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ACUMINATOPYRONE: REVISED STRUCTURE AND PRODUCTION BY *FUSARIUM CHLAMYDOSPORUM* AND *FUSARIUM TRICINCTUM*

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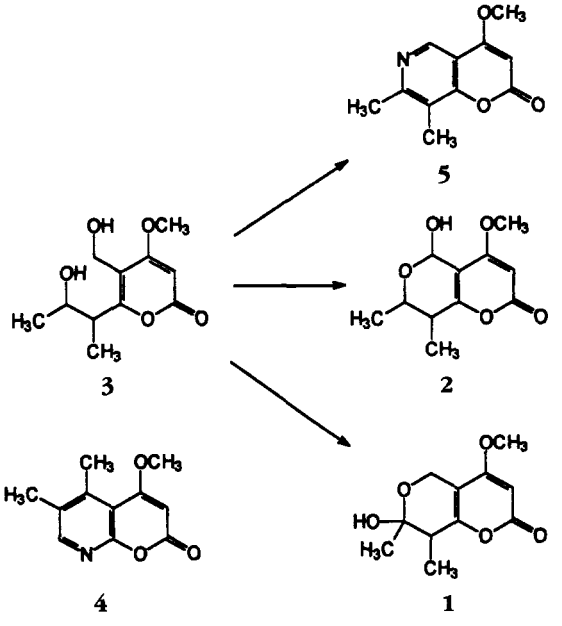
ABSTRACT.—Acuminatopyrone, a recently described metabolite of *Fusarium acuminatum*, was shown to be produced by several strains of *Fusarium chlamydosporum* and *F. tricinctum* isolated from various substrates in different countries. The structure of acuminatopyrone has been revised on the basis of NOEDIF and HETCOR nmr experiments, and shown to be 4-methoxy-7,8-dimethyl-2H-pyrano[4,3-*b*]pyridin-2-one. This structure is biosynthetically consistent with that of chlamydosporol and related compounds that are produced by the same fungal species.

Fusarium isolates of the *Sporotrichiella* section have been often associated with infected cereal grains and feed implicated in human and animal toxicoses, mainly because of their ability to produce trichothecenes (1). *Fusarium chlamydosporum* Wollenw. & Reinking and *Fusarium tricinctum* (Corda) Sacc. are two species belonging to this section which do not produce trichothecenes, and may show some biological activity due to the production of other secondary metabolites, such as chlamydosporol [**1**], moniliformin, enniatins, and visoltricin (1–5). Recently, we have isolated two new chlamydosporol-related compounds, namely, isochlamydosporol [**2**] and chlamydospordiol [**3**], from a culture of *F. chlamydosporum* strain T-826, and have shown their production by several strains of *F. chlamydosporum* and *F. tricinctum* (6). From the culture extract of *F. chlamydosporum* T-826, a biologically inactive fluorescent compound of composition $C_{11}H_{11}NO_3$ (mol wt=205) was also isolated. The spectroscopic properties of this compound clearly indicated that it was identical with acuminatopyrone, a compound recently isolated together with **1** from a culture of *F. acuminatum* (7), for which structure **4** was proposed. Our inspection of this structure suggested that it is not biosynthetically consistent with its co-occurrence with chlamydosporol [**1**] and related compounds **2** and **3**. Structure **5** appears to be a better representation based on biosynthetic reasoning. The previously suggested structure for acuminatopyrone [**4**] was proposed largely on the uv spectral data of its hydrolysis product compared to the reported spectra of related but not identical compounds. In this report we present nmr evidence that structure **5** represents acuminatopyrone.

RESULTS AND DISCUSSION

Standard ^{1}H- and ^{13}C-nmr spectra (broad-band and gate-decoupled) of acuminatopyrone are compatible with six structures that could present one CH α- to the pyridine nitrogen (a proton signal at 8.733 ppm correlated to the carbon signal at 142.14 ppm which shows a $^1J_{CH}$ of 184.9 Hz) and four quaternary carbon signals above 150 ppm. Successive irradiations of methyl signals at 2.330 and 2.583 ppm induced a strong positive nOe effect on the non-irradiated one, indicating their vicinal nature and leading therefore to the elimination of two possible structures. The absence of any enhancement



at 8.733 ppm (H adjacent to pyridine nitrogen) led to the rejection of two additional possible structures. The experiment which strongly indicated structure **5** for acuminatopyrone was the irradiation of the OMe signal at 3.984 ppm which induced a strong nOe on the signal at 5.623 ppm (H-3) and a small nOe at 8.733 ppm (the hydrogen adjacent to the pyridine nitrogen atom).

The long-range C-H correlations observed in an HETCOR nmr experiment not only confirmed the choice of structure **5** but allowed the assignments of all the carbon signals. In any of the possible structures for **5**, the signals at 89.70 and 166.12 ppm belong to C-3 and C-4 respectively, the latter showing a correlation with the -OMe hydrogen signal. In the HETCOR spectrum obtained in the study the signals at 161.71 and 161.07

TABLE 1. ^{1}H- and ^{13}C-Nmr Parameters of Acuminatopyrone [**5**] in $CDCl_3$ (Chemical Shifts are in ppm/TMS and Coupling Constants in Hz).

