J Ind Microbiol Biotechnol (2003) 30: 220–224
DOI 10.1007/s10295-003-0048-y

ORIGINAL PAPER

J. G. Ondeyka · A. W. Dombrowski
J. P. Polishook · T. Felcetto · W. L. Shoop
Z. Guan · S. B. Singh

Isolation and insecticidal activity of mellamide from *Aspergillus melleus*

Received: 3 January 2003 / Accepted: 21 February 2003 / Published online: 8 April 2003

Abstract Mellamide, a novel indole amide, was isolated from a fermentation of *Aspergillus melleus* using silica gel and high-performance liquid chromatographic methods. This allowed its separation from three known antiparasitic compounds (ochratoxin A, viomellin and xanthomegnin) also present in the potent extract. The structure was elucidated by ^{1}H, ^{13}C, COSY, DEPT, HMQC and HMBC NMR experiments. HR-FTMS aided in the molecular weight and formula determination. Mellamide showed in vitro insecticidal activity in bioassays against larvae of *Lucilia sericata* and *Aedes egypti* with LD_{90} of 1,000 and 50 μg/ml, respectively.

Keywords *Aspergillus melleus* · Mellamide · Insecticide

Introduction

Over the years, we have reported the discovery of several novel endo and ecto-antiparasitic compounds, such as avermectins [1], paraherquamides [6], and nodulisporic acids [7], isolated from bacterial and fungal sources. Despite intensive research, ecto- and endo-parasites, such as fleas, ticks, and intestinal worms, still pose significant health hazards to humans and their companion and food animals. With respect to insecticides, although there have been numerous recent entries onto the market, many are limited by their therapeutic index, environmental toxicity, development of resistance, or to topical application only. Therefore, discovery of safe antiparasitic drugs with new modes of action, preferably acting systemically, remains a critical need in human and animal medicine. Our continued interest in novel antiparasitic agents led us to screen extracts of fungal isolates in vitro using surrogate organisms such as blowfly larvae (*Lucilia sericata* [8]), mosquito larvae (*Aedes egypti* [3]) and *Haemonchus contortus* [5] as antiparasitic assays.

Screening of our internal fungal extract libraries led to the isolation of mellamide, a new indole amide with insecticidal properties, produced by *Aspergillus melleus* (Fig. 1). This novel compound was isolated from the methylethylketone extract of a solid fermentation grown in roller bottles, using silica gel and reverse phase high-performance liquid chromatography (HPLC) chromatography. Monitoring the fractionation with the in vitro assays, *L. sericata*, *A. egypti*, and *H. contortus*, also afforded known compounds with antiparasitic activity such as ochratoxin A [12], viomellin [9] and xanthomegnin [10]. Mellamide possessed insecticidal activity against mosquito larvae and blowfly larvae. This report describes details of the producing strain, isolation and structure determination, and biological activities of this compound.

J. G. Ondeyka (✉) · Z. Guan · S. B. Singh
Natural Products Chemistry, Merck Research Laboratories, R80Y-355, PO Box 2000, Rahway, NJ 07065, USA
E-mail: john_ondeyka@merck.com

A. W. Dombrowski · J. P. Polishook · T. Felcetto · W. L. Shoop
Human and Animal Infectious Disease Research, Merck Research Laboratories, Rahway, NJ 07065, USA

Materials and methods

HPLC and thin layer chromatography analysis

The chromatographic system consisted of an HP 1100 liquid chromatograph with a diode array detector and Chemstation software (Hewlett-Packard, Palo Alto, Calif.).

The extract was concentrated under reduced pressure and dried under vacuum. A small portion of the extract was dissolved in methanol and concentrated to 10×, and a 5 μl aliquot was injected onto a Zorbax RX-C8 HPLC column (4.6×150 mm). A linear gradient was used with 0.1% trifluoroacetic acid from 20% to 90% acetonitrile in 15 min at a flow rate of 1 ml min^{-1}. Preparative HPLC was performed on a Gilson system using Unipoint software and a Zorbax RX-C8 column (21×250 mm) at ambient temperature with an isocratic elution at 40% acetonitrile and a flow rate of 8 ml min^{-1}.

