Inhibition of Degranulation of RBL-2H3 Cells by Extracts and Compounds from *Armillaria ostoyae*

Version 1

Authors

Simon Merdivan¹, Kristina Jenett-Siems², Karsten Siems³,
Timo Niedermeyer⁴, Michael J. Solis^{5,6}, Martin Unterseher⁵,
Ulrike Lindequist¹

Affiliations

- 1 Institute of Pharmacy, Ernst-Moritz-Arndt University Greifswald, Greifswald, Germany
- 2 Institute of Pharmacy, Freie Universität Berlin, Berlin, Germany
- 3 AnalytiCon Discovery GmbH, Potsdam, Germany
- 4 Interfaculty Institute of Microbiology and Infection Medicine, Eberhard Karls University Tübingen, Tübingen, Germany
- 5 Institute of Botany and Landscape Ecology, Ernst-Moritz-Arndt University Greifswald, Greifswald, Germany
- 6 Department of Natural Sciences, College of Science and Information Technology, Ateneo de Zamboanga University, Zamboanga City, Philippines

Key words

Armillaria ostoyae, Physalacriaceae, RBL-2H3, sesquiterpene aryl esters, β -hexosaminidase assay, melleolide, cytotoxicity

received July 22, 2016

revised November 4, 2016

accepted November 9, 2016

Bibliography

DOI <http://dx.doi.org/10.1055/s-0042-121608>

Planta Med Int Open 2017; 4: 1–7 © Georg Thieme Verlag KG Stuttgart · New York | ISSN 2199-157X

Correspondence

Simon Merdivan

Institute of Pharmacy, Pharmaceutical Biology, Ernst-Moritz-Arndt University Greifswald

Felix-Hausdorff-Str. 1, 17487 Greifswald, Germany

Phone: +49 38 34 86 48 64, Fax: +49 38 34 86 48 85

merdivans@uni-greifswald.de

Supporting information available online at
<http://www.thieme-connect.de/products>

ABSTRACT

Armillaria ostoyae (Romagn.) Herink is an edible honey mushroom from the family Physalacriaceae (Agaricales, Basidiomycota). Dichloromethane extracts of mushroom mycelium and fruiting bodies exhibited a significant degranulation inhibiting effect on RBL-2H3 cells using noncytotoxic concentrations. Bioactivity-guided fractionation of the mycelial dichloromethane extract led to the isolation of sesquiterpene aryl esters. Methyl linoleate could also be isolated. These substances were obtained from *A. ostoyae* for the first time, with one compound representing an undescribed natural product. Purified compounds melleolide H and J inhibited degranulation significantly. *A. ostoyae* could be a candidate for support of allergy treatments.

ABBREVIATIONS

BSA	bovine serum albumin
CTAB	cetyltrimethyl ammonium bromide
DNP-HSA	2,4-dinitrophenyl human serum albumin
HBSS	Hank's balanced salt solution
ITS	internal transcribed spacer
NRU	neutral red uptake assay
p-NAG	4-nitrophenyl- <i>N</i> -acetyl- β -D-glucosaminide

Introduction

During our investigations of biologically active fungi, *Armillaria ostoyae* (Romagn.) Herink [1], a basidiomycete belonging to the family Physalacriaceae, came into focus, as it affected degranulation in RBL-2H3 cells. The species is commonly known as edible

mushroom, but it also occurs as a devastating pathogen of various tree species causing serious economic losses in forestry all over the world [2]. The mechanisms of host infection as well as host-pathogen interactions have been studied intensively [3–7]. In addition, *Armillaria* mushrooms attract a wider interest, for instance, as the world's biggest living organism [8] or as a producer of bioluminescence [9]. A broad variety of antibacterial and cytotoxic sesquiterpene aryl esters were isolated from the genus *Armillaria*, especially *Armillaria mellea* [10–20], but also *Armillaria novae-zealandiae* [21] and *Armillaria tabescens* [22]. In this manuscript, degranulation inhibiting effects of extracts from fruiting bodies and cultivated mycelium as well as bioactivity-guided isolation of compounds from the mycelium of *A. ostoyae* are described. Degranulation is the process in which cells release secretory products stored in secretory granules by exocytosis. It is an important feature of many immune cells, e.g., for release of mediators [23]. Inhibition of degranulation can result in the blocking of inflammatory or allergic reactions.

► **Fig. 1** Inhibition of degranulation of RBL-2H3 cells by extracts from the mycelium of *A. ostoyae* and medium; pos: antibody-stimulated (without test substance); neg: unstimulated (without antibody and test substance). Concentration = 90 μ g/mL, Welch's test against solvent, $n \geq 4$.

