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ORIGINAL ARTICLE

5′-*O*-methyl-14-hydroxyarmillane, a new armillane-type sesquiterpene from cultures of *Guyanagaster necrorhiza*

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Abstract

Protoilludene-type sesquiterpene aryl esters are a unique and very diverse compound class that were exclusively isolated from members of the genus *Armillaria* (*Agaricomycetes*, *Physalacriaceae*) up to this point. Herein, we describe the isolation and structural characterization of 5′-*O*-methyl-14-hydroxyarmillane (**1**), a new armillane-type derivative, that was obtained from submerged cultures of *Guyanagaster necrorhiza* (CBS 138623) together with the known congeners melleolide G (**2**), melleolide B (**3**), and 10-dehydroxy-melleolide B (**4**). ROESY data and coupling constants assigned the relative configurations of **1**, while common absolute configurations were confirmed from comparison of its ECD spectrum to the one of 10-hydroxy-5′-methoxy-6′-chloroarmillane (**5**). Additionally, the configuration of melleolide G (**2**) was revised based on observed ROESY correlations. It is the first time that protoilludene-type sesquiterpene aryl esters were isolated from another genus, namely, *Guyanagaster*, that is closely related to *Armillaria*. **1**–**4** were evaluated for their biological activities in a serial dilution assay against several yeast, fungi, and bacteria, as well as in a cytotoxicity assay against different cell lines. Compound **4** was moderately active against *Bacillus subtilis*, *Staphylococcus aureus*, and *Mucor hiemalis*. Furthermore, **1**, **3**, and **4** showed weak cytotoxic effects against the mouse fibroblast cell line L929 and the cervix carcinoma cell line KB3.1.

Keywords *Guyanagaster necrorhiza* · Protoilludene · Sesquiterpene · Armillane

Introduction

The protoilludene-type sesquiterpene aryl esters are a chemically very diverse class of compounds among the natural products that can be obtained from fungal sources. Up to this point, more than 70 congeners of this compound class were discovered and described in literature (Dörfer et al. 2019). Often casually summarized as melleolides, strictly speaking protoilludene-type aryl esters have to be divided into three groups according to the localization of the double bond within the protoilludene backbone. Melleolides are $\Delta^{2\text{-}3}$-unsaturated and armillyl orsellinates are $\Delta^{2\text{-}4}$-unsaturated, while armillanes are characterized by the absence of the double bound within the 6-membered ring (Engels et al. 2021). Alongside their chemical diversity, several members of the protoilludene-type aryl esters show a wide range of bioactivities, including antibacterial, antifungal, and cytotoxic effects (Dörfer et al. 2019). Interestingly, this class of natural products was exclusively isolated from cultures of the genus *Armillaria* (*Agaricomycetes*, *Physalacriaceae*) until now. *Armillaria* spp. are well-known and globally distributed parasitic fungi that affect a broad range of different hosts in timber and agronomic systems, where they play a critical role as root pathogens and decomposers (Baumgartner et al. 2011; Coetzee et al. 2018). In 2010, a gasteroid fungus that is closely related to *Armillaria* was described as *Guyanagaster necrorhiza* (Henkel et al. 2010) and together with *Desarmillaria*, these genera were later assigned to the Armillarioid clade (Koch et al. 2017; Kedves et al. 2021). Phylogenetic reconstruction showed that *Guyanagaster* species are extant taxa that diverged from early members of the Armillarioid clade when this lineage evolved in Eurasia about 51 million years ago (Koch et al. 2017).

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During this process, the mushroom ancestor of *Guyanagaster* underwent a process referred to as gasteromycation leading to the gasteroid species that were only found in the Guiana Shield region (Guyana) up to this point (Henkel et al. 2010; Koch et al. 2017). The close taxonomic relationship of the genera *Armillaria* and *Guyanagaster* led to the endeavor to investigate the secondary metabolism of *Guyanagaster* species for the production of protoilludene-type sesquiterpene aryl esters. Herein, we describe the isolation and structural characterization of 5′-*O*-methyl-14-hydroxyarmillane (**1**), a new armillane-type derivative, that was obtained from submerged cultures of *G. necrorhiza* (CBS 138623) together with the known congeners melleolide G (**2**), whose configuration we revise, melleolide B (**3**), and 10-dehydroxymelleolide B (**4**).