Silica gel 60 (E-Merck, Darmstadt, Germany) was used for normal phase column chromatography (200 cm^3) starting with hexane:ethyl acetate (1:1). Analytical silica gel thin layer chromatography (TLC) 60 F_{254} plates (E-Merck) were used to follow the

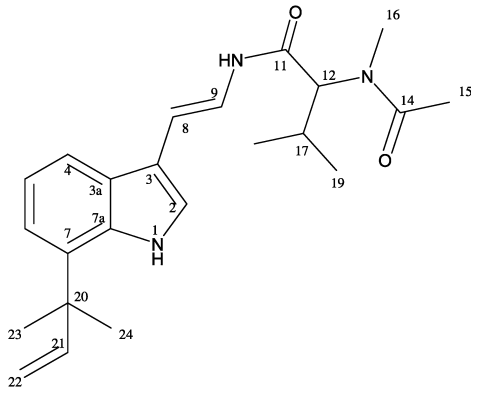

Fig. 1 Structure of mellamide with atom numbering system

column chromatographic separation using as eluent methylene chloride:methanol (9:1). Reverse phase (RP) C18 F_{254} plates (250 mm; 20×20 cm; Whatman) were developed in 80% aqueous methanol. The plates were developed in ascending mode in an unsaturated chamber. Spots were visualized under UV at 254 nm; bands were scraped off and eluted with methanol. The solutions were filtered through a 0.45 μm Millex filter.

Physical and chemical properties

NMR experiments were carried out on a Varian Unity 400 MHz instrument using a dual 3 mm probe. Many one- and two-dimensional techniques, such as ^{1}H, ^{13}C, COSY, DEPT, NOESY, HMQC and HMBC, were recorded. Deuterated acetonitrile was used as the NMR solvent and the residual solvent peak was used for internal reference. Liquid chromatography mass spectroscopy (LC-MS) in the electro-spray ionization mode was used for determination of molecular weight with a Thermo-Finnigan LCQ and high resolution (HR) mass measurement was made on a Thermo-Finnigan NewStar 3T FT/MS instrument. UV/V spectra were recorded on a Beckman DU 70 spectrophotometer. Optical rotation was determined on a Perkin-Elmer 241 polarimeter.

Antiparasitic assays

Details for the antiparasitic assays used in this report can be found in the following references: *Aedes egypti* [3]; *Lucilia sericata* [8]; *Haemonchus contortus* [5].

Growth conditions for *A. melleus* strain

A. melleus Yukawa MF6589, was isolated from soil collected in the Guanacaste Conservation Area, Costa Rica. This isolate produces an abundant amount of sclerotia, which are thick-walled masses of hyphae (Fig. 2). The culture is maintained at Merck Research Laboratories (Rahway, N.J.) and is available upon request.

The culture was inoculated into seed flasks by aseptically transferring a 1 ml aliquot of the frozen vegetative mycelium into a 250 ml Erlenmeyer flask containing 50 ml seed medium of the following composition (per liter of deionized water): Bacto neopeptone 10 g, maltose 40 g, yeast extract 10 g, agar 4 g. The seed medium was prepared with distilled water, and was dispensed into 250 ml Erlenmeyer flasks and capped with cotton plugs before being autoclaved at 121°C for 20 min. The seed culture was incubated at 25°C, 220 rpm and 85% relative humidity, for 2 days, and then transferred to the production medium.

Fermentations were performed on a solid substrate production medium. The production phase was grown in 4-l roller bottles, containing approximately 1,250 ml (measured by volume) of

Fig. 2 Eleven-day-old streak plate culture of *Aspergillus melleus* MF6589 on yeast-malt agar (Difco)

