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Isolation and Insecticidal/Anthelmintic Activity of Xanthonol, a Novel Bis-xanthone, from a Non-sporulating Fungal species

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Abstract Xanthonol, a novel dimeric xanthone, was isolated from a fermentation broth of a non-sporulating fungal species using Sephadex LH20 followed by HPLC and the structure elucidated by spectral analysis. Xanthonol exhibited insecticidal and anthelmintic activities against larvae of *Lucilia sericata*, *Aedes aegypti*, and *Haemonchus contortus* with LD_{90} of 33, 8, and 50 μg/ml, respectively.

Keywords non-sporulating fungus, xanthonol, insecticide, anthelmintic

Introduction

We have recently reported the discovery of a fungal metabolite mellamide [1] as an insecticidal agent which represented another compound in a long list of insecticidal and anthelmintic agents from our laboratories that include avermectins [2], paraherquamides [3], and nodulisporic acid [4], isolated from both prokaryotic and eukaryotic sources. Ecto and endo parasites, such as fleas, ticks, and intestinal worms, pose significant health hazards to humans and their domestic animals. Insecticides and anthelmintics, even with the latest approved treatments, are limited by their therapeutic index, environmental safety, development of resistance, and/or lack of systemic effects. Discovery of safe and efficacious systemic antiparasitic drugs with new modes of action are critically and continuously needed to effectively treat human and animal infections.

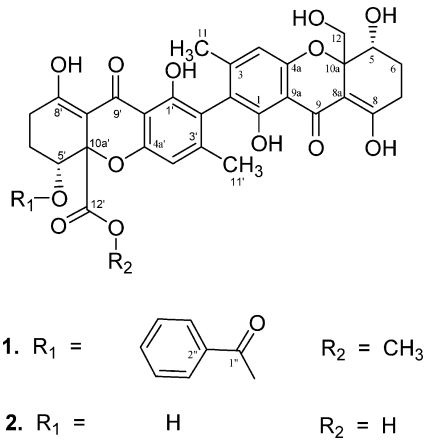

Fig. 1 Structure of xanthonol (**1**) and hydrolysis product (**2**).

Our continued interest in novel antiparasitic agents led us to screen extracts of fungal fermentations in *in vitro* antiparasitic assays using surrogate organisms such as blowfly larvae, *Lucilia sericata* [5], mosquito larvae, *Aedes aegypti* [6], and a parasitic nematode of sheep, *Haemonchus contortus* [7]. This screening strategy led to the isolation and identification of xanthonol (**1**), a new dimeric xanthone from an extract of a non-sporulating fungal species (Figure 1). This report describes the details of the producing strain, isolation, structure determination and biological activities of xanthonol (**1**).

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Materials and Methods

General Procedures

The NMR experiments were conducted on a Varian Unity

500 MHz instrument using a dual 3 mm probe. All 1D and 2D experiments such as ^{1}H, ^{13}C, COSY, DEPT, NOESY, HMQC and HMBC were recorded in CD_3CN or CD_3OD. Residual solvent peaks were used as internal reference (CD_3CN: δ_H 1.94 and δ_C 118.7 and CD_3OD: δ_H 3.30 and δ_C 49.0). ESI-LC-MS was used for determination of molecular weight with a Thermo-Finnigan LCQ. High resolution mass measurement was performed on a Thermo-Finnigan NewStar 3T FT/MS instrument. UV visible spectra were recorded on a Beckman DU 70 spectrophotometer. Optical rotation was determined on a Perkin-Elmer 241 polarimeter. Analytical HPLC was performed on HP 1100 liquid chromatograph with a diode array detector (DAD) and Chemstation software (Hewlett-Packard, Palo Alto, USA). Preparative HPLC was performed on a Gilson system using Unipoint software.

Antiparasitic Assays

Details of the antiparasitic assays have been described elsewhere for *Aedes aegypti* [5]; *Lucilia sericata* [6]; and *Haemonchus contortus* [7].

