolactarane, only two additional skeletons are encountered, i.e., the protoilludane and the aristolane-nardosinane ones. Again this consideration is true as far as the European taxa are concerned, but it might well be incorrect on a world perspective.

A third important remark is that there is no clear-cut chemical separation between *Russula* and *Lactarius*, and some *Lactarius* and *Russula* sections have very similar biochemical content. A clear example is given by *Lactarius* sect. Albati and *Russula* sect. Foetentinae, in which stearoylvelutinal (**1**) is transformed into a mixture of marasmanes and lactaranes. In particular, it seems that the pungent-acrid taste of Russulaceae originates from enzymatic transformations of tasteless esters, present in undamaged fruiting bodies, into pungent bioactive compounds found in injured mushrooms. This is considered to constitute a chemical defense mechanism that protects mushrooms against the attack of parasites and predators, including mammals [5,7]. Among the different types of this general mechanism, that based on the enzymatic conversions of velutinal esters into hot-tasting dialdehydes, like isovelleral (**6**) and velleral (**7**), seems to be the most widespread one in Russulaceae. Noticeably, however, velutinal esters are also contained in some mild tasting *Russula* as well. For example, we have observed that in *R. paludosa* and *R. xerampelina* they are enzymatically transformed into non-acrid compounds [45].

In particular, it seems that all the *Russula* containing cystidia positive to the "sulfoaldehyde test" (see the discussion under *R. lepida*), synthesize velutinal esters, and this may indicate their phylogenetic proximity. On the other hand, *R. lepida*, which is negative to the sulfoaldehyde test, produces completely different terpenoids. In addition, nothing is known about the chemistry of the mild tasting *Russula* species belonging to the sections Incrustatula and Amoenula, which are not provided with gloeocystidia and therefore, very likely should not synthesize velutinal esters.

Regarding the occurrence of the different molecular skeletons in the various morphological sections of Russulaceae, it appears that some molecular skeletons are present in one section of one genus only. This is the case, for instance, of guaianes in section Dapetes, and caryophyllanes in Olentes (*Lactarius*), as well as prenylated phenols in Plinthogali (*Lactarius*) [5]. In such cases, the different chemical contents of fruiting bodies nicely confirm the different positions of these mushrooms in morphological taxonomy.

In a few cases, a certain molecular skeleton is found in only one species. This happens, for instance, for *L. uvidus*, *L. porninsis*, *L. glutinopallens*, and *R. lepida*, from which drimane, farnesane, glutinopallane, and aristolane sesquiterpenoids, respectively, have been isolated [5]. Thus, chemical data indicate that these species possibly occupy an isolated taxonomic position.

Finally, some skeletons cross several sections and are present in both the *Lactarius* and *Russula* genera. One of the most striking examples is represented by the protoilludanes, which are found in *R. pseudodelica* and *R. japonica* (sect. Plorantes), in *L. violascens* and *L. repraesentaneus* (sect. Uvidi), and in *L. atlanticus* (sect. Rhysocybella). In these cases chemical data diverge from the morphological classification, as these species are unrelated from a morphological point of view, with the exception of *L. violascens* and *L. repraesentaneus*, both belonging to the same section Uvidi [3].

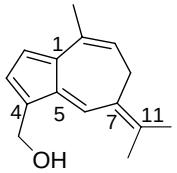
Actually, even the large number of species containing velutinal esters (**1**) in intact fruiting bodies can be clearly differentiated on the basis of the sesquiterpenoids formed enzymatically upon injury. In fact, in a few species, only metabolites maintaining the original marasmane skeleton are produced, i.e. isovelleral (**6**) and isovellerol (**82**); in others, both marasmane and lactarane sesquiterpenoids are generated; finally, in the largest number of species, only lactaranes and derivatives thereof, such as the secolactaranes, for example, alcohol **10**, are formed. These differences very likely reflect the existence of different enzymatic systems at work.

In addition, some chemical data would suggest that certain species are "intrusive" in some sections. For instance, *R. lepida*, producing aristolane sesquiterpenoids [26-28], does not seem to belong to subgenus Russula, a rather homogeneous group of species all producing marasmane and lactarane sesquiterpenes. Similarly, *L. atlanticus* does not fit into section Olentes, as it synthesizes protoilludanes instead of caryophyllanes [30], whereas *L. porninsis* does not match with other Zonarii, since it produces farnesane instead of marasmane sesquiterpenoids [5]. Another oddity is the presence of *L. uvidus*, *L. violascens*, and *L. repraesentaneus* in the same section Uvidi [3], since the first species produces drimanes, whereas the last two form protoilludanes.