Results

Three different extracts made with dichloromethane, methanol, and water were tested in a degranulation assay. Dichloromethane and methanol extracts from the mycelium of *A. ostoyae* exhibited significant degranulation inhibitory effects on RBL-2H3 cells (► **Fig. 1**). The Soxhlet dichloromethane extract was fractionated on a silica gel column with an *n*-hexane/ethanol/dichloromethane/methanol gradient. Fractions 2–4 were subjected to column chromatography using a toluene/acetone gradient from which fractions were also tested in the degranulation assay. Fractions 1, 2, 4, and 5 exhibited inhibitory activity, while fractions 3, 7, 9, and 11 did not. Known purified compounds melleolide H (purity $\geq 93.5\%$) and J (purity $\geq 95.4\%$), obtained from fraction 1, showed significant degranulation inhibition of RBL-2H3 cells. Other substances could be isolated only in much smaller amounts and were not tested. The potency of melleolide H was nearly one order of magnitude weaker than melleolide J. Again, the potency of the reference substance quercetin was one order of magnitude stronger than melleolide J. Quercetin had an IC_{50} of 6.7 μ M, a value in accordance with the literature [24]. The biological effects are summarised in ► **Table 1**.

Dichloromethane extracts and pure compounds obtained from *A. ostoyae* were tested for cytotoxic effects on RBL-2H3 cells using the NRU assay. The dichloromethane extract of the fruiting body exhibited no cytotoxic effect after 1 h (approximately the time necessary for degranulation testing) and a weak cytotoxic effect after 24 h. The IC_{50} of the cytotoxicity of the mycelial extracts was one order of magnitude higher than the IC_{50} for degranulation inhibition after 1 h and also after 24 h. Etoposid as a reference substance has an IC_{50} of 0.9 μ M. The cytotoxicity IC_{50} can be found in ► **Table 1**.

After repeated column chromatography on silica gel, from 11 fractions, 8 sesquiterpene aryl esters and methyl linoleate were

► **Table 1** Biological effects of extracts/substances from *A. ostoyae*.

Dichloromethane extracts/substances	IC_{50} Inhibition of degranulation	IC_{50} Cytotoxicity
Fruiting body <i>A. ostoyae</i>	115.1 μ g/mL	1 h: > 500 μ g/mL 24 h: 183.6 μ g/mL
Mycelium <i>A. ostoyae</i>	21.5 μ g/mL	1 h: 111.1 μ g/mL 24 h: 18.5 μ g/mL
Melleolide H	99.9 μ mol/L	24 h: 23.6 μ mol/L
Melleolide J	39.5 μ mol/L	24 h: 20.8 μ mol/L
Quercetin	6.7 μ mol/L	24 h: 185.2 μ mol/L
Etoposid	n. d.	24 h: 0.9 μ mol/L

isolated by semipreparative HPLC. The yields were between 0.0093 % and 0.091 % of dried biomass. Structures of isolated substances can be found in ► **Fig. 2**.

The isolated meroterpenes were identified as melleolide C [10] (2), H [11] (3) and J [21] (4), melleodonal C [12] (5), 10-hydroxymelleolide [15] (6), armillarin [22] (7), and armillaridin [25] (8) by means of mass spectrometry and NMR spectroscopy, while methyl linoleate (9) was identified by NMR spectroscopy. 13-Hydroperoxyarmillarinin (1) displayed a peak at m/z 503.1443 [$M + Na^+$] $^+$ ($\delta = -0.040$ ppm) in the HRESIMS in the positive mode, corresponding to a molecular formula of $C_{24}H_{29}ClO_8$ and identical to melleodonal C. The NMR spectra of 1 and 5 showed strong similarities, but, in contrast to 5, substance 1 possessed just one hydroxymethylene group at δ_H 5.60 (t, 8.7 Hz, H-5). An additional methylene residue was observed at δ_H 1.71 (dd, 2.0, 13.0 Hz, H-10 β) and δ_H 1.32 (dd, 11.6, 13.0 Hz, H-10 α), which coupled to H-9 at δ_H 2.41 (dd, 2.0, 11.6 Hz). Furthermore, a suspicious downfield shift of C-13 (δ_C 89.5 versus δ_C 77.6 in 5) was observed, leading to the assumption that there should be a hydroperoxide group

► Fig. 2 Structures of isolated substances.

in this position. Similar shift differences are described, e.g., for peroxides obtained from *Artemisia alba* [26]. For NMR data of **1** see ► Table 2. Thus, **1** was identified as the 13-hydroperoxy derivative of armillarinin [27]. All isolated compounds were described for the first time from the mycelium of *A. ostoyae*. Compound **1** represents an up to now unknown natural product. Other characteristics of isolated compounds can be found in “Characterisation of isolated compounds”.

