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In vitro cytotoxicity of melleolide antibiotics: Structural and mechanistic aspects

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ABSTRACT

Melleolide sesquiterpene aryl esters are secondary products of the mushroom genus *Armillaria*. We compared the cytotoxicity of eleven melleolides—five thereof are new natural products—against four human cancer cell lines. Armillaridin, 4-*O*-methylarmillaridin, and dehydroarmillylorsellinate were most active, at IC_{50} = 3.0, 4.1 and 5.0 μM, respectively, against Jurkat T cells for the former two compounds, and K-562 cells for the latter. Dehydroarmillylorsellinate did not inhibit respiration and RNA-synthesis of K-562 cells at 5 μM. However, replication of DNA dropped to 35% after 120 min at this concentration, and translational activity also decreased.

The basidiomycete genus *Armillaria* (honey mushroom) is recognized for the production of sesquiterpene aryl esters, referred to as melleolides.[1,2] Sporadically, antimicrobial properties have been described for these natural products.[3,4] Previously, we reported that arnamial **1** (Scheme 1) is active against human cancer cell lines and the mode of action involves apoptosis.[5] The results of this study also established a preliminary structure–activity relationship. It pointed to the degree of sesquiterpene hydroxylation and the position of the double bond as factors which impact bioactivity. However, our results contrasted published data[4] on antimicrobial activity as substitution of the aromatic moiety (5′-*O*-methylether, and/or 6′-chlorination) was not relevant for activity, according to our data.

Identification of new melleolides: Following up on our previous study we compared the inhibitory activity of eleven aryl esters against human cancer cells. The compounds were purified from a liquid culture of *A. mellea* FR-P75.[6] The combined MS and 1D and 2D NMR spectral data identified six previously published compounds: armillaridin **2**,[7] melleolide D **5**,[8] A52a **6**,[9] armellide B **8**,[10] 13-hydroxy-dihydromelleolide **10**,[11] and armillarin **12**.[7] Five out of these eleven aryl esters were recognized as new natural products (Table 1). 4-*O*-Methylarmillaridin **3** showed virtually identical chemical shifts as **2** in the ^{1}H and ^{13}C NMR spectra. However, the presence of an additional methyl group (δ_H 3.24 ppm; δ_C 52.8 ppm) and its mass (*m*/*z* 485.1688 $[M+Na]^+$) was suggestive of an O-methylated derivate of **2**. HMBC data showed coupling of the C-4 with the methyl hydrogens, thus proving the 4-*O*-methylether. The sesquiterpene backbone of 5′-methoxy-6′-chloroarmillane 4 is characterized by the absent double bond and, thus, identical to armillane **13**.[12] The combined NMR data (DEPT, ^{13}C and ^{1}H) proved a fully reduced C-2 at δ_C 48.2 ppm. Compounds 4 and **13** were dissimilar in that the aromatic moiety of **4** was modified with a 6'-chlorine (absent H-6′ doublet at δ_H 6.28 ppm, exact mass consistent with $C_{24}H_{33}O_7Cl$) and a 5-*O*-methyl group. The position of the methoxy group was confirmed by HMBC data which showed coupling of the C-5′ as the methyl hydrogens resonated at δ_H 3.85 ppm. **7** was identified as the 13,14-dihydroxylated derivative of A52a **6**. Modifications at these positions were evident as one CH_3 signal had disappeared, compared to **6**, and an additional CH_2 group at δ_C 72.0 ppm appeared instead. For C-13 we observed a downfield shift to δ_C 77.2 ppm in **7**. Moreover, a DEPT experiment confirmed C-13 as a quaternary carbon atom. 6′-chloro-13-hydroxy-dihydromelleolide 11 yielded virtually identical NMR spectra as 13-hydroxy-dihydromelleolide **10**, except for an aromatic proton at position 6', which is the chlorination site of melleolides. Consistently, a downfield shifted signal for C-6′ (δ_C 114.9 ppm), along with a mass difference of 34 Da and the characteristic MS isotope pattern for chlorinated compounds were found. Dehydroarmillylorsellinate **9** has not been described from natural sources, but been obtained by chemical