Results and discussion

Guyanagaster necrorhiza was cultivated in liquid YM6.3 medium. Extraction of the harvested mycelium and culture broth led to extracts that were purified by preparative HPLC. This resulted in the isolation and characterization of the new meroterpenoid 5′-*O*-methyl-14-hydroxyarmillane (**1**) that showed a molecular ion cluster at *m*/*z* 451.2333 $[M+H]^+$ (calcd. for $C_{24}H_{35}O_8$, 451.2326) in the HRESIMS spectrum revealing the molecular formula $C_{24}H_{34}O_8$. Its ^1H and ^{13}C NMR (Table 1) data were highly similar to those of armillane (Donnelly and Hutchinson 1990). The key difference is the methylation of the hydroxy group at C-5′ and the presence of an additional hydroxy group at C-14 for **1**. The relative configuration was confirmed by coupling constants and ROESY data: the large coupling constants $J_{H2,H3} = J_{H3,H13} = 11.0$ Hz, observed in the triplet of H-3, indicate that all of these three protons are in axial position on alternating sides of the ring structure. ROESY correlations between H_3-15/H-9 and H_3-15/H-13, as well as between H_2-14 and H-3, H-10α, H-12α confirm an unchanged configuration compared to **5**. The absolute configuration was assigned as 2*R*,3*S*,4*R*,5*R*,7*R*,9*S*,11*R*,13*R*, which is characteristic for melleoid sesquiterpenoids, since the ECD spectra of 5′-*O*-methyl-14-hydroxyarmillane (**1**) and the one of 10-hydroxy-5′-methoxy-6′-chloroarmillane (**5**) (Mándi and Kurtán 2019) are very similar. Due to the absence of the

Table 1 ^{13}C (175 MHz) and ^1H (700 MHz) NMR spectroscopic data of compound **1** in acetone-d_6 (δ in ppm)

No.	δ_C, type	δ_H (*J* in Hz)	COSY	C to H HMBC	N/ROESY
1	63.0, CH_2	3.86, dd (10.9, 5.1) 4.00, dd (10.9, 3.4)	2β, 1 2β, 1	2, 3, 4 3, 4	
2	46.4, CH	β: 2.09, m	3α, 1, 1	1, 5, 4	
3	68.7, CH	α: 3.74, t (11.0)	13β, 2β	12, 7, 2, 1	10α, 14
4	81.6, C				
5	76.8, CH	α: 5.41, t (8.5)	6α	6, 2, 4, 1′	8, 6α
6	34.4, CH_2	α: 1.94, d (2.4) β: 1.88, m	5α 8	8, 7, 4 8, 7, 9, 5	5α
7	39.0, C				
8	22.5, CH_3	1.18, s	6β	6, 7, 9, 4	5α
9	47.5, CH	β: 2.17, m	10β, 10α	6, 7, 10, 4	15
10	39.7, CH_2	α: 1.57, m β: 1.34, m	10β, 9β 10α, 9β	15, 7, 11, 9, 14 12, 13	14, 3α 15
11	42.9, C				
12	38.6, CH_2	α: 2.11, m β: 1.34, m	12β 12α, 13β	15, 10, 11, 9, 3, 14 15, 11, 3, 14	14 15
13	47.3, CH	β: 2.02, m	12β, 3α	10, 11, 3	
14	72.6, CH_2	3.34, m		15, 12, 10, 11	10α, 12α, 3α
15	27.5, CH_3	1.01, s		12, 10, 11, 14	10β, 12β, 9β, 13β
1′	172.4, C				
2′	106.1, C				
3′	166.7, C				
4′	99.7, CH	6.33, d (2.4)		2′, 6′, 5′	
5′	165.2, C				
6′	111.7, CH	6.36, d (2.4)		8′, 4′	
7′	144.2, C				
8′	24.7, CH_3	2.57, s		4′, 2′, 6′, 7′	
9′	55.9, CH_3	3.82, s		5′	

double bond in the 6-membered ring of the protoilludene backbone, **1** can be assigned to the armillane-type sesquiterpene aryl esters.