Carbon	$^{13}C/^{1}H$	Proton resonance[a]				
		2.330	2.583	3.984	5.623	8.733
2	161.71					
3	89.70				168.8[c]	
4	166.12			LRC[b]		
4a	109.97				LRC[b]	
5	142.13					184.9[c]
7	161.07	LRC[b]	LRC[b]			LRC[b]
8	118.52	LRC[b]	LRC[b]			
8a	156.71	LRC[b]				LRC[b]
OMe-4	56.45			146.9[c]		
Me-7	23.01		127.4[c]			
Me-8	10.92	129.0[c]				

[a]See text for individual ^{1}H-nmr assignments.

[b]LRC means that a long-range coupling constant has been detected with an HETCOR experiment using a D3 delay of 50 msec ($J=10$ Hz).

[c]The numerical values at the line/column crossings are $^{1}J_{CH}$ coupling constants.

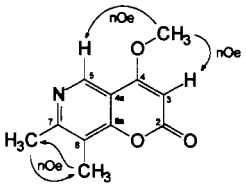


FIGURE 1. Observed nOes in Acuminatopyrone [**5**].

ppm were too close to be separated, but a GATEDEC experiment showed that the former is a sharp singlet which must belong to C-2, while the latter signal is broad. Thus, the three correlations observed with H-5, Me-7 and Me-8 concern the signal at 161.07 ppm (C-7). Similar correlations with H-5 and Me-8 were seen with the signal at 156.71 ppm (C-8a). Finally, the signal at 109.97 ppm was assigned to C-4a due to its correlation with H-3, and the remaining signal at 118.52 ppm to C-8 for its correlations with the Me-7 and Me-8 proton signals. These results are summarized in Table 1 and in Figure 1.

When the production of acuminatopyrone [**5**] by *Fusarium* isolates is considered, **5** was produced by six out of 11 strains of *F. chlamydosporum* and by six out of 24 strains of *F. tricinctum* at concentration levels ranging from 36 to 1800 mg/kg and from 3 to 37 mg/kg, respectively. The sources of the isolates producing acuminatopyrone are shown in Table 2 along with concentrations found in the cultures. By comparing the two fungal species it appears evident that acuminatopyrone producers occur with higher incidence within strains of *F. chlamydosporum* (55% compared to 25% of *F. tricinctum*), and the average production yield of *F. chlamydosporum* (738 mg/kg) is about fiftyfold higher than that of *F. tricinctum* (14.2 mg/kg) strains. The production of acuminatopyrone does not seem to be affected by the geographic area and the substrate of origin of the fungal isolates (Table 2). All strains producing acuminatopyrone also produced chlamydospordiol [**3**], and those lacking the capability to synthesize **3** did not produce acuminatopyrone [**5**]

TABLE 2. Production of Acuminatopyrone [**5**] by Strains of *Fusarium chlamydosporum* and *Fusarium tricinctum* Isolated from Various Substrates and Geographic Areas.[a]

Strain no.[b]	Substrate of origin	Geographic area	Acuminatopyrone content (mg/kg)
F. chlamydosporum			
T-729[c]	Soil sorghum field	Florida	1800
T-826[c]	Corn (*Zea mais*)	Georgia	1460
T-731[c]	Soil Sorghum field	Mississippi	459
T-513	Soil coconut grove	Philippines	441
T-669	Soil tapioca patch	Thailand	230
T-746	Soil sorghum	Puerto Rico	36
F. tricinctum			
ITEM-649[c]	Wheat kernel (*Triticum sativum*)	Poland	20
T-226[c]	Onion bulb (*Allium cepa*)	Hungary	6
T-387[c]	*Trifolium pratense*	Germany	13
T-511[c]	Mulberry stem (*Maurus alba*)	Australia	3
T-823[c]	Barley ear (*Hordeum vulgare*)	Denmark	6
T-460	Birdsfoot trefoil (*Ornithopus perpusillus*)	New York	37

[a]All strains produced chlamydospordiol **3** (see ref. 6).

[b]Accession number to the following collections: ITEM, Istituto Tossine e Micotossine, C.N.R., Bari, Italy; T, Fusarium Research Center, Pennsylvania State University, University Park, PA, USA.

[c]These strains produced also chlamydosporol **1** with or without isochlamydosporol **2** [see Solfrizzo *et al.* (6)].

either (6). Acuminatopyrone [**5**] therefore appears to be produced from the biosynthetic precursor chlamydospordiol [**3**] that leads also to chlamydosporol [**1**] and isochlamydosporol [**2**] (in our hands reaction of **1** with NH_3 under a variety of conditions did not lead to any detectable amount of acuminatopyrone [**5**]).

