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FUSARIC AND 9,10-DEHYDROFUSARIC ACIDS AND THEIR METHYL ESTERS FROM *FUSARIUM NYGAMAI*

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Key Word Index—*Fusarium nygamai*; Hyphomycetes; *Striga hermonthica*; Scrophulariaceae; sorghum; phytotoxins; herbicides; mycotoxins; fusaric acid and its methyl ester; 9,10-dehydrofusaric acid and its methyl ester; fumonisin B_1.

Abstract—Fusaric and 9,10-dehydrofusaric acids and their corresponding methyl esters were isolated from the culture filtrates of *Fusarium nygamai*. The methyl esters were characterized by chemical and spectroscopic methods and reported here for the first time as naturally occurring products. When assayed on tomato leaves and seedlings at 2.7×10^{-3} and 2×10^{-4} M, respectively, fusaric and 9,10-dehydrofusaric acids and their methyl esters showed wide chlorosis rapidly evolving into necrosis as well as a strong inhibition of root elongation, respectively. When assayed at 10^{-4} M on brine shrimps (*Artemia salina*), fusaric and 9,10-dehydrofusaric acids did not prove to be toxic, while their methyl esters showed a toxicity level of 50%, expressed as mortality.

INTRODUCTION

Striga hermonthica (Del.) Benth., commonly called witchweed, is a parasitic weed which causes severe losses in many important cereal crops, i.e. sorghum, corn, millet, rice and sugarcane. Recently, Abbasher and Sauerborn [1] reported a survey of microorganisms which are pathogenic to *S. hermonthica*, and suggested that these could be useful for biologically controlling this weed. Among these, a strain of *Fusarium nygamai* Burgess and Trimboli, which causes large leaf and stem necrosis on the host, proved to be particularly promising. Considering the possible direct application of phytoxins as herbicides to avoid any risks arising from the release of microorganisms into the field, it seemed interesting to consider the production of toxins by this strain of *F. nygamai*. This paper describes the isolation and the spectroscopic and biological characterization of the phytotoxic fusaric and 9,10-dehydrofusaric acids and their respective methyl esters. The latter appear to be novel.

RESULTS AND DISCUSSION

The phytotoxic culture filtrate of *F. nygamai* yielded toxic metobolites that were partially extracted with ethyl acetate at pH 4.6. Conversely, the toxins were exhaustively extracted with the same solvent at pH 2, suggesting an acid nature for some of them. The crude extract (7.67 g), which showed a high phytotoxicity, was fractionated on a silica gel column in 18 fractions (see Experimental). The residues of the fractions 3–4 and the most polar 17–18 showed considerable phytotoxic activity. TLC analysis (silica gel, eluent A and reverse phase, eluent B) of the latter showed—as main metabolites—one (**1**) with R_f values (0.40 and 0.33, respectively) which were identical (also demonstrated by co-chromatography) to those of an authentic sample of fusaric acid, while the other (**2**) demonstrated behaviour near (R_f 0.30 and 0.51, respectively) to **1**. Further purification of this residue under low pressure on a reverse phase column made it possible to obtain, respectively, both a moderate and abundant amount (12.2 and 121.6 mg l^{-1}, respectively) of **1** and **2** as pure solids.

Their ^{1}H NMR spectra confirmed the chemical nature of **1** as fusaric acid and allowed **2** to be identified as the 9,10-dehydrofusaric acid, as their signal patterns were very similar to those recently reported by Abraham and Henssen [2]. To integrate the values reported for **2**, the assignment and the multiplicity of the three olefinic protons were measured exactly (see Experimental). However, these authors, who also reported the isolation of a new diterpene from *F. oxysporum* Schl. together with the fusaric acids, were unable to separate **1** and **2** and characterized them as a mixture. Therefore, our purification procedure represents a convenient method to obtain satisfactory amounts of the two toxins as pure compounds.

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The identification of **1** and **2** was also corroborated by the molecular ions observed in their EI mass spectra at *m/z* 179 and 177, respectively. Moreover, in agreement with literature data, the characteristic fragmentation peaks of the aliphatic side chains at C-5 and of the pyridine ring were observed, as reported in detail in the Experimental section [3]. In addition, the FAB mass spectra—recorded in both positive and negative ions—of these compounds showed only two couples of intense peaks at *m/z* 180 and 178, and *m/z* 178 and 176, corresponding to the $[M + H]^+$ and $[M - H]^-$ pseudomolecular ions of **1** and **2**, respectively.