Discussion

Species determination within the genus *Armillaria* is very complex, as they represent more of a species aggregate than distinctive species. Sequence analysis showed the mycelial material to cluster in the *A. ostoyae* group, which was therefore taken as the species of the mycelium. From the mycelium of *A. ostoyae*, different metabolites were purified using bioactivity-guided isolation. Dichloromethane extracts from fruiting bodies and mycelium had significant degranulation inhibiting effects on RBL-2H3 cells. Purified compounds **3** and **4** exhibited an inhibition of degranulation in differing potencies, with the halogenated derivate showing a higher potency than the unhalogenated derivate. These compounds potentially contribute to the activity of the extract. Sesquiterpene aryl esters from *Armillaria* sp. were already found to possess antibacterial [10, 16], antifungal [28] and cytotoxic [29, 30] effects and to inhibit the growth of lettuce [25]. Inhibition of degranulation of RBL-2H3 cells was observed for the first time in *A. ostoyae*,

► Table 2 NMR data of 13-hydroperoxyarmillarinin (500 MHz, δ [ppm], J [Hz]) in CDCl₃.

Atom	δ_H	δ_C
1	9.57 s	196.4
2		137.8
3	6.98 s	150.2
4		75.6
5	5.60 t (8.7)	75.3
6	2.11 dd (8.7, 11.1)	32.6
2.04 dd (8.7, 11.1)		
7		37.4
8	1.32 s	22.1
9	2.41 dd (2.0, 11.6)	46.8
10	1.71 dd (2.0, 13.0)	43.6
1.32 dd (11.6, 13.0)		
11		35.9
12	2.38 d (14.5)	52.1
1.71 d (14.5)		
13		89.5
14	1.15 s	30.2
15	1.02 s	31.1
1'		n. d.
2'		106.7
3'		163.3
4'	6.43 s	99.3
5'		159.9
6'		116.2
7'		139.6
8'	2.48 s	20.0
OCH ₃	3.90 s	56.6
3'-OH	11.30 s	

and all purified compounds were also isolated from the mycelium of *A. ostoyae* for the first time.

Extracts and the pure compounds **3** and **4** exhibited certain cytotoxic activity. In the extracts, cytotoxicity was time dependent and stronger after 24 h than after 1 h. After 1 h, the IC₅₀ for cytotoxic activity was one order of magnitude higher than the IC₅₀ of degranulation inhibition. The IC₅₀ of degranulation inhibition, therefore, is not due to a cytotoxic effect. The enzyme β -hexosaminidase is a membrane-bound enzyme. If the membranal function is disturbed because of cell damage, the enzyme would no longer be placed in the membrane, but dissolved in the supernatant, resulting in a higher activity. This effect is used for standardisation of the test, as the β -hexosaminidase activity of the cell lysis is set to 100%. The effect also is not caused by direct inhibition of the reporter β -hexosaminidase, as extracts and substances showed no significant inhibition of this enzyme (data not shown).

A. ostoyae is an edible mushroom, but should be cooked before consumption to avoid gastrointestinal problems. Honey mush-

rooms are widely used as food and until now, no toxicological risks have been reported. Through the intake of *A. ostoyae*, no problematic health issues should emerge. Also, cytotoxicity has already been observed in this mushroom. In the future, *A. ostoyae* could play a supporting role in the therapy of allergies.

Materials and Methods

Cell lines, chemicals, and biochemicals

Adherent RBL-2H3 cells were obtained from DSMZ (Braunschweig, Germany). Cells were cultured in DMEM (Sigma; PAA) with 1% penicillin/streptomycin (Sigma) and 8% FCS (Sigma). Cells were incubated at 37 °C in a humidified atmosphere with 95% air and 5% CO₂.

The composition of Tyrode's buffer was as follows [31]: NaCl 130 mM, KCl 5 mM, CaCl₂ × 2H₂O 1.4 mM, MgCl₂ × 6H₂O 1 mM, HEPES 10 mM, glucose 5.6 mM, BSA 0.1% or 0.1 mL/L of a 7.5% BSA solution (Sigma). Anti-DNP IgE antibody was also obtained from Sigma (D8406).

Fungal material and species determination

Fresh fruiting bodies of *A. ostoyae* were collected in a forest near Greifswald, Germany (54°7' 31.51'' N; 13°18' 30.46'' E), within an environment consisting mainly of coniferous trees. Fruiting bodies were cut in pieces, washed, frozen, and lyophilised. A voucher specimen is stored at the Institute of Pharmacy of the Ernst-Moritz-Arndt University Greifswald (no. AoFrK_PMIO). Mycelium of *A. ostoyae* was available from the mushroom collection of the Institute of Pharmacy of the Ernst-Moritz-Arndt-University Greifswald (no. AoM_PMIO), Germany and cultivated for 3 weeks in Hagem medium pH 5.4 (composition: ammonium succinate 0.5 g, KH₂PO₄ 0.5 g, MgSO₄ × 7H₂O 0.5 g, 1% FeCl₃ solution 0.5 mL, glucose 5 g, malt extract R2 5 g, Aq. dest. 1 L) at room temperature in a day-night rhythm, filtered from the culture broth, frozen, and freeze-dried. Before extraction, freeze-dried fruiting bodies and mycelium were ground to a powder. The extraction of fungal DNA, PCR of the fungal ITS rDNA, the official fungal barcode [32], and Sanger sequencing followed approved protocols [33]. DNA was extracted using a CTAB/chloroform/isomyl alcohol/isopropanol protocol. After extraction and drying of DNA, amplification of the ITS region of the ribosomal DNA was done by PCR applying standard concentrations and conditions on a Mastercycler: 15.64 µL ddH₂O, 5 µL Mango buffer, 1.7 µL or 50 mM MgCl₂ solution, 0.5 µL 10 mM dNTP, 0.5 µL of each primer (10 pM), 0.16 µL Taq DNA polymerase (5 U/µL), and 1 µL template DNA. Time program: 5 min at 94 °C, followed by 35 cycles 35 s at 94 °C, 50 s at 52 °C, 90 s at 72 °C, and 5 min at 72 °C in the end. The PCR product was run on 0.8% agarose gel. Sequencing was done in the Botanical Institute of the Ernst-Moritz-Arndt University Greifswald, Germany.

