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PRODUCTION OF BIOACTIVE SECONDARY METABOLITES IN THE FRUIT BODIES OF MACROFUNGI AS A RESPONSE TO INJURY

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Key Word Index—Ascomycetes; Basidiomycetes; fruit bodies; chemical defence; enzymatic conversions; antifungal; antibacterial; nematicidal.

Abstract—The conversion of secondary metabolites as a response to injury in fruit bodies of 121 species of Ascomycetes and Basidiomycetes was investigated. Extracts of intact and injured specimens were compared by analysis with TLC, and by assaying their antifungal (against *Nematospora coryli*), antibacterial (against *Bacillus brevis*) and nematicidal (against *Caenorhabditis elegans*) activities. Significant conversions induced by injury were observed (by TLC comparison) in the extracts of 66 species. For 37 of these the extract of the injured specimen showed higher bioactivity. Of the remaining 55 species, in which no changes between the extracts of intact and injured fruit bodies were observed, 30 were found to contain bioactive compounds. The possibility that these findings reflect the presence of chemical defence systems in mushrooms, mediated by enzymatic conversions activated by physical injury, is discussed.

INTRODUCTION

A large number of bioactive and structurally diverse fungal metabolites have been isolated and characterised over the years, and some of these have been used for the development of valuable pharmaceuticals and pesticides. Most fungal metabolites have been reported from fermentations, but also compounds formed in fruit bodies have attracted attention for several reasons. The fruit body has a distinct role in the life of a fungus, it is normally short-lived and forms an entity that is separated from the rest of the organism. Therefore, the metabolites of the fruit bodies may be formed for completely other reasons that those found in the mycelium, and one possibility is that they protect the fruit body from parasites and predators. Recently, a study comparing the insecticidal activities of extracts of the fruit bodies of 175 species was published in this journal [1], and no less than 79 were found to contain metabolites that inhibit insect development. This has prompted us to compile the data of a similar screening, of the antimicrobial and nematicidal activities of extracts of fruit bodies. However, in this investigation we have taken into account the fact that the fruit bodies of several species have been shown to convert their secondary metabolites enzymatically to new compounds as a response to injury. Striking examples are the pungent species belonging to *Lactarius*, in which the biologically inactive precursor stearoylvelutinal (**1**) is converted in seconds to a strongly antibiotic and pungent sesquiterpenoid dialdehydes such as isovelleral (**2**) as a response to injury [2]. The pigments of several *Xerocomus* species and other Boletales are also the result of enzymatic conversions that take place in injured fruit bodies, due to the conversion of pulvinic acid derivatives by oxidases (e.g. **3** to **4**) [3]. The chanterelle, *Cantharellus cibarius*, produces the fatty acid derivative cibaric acid (**5**) from dehydrocrepenyic acid (**6**) [4], while, for instance, several biindoline derivatives (e.g. **7**) and a series of 2,4-dimethylindole derivatives (e.g. **8**) are formed in fruit bodies of *Tricholoma scalpturatum* [5] and *T. sciodes* [6].

The ecological significance, if any, of the metabolites formed in injured fruit bodies is still not understood, partly because lack of material has hampered the assaying of the relevant biological activities, and partly because it is not known which biological activities would be the most relevant. However, at least in the cases where antibiotic and pungent compounds are formed from less active precursors, the conversion will result in the protection of the fruit body against

