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Cytotoxic and antifungal activities of melleolide antibiotics follow dissimilar structure–activity relationships

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ABSTRACT

The fungal genus *Armillaria* is unique in that it is the only natural source of melleolide antibiotics, i.e., protoilludene alcohols esterified with orsellinic acid or its derivatives. This class of natural products is known to exert antimicrobial and cytotoxic effects. Here, we present a refined relationship between the structure and the antimicrobial activity of the melleolides. Using both agar diffusion and broth dilution assays, we identified the $\Delta^{2,4}$-double bond of the protoilludene moiety as a key structural feature for antifungal activity against *Aspergillus nidulans*, *Aspergillus flavus*, and *Penicillium notatum*. These findings contrast former reports on cytotoxic activities and may indicate a different mode of action towards susceptible fungi. We also report the isolation and structure elucidation of five melleolides (6′-dechloroarnamial, 6′-chloromelleolide F, 10-hydroxy-5′-methoxy-6′-chloroarmillane, and 13-deoxyarmellides A and B), along with the finding that treatment with an antifungal melleolide impacts transcription of *A. nidulans* natural product genes.

1. Introduction

The melleolides (Fig. 1) represent a large family of aryl ester secondary metabolites produced by species of the basidiomycete genus *Armillaria*. Specifically, these metabolites are composed of a sesquiterpene protoilludene alcohol esterified with an orsellinic acid moiety (Donnelly et al., 1982; Midland et al., 1982). Protoilludane derivatives have been described from numerous basidiomycetes (Abraham, 2001). Likewise, orsellinic acid is an even more common microbial natural product and produced both by bacterial and fungal species (Schroeckh et al., 2009; Shen, 2003). However, the melleolides are structurally distinct in that they represent hybrid molecules in which these two common elements are combined into a unique structure, which is not found outside the genus *Armillaria*. Biosynthesis of the sesquiterpene protoilludene moiety proceeds by cyclization of farnesyl diphosphate, catalyzed by protoilludene synthase, and results in the tricyclic $\Delta^{2,4}$-protoilludene backbone (Engels et al., 2011). In *Armillaria mellea*, orsellinic acid is biosynthesized by a seven-domain polyketide synthase whose terminal thioesterase domain also catalyzes polyketide-terpene coupling (Lackner et al., 2013). A variable combination of methylation, chlorination, and oxidation/reduction generates in excess of 50 melleolide analogs (Misiek and Hoffmeister, 2012), which makes them one of the most diverse groups of natural products known from fungi and an attractive model to study structure–activity-relationships of natural compounds.

Armillaria is a globally distributed genus and includes species that follow a saprotrophic or parasitic lifestyle (Baumgartner et al., 2011). For this reason and given that *Armillaria* species are extremely long-lived organisms, it is reasonable to expect that their natural products, described above, exert biological effects and serve to interact with organisms surrounding the producer. In fact, various melleolides have been shown experimentally to inhibit microbial growth (Arnone et al., 1986; Momose et al.,

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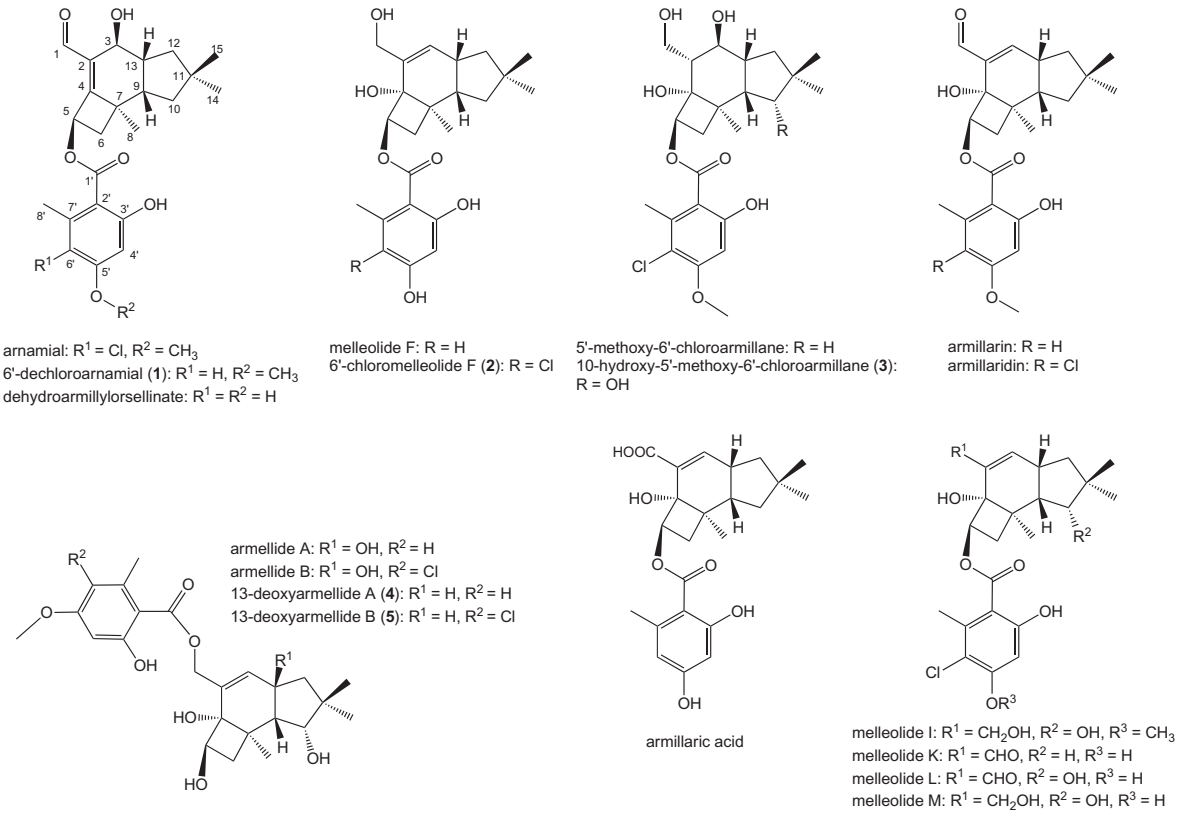