Published sequences were exported from GenBank [34], aligned by MAFFT [35, 36], and converted with Mesquite [37] into a NEXUS-file for the calculation of a phylogeny in MrBayes and RAxML. Graphical illustration was done by SeaView [38], MEGA [39], or TreeViewX 0.5 [40]. Species determination was realised with field observations (e.g., densely growing basidiomata, pres-

ence of rhizomorphs) and with macroscopic characteristics of fresh material (annulus, colorisation). ITS sequences were matched against published sequences. Similarity analysis with MrBayes (Bayesian inference approach) [41] and RAxML (Maximum likelihood approach) [42, 43] was used to confirm the secure placement of the own sequence within a clade of publicly available and published sequences of *A. ostoyae* (graphics can be found in Supporting Information).

Extraction of biomass and isolation of compounds

For preparation of the extracts for biological testing, biomass (fruiting bodies: ca. 25 g, mycelium: ca. 15 g) was extracted for 24 h with 500 mL solvent in a 250-mL Soxhlet apparatus. Successive extraction was in the order dichloromethane, methanol, water. Extracts were filtered through filter paper, the volume decreased under reduced pressure. Drying was done by evaporation of dichloromethane and methanol, followed by lyophilisation when the extracts were not completely dry. Water extracts were dried by lyophilisation only.

Extraction with succeeding isolation of the compounds was done with 500 mL dichloromethane in 1 L glass flasks at room temperature for 3 × 24 h. The solvent volume was 500 mL for each step. Extracts were filtered through filter paper and the volume decreased under reduced pressure at a temperature of ca. 40 °C. Drying was done by evaporation of dichloromethane at room temperature.

Sesquiterpene aryl esters were isolated from the dichloromethane extract (893 mg) prepared by the flask method. The biomass was extracted with 500 mL dichloromethane in a 1-L glass flask at room temperature for 3 × 24 h. Extracts were filtered through filter paper and the volume decreased under reduced pressure at a temperature of ca. 40 °C. Drying was done by evaporation of dichloromethane at room temperature. After that, a fractionation by column chromatography on silica gel 0.040–0.063 mm (Merck; ca. 155 g stationary phase, column dimensions: diameter 3 cm, height 41 cm) with an *n*-hexane/ethanol/dichloromethane/methanol (elution steps: *n*-hexane; *n*-hexane/ethanol 80:20; dichloromethane/methanol 90:10; methanol) gradient took place, yielding 5 fractions. Fractions 2, 3, and 4 (101 mg) were combined and subjected to another column chromatography on silica gel (ca. 50 g stationary phase, column dimensions: diameter 2 cm, height 39 cm) with a toluene/acetone gradient (toluene/acetone 90:10, 75:25, 50:50, methanol), resulting in 11 fractions. Isolation of methyl linoleate from the dichloromethane extract (749 mg) prepared at room temperature was done by C18E SPE (20 g cartridge; Phenomenex) with elution by an isopropanol/water gradient (elution steps: isopropanol 60%; isopropanol 70%; isopropanol 80%; isopropanol). The 70% isopropanol fraction (85 mg) was further purified by semipreparative HPLC, the last purification step for all substances. A Luna® C5 250 × 10 mm column, particle size 5 µm, 100 Å (Phenomenex) was used as the stationary phase. The mobile phase consisted of water as A and acetonitrile as B (VWR) in the gradient elution. In the later work, an addition of 0.1% formic acid to acetonitrile was used. The flow rate was 4 mL/min, and the detection channels were 190 nm and 272 nm. The time program was as follows: % B (time): 60 (0 min), 80 (23.5 min), 100 (24.5 min), 100 (29.5 min),

60 (31.5 min), 60 (37 min). The gradient was modified or shortened when possible, whereas the gradient slope was the same in all runs. The time program was as follows: % B (time) in isolation of methyl linoleate: 78 (0 min), 92 (23.5 min), 100 (24.5 min), 100 (29.5 min), 78 (31.5 min), 78 (37 min). After isolation, substances were tested for purity by HPLC with foregoing gradients.