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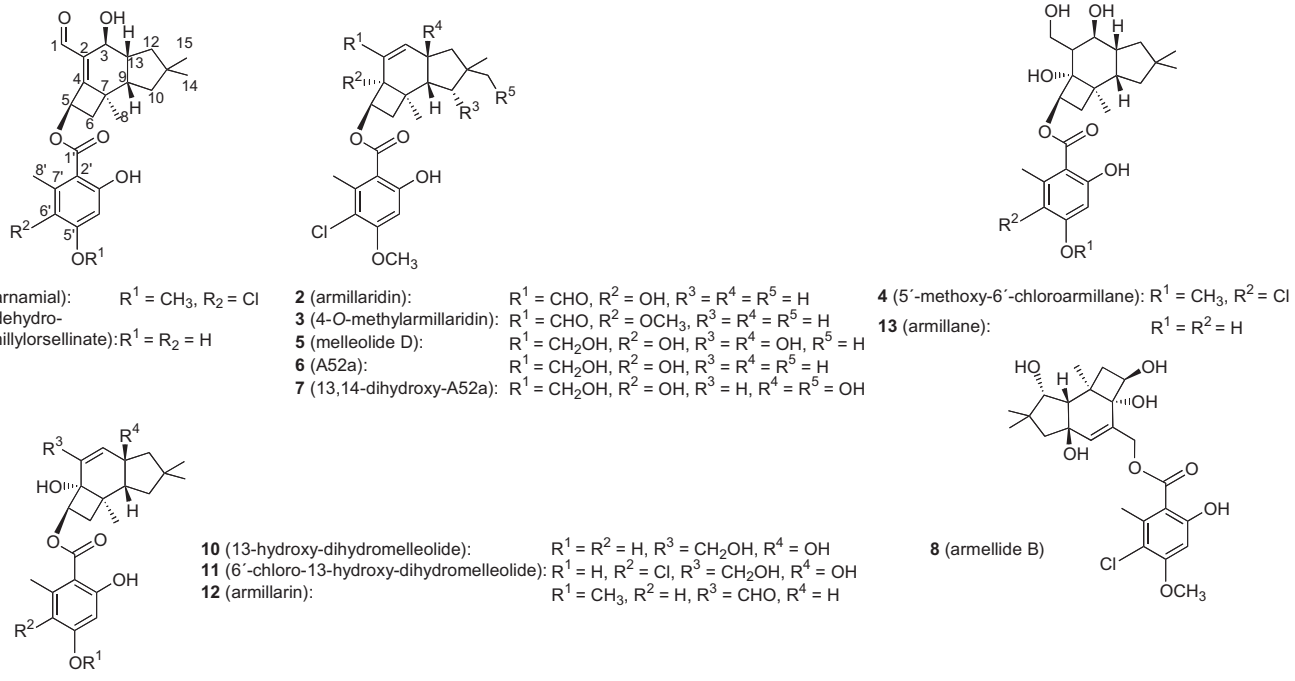

Scheme 1.

oxidation. Our ^{1}H NMR results were compatible to those reported,[1] Table 1 also shows ^{13}C NMR data which was not reported previously.