The purification of the less polar active fraction groups 3–4 of the initial column by a semi-preparative TLC step yielded the pure toxins **3** and **4** in low amounts (1.5 and 2.1 $mg\,l^{-1}$) with respect to **1** and **2**. The ^1H NMR spectrum of **3** and **4** essentially differed from that of **1** and **2**, respectively, because of the presence of a singlet typical of an aromatic acid methyl ester [4] appearing for both compounds at δ4.00. These data suggested that **3** and **4** were the methyl esters of fusaric and 9,10-dehydrofusaric acids, as was also confirmed by their IR and EI mass spectral data. In fact, the IR spectrum showed the typical absorption of ester carbonyl groups at 1724 and 1728 cm^{-1} in **3** and **4**, respectively [4].

The EI mass spectrum of **3** showed pseudomolecular and molecular ($[M + H]^+$ and $[M]^+$) ions at *m/z* 194 and 193, respectively, which generated a series of fragmentation peaks typical of 3-alkylpyridine and pyridinecarboxylic acids [3, 4]. In fact, when the pseudomolecular ion (*m/z* 194) lost MeO and CO residues in succession, it generated the ions at *m/z* 163 and 135, respectively. Similarly, when the molecular ion (*m/z* 193) lost MeO, C_3H_7, C_4H_9 or CH_3 followed by $CH_2{=}CHCH_2CH_3$ it yielded the ion at *m/z* 162, 150, 136 and 122, respectively. Moreover, peaks which are typical of a pyridine ring containing derivatives at *m/z* 79 $[C_5H_5N]^+$, 78 $[C_5H_4N]^+$, 77 $[C_5H_3N]^+$ and 66 $[C_5H_6]^+$ were observed [3, 4].

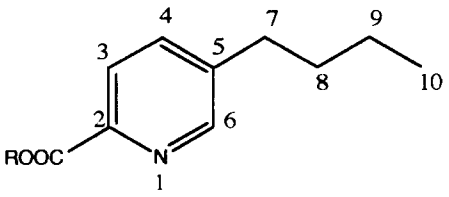
As expected, the EI mass spectrum of **4** showed the pseudomolecular and the molecular ($[M + H]^+$ and $[M]^+$) ions at *m/z* 192 and 191, respectively. Both ions, in agreement with literature on alkenylpyridines [3, 4] demonstrated fragmentation peaks similar to those observed in **3**, as well as those which are typical of a pyridine ring containing derivatives [3, 4]. Moreover, when the molecular ion lost the significant $CH_2{=}CH-CH_2$ or the CH_3 and $CH_2{=}CH{-}CH{=}CH_2$ residues in succession, it generated the ions at *m/z* 150 and 122, respectively. Also, metabolites **3** and **4** showed the same chromatographic and spectroscopic data as the derivatives obtained by esterification with diazomethane of the corresponding acids (**1** and **2**).

From these data the toxins **1–4** seemed to be satisfactorily identified as the fusaric and 9,10-dehydrofusaric acids and their corresponding methyl esters. When assayed on tomato leaves the phytotoxins caused widespread chlorosis to appear, rapidly evolving into necrosis. However, on tomato seedlings at 2×10^{-4} M, fusaric and 9,10-dehydrofusaric acids and their corresponding methyl esters showed an inhibition of root elongation of 67, 78, 55 and 70%, respectively. The slight differences of phytotoxicity observed among the toxins suggest that, most likely, the modifications such as the double bond [C(9)=C(10)] of the side-chain do not influence the activity as well as the esterification of the carboxyl group. The latter result is no surprise as the esters (**3** and **4**), have probably been enzymically hydrolysed into the corresponding free acids (**1** and **2**) in the plant. Such biological behaviour is frequently called 'lethal metabolism' [5].

When assayed on brine shrimps (*Artemia salina* L.) larvae at 10^{-4} M, fusaric and dehydrofusaric acids did not prove to be toxic, whereas their respective methyl esters showed a toxicity of 50%, expressed as a mortality percentage. This is probably due to the higher lypophilicity of the latter compounds compared to that of the acids.

