Structure and Cytotoxicity of Arnamial and Related Fungal Sesquiterpene Aryl Esters

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We report on the structure elucidation of arnamial, a new $\Delta^{2,4}$-protoilludane everninate ester from the fungus *Armillaria mellea*, and on the apoptotic activity of arnamial as well as the cytotoxic activity of structurally related compounds on selected human cancer cells. Arnamial showed cytotoxicity against Jurkat T cells, MCF-7 breast adenocarcinoma, CCRF-CEM lymphoblastic leukemia, and HCT-116 colorectal carcinoma cells at $IC_{50} = 3.9$, 15.4, 8.9, and 10.7 μM, respectively, and the related aryl ester melledonal C showed cytotoxic activity against CCRF-CEM cells ($IC_{50} = 14.75$ μM). [1,2-$^{13}C_2$]Acetate feeding supports a polyketide origin of the orsellinic acid moiety of arnamial.

Arnamial **1** and related esters melledonol **2**, melleolides C and D (**3**, **4**), melledonals A and C (**5**, **6**), and 10α-hydroxymelleolide **7** (Chart 1) are natural products of the homobasidiomycete genus *Armillaria*, whose conspicuous carpophores are colloquially referred to as "honey mushrooms".[1−4] Data on antimicrobial activities of this class of aryl esters have been published sporadically, indicating varied degrees of antibiosis.[3,5,6] As yet, inhibitory activities against human cell lines have not been described for any member of this class of natural products. Along with the structure elucidation of the new aryl ester **1** we report on the cytotoxic activity of known *Armillaria* aryl esters on human cancer cell lines. We also present evidence that the apoptotic pathway is involved in the cytotoxic effect.

Compounds **1**−**7**, including the new compound **1** and known compounds **2**−**7**, were isolated from liquid cultures of *Armillaria mellea* FR-P75. HRAPCIMS analysis of **1** in negative mode provided a molecular ion peak at *m/z* 447.1569 $[M - H]^-$ indicating a molecular formula of $C_{24}H_{29}ClO_6$. The ^{1}H NMR data (Table 1) indicated that **1** was closely related to aryl esters **2**−**7** as well as the arnamiol, armillol, and armillaribin series of compounds **8**−**11**.[5,7,8] The spectra were suggestive of an esterified 6′-chloroeverninic acid as the aromatic moiety, which was previously found in other aryl esters, such as arnamiol (**8**), melleolide D (**4**), and melledonal C (**6**).[7] The ^{1}H NMR data of the sesquiterpene moiety were very similar to those of **8**, except for one highly deshielded signal indicating an aldehyde (δ 9.84, 1H, s, H-1) and a lack of signals for a primary alcohol. The ^{13}C NMR data of **1** were also similar to data reported for **8**[5,7] with the exception of downfield shifted signals for carbons C-1 and C-4 at δ 190.9 and 169.7, respectively, indicating the presence of a vinyl aldehyde functionality. The combined NMR data suggest that **1** is the aldehyde of **8**, which is unknown from natural sources, but an analogue was described by Donnelly and co-workers as the manganese dioxide oxidation product of the prototypical armillylorsellinate (**10**).[5] 2D NMR spectra corroborated the 3-hydroxy $\Delta^{2,4}$-protoilludane moiety of **1**, as HMBC experiments established a ^2J-coupling between the aldehyde proton H-1 and C-2 (δ 133.2) as well as a ^3J-coupling with the oxygenated carbon at C-3 (δ 72.4), which was identified as a tertiary carbon by HSQC experiments and thus supports the presence of a hydroxy group at C-3. The $^3J_{H,H}$ coupling constants were consistent with previous data established by NMR and X-ray crystallographic analyses of armillylorsellinate **9**, a $\Delta^{2,4}$-protoilludane alcohol, and its oxidation product,[5] thus suggesting the identical relative configuration.