A close analysis of the sesquiterpenoids with the same skeleton produced by different species shows that the patterns of functional groups are often different, though differences may be small, thus making these metabolites rather specific for the species and, therefore, of taxonomic interest. For instance, in the section Dapetes, sangol (**121**) is found only in *L. sanguifluus*, while the regioisomer olefin **122** is found in all the species of the section [5]. In section Olentes, *L. camphoratus* synthesizes a caryophyllane oxidized at C(4) and C(5) [17], while the caryophyllane **19b** produced by *L. subumbonatus* is oxidized at C(6) [18].

A further example is cupreal (**83**), a marasmane aldehyde isolated so far only from *R. cuprea* [46], but not present in other marasmane synthesizing species.

The nature of the fatty acids. All the investigated species of Russulaceae, except *R. lepida*, contain, in intact fruiting bodies, a single or a mixture of fatty acids esterified to either alcoholic or, very rarely,



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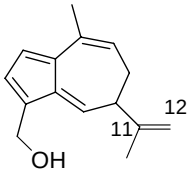

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phenolic moieties [5] of different secondary metabolites, including sesquiterpenes; the kinds of fatty acids appear to be useful chemotaxonomic markers too. Saturated and unsaturated C_{18} acids are by far the most commonly found, followed by C_{16} acids (mainly palmitic acid), while shorter or longer-chain acids are rarer.

It is immediately evident that the unusual 6-ketostearic acid (lactarinic acid) is present only in species of the genus *Lactarius*, but not in *Russula*. This acid occurs occasionally alone, as in *L. chrysorrheus*, but is usually in a mixture with other C_{18} acids, as in *L. rufus* and *L. necator* [5]. Of great interest is the recent isolation from Russulaceae of (6*S*)-hydroxystearic acid, the C(6)-reduced product of lactarinic acid [18]. Notably, *L. subumbonatus* is the only species from which this acid has been isolated till now. It should be stressed, however, that several *Lactarius* species do not synthesize lactarinic acid, but the more common stearic and oleic acids. It might be suggested that lactarinic acid is produced by the more evolved species of *Lactarius*, whereas this fatty acid is lacking in the more archaic ones. Unsaturated C_{18} fatty acids, like oleic and linoleic acids, are mainly found in *Russula* and in a handful of *Lactarius* [5].

The past and the future. The first chemical studies on sesquiterpenoids of Russulaceae started in the early 50s by Sorm's group in Prague. They did remarkable work on the elucidation of the structures of a few very fragile guaiane constituents of the pigments of *Lactarius* of the section Dapetes [5]. Since the early 70s the studies by Daniewski in Poland, the Swedish group at Lund and our group at Pavia greatly contributed to the determination of the structures of pungent sesquiterpenoids and a number of related compounds [5]. A remarkable breakthrough in the field was the discovery of velutinal esters by Gluchoff-Fiasson and Kühner's team in 1982 [8], which paved the way to the detailed comprehension of the biochemical mechanisms underpinning the so called "chemical defense system" of Russulaceae.

In conclusion, the history of sesquiterpenoids of Russulaceae spans over more than fifty years now but, as this review has illustrated, is still a lively field of research. The recent activities of research teams from Japan and China testify to the worldwide interest in the field. In fact, compounds with new structures and even with new skeletons continue to be discovered and often they display various and important biological activities. Extension of the studies to non-European species, which, except for a very few *Lactarius*, were the only ones studied till a decade ago, has greatly increased the possibility to achieve new important discoveries in the field. *Russula* species, in particular, are still largely unexplored, considering their great number and worldwide distribution [4,6,59]. Moreover, the likely discovery of other potent bioactive compounds is easily predictable, thanks to the increasing number of biological targets available nowadays and the use of more sophisticated bioassays than in the past.

Biosynthetic studies on the intricate pathways leading to the different sesquiterpenoid skeletons of Russulaceae have been almost neglected so far; therefore, we believe that the elucidation of the most intriguing backbone rearrangements, for example those involving the protoilludane and marasmane skeletons, should inspire several fascinating mechanistic experiments.

At the end of this review, we wish that different authors in the world would follow the same appropriate procedure for the isolation of sesquiterpenoids of Russulaceae, to prevent the formation of artifacts that hamper the correct comparison of the results obtained on different species or on the same species collected in different places. Indeed, it is mandatory for every scientist to avoid, whenever possible, the introduction of gross mistakes in the chemical and mycological literature.

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