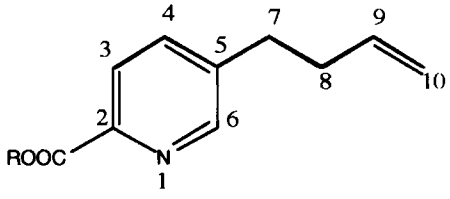
This paper illustrates that the main phytotoxins produced *in vitro* by *F. nygamai* were fusaric acid, 9,10-dehydrofusaric acid (**1** and **2**, respectively) and, in a lesser amount, their corresponding methyl esters (**3** and **4**, respectively). The phytotoxins **1** and **2** were already described as toxic metabolites produced from other species of *Fusarium* [2,6–8] as well as *Fusarium* wilt toxin [9], but this is the first report which describes **1** and **2** as the main toxic metabolites of *F. nygamai*. The phytotoxins **3** and **4** are reported here for the first time as naturally occurring compounds.

The *F. nygamai* strain ITEM 2141 was also assayed by its ability to produce other well known *Fusarium* toxins,



1 R=H

3 R=Me



2 R=H

4 R=Me

but it seemed unable to biosynthetize moniliformin, beauvericin, enniatin B, nivalenol, deoxynivalenol, 3-acetildeoxynivalenol, T-2 and HT-2 toxins, neosolaniol, fusarenone X, zearalenone and zearalenolo [10], whereas when grown on wheat kernels, it produced a very low amount of fumonisine B_1 [11]. In fact, in the TLC analysis (silica gel, eluent D) the main metabolite, which was present in the corresponding organic culture extract, showed a very similar behaviour to that of an authentic sample of fumonisin B_1. In addition, the FAB mass spectrum, which was recorded in negative ions on a partially purified sample, showed an intense negative molecular ion ($[M]^-$) ion at m/z 721. However, fumonisin B_1 seems to only be present in traces in the culture filtrates since the same sample was not detected by ^{1}H NMR analysis.

Conversely, considering the satisfactory amount obtained from a liquid culture of *F. nygamai*, the two fusaric acids represent the main phytotoxins produced from this fungus. They may prove to be of commercial interest due to their potential use as herbicides to combat *S. hermonthica*, as well as other noxious weeds.

EXPERIMENTAL

General. UV and IR spectra: MeCN and neat, respectively. ^{1}H and ^{13}C NMR spectra: 270 and 67.92 MHz, respectively, in CD_3OD or $CDCl_3$, using the same solvent as int. standard. EIMS: 70 eV; FABMS: 8.0 kV, using Cs^+ as bombarding atoms. Analyt. and prep. TLC were carried out on silica gel (Merck, Kieselgel F_{254} 0.25 and 0.50 mm, respectively) or on reverse phase (Whatman, KC18F, 0.20 mm) plates. Spots were visualized by exposure to UV radiation or by spraying with 17% *p*-anisaldehyde (0.6 ml anisaldehyde in 28 ml MeOH, 4 ml AcOH and 2 ml H_2SO_4). CC: silica gel (Merck, Kieselgel 60, 230–400 mesh) or reverse phase (Lobar, Lichroprep RP-18) at medium and low pressure, respectively. Solvent systems (A) EtOAc–MeOH–H_2O (8.5:2:1); (B) H_2O–MeCN (1:1); (C) EtOAc–petrol (2.3:1); (D) $CHCl_3$–MeOH–H_2O–AcOH (55:36:8:1). The sample of fusaric acid used as ref. compound was purchased from Fluka; neosolaniol, T2 and HT2 toxins, 3-acetyldeoxynivalenol, deoxynivalenol and nivalenol were from Macor Chemicals., Jerusalem; and moniliformin, enniatin B, fusarenone X, zearalenone, zearalenol, beauvericin and fumonisin B_1 were from Sigma.

Fungus. The strain of *F. nygamai* isolated from diseased *S. hermonthica* plants was kindly supplied by Dr J. Sauerborn, University of Giessen, Germany, and deposited in the fungal collection of the Istituto Tossine Micotossine da Parassiti Vegetali, CNR, Bari, Italy (ITEM 2141).