A small set of adherent (HCT-116 colorectal carcinoma and MCF-7 breast adenocarcinoma cells) and suspension (Jurkat T and CCRF-CEM acute lymphoblastic leukemia) cells was exposed to **1**−**7**, along with orsellinic acid (**12**) and 5-*O*-methylorsellinic acid (**13**). Among the aryl esters tested, **1** had the highest levels of cytotoxicity against all cell lines. IC_{50} values were 10.69, 15.40, 3.93, and 8.91 μM for HCT-116, MCF-7, Jurkat T cells, and CCRF-CEM cell lines, respectively (Table 2). Compounds **2**, **3**, and **5** did not inhibit any of the cell lines at the concentrations tested ($\leq$100 μM, data not shown). Comparison of the most active compound, **1**, with the closest analogue, melledonal C (**6**), suggests that the position of the terpene double bond and/or hydroxy groups is critical for activity. Neither free orsellinic acid (**12**) nor the 5-*O*-methyl derivative (**13**) showed any inhibitory effect. Cumulatively, these data imply a preliminary structure−activity relationship, with the position of the double bond (and thus consequences for the overall spatial structure of the protoilludane, such as a twisted cyclobutane[5,9]) appearing critical for cytotoxicity, while 6′-chlorination and 5-*O*-methyl ether formation contributed less.

Jurkat T cells treated with a 10 μM concentration of arnamial for up to 6 h showed remarkable cell shrinkage and blebbing. Consequently, we measured caspase-3 activity in Jurkat T cells, treated with either 10 μM arnamial or 0.3 μM actinomycin D as the control. Compared to untreated cells, an 18.1-fold ($\pm$1.0) increase in caspase-3 activity was observed in the arnamial-treated cells after 6 h. Actinomycin D-mediated activation of caspase-3 was 5.7-fold ($\pm$1.2) after this period of time. Furthermore, DNA fragmentation was measured by detecting histone−DNA complexes by means of ELISA. Jurkat T cells treated with 10 μM arnamial for 6 h showed a 4.5-fold ($\pm$0.42) increase in fragmented DNA compared to untreated cells, whereas actinomycin D induced a 4.3-fold ($\pm$0.57) increase. These data strongly suggest that arnamial induces cell death by apoptosis.

Feeding of sodium [1,2-$^{13}C_2$]acetate was performed to clarify the biosynthetic origin of **12** in basidiomycetes and *Armillaria* in particular. A very likely scenario includes a polyketide synthase (PKS), as streptomycete PKSs, such as AviM and CalO5, were shown to catalyze formation of this common polyketide.[10,11] It was also identified as an aberrant product of PKS1, an ascomycete PKS