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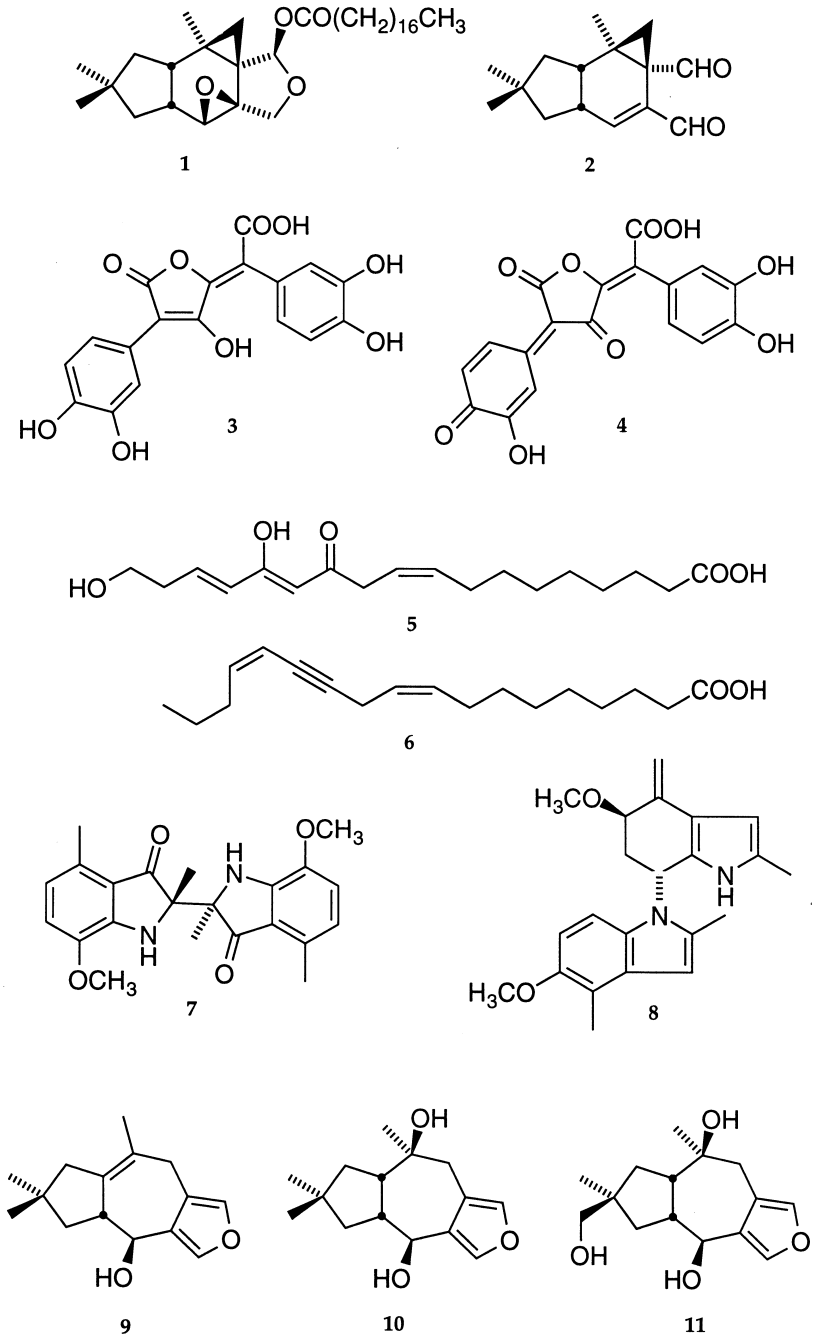

1 2 3 4 5 6 7 8 9 10 11

Fig. 1.

many parasites and predators. Despite this possibility, no comprehensive study of the frequency of this phenomenon in fruit body-forming higher fungi has so far been carried out. In this study, we have prepared extracts of intact as well as injured fruit bodies of 121 representative species within the German and Swedish mycoflora, and compared them by analysis with TLC and by assessing their antimicrobial and nematicidal activities. Antimicrobial assays were chosen as many micro-organisms such as mycophilic fungi are specialising on fungal tissue as nutrient sources, whereas the assay for nematicidal activities was employed as nematodes are the most abundant soil animals and many nematode species are feeding on fungal tissue. The test organisms, however, are not parasites but were selected according to our previous experience during investigations of bioactive secondary metabolites. They represent rather sensitive strains of their respective taxa and will consequently respond even to weakly active compounds and/or such metabolites that are only present in a given extract in small amounts.