Growth Conditions for the Fungal Strain

The non-sporulating fungus, MF6460, was isolated from a leaf litter of *Manilkara bidentata* collected in El Verde, Puerto Rico. The culture is maintained in the Merck culture collection at Merck Research Laboratories, Rahway, New Jersey 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: corn steep powder 2.5 g/liter, tomato paste 40 g/liter, glucose 10 g/liter, oat flour 10 g/liter and trace elements 10 ml consisting of $FeSO_4 \cdot 7H_2O$ 1.0 g/liter, $MnSO_4 \cdot H_2O$ 1.0 g/liter, $CuCl_2 \cdot 2H_2O$ 0.025 g/liter, $CaCl_2$ 0.1 g/liter, H_3BO_3 0.056 g/liter, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 0.019 g/liter, $ZnSO_4 \cdot 7H_2O$ 0.2 g/liter and adjusted to pH 6.8. The seed medium was prepared in distilled water, and was dispensed into 250 ml Erlenmeyer flasks and capped with cotton plugs before being autoclaved at 121°C for 20 minutes. 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 either in solid state on vermiculite or in liquid media. The production phase, for solid state fermentations, was grown in 4-liter roller bottles, containing approximately 1250 ml (measured by volume) of 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 was formulated as follows: glycerol 40 g/liter, glucose 300 g/liter, yeast extract 8.0 g/liter, Na_2HPO_4 2.0 g/liter, monosodium glutamate 6.0 g, $NaNO_3$ 2.0 g/liter, $MgSO_4 \cdot 7H_2O$ 2.0 g/liter, $CaCO_3$ 16.0 g/liter, and K elements 1 ml. (The K element solution was prepared as follows: $FeCl_3 \cdot 6H_2O$ 5.8 g/liter, $NaMoO_4 \cdot 2H_2O$ 0.5 g/liter, $MnSO_4 \cdot H_2O$ 0.1 g/liter, $CuSO_4 \cdot 5H_2O$ 0.015 g/liter, $CoCl_2 \cdot 6H_2O$ 0.02 g/liter, $ZnCl_2$ 0.02 g/liter, $SnCl_2 \cdot 2H_2O$, H_3BO_3 0.01 g/liter, KCl 0.02 g/liter in conc. HCl). The pH of the medium was adjusted to 7.0 before adding $CaCO_3$. The production medium was dispensed in 110 ml aliquots in 500 ml bottles and autoclaved 15 minutes at 121°C. The solid and liquid portions of the production medium were combined and inoculated with 20～24 ml of the seed culture. The 4-liter 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. Production of active component was low.

The production phase for liquid fermentation was grown in 250 ml shake flasks, containing approximately 50 ml of liquid medium with inoculums. The nutrient solution was formulated as follows: glycerol 75 g/liter, glucose 50 g/liter, ardamine pH 5.0 g/liter, soybean meal 5.0 g/liter, tomato paste 5.0 g/liter, sodium citrate 2.0 g/liter, $(NH_4)_2SO_4$ 2.0 g/liter. The production medium was dispensed in 250 ml shake flasks and autoclaved for 15 minutes at 121°C. An aliquot of 2.0 ml of seed fermentation was added to the 50 ml of production medium and allowed to incubate at 22°C in a Forma room on a shaker at 220 rpm and 70% relative humidity for 21 days.

Isolation of Xanthonol (1)

A 3-liter fermentation of the fungus grown in shake flasks was extracted with 3-liters of methyl ethyl ketone and the organic layer separated and concentrated under reduced pressure using a rotary evaporator to an aqueous solution. The yellow aqueous solution was sequentially extracted with 75 ml of CH_2Cl_2, followed by 200 ml of EtOAc and the remaining aqueous layer was freeze dried. The CH_2Cl_2 and EtOAc extracts were combined and concentrated to give an oil which was triturated with hexane and filtered to yield 350 mg of a solid. The freeze dried solid (4.3 g) was triturated with hexane (200 ml) and filtered to yield 3 g of a solid. Both solids were combined and dissolved in 120 ml of MeOH and charged to a 4-liter Sephadex LH20 column in MeOH and eluted at a flow rate of 20 ml/minute resulting in 1.52 g of highly enriched xanthonol in 1.15～1.25 column volumes. This material was further purified by reversed phase preparative HPLC using Waters Symmetry C_{18} column (21×250 mm) eluting with 50% aqueous CH_3CN at a flow rate of 8 ml/minute at ambient temperature

Table 1 ^{13}C and ^{1}H NMR assignments of xanthonol (**1**) in CD_3CN (500 MHz, δ in ppm)