Structure–activity-relationship: For the present study we included six aryl esters whose aromatic system is identical to **1**, that is, an ester of 6′-chloroeverninic acid, and further four derivatives whose aromatic system differs structurally. We used human cancer cell lines (K-562 leukemia, MCF-7 breast adenocarcinoma, Jurkat T cells, and HeLa cervix carcinoma cells) to compare inhibitory activity in vitro (Table 2), following an established procedure.[13] Based on our previous findings for **1** ($\Delta^{2,4}$-protoilludane), the relevance of the double bond was explored with **2** ($\Delta^{2,3}$) and **4** (fully reduced). Compounds 1 and **2** solely differ in the double bond position, and IC_{50} values of those two isomers were comparable for Jurkat and MCF-7 cells (**1**: 3.9 (±0.4) and 15.4 (±0.3) μM; **2**: 3.0 (±0.3) and 7.8 (±0.9) μM for Jurkat and MCF-7 cells, respectively). Contrasting our initial assumption, the above data shows that the position of the double bond is not relevant for bioactivity against cancer cells. An about threefold decrease in activity was observed with **4** which lacks a terpene double bond (Table 2) and also carries an alcohol functionality at C-1. Compound 2 represents the 6′-chlorine derivative of **12**, and both compounds show nearly identical activity (**2**: 7.8 (±0.9) and 8.9 (±1.3) μM; 12: 11.6 (±0.5) and 9.9 (±0.6) μM for MCF-7 and K-562 cells, respectively) suggesting 6′-chlorination does not determine cytotoxicity. Compounds **10** and **11** supported this notion, as neither substance showed activity at <100 μM, except with Jurkat cells, which were very moderately inhibited by 11 (IC_{50} = 46.6 (±3.1) μM), but not by **10**. Compounds **2** and **6** solely differ in the functionality at C-1 (aldehyde vs alcohol). The aldehyde **2** with its Michael acceptor system is about three times more active against our set of human cell lines than **6**. Previous data on cytotoxicity of other C-1 alcohols is not available in the literature, however, various reports do not point to the aldehyde as a prerequisite for antimicrobial activity.[4,8,14] At <100 μM, we did not observe activity with **5**, **7**, **8**, and **10**. These compounds represent melleolide derivatives with a higher degree of terpene hydroxylation. Plausible scenarios may include poor uptake and transport across biological membranes, due to the additional hydroxyls, or alternatively, that these modifications prevent the molecule from closely interacting with the cellular target.

Mechanism of action: Using K-562 cells we monitored cellular respiration and investigated DNA- and RNA-synthesis, and translation, in the presence of **9** by measuring uptake of 2-^{14}C-thymidine, 2-^{14}C-uridine and ubiquitously labeled ^{14}C-L-leucine, respectively.[15] A concentration range of 1.25–5.0 μM turned out to be appropriate for these assays, as the activity followed a sharp sigmoidal dose–effect-relationship (Fig. 1). Exposure to **9** did not inhibit respiration at the concentrations tested (data not shown). Cellular RNA-synthesis was also virtually unaffected after 120 min and up to 5.0 μM. However, exposed cells responded with a decrease in DNA-synthesis. At 1.25 [5.0] μM the rate dropped to 90% (±2.1) [70% (±1.2)] after 60 min, and to 71% (±2.4) [35% (±1.7)] after 120 min. At 1.25 μM protein synthesis remained unaffected after 60 min and was slightly reduced (96% (±0.7)) after 120 min. Compared with DNA inhibition, a delayed decrease in protein synthesis set in at 5.0 μM. The observed rate was 76% (±3.0) after 60 min, and dropped to 35% (±3.9) after 120 min, compared to untreated cells. Controls (120 min, end point values) treated with 0.47 μM doxorubicin showed a similar profile of inhibited DNA synthesis (46% (±2.3)) and decreased translation (89% (±10.7)). Transcription decreased to 79% (±0.2). After exposure to 4.0 μM actinomycin D transcription had dropped to 2% (±0.2), whereas DNA synthesis had decreased to 58% (±0.9). The cycloheximide control (3.5 μM) resulted in inhibited translation (26% (±1.7)) while transcription remained unchanged (99% (±0.5)).

