Notes

XANTHOFUSIN, AN ANTIFUNGAL TETRONIC ACID FROM *Fusicoccum* sp.: PRODUCTION, ISOLATION AND STRUCTURE

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During ongoing screening for antifungal metabolites of potential interest for agricultural applications, an unidentified species of *Fusicoccum* (IMI CC No. 351573), isolated from Mediterranean plant material, was discovered as an efficient source of a novel tetronic acid, xanthofusin (*E*-3,4-dihydroxy-2-methyl-6-oxo-2,4-heptadienoic acid-γ-lactone), which exhibited interesting antifungal activities. We report on its production, isolation, chemical structure and biological activity.

Characterization of *Fusicoccum* sp. IMI CC No. 351573

Symptom-bearing plant material of the Lilliaceae, *Ashodelus macrocarpus*, was collected July 1991 in Greece. After surface sterilization, small segments of stem, leaf and fruit were plated on Potato Dextrose Agar (PDA, Difco) plates and incubated for 2 days at 26°C. Pure cultures could be obtained from the plates by one additional transfer to PDA plates. The culture was maintained on PDA slants and deposited with the International Mycological Institute Culture Collection, Kew, England (IMI CC No. 351573).

The characteristics of a three day old colony are a whitish outer zone, made up of young transparent hyphae, a darker inner zone, and a center with pycnidia. The dark zone is made up of coarse, wavy, pigmented hyphae. The pycnidia mature and ooze with conidia after three days of incubation at 26°C. The pycnidia are cream colored, often irregular in shape, especially whenever developed tightly together in clusters. The ostiole has a ring of dark pigmented cells, which can easily be observed at low magnifications in the stereoscopic microscope. The conidia are non-septate, varying significantly in length and with both ends rounded (fusiform to bacilliform). The isolate was identified as a species of the plant inhabiting Deuteromycete genus *Fusicoccum*.

Production and Isolation

Fusicoccum sp. (IMI CC No. 351573) was fermented in shake flasks. Each flask contained 100 ml of a yeast extract - sucrose medium prepared by mixing 1 liter distilled water, 20 g yeast extract (Difco), 150 g sucrose, and 1 ml trace metal solution (8.9 g $ZnSO_4 \cdot 7H_2O$ and 3.9 g $CuSO_4 \cdot 5H_2O$ dissolved in 500 ml distilled water). The pH was adjusted to 6.4 using 4 M HCl and the medium was autoclaved at 121°C for 40 minutes. A slant culture of the fungus grown on PDA (12 ml/slant) for 6 days at room temperature was shaken with 10 ml of sterile water. Three ml of the resulting suspension was used for inoculation of one shake flask. After shaking (200 rpm) for 6 days at 26°C, the mycelium from 20 flasks was separated by centrifugation (3,500 × *g*, 20 minutes) and extracted three times with 0.75 liter portions of EtOAc for two hours using vigorous mechanical stirring. The combined extracts were filtered to remove residual solids and dried by freezing. Ice was removed by filtration. Evaporation to dryness under reduced pressure yielded a brownish, semi-crystalline residue (2.1 g) which was taken up in a small volume of chloroform and applied to a silica gel column (Merck SiO_2 60, 63 ~ 200 μm, 300 × 40 mm) packed in EtOAc. Elution with EtOAc and collection of the fast moving yellow band yielded 1.05 g of the crude metabolite. Crystallization from EtOAc, by slow evaporation of the solvent at 4°C, yielded pure xanthofusin (470 mg) as long, yellow needles, mp 113 ~ 114°C (Found: C 57.21; H 4.78. Calcd for $C_8H_8O_4$: C 57.14; H 4.80); UV λ_{max} nm (ε) in MeOH: 283 (13,400), 330 (sh); IR ν_{max} cm^{-1} in KBr: 1776, 1686, 1646, 1580, 1451, 1435, 1376, 1354, 1285, 1244, 1157, 1022, 966, 862, 754, 616; EI-MS *m*/*z* (relative intensity %): 168 (20), 153 (2), 126 (3), 85 (100), 83 (19), 69 (11), 55 (7), 43 (34).

Fig. 1. Structure of xanthofusin (1), with long-range CH-couplings (two and three bonds) indicated by arrows.