Carbon	δ_C	Type	δ_H (mult, J in Hz)	HMBC
1	160.3	Q		
1′	160.5	Q		
2	118.3	Q		
2′	116.8	Q		
3	151.3	Q		
3′	150.3	Q		
4	109.7	CH	6.36 (s)	2, 4a, 9a, 11
4′	110.5	CH	6.45 (s)	2′, 4a′, 9a′, 11′
4a	158.0	Q		
4a′	158.8	Q		
5	66.6	CH	4.26 (dd, 2.0, 4.5)	7, 10a, 8a
5′	68.9	CH	5.88 (dd, 2.0, 4.5)	1″, 8a′, 7′, 10a′
6	23.0	CH_2	2.00 (m)	
			2.00 (m)	
6′	24.0	CH_2	2.12 (m)	
			2.22 (m)	5′, 8′, 10a′
7	25.6	CH_2	2.30 (dd, 20, 6.5)	5, 6, 8, 8a
			2.65 (ddd, 19, 8.0, 6.5)	6, 8, 8a
7′	25.5	CH_2	2.52 (dd, 20, 6.5)	5′, 8′, 8a′
			2.83 (ddd, 19, 8.0, 6.5)	6′, 8′, 8a′
8a	102.6	Q		
8a′	101.7	Q		
8	180.3	Q		
8′	180.2	Q		
9a	105.4	Q		
9a′	105.4	Q		
9	188.2	Q		
9′	188.7	Q		
10a	84.9	Q		
10a′	83.3	Q		
11	20.8	CH_3	1.97 (s)	2, 3, 4
11′	20.8	CH_3	1.95 (s)	2′, 3′, 4′
12	65.8	CH_2	3.50 (dd, 12.0, 7.5)	5, 10a
			3.78 (dd, 12.0, 5.5)	5, 10a
12′	171.3	Q		
13′	54.3	CH_3	3.73 (s)	12′
1″	166.1	Q		
2″	130.7	Q		
3″	130.6	CH	8.01 (dd, 8.0, 1.5)	1″, 5″, 7″
4″	129.7	CH	7.47 (t, 7.5)	2″, 6″
5″	134.5	CH	7.61 (t, 8.0)	3″, 7″
6″	129.7	CH	7.47 (t, 7.5)	2″, 4″
7″	130.6	CH	8.01 (dd, 8.0, 1.5)	1″, 3″, 5″
12-OH			3.12 (dd, 7.5, 5.5)	12
5-OH			3.25 (m)	6
1-OH			11.59 (s)	1, 2, 9a
1′-OH			11.48 (s)	1′, 2′, 9a′
8-OH			14.01 (s)	7, 8, 8a
8′-OH			14.03 (s)	7′, 8′, 8a′

yielding 1.5 g (500 mg/liter) of xanthonol (**1**), as a yellow amorphous powder.

Hydrolysis of Xanthonol (1)

A 50 mg aliquot of **1** was dissolved in tetrahydrofuran. A 3 ml saturated solution of LiOH was added slowly at room temperature while stirring. After 2 hours compound **1** was consumed and one major compound was formed and found in the aqueous layer. The aqueous layer was separated and diluted with water and neutralized with 1 N HCl. The aqueous layer was extracted twice with EtOAc and concentrated to dryness giving a solid which was purified by prep HPLC. An RP-18 Symmetry column (21×250 mm) was employed and eluted with 25% aqueous CH_3CN for 30 minutes followed by 50% aqueous CH_3CN for 30 minutes eluting **2** at 40 minutes which was lyophilized to give 4 mg of **2** as a yellow amorphous powder.

Xanthonol (**1**)

$[\alpha]_D^{23}$ +249° (*c*, 1.0, MeOH): soluble in MeOH, DMSO, $CHCl_3$; HRESI-FTMS (*m/z*) 715.2014 (calcd for $C_{38}H_{34}O_{14}$+H: 715.2027), UV(MeOH) λ_{max} 227 (ε 34,560), 275 (ε 7,940) and 337 (ε 29,500) nm; IR (ZnSe) ν_{max} 3421, 2957, 1730, 1604, 1582, 1450, 1362, 1271, 1241, 1173, 1091, 1071, 1027 cm^{-1}; For ^{1}H and ^{13}C NMR data, see Table 1.

Xanthonolic Acid (**2**)

HRESI-FTMS (*m/z*) 597.1617 (calcd $C_{30}H_{28}O_{13}$+H: 597.1608), UV (MeOH) λ_{max} 230, 275, 340 nm; ^{1}H NMR (CD_3OD, 400 MHz) δ 6.53 (1H, s), 6.50 (1H, s), 4.38 (1H, s), 4.33 (1H, s), 3.86 (1H, d, J=12.8 Hz), 3.57 (1H, J=12.8 Hz), 2.75 (2H, m), 2.36 (2H, m), 2.15 (2H, m), 2.02 (2H, m), 2.02 (3H, s), 1.99 (3H, s).

Results and Discussion

Isolation

Solid state fermentation grown in roller bottles or liquid fermentations grown in shake flasks of the unidentified fungus (MF6460) was extracted with methyl ethyl ketone and the extract was chromatographed on Sephadex LH20 followed by reversed-phase HPLC. The purification was followed by evaluation of each fraction in *in vitro* assays, using whole organism *L. sericata*, *A. aegypti*, and *H. contortus*, and led to the isolation of xanthonol (**1**, 500 mg/liter) as a pale yellow powder.