large-particle vermiculite (sterilized separately from the liquid), with 440 ml of a liquid nutrient solution poured over it at the time of inoculation. The nutrient solution contained (per liter): glycerol 100 g, glucose 70 g, L-tryptophan 0.7 g, NH_4Cl 3 g, monosodium glutamate 10 g, Amicase (Sheffield Products, Norwich, N.Y.) 8 g, 2-[*N*-morpholino]ethanesulfonic acid (MES) 20 g, K_2HPO_4 1 g, $MgSO_4{\cdot}7H_2O$ 0.5 g, $CaCO_3$ 1 g, 85% lactic acid 5 ml l^{-1}, and 50× salt solution 20 ml l^{-1}. The 50× salt solution contained (per liter): $FeSO_4{\cdot}7H_2O$ 0.5 g, $ZnSO_4{\cdot}7H_2O$ 0.5 g, $MnSO_4{\cdot}H_2O$ 0.1 g, $CuSO_4{\cdot}5H_2O$ 0.05 g, $CoCl_2{\cdot}6H_2O$ 0.04 g, in 0.6 N HCl. The pH of the medium was adjusted to pH 6.0 before adding $CaCO_3$. The production medium was dispensed as 220 ml aliquots in 500 ml bottles and autoclaved for 15 min at 121°C. The solid and liquid portions of the production medium were combined and inoculated with 20–24 ml seed culture. The roller bottle was shaken to coat the vermiculite with the seed growth and nutrient solution. Incubation was on a Wheaton roller apparatus rotating at approximately 4 rpm, at 22°C and 70% relative humidity, for 19 days.

Isolation

A 2-l fermentation of *A. melleus* grown in roller bottles was extracted with 2-l methylethylketone and filtered. The filtrate was concentrated under reduced pressure using a rotary evaporator. The residue weighed 5.8 g and was sequentially triturated starting with 125 ml of hexane, followed by 200 ml acetonitrile and 50 ml methylene chloride. The acetonitrile-soluble fraction showed activity in all three of the antiparasitic assays, while the methylene chloride-soluble fraction showed activity in only the mosquito larvae assay. The acetonitrile and methylene chloride fractions were combined, concentrated to dryness, redissolved in methanol, and filtered. The solid was washed with cold methanol and the solutions were combined. The purple solid (290 mg) that remained after washings, was soluble in methylene chloride.

The methanol-soluble material was concentrated to dryness to produce an oily residue, which was dissolved in ethyl acetate (10 ml) and charged to a silica gel column. The column was eluted with one column volume (cv) of hexane-ethyl acetate (1:1), followed by 1.5 cv of ethyl acetate and 1 cv of methanol, collecting 5 ml fractions. Fractions 75–85 showed activity in the mosquito larvae assay, while fractions 150–158 exhibited activity in all three assays. The major component present in the late-eluting silica gel fraction was determined to be ochratoxin A (Fig. 3) by ^{1}H NMR, LC/MS and co-injection with an authentic sample. The early eluting active fraction from the column was enriched with mellamide. This

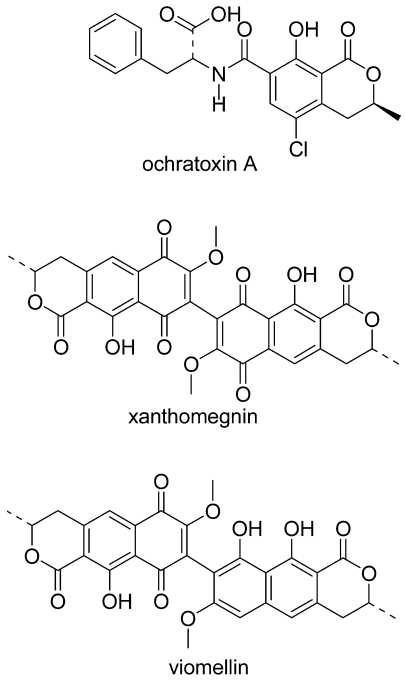

Fig. 3 Structures of known mycotoxins isolated from *A. melleus*

material was further purified by preparative RP C-18 TLC to give 120 mg "mellamide (1)" (titer of 60 mg l^{-1}). The purple solid mentioned above contained two major compounds identified as xanthomegnin and viomellin (Fig. 3) as determined by analytical HPLC, ^{1}H NMR, LC/MS and co-injection with an authentic sample.