RESULTS AND DISCUSSION

The results of the screening are presented in Tables 1 and 2. In Table 1, the data for the individual species are given, while Table 2 gives an overview of the results. It is noteworthy that differences in the chemical composition between extracts of intact and injured fruit bodies can be observed (by TLC analyses) for a majority of the species that have been investigated. The question whether the TLC differences observed are the result of enzymatic conversions or simply of chemical transformations has not been investigated. However, previous investigations in this area (*vide supra*) have indicated that rapid (minutes) enzymatic conversions of secondary metabolites in injured fruit bodies as a response to physical injury is a widespread phenomenon in the kingdom of fungi. Table 2 clearly shows that there are large differences between the orders. In all Russulales species investigated here, and in 15 of 18 Boletales species, the extracts of injured fruit bodies differ from those of intact, while this is the case for only a few (6 of 27) of the Agaricales, Tricholomataceae excepted. For the Tricholomataceae species, changes were observed with approximately 50% of the investigated species.

In Table 2, the results have also been divided according to the following criteria; (a) The extracts, both of intact and injured specimens, are inactive in our three assays; (b) Both types of extracts possess approximately the same bioactivity; (c) The bioactivity decreases as a response to injury; (d) The bioactivity increases as a response to injury.

In most of the 55 species for which the TLC of the extracts of the intact and the injured mushrooms did not differ significantly, the bioactivities were, as expected, approximately the same. The 10 cases in which the bioactivity decreased (4) or increased (6) may be due to the degradation/formation of compounds that were not detected in our TLC system. For 25 of the 66 species in which the metabolites are converted (or transformed) as a response to injury, the two types of extracts were found to possess approximately the same bioactivity. Either the bioactivities of the compounds that are degraded and formed in these species are balanced, or they are not active in the assays used here. In two species, both belonging to the Tricholomataceae, the antibacterial activity is lost as a result of injury, but in no less than 37 species (i.e. 30% of the investigated fungi) the bioactivities increase in response to injury.

Most Russulales investigated (12 of 15) belong to group 2D, which is expected as injured fruit bodies of pungent *Russula* and *Lactarius* species have been shown to produce antibiotic sesquiterpenoid unsaturated dialdehydes (e.g. isovelleral (**2**)) from velutinal esters (e.g. stearoylvelutinal (**1**)). The nematicidal activity of isovelleral (**2**) towards *C. elegans* is approximately 10 μg ml (LD_{50}). In addition to the dialdehydes, these conversions also generate free fatty acids, which are well-known to exhibit nematicidal activity and have been identified as the nematicidal principles of fungi as well as plants [7–9]. There may therefore be an ecological role also for the fatty acid of the precursor stearoylvelutinal (**1**), in protection against invertebrate parasites. The mild-tasting *Lactarius mitissimus* produces the three furan sesquiterpenoids **9**–**11** [10], they were re-isolated in this investigation and shown to possess weak nematicidal activity towards *C. elegans* (LD_{50} is approximately 100 μg ml for all three). In the agar diffusion assay, however, the compounds were devoid of activities towards *N. coryli* and *B. brevis* up to 100 μg disk.

In *Tricholoma terreum* and in all *Lepista* species investigated here the nematicidal activity increases in response to injury, and it could be shown that *T. terreum* produces considerable amounts of linoleic acid and S-coriolic acid while *Lepista* spp. produce free linoleic acid, along with unidentified metabolites. Also the *Melanoleuca* species investigated, *Laccaria amethystina* and *Marasmius wynnei*, produce fatty acids upon injury, demonstrated by the isolation and spectral characterisation of the nematicidal principle. In *Clitocybe odora*, *Hohenbuehelia grisea*, *Laccaria amethystina*, *Psathyrella velutina*, *Stropharia aeruginosa* and *Tricholoma terreum* the nematicidal activities caused by the fatty acids are accompanied by antibiotic effects, mediated by other metabolites. The Boletales species differ from the Russulales in that the extracts in most cases are inactive and that only few (4 of 15) of the species in which changes are observed increase the bioactivity as response to injury. No changes in composition or bioactivity could be observed in the few Ascomycetes investigated here, and if this holds for a larger number of species it could indicate a difference between the two sub-divisions of fungi.

In the mild-tasting *L. deterrimus*, we also observed nematicidal activities in the extracts of the injured specimens. As guaiane sesquiterpenes were isolated from this species before [11] and were detected by TLC (comparison with authentic material), they might be responsible for these activities. The amounts of these unstable compounds available, however, did not allow the evaluation of their nematicidal activities. Their antimicrobial and cytotoxic effects have previously been described [12].