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Uv spectra (MeOH) and ir spectra (KBr tablet) were performed on a Beckman DU-65 spectrophotometer and a Perkin Elmer Ft-ir spectrometer, respectively. Mass spectra were obtained at 70 eV on a Finnigan MAT 4500 gc/ms system with an INCOS data system, with the probe temperature programmed from 40 to 350°. ^{1}H- and ^{13}C-nmr spectra were recorded in $CDCl_3$ at 500.13 MHz and 125.76 MHz with digital resolution of 0.076 and 0.8 Hz/pt respectively, on a Bruker WM-500 instrument using TMS as internal standard. NOEDIF and HETCOR nmr experiments were performed with the standard Bruker programs.

Hplc analyses were performed with a Waters 625 lc system equipped with a HP 1040 uv diode array detector connected to a HP 9000 series 300 computer. An RP-18 PLRP-S 5 μm 100 A (150×4.6 mm) polymeric column, preceded by a PS-DVB guard cartridge (5×3 mm) with same packing material (Polymer Laboratories, UK), was used with the mobile phase MeCN-0.01 NH_4OH (35:65) at flow rate of 1 ml/min.

ISOLATION OF ACUMINATOPYRONE [**5**].—*F. chlamydosporum* strain T-826 isolated from corn in the United States was cultured on autoclaved corn (brought up to 45% moisture content) for 4 weeks at room temperature (5×1 liter Erlenmeyer flasks, each containing 200 g of corn). The culture material was dried at 60° for 24 h then extracted with 2 liters of MeOH/1% aqueous NaCl and filtered. The filtrate was defatted with *n*-hexane (2×0.5 liters), and then extracted with CH_2Cl_2. The residue, after solvent evaporation, was reconstituted with toluene (30 ml) and passed through a column of neutral aluminium oxide (J.T. Baker, Hayes, UK; 30 g in a 3 cm i.d. glass column). Acuminatopyrone (fluorescent) was eluted from the column with 600 ml of toluene, and crystallized twice from EtOAc (yield 250 mg). Acuminatopyrone [**5**] appeared as a yellow fluorescent spot at R_f=0.85 on pre-coated Si gel hptlc plates (10×10 cm, Merck, Darmstadt, Germany) eluted with toluene-Me_2CO (12:7).

The uv spectrum of acuminatopyrone [**5**] showed absorption maxima at 269, 277 (s), 291, and 305 nm. Ir ν max 3084, 1736, 1620, 1395, and 1253 cm^{-1}. Ms *m/z* 205 (100), 177, 162, 147, and 135. The nmr data are reported in Table 1.

PRODUCTION OF ACUMINATOPYRONE BY *FUSARIUM* ISOLATES.—Eleven strains of *F. chlamydosporum* and 24 strains of *F. tricinctum* isolated from different substrates in various geographical areas were tested for acuminatopyrone [**5**] production [a complete list of these strains is reported in ref. (6). In particular, with the exception of one strain (*F. tricinctum* ITEM-649, KF-260) taken from the collection of the Istituto Tossine e Micotossine, C.N.R., Bari, Italy (taxonomist A. Logrieco), all the fungal strains considered in this study were obtained from the collection of the Fusarium Research Center, Pennsylvania State University, University Park, PA (taxonomist P.E. Nelson). The geographic area and substrate of origin of the strains producing acuminatopyrone are reported in Table 2]. Cultures on autoclaved corn (brought up to 45% moisture content) were obtained as described in ref. (6), where the same isolates were used for screening the production of chlamydosporol **1** and related compounds **2** and **3**. Culture material was extracted and analyzed as follows: Dried culture material (20 g) was extracted with 100 ml of MeOH-1% aqueous NaCl (55:45) in a blender, and filtered. The filtrate (50 ml) was defatted twice with 25 ml of *n*-hexane, and partitoned with CH_2Cl_2 (3×25 ml). The residue after solvent evaporation was brought up to 1 ml with MeOH. The MeOH extract (50 μl) was submitted to solid-phase extraction through a 500 mg C-18 minicolumn (LC-18 Supelclean SPE Tube, Supelchem, Milan, Italy) previously conditioned with MeOH (5 ml) and MeOH-H_2O (30:70) (5 ml). The extract was adsorbed onto the minicolumn, and then washed with 2 ml of MeOH-H_2O (30:70). Acuminatopyrone was eluted from the column with 2×2 ml of MeOH-0.01% NH_4OH (80:20). The residue, after solvent evaporation, was reconstituted with MeOH and analyzed by hplc. Acuminatopyrone [**5**] was detected at 269 nm (uv absorption maximum) as a well-resolved peak with a R_t of 4.6 min.

A time course of acuminatopyrone [**5**] production by *F. chlamydosporum* strain T-826 was performed by harvesting cultures (3 replicates) at one-week intervals for a period of 6 weeks. Acuminatopyrone [**5**] started to form at week 3 and continued to increase until week 6. The mean concentrations of acuminatopyrone in cultures harvested at weeks 3, 4, 5, and 6 were 656, 1437, 3219, and 3875 mg/kg, respectively.

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