Fig. 1. Chemical structures of melleolides. Compounds **1**–**5** represent new natural products. The terpene moiety is numbered 1–15, the orsellinic acid moiety is numbered 1′–8′.

2000), and display phytotoxicity (Peipp and Sonnenbichler, 1992). More recently, the cytotoxic effects of melleolides on human cancer cell lines were demonstrated and it was found that the mode of action involves decreased DNA synthesis (Bohnert et al., 2011). Here, we show that the structure–activity-relationship of the melleolides regarding their antifungal activity is dissimilar from that of their cytotoxic effects against human cancer cell lines. Sharply contrasting the structure–activity-relationship of cytotoxicity against human cells, it is the position of the double bond within the sesquiterpene moiety that is critical for antifungal activity. For our investigation we used known melleolides along with new members of this family of compounds, whose isolation and structural elucidation is also described here. Based on *Aspergillus nidulans* transcriptomic data, we hypothesize that melleolides may play a role in intermicrobial communication as exposure to an antifungal melleolide (dehydroarmillylorsellinate) affected expression of genes related to natural product biosynthesis.

2. Results

2.1. Isolation and structural elucidation of new melleolides

Melleolides were purified from the supernatant of a liquid culture of *A. mellea* FR-P75. A primary criterion in identification of melleolides in complex samples and crude extracts was their signature UV spectra (Bohnert et al., 2011; Cremin et al., 1995) with three absorption maxima typically appearing at wavelengths of $\lambda = 217$, 260, and 310 nm, respectively. Subsequent mass spectrometry results, combined with extensive 1D and 2D NMR spectral data (Table 1 and Supplementary data) identified five new natural products belonging to the melleolide family, namely 6′-dechloroarnamial (**1**), 6′-chloromelleolide F (**2**), 10-hydroxy-5′-methoxy-6′-chloroarmillane (**3**), which features a reduced terpene, and 13-deoxyarmellides A (**4**) and B (**5**, Fig. 1). Further melleolides that were purified from the fermentation broth and identified by NMR were the previously described compounds arnamial (Misiek et al., 2009), dehydroarmillylorsellinate (Bohnert et al., 2011), as well as armillarin and armillaridin (Yang et al., 1984).

With regards to ^{1}H and ^{13}C NMR spectra (Fig. S1 and Fig. S2), compound **1** showed chemical shifts virtually identical to those of arnamial (Misiek et al., 2009). The slight upfield shift of the C-6′ signal in **1** (δ_C 111.7 compared to 116.0 in arnamial) could be ascribed to a hydrogen atom replacing the chlorine substituent of arnamial at this position. The corresponding proton signal (δ_H 6.27) was specific for **1** and showed a coupling to H-4′ ($J = 2.5$ Hz) as expected. The identification of **1** as 6′-dechloroarnamial was in accordance with high resolution (HR)ESIMS data (m/z 415.2119 $[M+H]^+$), which confirmed the absence of a chlorine atom (Fig. S3). Conversely, the spectra of melleolide F (Arnone et al., 1988a) and compound **2** differed only in that the aromatic signal for H-6′ was absent and that the resonance of the C-6′ carbon appeared at lower field (Fig. S4 and Fig. S5, δ_C 114.0 compared to 111.7 in melleolide F). We therefore conclude that the orsellinic acid moiety of **2** carries a chlorine atom at C-6′ and, hence, represents 6′-chloromelleolide F. This interpretation is supported by the isotopic pattern indicating a monochlorinated molecule, and by the exact mass (Fig. S6) m/z 435.1579 $[M-H]^-$, consistent with $C_{23}H_{28}O_6Cl$.

^{1}H and ^{13}C NMR data of compound **3** (Fig. S7 and Fig. S8) suggested the absence of a double bond in the sesquiterpene moiety, since the signal of C-2 was significantly shifted upfield (δ_C 45.3). Furthermore, this carbon carried one proton (δ_H 2.01), as observed in the HSQC spectrum. The structure was elucidated by comparison with spectral data of the known natural product 5′-methoxy-6′-chloroarmillane (Bohnert et al., 2011) whose NMR signals slightly differed: for **3**, the ^{13}C chemical shifts of C-10 and C-15 are