Structure Determination

Xanthonol (**1**) showed absorption maxima at λ_{max} 340, 275 and 230 nm in the UV spectrum indicating that it contained an extended chromophore. HR-FTMS gave a protonated pseudo molecular ion [M+H] at *m/z* 715.2014 suggesting a molecular formula of $C_{38}H_{34}O_{14}$+H (calculated 715.2027) for **1**. The ^{13}C NMR spectrum (Table 1) of **1** revealed the

presence of 38 carbons and supported the molecular formula with 22 degrees of unsaturation. The DEPT spectrum suggested the presence of 3 methyl, 5 methylene and 9 methine carbons which were corroborated by HMQC correlations. The NMR spectra suggested that it was an asymmetrical dimer.

The two methyl groups appeared as singlets at δ_H 1.95 (δ_C 20.8) and δ_H 1.97 (δ_C 20.8) in the ^{1}H NMR spectrum indicating that they were attached to aromatic carbons C-3 and C-3′ which was confirmed by HMBC correlations to three carbons each (Table 1). The third methyl group appeared at δ_H 3.73 (δ_C 54.3) and produced an HMBC correlation to the carboxyl carbon δ_C 171.3. The COSY spectrum of xanthonol revealed the presence of two $-CH_2-CH_2-CH-(O)-$ units and a phenyl ring. Additionally, the ^{1}H NMR spectrum displayed signals for two aromatic proton singlets, one each at δ_H 6.36 and 6.45, a pair of methine multiplets at δ_H 4.26 and 5.88 assigned to oxygen bearing carbon atoms, two pairs of chelated phenolic groups at δ_H 14.01, 14.03 and δ_H 11.59, 11.48, and a pair of hydroxy groups at δ_H 3.12 and 3.25. The ^{13}C NMR spectrum of **1** indicated the presence of two carbonyls (δ_C 166.1, C-1″; δ_C 171.3, C-12′), two enolic carbons (δ_C 180.3, C-8; δ_C 180.2, C-8′) and two ketones (δ_C 188.2, C-9, δ_C 188.7, C-9′). The phenolic group appearing at δ_H 11.59 gave HMBC correlations to δ 160.3 (C-1), 118.3 (C-2) and 105.4 (C-9a) confirming its substitution at C-1 and analogous correlations of OH appearing at δ_H 14.48 confirmed its connectivity at C-1′. HMBC correlations of H-5′ (δ 5.88) to C-1″ (δ 166.1) indicated that the benzoyl group is attached to C-5′ (δ 68.9). Similar HMBC correlations of other protons to respective carbons (Table 1) helped assignment of structure **1** to xanthonol.

The coupling constants between H-5 and H-6 of 2.0 and 4.5 Hz are consistent with an equatorial orientation of H-5, indicating axial orientation of the C-5 OH. The same is true for H-5′ and C-5′ OCH_3. This is consistent with data reported for structurally similar dimeric xanthones such as dicerandrols [8], neosartorin [9], ascochrome [10] and secalonic acids [11] reported to have antibiotic and cytotoxic activitied.

Xanthonol was hydrolyzed with LiOH to give xanthonolic acid (**2**) (Figure 1). The hydrolytic product indicated the absence of the signals of phenyl ring and the methoxy group in the ^{1}H NMR spectrum. In addition, the H-5′ (δ 5.88) was shifted upfield to δ 4.38 indicating the loss of the benzoate group and confirming that the benzoate was located at C-5′.

Antiparasitic Activity

Xanthonol (**1**) displayed moderate insecticidal and anthelmintic activities against *A. aegypti*, *L. sericata*, and *H. contortus* exhibiting LD_{90} values of 8, 33, and 50 μg/ml, respectively (Table 2). The hydrolytic product (**2**) was not active at the highest levels (50∼500 μg/ml) tested. The corresponding biological activities of nodulisporic acid A, paraherquamide, and ivermectin are listed for comparison in Table 2.

Table 2 Biological activities of xanthonol (**1**) and xanthonolic acid (**2**)

Compound	LD_{90}		
	A. aegypti, μg/ml	*L. sericata*, μg/ml	*H. contortus*, μg/ml
1	8	33	50
2	NA@50	NA@500	NA@200
Nodulisporic acid A	0.5	0.3	NA
Paraherquamide	50	50	100
Ivermectin	0.005	0.040	0.005

NA (not active).

Conclusion

Xanthonol (**1**), isolated from a non-sporulating fungus, exhibited insecticidal and anthelmintic activities against *L. sericata*, *A. egypti*, and *H. contortus*. This dimeric compound belongs to a growing list of bis-xanthones that exhibit varying biological activities.

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