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Chart 1

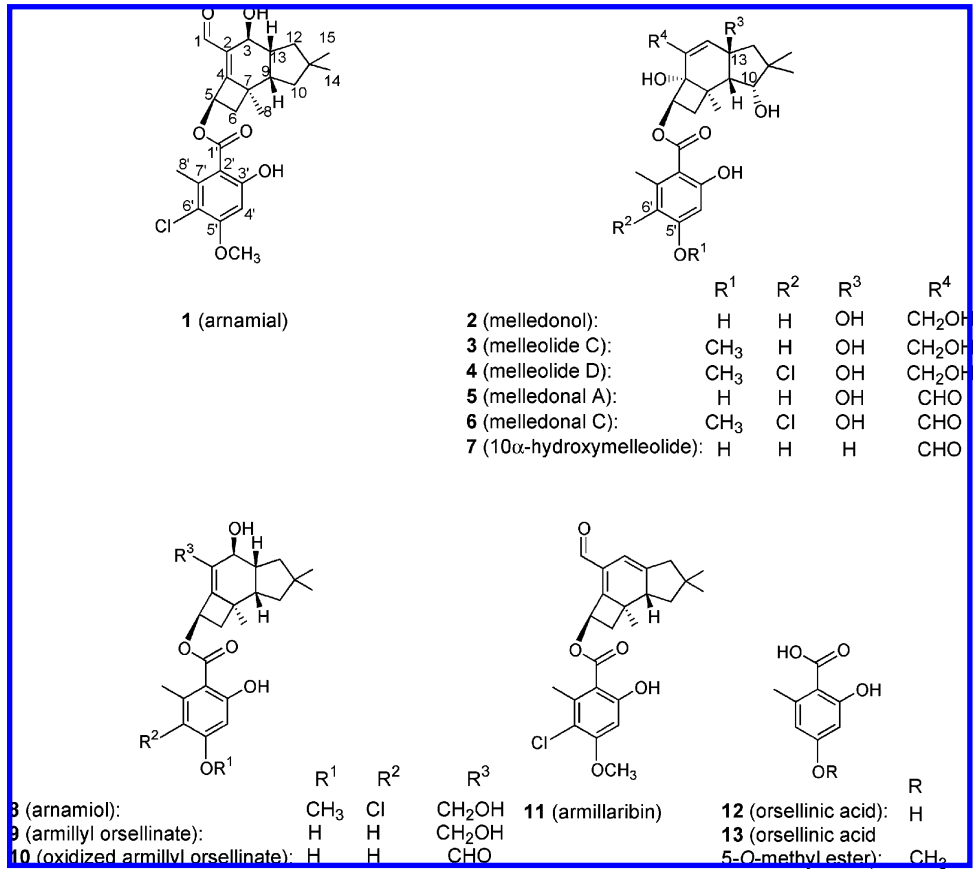

Table 1. NMR Data of Arnamial ($CDCl_3$, 400 MHz for ^1H NMR, 100 MHz for ^{13}C NMR)

position	δ_C	δ_H (J in Hz)	HMBC
1	190.9, CH	9.84, s	2, 3
2	133.2, C		
3	72.4, CH	4.42, dd (7.0, 2.8)	2, 4, 9, 13
4	169.7, C		
5	70.3, CH	6.31, m	4, 6
6	45.5, CH_2	6a: 2.83, dd (11.3, 8.5)	4, 5, 8
		6b: 2.16, dd (11.3, 7.3)	5, 7, 8, 9
7	40.4, C		
8	21.2, CH_3	1.28, s	4, 6, 7
9	46.2, CH	2.4−2.5, m	3, 8, 13
10	46.2, CH_2	10a: 1.96, dd (12.5, 5.5)	9, 11
		10b: 1.28, m	8, 9
11	40.9, C		
12	39.7, CH_2	12a: 1.53, dd (12.7, 5.9)	9, 10, 13
		12b: 1.43, dd (12.7, 9.7)	9, 10
13	48.4, CH	2.4−2.5, m	3, 8, 13
14	29.7, CH_3	1.14, s	9, 10, 12, 15
15	27.3, CH_3	1.02, s	9, 10, 12, 14
1′	169.9, C		
2′	105.4, C		
3′	163.5, C		
4′	98.6, CH	6.46, s	1′, 2′, 3′, 5′, 6’
5′	160.2, C		
6′	116.0, C		
7′	139.4, C		
8′	20.1, CH_3	2.64, s	2′, 6′, 7′
3′-OH		11.36, br s	2′, 3′, 4′, 5′, 6′
5′-OCH_3	56.4, CH_3	3.94, s	5′

from *Colletotrichum lagenarium*[12] that falls into an evolutionarily different clade of PKSs.[13] However, current knowledge on polyketide assembly in basidiomycetes is scarce. Therefore, we could not discount a route via the shikimate pathway, which seemed chemically plausible.[14,15] If a PKS catalyzes aromatic ring formation, doubly ^{13}C-labeled acetate units would be incorporated in all carbon atoms of **12**. Thus, strong couplings between the ^{13}C atoms of each incorporated [1,2-^{13}C]acetate unit would be detectable in the 2D INADEQUATE NMR experiment. Even if some ^{13}C-labeled precursor is routed into a shikimate-derived product, the $^{13}C-^{13}C$ coupling of adjacent carbon atoms C-7′ and C-8′ will still umambiguously indicate the polyketide origin. In the case of a PKS-directed biosynthesis, these carbons originate from the first acetate unit of the polyketide chain. In the alternative scenario of a shikimate origin, C-8′ is introduced by a methylation step that excludes $^{13}C-^{13}C$ coupling from an intact doubly labeled acetate. As the ^{13}C signals of the aromatic orsellinate moiety do not interfere with signals from fatty acids and other aliphatic compounds that may have become labeled, we used crude EtOAc extracts dissolved in $CDCl_3$ for the NMR experiments.

The 2D INADEQUATE NMR unambiguously identified strong couplings between carbon atoms of orsellinate in a pattern consistent with the incorporation of intact acetate units. For the aromatic carbons we observed twin signals due to the presence of differently substituted orsellinic acid, i.e., a chlorine atom at C-6′ and methoxy group at C-5′, as shown in Chart 1, and some chemical shifts slightly differed by up to ~3 ppm. Nevertheless, $^{13}C-^{13}C$ couplings of acetate units incorporated into the orsellinate moiety of **1** can be assigned distinctly (Table 3). The strong coupling between C-8′ (δ 19.4) and C-7′ (δ 139.2) unambiguously supports the polyketide origin of the orsellinate moiety.