Furthermore, with melleolide G (**2**), melleolide B (**3**), and 10-dehydroxy-melleolide B (**4**) three known congeners, previously described from *Armillaria* spp. in the literature, were isolated from the same batch of submerged *G. necrorhiza* cultures. While **2** and **3** were reported from cultures of *A. mellea* (Arnone et al. 1986; Arnone et al. 1988), **4** was obtained from an undefined species *Armillaria* sp. (Yin et al. 2012). As their names already imply, **2**–**4** belong to the subclass of melleolides, due to their characteristic double bond localization ($\Delta^{2\text{-}3}$-unsaturated). Although our ^{13}C data of **2** is virtually identical to those of Arnone et al. (1988), the assignment of C-2 and C-3 has to be exchanged based on HMBC data (Table S1, Figure S10). More importantly, the published confirmation of **2** is not consistent with the ROESY correlations we observed: while methyl H_3-15 shows ROESY correlations to both methines H-9 and H-13, oxymethylene H_2-14 has correlations with H-10α, H-12α, and H_3-8 (Table S1, Figure S11). Therefore, the stereoconfigurational assignment of C-11 has to be revised, with the oxymethylene (CH_2-14) pointing below the main molecular plane.

Protoilludene-type sesquiterpene aryl esters are well known for their broad spectrum of biological activities and several derivatives were reported as antibacterial, antifungal, or cytotoxic (Dörfer et al. 2019). Therefore, the potential bioactivities of compounds **1**–**4** were evaluated in a serial dilution assay against several yeast, fungi, and bacteria, as well as in a cytotoxicity assay against different cell lines. In the serial dilution assay, only **4** showed moderate activities against *B. subtilis*, *S. aureus*, and *M. hiemalis* (Table 2). The relationship between structures and biological activities of this compound class has not been systematically investigated yet; thus, no conclusions to explain the activities of **4** can be made. Solely the weak or missing cytotoxic effects of **1**–**4** (Table 3) can be associated with the hydroxylation of C-1, since especially derivatives with an aldehyde function at this position are reported to be the ones with the highest potential for cytotoxicity (Dörfer et al. 2019).

For the first time, protoilludene-type sesquiterpene aryl esters are reported from another genus than *Armillaria*. Our data confirm previous results describing the genus *Guyanagaster* as closely related to *Armillaria* (Henkel et al. 2010; Koch et al. 2017; Kedves et al. 2021).

Table 3 Half inhibitory concentration (IC_{50} in µM) of compounds **1**–**4** isolated from *Guyanagaster necrorhiza* (CBS 138623) to assess the cytotoxicities

Compound	KB3.1	L929
1 (5′-*O*-methyl-14-hydroxyarmillane)	37.8	10.9
2 (melleolide G)	–	–
3 (melleolide B)	16.2	46.3
4 (10-dehydroxy-melleolide B)	13.7	18.3
Epo B (positive control)	3.8×10^{-5}	1.1×10^{-3}

The assay was performed with stock solutions of **1**–**4** (1 mg/mL in acetone). Epothilone B was used as positive control, while acetone (20 µL) was used as negative control and did not show inhibitory effects

IC_{50} in µM; – no activity

Table 2 Minimum inhibitory concentrations (MIC in µg/mL) of compounds **1**–**4** isolated from *Guyanagaster necrorhiza* (CBS 138623) to assess the antimicrobial activities

Compound	*Bacillus subtilis* DSM 10	*Staphylococcus aureus* DSM 346	*Mycobacterium smegmatis* ATCC 700084	*Schizosaccharomyces pombe* DSM 70572	*Pichia anomala* DSM 6766	*Mucor hiemalis* DSM 2656	*Rhodotorula glutinis* DSM 10134
1 (5′-*O*-methyl-14-hydroxyarmillane)	33.3	–	–	–	–	–	–
2 (melleolide G)	66.6	–	–	–	–	–	–
3 (melleolide B)	66.6	33.3	–	33.3	66.7	33.3	33.3
4 (10-dehydroxy-melleolide B)	16.6	16.6	66.6	33.3	–	16.6	66.6
Positive control	16.6 [O]	0.21 [G]	1.7 [K]	4.2 [N]	4.2 [N]	1.0 [N]	1.0 [N]

Stock solutions of **1**–**4** were used to perform the serial dilution assay (1 mg/mL in acetone). As positive controls, the antimicrobials [O] oxytetracycline, [G] gentamycin, and [K] kanamycin were used for bacteria and [N] nystatin was used for filamentous fungi and yeast. Acetone (20 µL) was used as negative control and did not show inhibitory effects. All tested compounds did not show activities against *Candida albicans* (DSM 1665), *Escherichia coli* (DSM 1116), *Pseudomonas aeruginosa* (DSM PA14), and *Chromobacterium violaceum* (DSM 30191)