Results and discussion

Structure determination

Mellamide (1), a pale yellow amorphous solid, showed absorption maxima at λ_{max} 300, 280 230 and 210 nm in the UV spectrum, indicating an extended indolic chromophore. HR-FTMS gave an exact mass of 382.2484 for $[M + H]^+$ (calculated 382.2494) suggesting a molecular formula of $C_{23}H_{31}N_3O_2$ + H for the indole amide, mellamide (1). The ^{13}C NMR spectrum (Table 1) revealed the presence of 23 carbons and supported the molecular formula. The HMQC experiment indicated 16 proton-bearing carbons. The DEPT spectrum suggested the presence of 6 methyl, 1 methylene and 9 methine carbons. The ^{1}H NMR spectrum (Fig. 4) along with the 2D-COSY spectrum indicated 6 methyl groups, two of which appeared as a singlet, indicative of connectivity to a quaternary carbon and two methyl doublets as part of an isopropyl group extending to an α-methine and revealing the valine residue. The remaining two methyl groups were elucidated to be an *N*-methyl (δ 3.0) and an acetyl group (δ 2.1). The COSY spectrum also revealed the presence of a vinyl and a trans-olefin in addition to a 1,2,3-coupled aromatic residue and an olefinic doublet (δ 7.23). The ^{13}C NMR spectrum of mellamide indicated the presence of two amide carbonyls (δ 172.8, C-14; δ 168.3, C-11). The structural fragments were assembled by an HMBC experiment (Table 1) to reveal structure 1 for mellamide.

Table 1 ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR assignments of mellamidein CD_3CN

Carbon no.	δC	Type	δH	HMBC
2	123.32	CH	7.23 (d, 2.4)	3, 3a, 7a, 8
3	113.05	Q		
3a	127.27	Q		
4	118.95	CH	7.58 (d, 7.6)	3, 3a, 6, 7a
5	120.92	CH	7.09 (dd, 7.2, 7.6)	3a, 7
6	119.81	CH	7.15 (d, 7.2)	4, 7a, 20
7	132.13	Q		
7a	135.54	Q		
8	107.68	CH	6.45 (d, 14.8)	2, 3a, 9
9	120.52	CH	7.35 (dd, 10.4, 14.8)	3, 8, 11
11	168.33	Q		
12	63.09	CH	4.70 (d, 10.8)	11, 14, 16, 17
14	172.83	Q		
15	22.47	CH_3	2.10 (s)	14
16	32.20	CH_3	3.00 (s)	12, 14
17	27.48	CH	2.30 (m)	11, 12, 18, 19
18	20.40	CH_3	0.96 (d, 6.4)	12, 17, 19
19	19.30	CH_3	0.85 (d, 6.8)	12, 17, 18
20	41.36	Q		
21	148.23	CH	6.11 dd, 10.4, 17.6)	7, 20, 23, 24
22	112.71	CH_2	5.20, 5.30 (dd, 10.4, 17.6)	20, 21
23	27.65	CH_3	1.49 (s)	7, 20, 21, 24
24	27.65	CH_3	1.49 (s)	7, 20, 21, 23
N-10		NH	8.80 (d, 10.0)	11
N-1		NH	8.96 (br s)	

The structure of the dimethyl allyl substituted indole and the *N*-methyl, *N*-acetyl valine unit was assigned by the HMBC correlations. The C-8 olefinic proton showed 3-bond correlations to C-2 and C-3a of the indole unit while the C-9 olefinic proton showed 3-bond correlations to C-3 of the indole and the C-11 carbonyl of *N*-methyl *N*-acetyl valine indicating that C-8 and C-9 bridged the indole unit with valine. The stereochemistry of the C-8–C-9 olefin was assigned (*E*) based on the observation of a large coupling constant ($J = 14.8$ Hz) between H-8 and H-9.

Mellamide is structurally similar to various indole amides (Fig. 5) such as tryptostatins [2], terpeptins [4], and aspergillamide [11] isolated from other *Aspergillus* spp.; the former two as cell cycle inhibitors and the latter as a cytotoxic agent against tumor cells.

Antiparasitic activity

The indole amide displayed a moderate insecticidal profile with activity (LD_{90}) against *A. egypti* at 50 μg/ml and *L. sericata* at 1,000 μg/ml (Table 2). Mellamide possessed no activity against the endoparasite *H. contortus*.