Characterisation of isolated compounds

^1H NMR spectra were recorded at 400 MHz and 27 °C; solvent deuteriochloroform. 2D spectra were measured at 500 MHz, 27 °C; solvent deuteromethanol or deuteriochloroform. High-resolution mass spectra were obtained from a maXis 4G TOF-MS system (Bruker) by flow injection analysis of the compounds. The CD spectrum in the wavelength range of 200–400 nm of 13-hydroperoxyarmillarinin was measured with a JASCO T-810 system (JASCO) using isopropanol as the solvent. The IR spectrum of 13-hydroperoxyarmillarinin was recorded on an Alpha FTIR spectrometer (Bruker). Spectral data of the known compounds can be found in the literature indicated.

Compound 1 from fraction 1 (1.4 mg): Resin. UV [$\text{L} \times \text{mol}^{-1} \times \text{cm}^{-1}$]: $\epsilon_{219} = 8272$, $\epsilon_{238} = 4440$, $\epsilon_{271} = 1678$, $\epsilon_{283} = 1206$, $\epsilon_{326} = 617$. CD [$1 \times \text{mol}^{-1} \times \text{cm}^{-1}$]: $\Delta\epsilon_{219} = 0.29$, $\Delta\epsilon_{238} = -0.91$, $\Delta\epsilon_{271} = 0.023$, $\Delta\epsilon_{283} = -0.044$, $\Delta\epsilon_{326} = 0.34$. Absolute configuration was as described by Kobori et al. [25]. IR ν [$1 \times \text{cm}^{-1}$]: 1726 s (C=O st, Ester), 1666 s (C=O st, aldehyde), 2926 m (=CH st), 2854 m (-CH st), 3353 b (O-H st, coordinated). Monoisotopic mass 480.1551. (+)-HRESIMS 503.1443 [$\text{M} + \text{Na}^+$] ($\delta = -0.040$ ppm). Molecular formula $\text{C}_{24}\text{H}_{29}\text{ClO}_8$.

Compound 2 from fraction 7 (3.6 mg) [10]: Monoisotopic mass 446.1941. Resin. (-)-HRESIMS 445.1864 [$\text{M} - \text{H}^+$] $^+$ ($\delta = -0.880$ ppm). Molecular formula $\text{C}_{24}\text{H}_{30}\text{O}_8$.

Compound 3 from fraction 4 (11.9 mg) [11]: Monoisotopic mass 430.1992. Resin. (+)-HRESIMS 453.1881 [$\text{M} + \text{Na}^+$] $^+$ ($\delta = -0.619$ ppm). Molecular formula $\text{C}_{24}\text{H}_{30}\text{O}_7$.

Compound 4 from fraction 4 (13.7 mg) [25]: Monoisotopic mass 464.1602. Resin. (+)-HRESIMS 487.1494 [$\text{M} + \text{Na}^+$] ($\delta = -1.133$ ppm). Molecular formula $\text{C}_{24}\text{H}_{29}\text{ClO}_7$.

Compound 5 from fraction 6 (3.3 mg) [12]: Monoisotopic mass 480.1551. Resin. (-)-HRESIMS 479.1478 [$\text{M} - \text{H}^+$] $^+$ ($\delta = -0.116$ ppm). Molecular formula $\text{C}_{24}\text{H}_{29}\text{ClO}_8$.

Compound 6 from fraction 7 (2.9 mg) [15]: Monoisotopic mass 416.1835. Resin. (-)-HRESIMS 415.1755 [$\text{M} - \text{H}^+$] $^+$ ($\delta = -1.734$ ppm). Molecular formula $\text{C}_{23}\text{H}_{28}\text{O}_7$.

Compound 7 from fraction 4 (2.4 mg) [22]: Resin. Monoisotopic mass 414.2042. (+)-HRESIMS 437.1935 [$\text{M} + \text{Na}^+$] $^+$ ($\delta = 0.093$ ppm). Molecular formula $\text{C}_{24}\text{H}_{30}\text{O}_6$.

Compound 8 from fraction 4 (3.9 mg) [25]: Resin. Monoisotopic mass 448.1653. (+)-HRESIMS 471.1548 [$\text{M} + \text{Na}^+$] $^+$ ($\delta = 0.693$ ppm). Molecular formula $\text{C}_{24}\text{H}_{29}\text{ClO}_6$.

Compound 9 was isolated by C18 SPE followed directly by semipreparative HPLC (3.6 mg): Oil. Molecular formula $\text{C}_{19}\text{H}_{34}\text{O}_2$.