IC_{50} in µg/mL; – no activity

Material and methods

General experimental procedures

A PerkinElmer 241 polarimeter was used to measure the optical rotation. To record the UV spectra, a Shimadzu UV-Vis spectrophotometer UV-2450 (Duisburg, Germany) was used. NMR spectra were measured with the Bruker Avance III 500-MHz spectrometer (Bremen, Germany) equipped with a BBFO (plus) SmartProbe (^{1}H 500 MHz, ^{13}C 125 MHz) and the Bruker Avance III 700-MHz spectrometer (Bremen, Germany) equipped with a 5-mm TCI cryoprobe (^{1}H 700 MHz, ^{13}C 175 MH). Selected chemical shifts of acetone-d_6 (^{1}H: 2.05 ppm, ^{13}C: 29.92 ppm) were used to reference the NMR data. The Agilent 1200 series HPLC-UV system (Santa Clara, CA, USA) in combination with an ESI-TOF-MS (Maxis, Bruker, Bremen, Germany) was used to record HRESIMS data. These measurements were performed with the 2.1 × 50 mm, 1.7 µm, C18 Acquity UPLC BEH (Waters, Milford, MA, USA) column, using MilliQ water + 0.1% formic acid as solvent A and acetonitrile + 0.1% formic acid as solvent B (gradient: 5% B for 0.5 min increasing to 100% B in 19.5 min and maintaining 100% B for 5 min, flow rate: 0.6 mL/min, UV/Vis detection: 200–600 nm).

Fungal material

The culture of *G. necrorhiza* we studied (original code MCA 3950) was kindly provided by M. Cathie Aime, who also deposited the strain at the Westerdijk Fungal Biodiversity Institute (Utrecht, Netherlands) under the accession number CBS 138623. The strain was previously used in the study by Koch and Aime (2018).

Fermentation of *G. necrorhiza* and extraction

Forty 500-mL Erlenmeyer shape culture flasks, each of them containing 200 mL of YM6.3 medium (10 g/L malt extract, 4 g/L D-glucose, 4 g/L yeast extract, pH 6.3), were inoculated with three 50-mm^2 sized pieces of well-grown mycelium from YM6.3 agar plates per flask. The cultures were incubated on a rotary shaker at 23 °C and 140 min^{-1}. To monitor the consumption of the glucose, test strips (Medi-Test Glucose, Macherey-Nagel, Düren, Germany) were used. After 24 days, the culture broth was tested negative for glucose and 2 days later the cultures were harvested (26 days in total). Mycelium and supernatant were separated by centrifugation at 5100 rpm for 30 min. The mycelium was extracted with acetone in an ultrasonic bath for 30 min, twice. Subsequently, the mycelium was separated from the liquid phase by filtration and the organic solvent was evaporated at 40 °C on a rotary evaporator. Dilution of the remaining aqueous phase with H_2O was followed by an extraction against EtOAc (1:1, twice). The obtained organic phase was evaporated to dryness on a rotary evaporator at 40 °C leading to 126 mg mycelial extract. Extraction of the supernatant was carried out against EtOAc (1:1) in a separatory funnel, twice. After phase separation, the organic phase was kept and evaporated to dryness (40 °C) leading to 360 mg of supernatant extract.

Analytical HPLC

The extracts that were obtained from the mycelium and supernatant of *G. necrorhiza* were dissolved with acetone to yield a concentration of 10 mg/mL. This was followed by a filtration of 100 µL of the solution with Whatman Mini-UniPrep™ syringeless filter devices (cytiva, Marlborough, MA, USA). All samples were analyzed using an analytical HPLC device (Dionex UltiMate 3000 series) coupled to an ion trap mass spectrometer (amazon speed by Bruker). After injection of 2 µL of the samples, the separation was carried out with an ACQUITY-UPLC BEH C18 column (50 × 2.1 mm; particle size: 1.7 µm) by Waters. As mobile phase HPLC grade H_2O and MeCN with 0.1% formic acid were used at a flow rate of 600 µL/min. The gradient started at 5% of MeCN, continued with an increase to 100% MeCN in 20 min, and remained at 100% MeCN for 5 min. All obtained chromatograms were analyzed with the software Data Analysis 4.4 (Bruker).