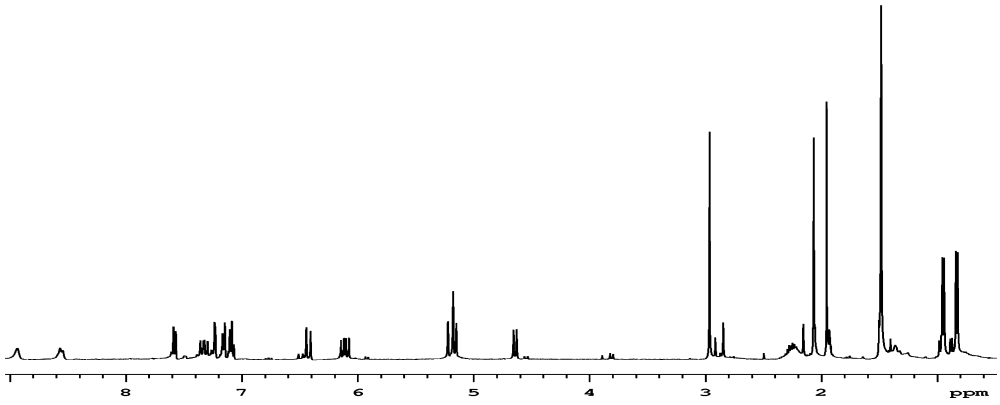

Fig. 4 ^{1}H NMR of mellamide (1) in CD_3CN at 400 MHz

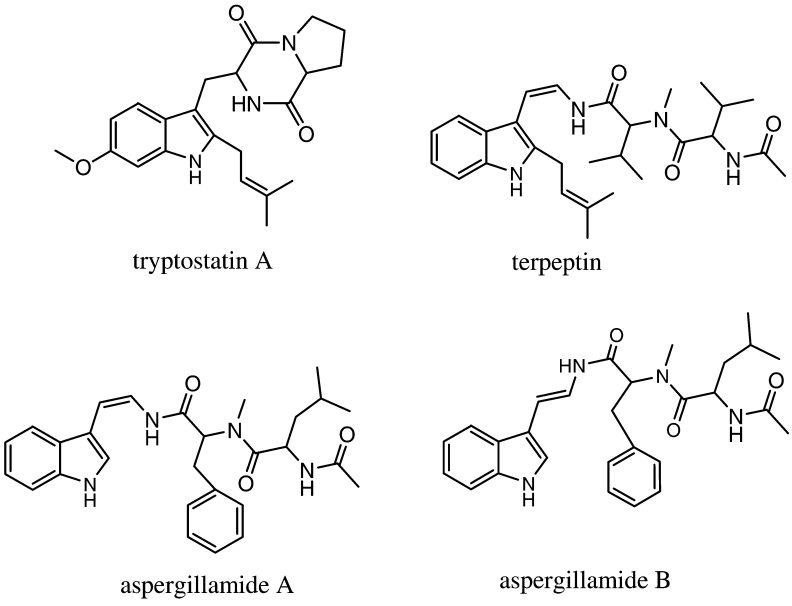

Fig. 5 Indole peptides reported from *Aspergillus* sp.

The bulk of the biological activity of the extract of *A. melleus* was due to ochratoxin A, viomellin and xanthomegnin, all known to possess insecticidal activity. They are known to be active in mosquito larvae assays and all three have been reported from *A. melleus* in the literature. Ochratoxin A accounted for most of the activity of the extract and showed LD_{90} values of 10, 1,000, and 2 μg/ml against *A. egypti*, *L. sericata*, and *H. contortus*, respectively. The other two compounds exhibited variable activities as shown in Table 2. For comparison, nodulisporic acid A, paraherquamide, and ivermectin, some of the most important insecticidal agents are also listed in Table 2.

Table 2 Biological data of isolated compounds and others

Compound	LD_{90}		
	Aedes aegypti μg/ml	*Lucilia sericata* μg/ml	*Haemonchus contortus* μg/ml
Mellamide	50	1,000	na[a]
Ochratoxin A	10	1,000	2
Viomellin	10	na	100
Xanthomegnin	10	na	100
Nodulisporic acid A	0.5	0.3	na
Paraherquamide	50	50	100
Ivermectin	0.005	0.040	0.005

[a]Not active

Conclusion

A new indole amide, mellamide (1), was isolated as a modest insecticidal agent from a culture of *A. melleus* using *H. contortus*, *L. sericata* and *A. egypti* in vitro antiparasitic assays. The structure of this compound was determined by one and two-dimensional NMR and HR-FTMS.

Acknowledgements We thank Bruce Michael for running the *Haemonchus contortus* assay during the screening and providing the bioassay-guided fractionation support, and Marta Arocho for the initial library extract preparation.

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