Production, extraction and purification of phytotoxins. Single spore cultures of *F. nygamai* were kept on a potato–dextrose–agar medium, with subculturing at monthly intervals. 1 l Erlenmeyer flasks (containing 200 ml M-1D medium) [12] were inoculated with 1 ml of an abundant conidia suspension for the production of toxic metabolites. The flasks were incubated at 25°, in static conditions, for 4 weeks. The culture filtrates (6.4 l), which had high phytotoxicity levels, were lyophilized. The residue dissolved in 1/10 of original vol. with H_2O was acidified with HCO_2H at pH 2 and extracted with EtOAc (4 × 640 ml). The combined organic extracts were dried and evapd under red. pres. to give a brown-red oil (7.6 g) demonstrating considerable phytotoxic activity. The latter was submitted to CC on silica gel (eluent A) at medium pres. (20 bar) to give 18 fr. groups (F1–F18). Only the residue of the frs F3, F4, F17 and F18 demonstrated phytotoxicity; TLC analysis (silica gel, eluent A and reverse phase, eluent B) of the latter two (F17 and F18) showed they contained two very polar substances as main metabolites: one with R_f (0.40 and 0.33, respectively), which were identical to those of an authentic sample of fusaric acid, while the other demonstrated similar behaviour (R_f 0.30 and 0.51, respectively). The combined residues (1.2 g) were further fractioned by two steps on a reverse phase column (eluent B) at low pres. (3 bar) to give two pure solid compounds, identified as **1** (77.8 mg, 12.2 mg l^{-1}) and **2** (778 mg, 121.6 mg l^{-1}), respectively. Moreover, the combined residues (50 mg) of the active less polar fr. groups (F3 and F4) of the initial column were purified by prep. TLC (silica gel, eluent C) to yield the oily and homogeneous compounds, identified as the methyl esters of **1** (**3**, 9.7 mg, 1.5 mg l^{-1}) and **2** (**4**, 13.4 mg, 2.1 mg l^{-1}), respectively.

Fusaric acid (**1**). ^{1}H NMR (CD_3OD): the same signal pattern reported in ref. [2]; EIMS m/z (rel. int.): 179 $[M]^+$ (5), 149 $[M - C_2H_6]^+$ (5), 136 $[M - C_3H_7]^+$ (50), 135 $[M - C_3H_8]^+$ (100), 133 $[M - C_2H_5 - OH]^+$ (15), 119 $[M - CO_2 - CH_4]^+$ (39), 105 $[M - CO_2 - C_2H_6]^+$ (5), 91 $[M - CO_2 - C_3H_8]^+$ (60), 79 $[C_5H_5N]^+$ (13), 78 $[C_5H_4N]^+$ (13), 77 $[C_5H_3N]^+$ (22); FABMS(+) m/z: 180 $[M + H]^+$; FABMS(−) m/z: 178 $[M - H]^-$.

9,10-*Dehydrofusaric acid* (**2**). ^{1}H NMR, differed from that reported in ref. [2] for the following signal systems: δ 5.84 (1H, *ddt*, $J_{8,9} = 6.6$ Hz, $J_{9,10A} = 17.0$ Hz, $J_{9,10B} = 10.3$ Hz, H-9), 5.00 (1H, *ddt*, $J_{8,10A} = J_{10A,10B} = 1.4$ Hz, $J_{9,10A} = 17.0$ Hz, H-10A), 4.98 (1H, *ddt*, $J_{8,10B} = J_{10A,10B} = 1.4$ Hz, $J_{9,10B} = 10.3$ Hz, H-10B); EIMS m/z (rel. int.): 178 $[M + H]^+$ (45), 177 $[M]^+$ (69), 160 $[M + H - H_2O]^+$ (20), 159 $[M - H_2O]^+$ (28), 137 $[M + H - C_3H_5]^+$ (73), 136 $[M - C_3H_5]^+$ (97), 133 $[M - CO_2]^+$ (100), 131 $[M - HCO_2H]^+$ (73), 119 $[M + H - CO_2 - CH_3]^+$ (87), 108 $[C_5H_5NCHO]^+$ (86), 105 $[M - C_4H_7 - OH]^+$ (45), 91 $[M - CO_2 - C_3H_6]^+$ (86), 80 $[C_5H_6N]^+$ (74), 79 $[C_5H_4N]^+$ (39), 78 $[C_5H_5N]^+$ (66), 77 $[C_5H_3N]^+$ (77), 65 $[C_5H_5]^+$ (77); FABMS (+) m/z: 178 $[M + H]^+$; FABMS (−) m/z: 176 $[M - H]^-$.