Isolation of compounds 1–4 via reversed phase liquid chromatography

Purification of the obtained extracts was carried out using a Gilson PLC 2250 purification system coupled to a Gemini LC column 250 × 50 mm, 110 Å, 10 µm (Phenomenex, Torrance, CA, USA). HPLC grade H_2O + 0.1% formic acid (solvent A) and MeCN + 0.1% formic acid (solvent B) were used as mobile phase at a flow rate of 50 mL/min. The gradient for the purification of the supernatant extract started with isocratic conditions at 15% B for 5 min, followed by an increase to 80% B in 60 min, another increase to 100% B in 5 min and remained at 100% B for 10 min. The fraction at 37.8–38.5 min led to 2.6 mg of **1**, the fraction at 45.5–46.5 min led to 1.5 mg of **2**, the fraction at 52.0–53.0 min led to 1.1 mg of **3**, and the fraction at 60.3–61.0 min led to 0.2 mg of **4**. The gradient for the purification of the mycelial extract started with isocratic conditions at 25% B for 5 min, followed by an increase to 100% B in 75 min and remained at 100% B for 10 min. The fraction at 35.0–36.5 min led to 0.5 mg of **2**, the fraction at 39.5–41.0 min led to 0.5 mg of **3**, and the fraction at 71.0–72.5 min led to 0.5 mg of **4** (Fig. 1).

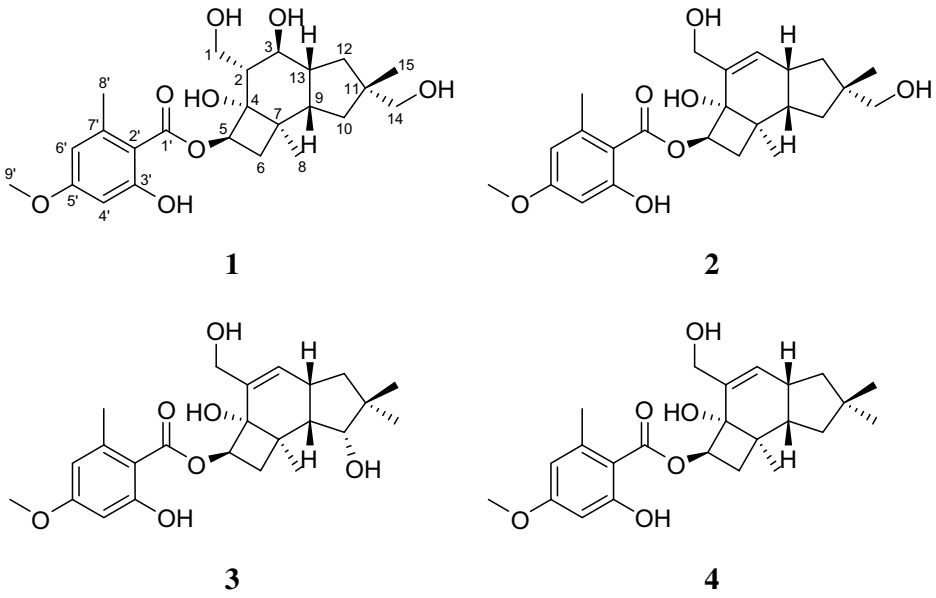

Fig. 1 Protoilludene-type sesquiterpene aryl esters isolated from cultures of *G. necrorhiza*. **1**: 5′-*O*-methyl-14-hydroxyarmillane; **2**: melleolide G; **3**: melleolide B; **4**: 10-dehydroxy-melleolide B

Spectral data

5′-*O*-methyl-14-hydroxyarmillane (**1**): colorless oil; $[\alpha]^{20}_{D} = -13$ (*c* 0.1, MeOH); UV/Vis (0.01 mg/mL, MeOH) $\lambda_{max}(\log\varepsilon)$: 301 (4.30), 265 (4.01), 212 (3.59) nm; ECD (0.25 mg/mL, MeOH) $\lambda(\Delta\varepsilon)$ 302 (1.1), 263 (−1.1), 238 (0.0), 219 (−0.7), 198 (1.8) nm, Fig. 2; ^{1}H and ^{13}C NMR data (acetone-d_6), Table 1; ESIMS *m/z* 451.25 $[M+H]^+$, 449.30 $[M-H]^-$; HRESIMS *m/z* 451.2333 $[M+H]^+$ (calcd. for $C_{24}H_{35}O_8$, 451.2326); t_R = 9.2 min (analytical HPLC).