Our preliminary data point to DNA, or a protein associated with it, as primary target for interaction and as reason for cytotoxicity of **9**. As these assays only reflect total protein or RNA biosynthesis, and do not resolve transcriptional or translational activity of specific loci or mRNA species, further work is warranted and under way to identify the precise cellular target of the melleolide antibiotics. The structures of the fungal sesquiterpene illudin S and its semisynthetic derivative irofulvene are distantly related to the melleolide terpene moiety and known to generate DNA strand breakages.[16] Strong evidence exists that stalled transcription forks are the primary trigger for apoptosis of cells exposed to illudin S and that nucleotide excision repair, but not global repair mechanisms, are

Table 1
Physical and spectral data of new melleolides **3**, **4**, **7**, **9** and **11**

	(**3**)		(**4**)		(**7**)		(**9**)		(**11**)	
	4-*O*-Methylarmillaridin		5′-Methoxy-6′-chloroarmillane		13,14-Dihydroxy-A52a		Dehydroarmillylorsellinate		6′-Chloro-13-hydroxy-dihydromelleolide	
	Found	Calcd	Found	Calcd	Found	Calcd	Found	Calcd	Found	Calcd
HRMS Ion Formula	485.1688 $[M+Na]^+$ $C_{25}H_{31}O_6Cl$	485.1701	467.1840 $[M-H]^-$ $C_{24}H_{33}O_7Cl$	467.1842	481.1638 $[M-H]^-$ $C_{24}H_{31}O_8Cl$	481.1635	401.1973 $[M+H]^+$ $C_{23}H_{28}O_6$	401.1959	451.1531 $[M+H]^+$ $C_{23}H_{29}O_7Cl$	451.1529
Position	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C
1	9.50 s	193.3	3.96 dd (3.4, 10.9) 3.87 dd (4.7, 10.9)	63.5	4.33 d (14.4) 4.11 d (14.4)	63.4	9.90 s	192.8	4.34 d (14.5) 4.11 d (14.5)	63.4
2	–	133.7	1.95 m	48.2	–	138.5	–	135.0	–	137.8
3	7.17 d (2.3)	159.7	3.69 t (11.1)	70.0	5.86 s	130.9	4.36 dd (2.9, 8.8)	73.8	5.89 s	131.2
4	–	80.0	–	82.4	–	78.1	–	170.5	–	77.2
5	5.71 t (8.8)	74.4	5.28 t (8.4)	77.5	5.36 t (8.7)	77.2	6.34 ddd (2.8, 7.2, 8.7)	71.5	5.35 t (8.7)	77.1
6	2.11 dd (8.8, 11.4) 1.71 dd (8.8, 11.4)	33.1	1.96 dd (8.2, 10.8) 1.78 dd (8.8, 10.8)	35.3	2.56 m 1.87 dd (8.7, 10.9)	32.5	2.15 dd (7.2, 11.4) 2.78 dd (8.7, 11.4)	47.0	2.53 dd (8.7, 11.0) 1.86 m	32.7
7	–	37.9	–	37.6	–	40.2	–	41.1	–	34.7
8	1.29 s	20.1	1.13 s	22.9	1.21 s	22.7	1.22 s	21.8	1.20 s	22.6
9	2.37 m	42.8	2.13 q (6.9, 13.3, 12.6)	49.0	2.17 dd (7.6, 12.3)	49.5	2.53 m	48.1	2.17 dd (7.5, 12.8)	50.6
10	1.60 m 1.28 m	42.8	1.42–1.48	45.3	1.46 dd (7.6, 13.1) 1.29 m	39.1	1.49 m 1.55 ddd (1.7, 7.9, 12.6)	42.3	1.56 dd (7.5, 12.8) 1.34 t (12.8)	44.0
11	–	38.7	–	40.0	–	40.0	–	41.6	–	40.1
12	2.12 dd (9.9, 13.5) 1.59 m	45.7	1.92 m 1.55 dd (7.5, 13.9)	44.3	2.02 m 1.72 m	54.4	1.28 dd (10.7, 12.6) 1.89 ddd (1.7, 7.4, 12.6)	47.8	1.89 d (13.6) 1.84 d (13.6)	59.5
13	3.16 m	38.9	2.02 m	46.9	–	77.2	2.38–2.43 m	50.7	–	78.4
14	1.09 s	29.9	1.12 s	33.3	3.18 d (2.4)	72.0	1.16 s	30.4	1.08 s	32.0
15	1.11 s	28.7	0.99 s	33.0	1.09 s	27.2	1.05 s	28.1	0.95 s	31.6
1′	–	168.7	–	171.6	–	170.6	–	171.9	–	170.8
2′	–	108.7	–	111.2	–	111.5	–	105.9	–	110.6
3′	–	160.1	–	160.5	–	160.5	–	166.8	–	160.4
4′	6.49 s	97.8	6.45 s	99.8	6.45 s	99.2	6.21d (2.4)	102.3	6.32 s	102.4
5′	–	158.6	–	161.7	–	159.8	–	164.5	–	158.6
6″	–	114.2	–	116.3	–	115.7	6.26 d (2.4)	113.1	–	114.9
7′	–	137.6	–	139.5	–	139.0	–	144.9	–	139.4
8′	2.40 s	17.8	2.50 s	19.8	2.41 s	19.1	2.44 s	24.9	2.42 s	19.3
4-OCH_3	3.24 s	52.8	–	–	–	–	–	–	–	–
5'-OCH_3	3.89 s	55.2	3.85 s	57.2	3.85 s	56.7	–	–	–	–
IR (neat), ν in cm^{-1}	2955, 2866, 2836, 2721, 1695, 1645,1599, 1245		3348 (br), 2948, 2866, 1647, 1600, 1239		3358 (br), 2946, 2869, 1644, 1600, 1237		3260 (br), 2935, 2865, 1645, 1619, 1588, 1506, 1251		3340 (br), 2951, 2867, 1647, 1609, 1584, 1239	
$[\alpha]_D^{25}$	104		23		11		−107		23	
UV, λ in nm	220, 260, 310		218, 263, 309		209, 261, 299		217, 262, 302		210, 262, 311	