EXPERIMENTAL

Extraction of fruit bodies

The fruit bodies were collected in 1993 and 1994 during filed excursions in the south of Sweden (Skåne, Småland) and south-west Germany (vicinity of Kaiserslautern and Pirmasens) and brought immediately to the laboratory. The fungi were identified according to [13–17]. The extractions were essentially carried out as described in reference 2. Briefly, the fruit bodies were extracted with freshly distilled ethyl acetate (ca 100 ml per 100 g fresh fruit body), by grinding them in a mortar. One portion was ground under the

Table 1.

Species	Intact N*	Intact AF†	Intact AB‡	Injured N*	Injured AF†	Injured AB‡	TLC-change§
AGARICALES							
Tricholomataceae							
Calocybe gambosa (Fr.) Donk	−	−	−	−	−	−	(+)
Clitocybe cerussata (Fr.) Kummer	−	−	−	(+)	−	−	−
Clitocybe dicolor (Pers.) Lge.	−	−	−	−	−	−	−
Clitocybe ditopa (Fr. ex Fr.) Gill.	−	−	−	−	−	−	−
Clitocybe obsoleta (Batsch ex Fr.) Quel.	−	+	+	−	+	++	+
Clitocybe odora (Bull. ex Fr.) Kummer	−	+	(+)	+	+	+	+
Clitocybe rivulosa (Pers. ex Fr.) Kummer	−	−	−	−	−	−	+
Collybia acervata (Fr.) P. Karst.	−	−	−	−	−	−	+
Collybia butyracea (Bull. ex Fr.) Quel.	−	−	−	−	−	−	−
Collybia dryophila (Bull. ex Fr.) Kummer	−	−	−	−	−	−	−
Collybia peronata (Bolt. ex Fr.) Sing.	−	−	−	−	−	−	−
Hohenbuehelia grisea (Pk.) Sing.	−	+	+	+	++	+	+
Laccaria amethystina (Bolt. ex Hooker) Murr.	−	−	−	+	+	−	+
Lepista gilva (Pers. ex Fr.) Roze	−	−	−	(+)	−	−	−
Lepista inversa (Scop. ex Fr.) Pat.	−	(+)	−	(+)	−	−	+
Lepista irina (Fr.) Bigelow	(+)	−	−	+	−	−	+
Lepista nebularis (Fr.) Harmaja	−	−	−	(+)	−	−	+
Lepista nuda (Bull. ex Fr.) Cke.	−	−	−	+	−	−	+
Lepista personata (Fr. ex Fr.) Cke.	−	−	−	+	−	−	+
Marasmius alliaceus (Jacq. ex Fr.) Fr.	(+)	+	+	(+)	+	−	+
Marasmius wynnei Bk. and Br.	−	−	−	+	−	−	+
Melanoleuca cognata (Fr.) K. and M.	−	−	−	+	−	−	+
Melanoleuca melaleuca (Pers. ex Fr.) Mre.	−	−	−	(+)	−	−	+
Mycena amicta (Fr.) Quel.	−	−	+	−	−	−	−
Mycena epipterygia (Scop.) S. F. Gray	−	−	−	−	−	−	−
Mycena polygramma (Bull. ex Fr.) S. F. Gray	−	−	−	−	−	−	−
Mycena pura (Pers.) Kummer	+	−	−	+	−	−	−
Mycena tintinabulum (Fr.) Quel.	−	−	+	−	−	+	−
Mycena sepia Lge. (syn. *M. vitrea* (Fr.) Quel.)	(+)	−	−	(+)	−	−	−
Oudemansiella platyphylla (Pers. ex Fr.) Mos.	−	−	+	+	−	+	+
Oudemansiella longipes (Bull.)	−	−	+	+	−	+	+
Tricholoma albobrunneum (Pers. ex Fr.) Kummer	−	−	−	−	−	−	−
Tricholoma columbetta (Fr.) Kummer	+	−	+	+	−	+	−
Tricholoma flavovirens (Pers. ex Fr.) Lund and Nannf.	+	−	−	+	−	−	−
Tricholoma saponaceum (Fr.) Kummer	−	+	+	−	+	+	−
Tricholoma terreum (Schff. ex Fr.) Kummer	−	−	+	+	+	+	+
Tricholoma ustale (Fr. ex Fr.) Kummer	+	−	(+)	+	−	−	(+)
Tricholomopsis decora (Schff. ex Fr.) Sing.	−	−	+	−	−	+	−
Agaricaceae							
Agaricus bitorquis (Quel.) Sacc.	−	−	−	−	−	−	−
Agaricus bisporus (Lge.) Sing.	−	−	−	−	−	−	+
Cystoderma amiantinum (Scop. ex Fr.) Fay.	−	−	−	−	−	−	−
Cystoderma carcharias (Pers.) Fay.	−	−	−	−	−	−	+
Amanitaceae							
Amanita citrina (Schff.) S. F. Gray	−	−	−	−	−	−	−
Amanita fulva (Schff. ex) Pers.	−	−	−	−	−	−	−
Amanita rubescens (Schff. ex Fr.) S. F. Gray	−	−	−	−	−	−	−
Hygrophoraceae							
Hygrophorus mesotephrus Berk. and Br.	(+)	++	−	(+)	++	−	+
Hygrophorus piceae Kühn.	+	+	+	+	+	+	−
Hygrophorus pustulatus (Pers. ex Fr.) Fr.	+	+	++	+	+	++	−
Cortinariaceae							
Cortinarius balteatoalbus R. Hry.	−	+	−	−	+	−	−
Dermocybe palustris (Mos.) Mos.	−	−	−	−	−	(+)	−
Dermocybe semisanguineus (Fr.) Mos.	−	(+)	+	−	+	+	−