Evaluation of antimicrobial activity

To determine minimum inhibitory concentrations (MICs) against several yeast, fungi, and bacteria, serial dilution assays in 96-well microtiter plates were performed as described by Harms et al. (2021). Compounds **1**–**4** were tested against *Bacillus subtilis*, *Staphylococcus aureus*, *Mycobacterium smegmatis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Chromobacterium violaceum*, *Schizosaccharomyces pombe*, *Candida albicans*, *Pichia anomala*, *Mucor hiemalis*, and *Rhodotorula glutinis*.

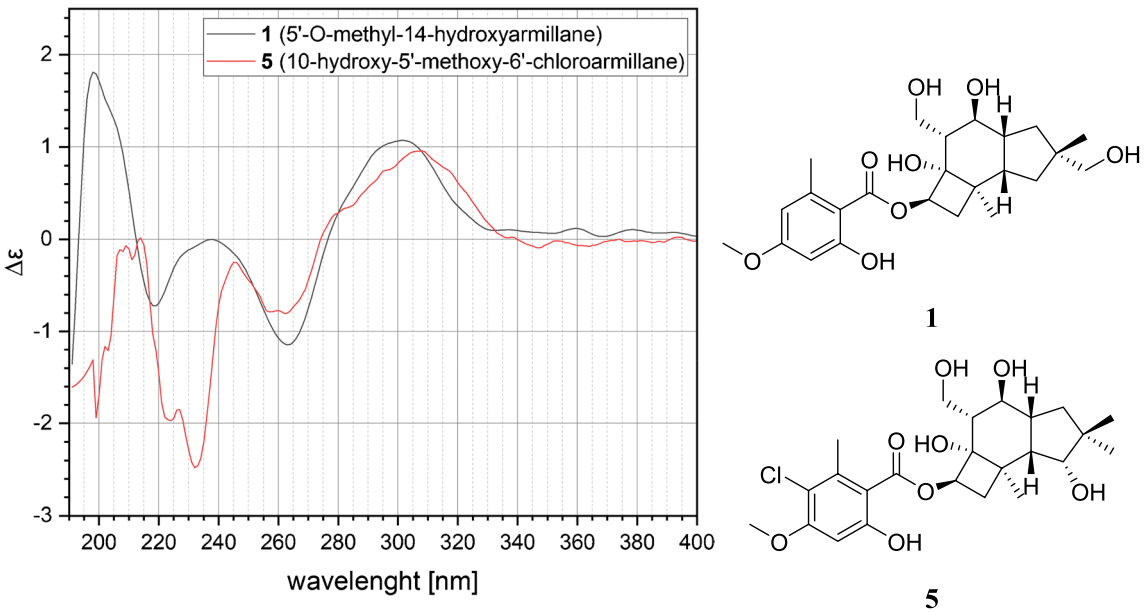

Fig. 2 ECD spectrum of 5′-*O*-methyl-14-hydroxyarmillane (**1**) in comparison to the one of 10-hydroxy-5′-methoxy-6′-chloroarmillane (**5**) that was previously isolated from cultures of *Armillaria ostoyae* (DSM 115711)

Evaluation of cytotoxicity

The cytotoxicities of compounds **1**–**4** were assessed by in vitro assays in 96-well plates as described by Harms et al. (2021). They were tested against the mouse fibroblast cell line L929 and the cervix carcinoma cell line KB3.1.

Conclusions

With 5′-*O*-methyl-14-hydroxyarmillane (**1**), a new armillane-type derivative, and the known congeners melleolide G (**2**), melleolide B (**3**), and 10-dehydroxy-melleolide B (**4**), four protoilludene-type sesquiterpene aryl esters were obtained from submerged cultures of *G. necrorhiza* (CBS 138623). It is the first time that members of this compound class were isolated from cultures of another genus than *Armillaria*. These results confirmed the close relationship between *Armillaria* and *Guyanagaster* leading to the assumption that more congeners, including new derivatives, can be found during further investigations on the secondary metabolism of *Guyanagaster* species. Therefore, *G. necrorhiza* (CBS 138623) can be cultivated under different conditions or other specimen can be investigated for their secondary metabolite production.

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Author contribution The study including cultivation, extraction, and compound isolation was conducted by D. Nedder with support from S. Pfütze. Analysis and structure elucidation was conducted by S. Pfütze. The first draft of the manuscript was written by S. Pfütze and revised by F. Surup and M. Stadler. All authors approved the final version.

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Declarations

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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