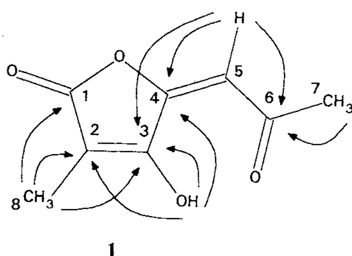

Table 1. ^{13}C and ^{1}H NMR data ($CDCl_3$) for xanthofusin.

No.	δ_C [a]	δ_H [b]	J_{CH} [c]
1	168.6	—	$J_{1,8}=5.0$
2	104.0	—	$J_{2,8}=7.0$, $J_{2,OH}=6.0^d$
3	161.3	—	$J_{3,8}=4.4$, $J_{3,OH}=4.8^d$, $J_{3,5}=6.4$
4	159.6	—	$J_{4,OH}\approx 1.0^d$, $J_{4,5}=6.2$
5	107.1	6.33 (s)	$J_{5,5}=161.2$, $J_{5,7}=1.7$
6	203.5	—	$J_{6,5}=2.9$, $J_{6,7}=5.9$
7	31.5	2.48 (s)	$J_{7,7}=128.6$
8	6.4	1.86 (s)	$J_{8,8}=130.1$

a In ppm relative to TMS (0.0 ppm) at 75.47 MHz (297 K).
b In ppm relative to TMS (0.0 ppm) at 300.13 MHz (297 K).
c $J_{n,m}$ denotes the coupling constant in Hz between C-n and H-m (or OH at δ 13.09).
d Non-observable after exchange with D_2O.

Structure

The ^{1}H NMR spectrum (Table 1) signalled the presence of two methyl groups, one vinylic and one hydroxy proton, slowly exchangeable with D_2O. The eight signals present in the ^{13}C NMR spectrum (Table 1) were assigned to two methyl groups, four sp^2 carbons (one of which carried a proton), and two carbonyl groups.

The deduced structure of xanthofusin (1) is in agreement with the observed direct and long range CH-connectivities (as indicated in Fig. 1), established by selective proton decoupling of the ^{13}C NMR spectrum. The one-, two- and three-bond C-H coupling constants are listed in Table 1. The *E*-configuration at the exocyclic double bond, which allows intramolecular hydrogen bonding between the 3-hydroxy and the 6-oxo group, is reflected in the sharp, low-field resonance (13.09 ppm) of the 3-hydroxy proton. The slow exchange rate observed at room temperature for this proton, permits observation of coupling between the hydroxy proton and C-2, C-3 and C-4. The chemical shifts for C-4 and C-5, which are sensitive to the geometry of the exocyclic double bond, are in good agreement with the corresponding shifts reported for other tetronic acids having an exocyclic *E*-double bond at C-4.[1)]

Biological Assays

Inhibition of *Phytophthora infestans* was observed in a sporangia suspension assay. To each well of a 24 well microtiter plate (wells, depth 15 mm and diameter 15 mm) 900 μl dilute salt solution (DS)[2)], 100 μl sporangia suspension ($10^4 \sim 10^5$/ml in DS) and 100 μl pea supernatant was added. The pea supernatant was prepared by boiling 500 g of frozen green peas in 1 liter of distilled water. The boiled mixture was cooled, blended and added to 100 ml DS and 20 g sucrose. The pea supernatant was obtained by centrifugation (3,500 × *g*, 30 minutes) and autoclaving (120°C, 20 minutes). Test solutions of xanthofusin in acetone were added to the wells in 20 μl alliquots. The plates were incubated at 20°C for 3 days in artificial daylight, and assessment (growth/no growth) relative to a untreated control was made.

Biological Activities

Xanthofusin strongly inhibits the growth of various plant pathogenic *Oomycetes*, especially *Phytophthora infestans*. The minimal inhibitory concentration for xanthofusin using the sporangia suspension assay described above is 0.2 μg/ml. Weak activity was observed against other fungi including *Botrytis cinerea* and *Rhizoctonia solani*. No activity was observed against bacteria and yeast.

The LD_{50} for oral administration to mice is estimated to be 250 mg/kg.

Discussion

Xanthofusin belongs to a group of tetronic acids characterized by an exocyclic double bond at the 4-position. This group of compounds, known as the 4-ylidenetetronic acids, has been reviewed by PATTENDEN[3)] with respect to distribution, synthesis, biosynthesis and biological activities. At least two separate biosynthetic pathways have been established for fungal tetronic acids[4)], and feeding experiments with appropriately labeled precursors have been initiated in order to elucidate the biosynthetic pathway for xanthofusin in *Fusicoccum*.

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