Degranulation assay

After trypsinisation, the cell concentration was adjusted to 5×10^5 cells/mL with DMEM. Four hundred μL of this suspension were given into a well of a 24-well plate (i.e., 2×10^5 cells/well). Subsequently, 100 μL of a 495 ng/mL solution of IgE in PBS (phosphate

buffered saline) (without Ca and Mg, PAA) were added into the wells for the determination of the positive control and test samples, and 100 μL PBS (without Ca and Mg) were added into the wells for determination of spontaneous degranulation and cell lysis. Cells were incubated overnight at 37 °C in a humidified atmosphere with 95 % air and 5 % CO_2 .

The supernatant was removed and the cells were washed with 500 μL of Tyrode's buffer. Then, 497 μL (for testing of raw extracts) or 498 μL (fractions and purified compounds) were added to the wells for tests samples, 500 μL for the positive control ("pos"), and 510 μL for the determination of spontaneous degranulation ("neg"). In the wells for cell lysis, 510 μL of a 0.1 % solution of Triton X-100 in Tyrode's buffer was pipetted. Cells were incubated for 10 min. Then, 3 μL (raw extracts) or 2 μL (fractions, purified compounds) of test samples were added into corresponding wells followed by incubation for 10 min. Cell stimulation was done by 10 μL of a 50 $\mu\text{g}/\text{mL}$ solution of DNP-HSA (Sigma) in PBS for all cells except those for spontaneous degranulation and cell lysis. A blank was measured with 510 μL Tyrode's buffer without cells.

For the β -hexosaminidase assay, 50 μL of the supernatant of each well were transferred to a 96-well plate and 50 μL of the β -hexosaminidase substrate p-NAG, 1.2 mM in 0.1 M sodium acetate buffer (pH 4.5), were added followed by incubation for 2 h. The reaction was stopped with 150 μL of 0.4 M glycine buffer pH 10.7 (glycine was from Merck). Absorption was measured in a microtitre plate reader (BMG Labtech) at 405 nm. From all values, the blank was subtracted. An IC_{50} value was determined for the reference substance quercetin (purity $\geq 95\%$; Sigma).

For determination of the direct inhibition of β -hexosaminidase, after seeding of the cells, 100 μL PBS (without Ca and Mg, PAA) were added to each well. After incubation overnight, cells were washed with 500 μL of Tyrode's buffer and 0.1 % Triton X-100 (Sigma) solution in Tyrode's buffer and added as follows: 510 μL for cell lysis and blank, 508 μL to the wells destined for test samples. After incubation for 10 min, 2 μL of test samples were added, followed by incubation for 10 + 30 min (time period necessary for the degranulation assay). Fifty μL of the supernatant were transferred to a 96-well plate for determination of β -hexosaminidase activity as described above.

Neutral red uptake assay

The assay was conducted in a 96-well plate. In brief, 15000 cells suspended in 100 μL medium were sown into each well. Wells on the periphery of the plate were filled with medium only, followed by incubation overnight. On the next day, the medium was removed from all wells and in all columns, except for columns 3 and 11, 150 μL of medium were added. Columns 3 and 11 were filled with 100 μL of medium. Into column 4, 150 μL of test sample were given and mixed thoroughly. Then, 150 μL of the mixture were transferred from column 4 to column 5, mixed thoroughly and 150 μL was transferred to column 6, etc. From the last column, 150 μL were removed and discarded. Into column 3, 50 μL of the positive control (etoposid in medium) were pipetted, and into column 11, 50 μL of the solvent in the concentration used in column 3 were pipetted. Cells were incubated for 24 h. Subsequently, the medium was removed and cells were washed with

200 μL of HBSS (Sigma). Into all wells, 100 μL of a solution of neutral red in medium were added and cells were incubated for 3 h. Neutral red can be accumulated by living cells only. After the incubation period, the neutral red solution was aspirated and cells were washed two times with 100 μL HBSS each. Next, 100 μL ethanol/glacial acetic acid solution were added to each well for cell lysis and dissolution of the dye. Microwell plates were shaken for 45 min, followed by absorption measurement at 540 nm in a microtitre plate reader (BMG Labtech). An IC_{50} value of cytotoxicity was reported for the reference substance etoposid (purity $\geq 98\%$; Sigma).

For testing of an effect of substances, extracts, or fractions after 1 h, cells were incubated for 48 h after seeding. Then the test was conducted as described above.

Statistical analysis

Determination of IC_{50} values was done with the software GraphPad Prism® version 6 using nonlinear regression with four parameters and a variable slope option. Significance was tested by unpaired Welch's t-test.

Supporting information

Graphics from RAXML and MrBayes for species determination of the mycelium of *A. ostoyae* (Fig. S1–S2), CD, ATIR, and UV spectra and ^1H - as well as ^1H , ^1H -COSY NMR spectra of **1** (Fig. S3–S8), and LC chromatograms of crude extracts and active fractions (Fig. S9–S11) are available as Supporting Information.