Table 1. *Continued.*

Species	Intact N*	Intact AF†	Intact AB‡	Injured N*	Injured AF†	Injured AB‡	TLC-change§
Cortinariaceae							
Dermocybe sanguineus (Wulf. ex Fr.) Wünsche	−	(+)	+	−	+	+	−
Gymnopilus spectabilis (Fr.) Sing.	−	(+)	+	−	−	+	−
Hebeloma mesophaeum (Per. ex Fr.) Quel.	−	++	++	−	++	++	−
Hebeloma sinapizans (Paulet ex Fr.) Gill.	−	++	++	−	++	+	−
Inocybe maculata Boud.	−	−	−	−	−	−	−
Rozites caperata (Pers. ex Fr.) Sing.	−	−	−	−	−	−	−
Coprinaceae							
Coprinus comatus (Müll. ex Fr.) Pers.	−	−	−	−	−	−	−
Psathyrella hydrophila (Bull. ex Merat) R. Mre.	−	−	−	−	−	−	−
Psathyrella velutina (Pers. ex Fr.) Sing.	(+)	−	+	+	++	++	+
Strophariaceae							
Kuehneromyces mutabilis (Schff. ex Fr.) Sing. and Smith	−	−	−	−	−	+	+
Pholiota squarrosa (Pers. ex Fr.) Kummer	−	−	−	−	−	−	−
Pholiota flammans (Fr.) Kummer	−	(+)	−	−	(+)	−	−
Stropharia aeruginosa (Curt. ex Fr.) Quel.	−	−	+	+	+	+	+
Stropharia hornemannii (Weinm. ex Fr.) Lund. and Nannf.	−	(+)	(+)	−	(+)	(+)	−
BOLETALES							
Boleuts edulis Bull. ex Fr.	−	−	−	−	−	−	−
Boletus erythropus (Fr. ex Fr.) Pers.	−	−	−	−	−	−	+
Boletus luridus Schff. ex Fr.	−	−	−	−	−	−	+
Chroogomphus rutilus (Schff. ex Fr.) O. K. Miller	(+)	−	−	+	−	−	+
Gomphidius glutinosus (Schff.) Fr.	−	++	+	−	++	+	(+)
Hygrophoropsis aurantiaca (Wulf. ex Fr.) R. Mre.	−	−	+	−	+	++	+
Leccinum scabrum (Bull. ex Fr.) S. F. Gray	−	−	−	−	−	−	+
Leccinum testaceoscabrum (Secr.) Sing.	−	−	−	−	−	−	+
Paxillus atrotomentosus (Batsch) Fr.	−	+	−	−	+	−	(+)
Paxillus involutus (Batsch) Fr.	−	+	−	−	+	−	(+)
Paxillus panuoides Fr.	−	−	(+)	−	−	(+)	(+)
Porphyrellus pseudoscaber (Secr.) Sing.	+	−	−	+	−	−	−
Suillus luteus (L. ex Fr.) S. F. Gray	−	−	−	−	−	−	(+)
Tylopilus felleus (Bull. ex Fr.) P. Karst.	−	−	−	−	−	−	−
Xerocomus badius (Fr.) Kühn. ex Gilb.	−	−	−	+	−	−	+
Xerocomus chrysenteron (Bull. ex St. Amans) Quel.	−	−	(+)	(+)	−	−	+
Xerocomus rubellus (Krbh.) Quel.	−	−	−	+	−	−	(+)
Xerocomus subtomentosus (L. ex Fr.) Quel.	−	−	−	−	−	−	+
RUSSULALES							
Lactarius deterrimus Gröger	+	−	−	+	−	−	+
Lactarius helvus Fr.	(+)	(+)	(+)	(+)	+	+	+
Lactarius mitissimus Fr.	−	−	−	+	+	+	+
Lactarius necator (Bull. em. Pers. ex Fr.) Karst.	+	+	+	+	+	+	+
Lactarius porninsis Roll.	−	−	−	+	−	−	+
Lactarius rufus (Scop.) Fr.	−	+	+	++	+	++	+
Lactarius torminosus (Schff. ex Fr.) S. F. Gray	+	+	+	++	+	+	+
Lactarius trivialis Fr.	+	+	+	++	+	+	+
Lactarius vellereus (Fr.) Fr.	+	+	+	++	+	+	+
Russula albonigra Krbh.	+	−	+	+	(+)	++	+
Russula decolorans Fr.	(+)	−	−	+	−	−	+
Russula emetica Fr.	−	−	−	+	+	+	+
Russula fellea Fr.	−	−	−	+	+	+	+
Russula nigricans (Bull.) Fr.	−	−	−	+	−	−	+
Russula vinosa Linbl.	−	−	−	−	−	−	+
APHYLLOPHORALES							
Albatrellus ovinus (Schff. ex Fr.) Kotl. and Pouz	−	+	+	−	+	+	+
Cantharellus cibarius Fr.	−	−	−	−	−	−	(+)
Cantharellus tubaeformis Fr.	−	−	−	(+)	−	−	+