Irofulvene, the semisynthetic derivative of the sesquiterpene illudin S is currently being investigated as an anticancer drug in phase I and II clinical trials.[16,17] The sesquiterpene moiety of **1** and illudin share a common biosynthetic origin;[18] however, the late biosynthetic steps result in divergent ring structures. Specifically, the cyclopropane ring of illudin S and irofulvene has been shown essential for activity, as adduct formation to cellular nucleophiles, such as DNA, takes place via attack of this structural

Table 2. Cytotoxicity of *Armillaria* Protoilludanol Sesquiterpene Aryl Esters

	IC_{50} (μM)[a]			
	arnamial (**1**)	melleolide D (**4**)	melledonal C (**6**)	10α-hydroxymelleolide (**7**)
HCT-116 (colon carcinoma)	10.69	>100	>100	>100
MCF7 (breast adenocarcinoma)	15.4	>100	>100	>100
Jurkat (leukemia)	3.93	>100	58.75	86.17
CCRF-CEM (leukemia)	8.91	61.66	14.75	63.23

[a] The values represent the means of three replicate experiments of each compound. Substances **2**, **3**, **5**, **12**, and **13** were not active at concentrations ≤ 100 μM.

Table 3. 2D INADEQUATE NMR Data ($CDCl_3$, 100 MHz for ^{13}C NMR) for Arnamial (**1**)

position	INADEQUATE $\delta_C-\delta_C$ (in ppm)
1′−2′	172.3−105.4
3′−4′	160.8−99.2
5′−6′	160.1−115.8
7′−8′	139.2−19.4

feature.[19] Therefore, we expect that **1** exerts its activity through a different mechanism.

Experimental Section

General Experimental Procedures. The optical rotation was recorded on a JASCO P-1020 instrument; IR spectra were measured on a Bruker IFS55 instrument. One- and two-dimensional NMR spectra of pure compounds were recorded on a Bruker AMX 400 spectrometer, with $CDCl_3$ as solvent and internal standard (δ_C 77.0, δ_H 7.29). Chemical shifts are given in ppm, and coupling constants (*J*) in Hz. LC-MS was performed on an Agilent 1200 LC-time-of-flight (TOF) instrument. Mass spectrometry was performed by atmospheric pressure chemical ionization (APCI), in negative mode. The chromatograph was equipped with a Varian Polaris column (150 × 2 mm, 3 μm particle size), a guard column, and a diode array detector. UV spectra were extracted from the diode array data obtained during LC-MS analysis. The following gradient was run for analytical purposes (solvent A: H_2O, solvent B: 90% MeCN in H_2O): initial hold for 2 min at 15% B, then linear increase to 95% B over 33 min, at a flow rate of 0.3 mL/min. Chromatograms were recorded at λ = 254 nm. Chemicals, solvents, media, reagents, and antibiotics were purchased from Becton-Dickinson, Biorad, Fisher, Gibco-BRL, Roth, and Sigma-Aldrich; compounds **12** and **13** were synthesized following literature procedures.[20]

Culture Conditions. *Armillaria mellea* isolate FR-P 75 was grown in potato dextrose broth incubated at 25 °C in the dark and shaken at 180 rpm. Liquid seed cultures were grown in 25 mL of the same medium, supplemented with EtOH (0.15%, v/v), in 100 mL Erlenmeyer flasks, for 12−14 days. The main culture (4 L, dispensed into 12 × 1000 mL Erlenmeyer flasks) was incubated for another 18−20 days and amended twice with 2.5 mM (final) NaOAc.