Acknowledgements

We thank Michael Eccius (EMAU Greifswald) for measurement of the IR spectrum of 13-hydroperoxyarmillarinin and Jonathan Dickerhoff (EMAU Greifswald) for support in the recording of the CD spectra. Likewise, we thank Mrs. Monika Matthias (EMAU Greifswald) for excellent technical support, especially in the cultivation of the mycelium of *A. ostoyae*.

Conflict of Interest

The authors declare no conflict of interest.

References

- [1] Romagnesi H. Observations on *Armillaria*. Bull Trimest Soc Mycol Fr 1970; 86: 257–265
- [2] Dobbertin M, Baltensweiler A, Rigling D. Tree mortality in an unmanaged mountain pine (*Pinus mugo* var. *uncinata*) stand in the Swiss National Park impacted by root rot fungi. For Ecol Manage 2001; 145: 79–89
- [3] Cleary MR, van der Kamp BJ, Morrison DJ. Effects of wounding and fungal infection with *Armillaria ostoyae* in three conifer species. II. Host response to the pathogen. Forest Pathol 2011; 42: 109–123
- [4] Cleary MR, van der Kamp BJ, Morrison DJ. Effects of wounding and fungal infection with *Armillaria ostoyae* in three conifer species. I. Host response to abiotic wounding in non-infected roots. Forest Pathol 2012; 42: 100–108
- [5] Cruickshank M, Morrison DJ, Lalumière A. The interaction between competition in interior Douglas-fir plantations and disease caused by *Armillaria ostoyae* in British Columbia. For Ecol Manage 2009; 257: 443–452
- [6] Entry JA, Cromack jr. K, Hansen E, Waring R. Response of western coniferous seedlings to infection by *Armillaria ostoyae* under limited light and nitrogen. Phytopathology 1991; 81: 89–94
- [7] Pearce RB. Tansley Review No. 87. Antimicrobial defences in the wood of living trees. New Phytol 1996; 132: 203–233
- [8] Schmitt CL, Tatum ML. The Malheur national forest: Location of the world's largest living organism [The humongous fungus]. Portland, Oregon: United States Department of Agriculture (USDA), Forest Service, Pacific Northwest Region; 2008
- [9] Mihail JD. Bioluminescence patterns among North American *Armillaria* species. Fungal Biol 2015; 119: 528–537
- [10] Arnone A, Cardillo R, Nasini G. Structures of melleolides B–D, three antibacterial sesquiterpenoids from *Armillaria mellea*. Phytochemistry 1986; 25: 471–474
- [11] Arnone A, Cardillo R, Di Modugno V, Nasini G. Secondary mold metabolites. XXII. Isolation and structure elucidation of melleolones D and E and melleolides E–H, novel sesquiterpenoid aryl esters from *Clitocybe elegans* and *Armillaria mellea*. Gazz Chim Ital 1988; 118: 517–521
- [12] Arnone A, Cardillo R, Nasini G. Secondary mold metabolites. Part 19. Structure elucidation and absolute configuration of melleolones B and C, novel antibacterial sesquiterpenoids from *Armillaria mellea*. X-ray molecular structure of melleolone C. J Chem Soc Perkin Trans I 1988; 503–510
- [13] Chen CC, Kuo YH, Cheng JJ, Sung PJ, Ni CL, Chen CC, Shen CC. Three new sesquiterpene aryl esters from the mycelium of *Armillaria mellea*. Molecules 2015; 20: 9994–10003
- [14] Donnelly DMX, Sanada S, O'Reilly J, Polonsky J, Prangé T, Pascard C. Isolation and structure (X-ray analysis) of the orsellinate of armillol, a new antibacterial metabolite from *Armillaria mellea*. J Chem Soc Chem Commun 1982; 135–137
- [15] Donnelly DMX, Hutchinson RM, Coveney D, Yonemitsu M. Sesquiterpene aryl esters from *Armillaria mellea*. Phytochemistry 1990; 29: 2569–2572
- [16] Donnelly DMX, Abe F, Coveney D, Fukuda N, O'Reilly J. Antibacterial sesquiterpene aryl esters from *Armillaria mellea*. J Nat Prod 1985; 48: 10–16
- [17] Liu TP, Chen CC, Shiao PY, Shieh HR, Chen YY, Chen YJ. Armillaridin, a honey medicinal mushroom, *Armillaria mellea* (higher basidiomycetes) component, inhibits differentiation and activation of human macrophages. Int J Med Mushrooms 2015; 17: 161–168
- [18] Midland SL, Izac RR, Wing RM, Zaki A, Munnecke D, Sims JJ. Melleolide, a new antibiotic from *Armillaria mellea*. Tetrahedron Lett 1982; 23: 2515–2518
- [19] Momose I, Sekizawa R, Hosokawa N, Iinuma H, Matsui S, Nakamura H, Naganawa H, Hamada M, Takeuchi T. Melleolides K, L and M, new melleolides from *Armillaria mellea*. J Antibiot 2000; 53: 137–143
- [20] Yang JS, Su YL, Wang YL, Feng XZ, Yu DQ, Liang XT. Two novel protoilludane norsesterpenoid esters, armillasin and armillatin, from *Armillaria mellea*. Planta Med 1991; 57: 478–480
- [21] Arnone A, Cardillo R, Nasini G. Secondary mold metabolites. XXIII. Isolation and structure elucidation of melleolides I and J and armellides A and B, novel sesquiterpenoid aryl esters from *Armillaria novae-zelandiae*. Gazz Chim Ital 1988; 118: 523–527
- [22] Donnelly DMX, Konishi T, Dunne O, Cremin P. Sesquiterpene aryl esters from *Armillaria tabescens*. Phytochemistry 1997; 44: 1473–1478
- [23] Cell Degranulation – MeSH – NCBI. Available at <https://www.ncbi.nlm.nih.gov/mesh/?term=cell+degranulation>. Accessed October 5, 2016
- [24] Mastuda H, Morikawa T, Ueda K, Yoshikawa M. Structural requirements of flavonoids for inhibition of antigen-induced degranulation, TNF- α and