Table 1. *Continued*

Species	Intact N*	Intact AF†	Intact AB‡	Injured N*	Injured AF†	Injured AB‡	TLC-change§
APHYLLOPHORALES							
Clavulinopsis corniculata (Fr.) Corner	+	++	−	+	++	−	+
Hydnum rufescens Fr.	+	−	−	+	(+)	+	+
Meripilus giganteus (Pers. ex Pers.) Karst.	−	−	−	++	−	−	+
Pterula multifida Fr. ex Fr.	+	+	+	+	+	+	−
Pycnoporus cinnabarinus (Jacq. ex Fr.) Karst.	−	−	−	(+)	−	−	−
Ramaria gracilis (Fr.) Quel.	−	−	−	−	−	−	−
Ramaria stricta (Fr.) Quel.	−	−	(+)	−	−	−	−
Sparassis crispa Wulf. ex Fr.	+	−	−	+	−	−	+
Trametes versicolor (Fr.) Pil.	−	−	−	−	−	−	−
Tyromyces leucomalellus Murr.	−	−	−	−	−	−	+
Tyromyces stipticus (Pers. ex Fr.) Kotl. and Pouz.	−	−	−	−	−	−	+
HETEROBASIDIOMYCETES							
Calocera viscosa Pers. ex Fr.	−	+	−	++	(+)	−	(+)
Pseudohydnum gelatinosum (Scop. ex Fr.) Karst.	−	−	−	−	−	−	−
GASTEROMYCETES							
Calvatia excipuliformis (Pers.) Perd.	(+)	−	−	(+)	−	−	−
Lycoperdon pyriforme Schff. ex Pers.	(+)	−	−	(+)	−	−	−
Scleroderma citrinum Pers.	−	−	−	−	−	−	−
ASCOMYCETES							
Bulgaria inquinans Fr.	−	(+)	++	−	(+)	++	−
Cordyceps ophioglossoides (Ehrh. ex Fr.) Link	+	+	(+)	+	+	−	−
Neobulgaria pura (Fr.) Petrak	−	−	−	−	−	−	−
Peziza badia Pers. ex Mer.	−	++	++	−	++	++	−