HRESI-MS data were generated on an Exactive Orbitrap instrument (Thermo Scientific). NMR spectra of compounds **3** and **11** were recorded on a Bruker Avance III 500 MHz instrument, **4** and **9** on a Bruker Avance III 600 MHz instrument, **7** on a Bruker Avance II 300 MHz instrument. Compounds were solved in CD_3OD, and chemical shifts were referenced to residual solvent signals. The optical rotation (*c* 0.1, MeOH) was recorded on a JASCO P-1020 instrument; IR spectra were measured on a Bruker IFS55 instrument, UV spectra were extracted from the diode array data obtained during LC–MS analysis.

Table 2
IC_{50} values (μM) of *Armillaria* sesquiterpene aryl esters.

	(**1**) Arnamial	(**2**) Armillaridin	(**3**) 4-*O*-Methyl-armillaridin	(**4**) 5′-Methoxy-6′-chloroarmillane	(**6**) A52a	(**9**) Dehydroarmillyl-orsellinate	(**11**) 6′-Chloro-13-hydroxydihydro-melleolide	(**12**) Armillarin
MCF-7	15.4 (±0.3)*	7.8 (±0.9)	6.7 (±1.1)	26.5 (±0.4)	21.3 (±0.5)	8.0 (±0.5)	>100	11.6 (±0.5)
Jurkat	3.9 (±0.4)*	3.0 (±0.3)	4.1 (±0.8)	13.3 (±0.5)	10.4 (±0.2)	16.9 (±0.1)	46.6 (±3.1)	nd
HeLa	nd	9.2 (±1.6)	18.8 (±2.9)	35.8 (±2.6)	40.0 (±1.1)	15.2 (±2.0)	>100	16.7 (±2.1)
K-562	nd	8.9 (±1.3)	20.6 (±2.2)	25.0 (±0.9)	38.9 (±0.7)	5.0 (±0.3)	>100	9.9 (±0.6)

The values represent the means of three replicate experiments of each compound, the standard deviation is given in parentheses. Substances **5**, **7**, **8**, and **10** were not active at concentrations ⩽100 μM; nd: not determined; previously published values are indicated by an asterisk.