Extraction and Isolation. Upon harvest, the culture was filtered to separate the biomass from broth, which was extracted three times with an equal volume of EtOAc. The organic phase was dried over Na_2SO_4. Acetone was used to wash the mycelial cake and the filter, and added to the EtOAc extract, which was then evaporated *in vacuo*. Dry crude extracts were dissolved in MeOH and adsorbed onto a SepPak C-18 cartridge (Waters). Elution was done with a step gradient of 25, 50, and 75% MeOH in H_2O and 100% MeOH. The fractions were dried and applied to preparative HPLC (Varian ProStar, Dynamax column (250 × 41.4 mm i.d., Microsorb C-18, 8 μm particle size). Solvent A was H_2O; solvent B was MeCN. Aryl esters **2**−**7** were purified as follows: elution was at 40 mL/min with 50% B, for 1 min, a gradient 50−60% B over 25 min, and a subsequent increase to 100% B within 1 min. Workup of **1** was accomplished with a modified gradient: 85% B, for 1 min, gradient 85−98% B over 26 min, to 100% B within 1 min. The yield of **1** was 3 mg/L.

Arnamial (1): amorphous, white solid; $[\alpha]^{25}_D$ −22 (*c* 0.1, MeOH); UV (MeOH) λ_{max} 217, 263, 307 nm; IR (neat) 3376 (br), 2918, 2849, 1724, 1649, 1602, 1237, 1041 cm^{-1}; NMR data (see Table 1); HRAPCIMS *m/z* 447.1569 $[M - H]^-$ calcd *m/z* 447.1574 $[M - H]^-$ for $C_{24}H_{28}ClO_6$.

Feeding Experiment. For ^{13}C-labeling of aryl esters, we essentially followed the protocol above, but with a main culture volume of 4 × 150 mL, pulsed twice with 2.5 mM [1,2-$^{13}C_2$]acetate (final), added upon inoculation and after 7 days, respectively. The main culture was harvested and extracted after 14 days.

MTT Assay. HCT-116 (colon cancer), MCF7 (breast cancer), Jurkat T (leukemia), and CCRF-CEM (leukemia) cells were maintained in RPMI 1640 media supplemented with 10% fetal bovine serum, 1% GlutaMAX, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were plated in 96-well plates at (2.5−5.0) × 10^4 cells per well (250 μL). After overnight incubation at 37 °C, media was removed and the individual aryl esters were added to the plate in the appropriate medium for the cell type. 2-Fold dilutions of compounds were made in DMSO, starting at 20 mM, and 1 μL of compound solution was added to each well in 200 μL of media, yielding a final DMSO concentration of 0.5%. The highest final concentration tested was 100 μM. Control wells contained 0.5% DMSO (negative control) or, as positive controls, 100 μM 5′-*O*-(sulfamoyl)adenosine, or 50% DMSO. All reactions were done in triplicate. The plate was incubated for 48 h at 37 °C in a 5% CO_2/95% air humidified atmosphere.

Measurement of cell viability was carried out using a modified method of Mosmann based on 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT).[21] MTT was prepared fresh at 1 mg/mL in serum-free, phenol red-free RPMI 1640 media. MTT solution (200 μL) was added to each well, and the plate was incubated as described above for 3 h. The MTT solution was removed, and the formazan crystals were solubilized with 200 μL of 2-propanol. The plate was read on an M5e spectrophotometer (Molecular Devices) at 570 nm for formazan and 650 nm for background subtraction. IC_{50} values were calculated by fitting the data in GraphPad Prism software.

Caspase-3 Activity Assay. Jurkat T cells (1 × 10^6 cells/mL) were incubated with **1** (10 μM final) or with actinomycin D (0.33 μM final) for 6 h. Then the cell suspension was centrifuged (4 °C, 3 min, 1200 rpm), washed, and homogenized in 50 μL of homogenization buffer. The caspase-3 activity assay was performed exactly as described[22] using the caspase-3 substrate DEVD-AMC (Alexis) at a concentration of 200 nM. Relative fluorescence units (RFU) values were calculated via the ratio of average rate of the fluorescence increase and protein concentration determined by the Bradford assay. To compare different experiments, RFU sample values were referred to negative control (untreated cells) and given as fold increase values. Three independent experiments were carried out.

Cell Death Detection by ELISA. DNA fragmentation was measured by a histone ELISA (Roche Diagnostics) according to the manufacturer's instructions. Briefly, Jurkat T cells (1 × 10^6 cells/mL) were incubated with **1** (10 μM final) or with actinomycin D (0.33 μM final), for 6 h. After cell lysis, histone−DNA complexes in the supernatant were detected by ELISA. Measured values were referred to untreated cells and given as fold increase. Two independent replica experiments were carried out.

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Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

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