* Nematicidal activity towards *Caenorhabditis elegans* (LD_{90}, μg crude extract ml) after 18 h (see Ref. 8 for details of assay). −: $LD_{90} > 200$ μg ml. (+): $100 < LD_{90} < 200$ μg ml. +: $25 < LD_{90} < 100$ μg ml. ++: $LD_{90} < 25$ μg ml.

† Antifungal activity towards *Nematospora coryli* by 20 μg crude extract/paper disk (diameter 6 mm) in the agar diffusion assay after 24 h. −: No inhibition zone. (+): Inhibition zone 7–9 mm. +: Inhibition zone 10–15 mm. ++: Inhibition zone > 15 mm.

‡ Antibacterial activity towards *Bacillus brevis* by 20 μg crude extract/paper disk (diameter 6 mm) in the agar diffusion assay after 24 h. −: No inhibition zone. (+): Inhibition zone 7–9 mm. +: Inhibition zone 10–15 mm. ++: Inhibition zone > 15 mm.

§ The differences between the TLC's of the extracts of intact and injured specimens were judged as −, (+) or +. −: No significant change could be observed. (+): Relative differences in the amounts of the constituents were observed. +: Compounds present in the extract of the intact mushroom disppeared upon injury, and/or new compounds were formed in the injured mushroom.

Table 2.

	1 No changes observed*				**2** Changes observed*				
	A†	B‡	C§	D¶	A†	B‡	C§	D¶	Σ
Tricholomataceae	8	7	1	2	3	1	2	14	38
Other Agaricales	10	7	1	3	2	1	0	3	27
Boletales	2	1	0	0	6	5	0	4	18
Russulales	0	0	0	0	1	2	0	12	15
Other fungi	5	5	2	1	3	3	0	4	23
All fungi	25	20	4	6	15	12	2	37	121

* Differences between the TLC's of the extracts of intact and injured fruit bodies, see also Table 1 and Experimental.

† Both the extracts of intact and injured fruit bodies are inactive in the assays used here.

‡ The extracts possess some activity, which is approximately equal in both types of extracts.

§ The extracts of the injured fruit bodies are less bioactive in this investigation.

¶ The extracts of the injured fruit bodies are more bioactive in this investigation.

solvent, to produce the extract of the intact specimen, while another portion was ground and left for 30 min at room temperature before ethyl acetate was added and the extraction to produce the extract of the injured specimen took place. The ethyl acetate solutions were decanted from the mortar, dried over anhydrous sodium sulphate, and concentrated *in vacuo*.

TLC analysis

The samples were transferred to small vials, and a comparison between the extract of the intact and the injured specimens was made immediately by TLC analysis (SiO_2, toluene:acetone 7:3, detection by the UV absorption at 254 nm and by the colour reactions with anisaldehyde spraying reagent [18] after heating to 120°). The remaining solvent was removed by a stream of nitrogen, the weight of the oily residues was determined, and the samples were kept at $-20°$ until the biological assays were performed.

Biological assays

The antimicrobial activity was determined with 20 μg of the extracts in the agar diffusion assay [7] towards the yeast *Nematospora coryli* (antifungal activity) and the gram-positive bacterium *Bacillus brevis* (antibacterial activity). 50, 100 and 200 μg ml of the crude extracts were tested in the microwell plate assay towards the free-living nematode *Caenorhabditis elegans* for evaluation of nematicidal activity [8].

Isolation and identification of metabolites

Fatty acids and the bioactive metabolites from *L. mitissimus* were isolated by means of preparative HPLC (Jones chromatography; APEX Prepsil ODS 8 μm; column size: 250×25 mm) with water-methanol gradients as mobile phase. Their structures were confirmed by NMR spectroscopy and mass spectrometry. Isovelleral and other *Lactarius* metabolites were identified by comparison of TLC R_f values with those of authentic materials or comparison with literature data, respectively.

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