- IL-4 production from RBL-2H3 cells. *Bioorg Med Chem* 2002; 10: 3123–3128
- [25] Kobori H, Sekiya A, Suzuki T, Choi JH, Hirai H, Kawagishi H. Bioactive sesquiterpene aryl esters from the culture broth of *Armillaria* sp. *J Nat Prod* 2015; 78: 163–167
- [26] Appendino G, Gariboldi P, Menichini F. Oxygenated nerolidol derivatives from *Artemisia alba*. *Phytochemistry* 1985; 24: 1729–1733
- [27] Yang JS, Su YL, Wang YL, Feng XZ, Yu DQ, Liang XT. Chemical constituents of *Armillaria mellea* mycelium. V. Isolation and characterization of armillarilin and armillarinin. *Yao Xue Xue Bao* 1990; 25: 24–28
- [28] Bohnert M, Nützmann HW, Schroeckh V, Horn F, Dahse HM, Brakhage AA, Hoffmeister D. Cytotoxic and antifungal activities of melleolide antibiotics follow dissimilar structure – activity relationships. *Phytochemistry* 2014; 105: 101–108
- [29] Bohnert M, Miethbauer S, Dahse HM, Ziemer J, Nett M, Hoffmeister D. *In vitro* cytotoxicity of melleolide antibiotics: structural and mechanistic aspects. *Bioorg Med Chem Lett* 2011; 21: 2003–2006
- [30] Misiek M, Williams J, Schmich K, Hüttel W, Merfort I, Salomon CE, Aldrich CC, Hoffmeister D. Structure and cytotoxicity of armillarilin and related fungal sesquiterpene aryl esters. *J Nat Prod* 2009; 72: 1888–1891
- [31] Abramson J, Pecht I. Clustering the mast cell function-associated antigen (MAFA) leads to tyrosine phosphorylation of p62Dok and SHIP and affects RBL-2H3 cell cycle. *Immunol Lett* 2002; 82: 23–28
- [32] Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, Levesque CA, Chen W. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for fungi. *Proc Natl Acad Sci U S A* 2012; 109: 6241–6246
- [33] Solis MJL, Yurkov A, dela Cruz TE, Unterseher M. Leaf-inhabiting endophytic yeasts are abundant but unevenly distributed in three *Ficus* species from botanical garden greenhouses in Germany. *Mycol Prog* 2015; 14: 1019
- [34] Benson DA, Cavanaugh M, Clark K, Karsch-Mizrachi I, Lipman DJ, Ostell J, Sayers EW. GenBank. *Nucleic Acids Res* 2012; 41: D36–D42
- [35] Katoh K, Misawa K, Kuma K, Miyata T. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Res* 2002; 30: 3059–3066
- [36] Katoh K, Standley DM. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol Biol Evol* 2013; 30: 772–780
- [37] Maddison WP, Maddison DR. Mesquite: a modular system for evolutionary analysis, Version 2.75. Available at <http://mesquiteproject.org>
- [38] Gouy M, Guindon S, Gascuel O. SeaView version 4: A multiplatform graphical user interface for sequence alignment and phylogenetic tree building. *Mol Biol Evol* 2010; 27: 221–224
- [39] Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol* 2013; 30: 2725–2729
- [40] Page RD. TreeView: an application to display phylogenetic trees on personal computers. *Comput Appl Biosci* 1996; 12: 357–358
- [41] Huelsenbeck JP, Ronquist F. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* 2001; 17: 754–755
- [42] Stamatakis A. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 2014; 30: 1312–1313
- [43] Silvestro D, Michalak I. raxmlGUI: a graphical front-end for RAxML. *Org Divers Evol* 2012; 12: 335–337