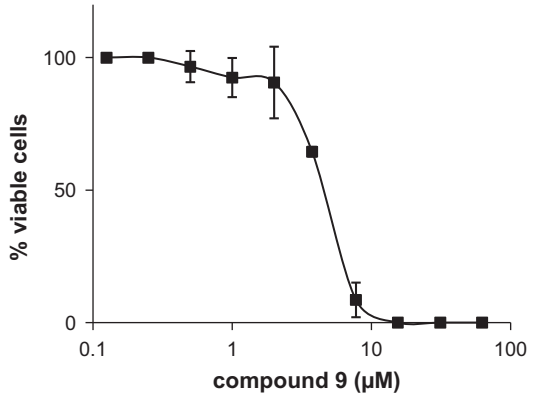

Figure 1. Antiproliferative effect of **9** on K-562 cells. Results were obtained following a described procedure[13] and are expressed as the percent of viable cells, compared to control, after 72 h. Bars indicate the standard deviation.

required to reverse illudin S-induced DNA lesions.[17,18] However, our data points to a dissimilar mechanism as RNA synthesis remained unaffected with **9**. Melleolides do not have a cyclopropane ring. However, the current model on the illudin/irofulvene mode of action relies on such a highly strained system, as it represents the target where cellular nucleophiles, such as thiols, attack and thereby promote alkylation of DNA and proteins.[19]

Comparing known and new melleolides we conclude that terpene hydroxylation is of major relevance, whereas the position of the terpene double bond and 6′-chlorination of the aromatic ring do not contribute to cytotoxicity. We also conclude that melleolides exert their cytotoxicity primarily via inhibition of DNA-biosynthesis.

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6. Fermentation and purification followed largely described procedures (see Ref. 5). Relevant modifications were made in that the seed culture mycelium was homogenized prior to inoculation of the main culture, and that the total culture volume was 19 L, dispensed into wide-necked flat-bottom culture flasks. Semi-preparative HPLC of **2**, **3**, **5–8**, and **10–12** was accomplished on a Shimadzu VP series instrument (column: Phenomenex Luna C18, 15 × 250 mm, 5 μm particle size, solvent A: H_2O, solvent B: MeCN, elution: flow at 6.5 mL/min, initial hold for 1 min at 80% B, then within 27 min to 100% B. The gradient to purify **4** and **9** was 40–60% B within 50 min, with 7.5 mL/min flow, on the above instrument and column. Additional purification of **4** and **9** was achieved on an Agilent 1200 instrument (Zorbax Eclipse XDB C-18 column 9.4 × 250 mm, 5 μm particle size, solvent A: H_2O, solvent B: MeCN, elution: flow 5 mL/min, isocratic conditions at 65% B maintained for 14 min, then to 100% B within 1 min. All chromatograms were recorded at λ = 254 nm.
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15. K562 leukemia cells were supplied in PBS buffer at a concentration of 1×10^6 cells per mL. The cell suspension was dispensed into 2 mL reaction tubes, and 9 (solved in DMSO) added at a final concentration of 1.25, 2.5, or 5.0 μM. For each ^{14}C-labeled precursor assays were set up in triplicate and one tube per concentration was left untreated as control (100% value). The cells were incubated at 37 °C, with the lid open, for 15 min. Then, 0.05 μCi of either 2-^{14}C-thymidine, ubiquitously labeled ^{14}C-L-leucine or 2-^{14}C-uridine was added to the tubes. After gentle mixing the tubes were incubated for another two hours under identical conditions. The reaction was stopped by adding 1 mL of ice cooled 10% (w/v) trichloroacetic acid. The cells were separated from the medium using a glass fiber filter (1.2 μm pore size, Whatman GF/C). The filter was washed with ice cold solution of 5% (w/v) trichloroacetic acid and then transferred into a scintillation vial. After adding 2.5 mL of scintillation cocktail (Rotiszint® eco plus, Roth) the samples were measured using a Beckman Coulter LS 6500 scintillation counter. For positive control, a specific inhibitor of each process was added: replication was inhibited by doxorubicin (0.05–0.47 μM), translation by cycloheximide (3.5–178 μM), and transcription by actinomycin D (0.4–4.0 μM).
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