Methyl ester of fusaric acid (**3**). Compound **3** had: UV λ_{max} (log ε) nm: 264 (3.39), 225 (3.77); IR ν_{max} 1724 (C=O), 1592, 1572 (Ar, C=C), 1313 (CO–O) cm^{-1}; ^{1}H NMR: Table 1; EIMS m/z (rel. int.): 194 $[M + H]^+$ (48), 193 $[M]^+$ (56), 164 $[M - C_2H_5]^+$ (67), 163 $[M + H - MeO]^+$ (100), 162 $[M - MeO]^+$ (85), 150 $[M - C_3H_7]^+$ (85), 136 $[M - C_4H_9]^+$ (93), 135 $[M + H - MeO - CO]^+$ (100), 122 $[M - MeO - CH_2{=}CHCH_2CH_3]^+$ (100),

Table 1. ^{1}H NMR data ($CDCl_3$) for the methyl esters of fusaric and 9,10-dehydrofusaric acids (**3** and **4**, respectively). The chemical shifts are in δ values (ppm) from TMS

H	3	4
3	8.05 *dd*	8.06 *d*
4	7.62 *dd*	7.64 *dd*
6	8.55 *dd*	8.56 *d*
7	2.69 *t*	2.80 *t*
8	1.61 *tt*	2.42 *dtdd*
9	1.38 *qt*	5.80 *ddt*
10A	0.94 *t* (3H)	5.02 *ddt*
10B		5.01 *ddt*
OMe	4.00 *s*	4.00 *s*

J(Hz); **3** and **4**: 3,4 = 8.0; 4,6 = 2.1; 7,8 = 7.3; 8,9 = 7.3; **3**: 3,6 = 0.5; 9,10 = 7.3; **4**: 8,10A = 8, 10B = 10A, 10B = 1.4; 9,10A = 16.8; 9,10B = 10.6.

119 $[M - MeO - CO - CH_3]^+$ (67), 106 $[C_6H_4NO]^+$ (65), 104 $[C_6H_2NO]^+$ (75), 92 $[C_6H_6N]^+$ (90), 91 $[C_6H_5N]^+$ (100), 79 $[C_5H_5N]^+$ (84), 78 $[C_5H_4N]^+$ (80), 77 $[C_5H_3N]^+$ (86), 66 $[C_5H_6]^+$ (63), 65 $[C_5H_5]^+$ (81).

Methyl ester of 9,10-dehydrofusaric acid (**4**). Compound **4** had: UV λ_{max} (log ε) nm: 264 (3.50), 225 (3.89); IR ν_{max} 1728 (C=O), 1642 (C=C), 1572, 1593 (Ar, C=C), 1313 (CO–O) cm^{-1}; ^{1}H NMR: Table 1; EIMS *m/z* (rel. int.): 192 $[M + H]^+$ (83), 191 $[M]^+$ (90), 162 $[M - C_2H_5]^+$ (62), 161 $[M + H - MeO]^+$ (100), 150 $[M - CH_2{-}CH = CH_2]^+$ (100), 133 $[M + H - MeO - CO]^+$ (100), 122 $[M - Me - CH_2 = CH - CH{=}CH_2]^+$ (100), 106 $[C_6H_4NO]^+$ (61), 92 $[C_6H_6N]^+$ (95), 91 $[C_6H_5N]^+$ (100), 80 $[C_5H_6N]^+$ (39), 79 $[C_5H_5N]^+$ (76), 78 $[C_5H_4N]^+$ (78), 77 $[C_5H_3N]^+$ (94), 65 $[C_5H_5]^+$ (90).

Esterification of fusaric acids. An ethereal CH_2N_2 soln (0.5 ml) was added to a soln of **1** (8 mg) in MeOH (0.5 ml). The reaction was left for 1 hr at room temp. and was stopped by evapn. The oily residue produced was the methyl ester (**3**) of **1** (8 mg). The same procedure was used to convert **2** (10 mg) into the corresponding methyl ester (**4**, 10 mg).

Mycotoxin production and extraction. The ability of *F. nygamai* to produce mycotoxins was determined by cultures on corn kernels. Yellow kernels cv. Plata (100 g), brought overnight to about 45% moisture in 500 ml flasks, were autoclaved for 30 min at 120°. 1 ml of an abundant conidial suspension from an actively growing fungal plate was used to inoculate the flasks. The cultures were incubated at 25° for 4 weeks. The harvest culture was dried in a forced draught oven at 50° for 48 hr, finely ground and stored at 4° until use. Control corn meal was produced in the same way, except that it was not inoculated. For the extraction of zearalenones, trichotecenes, moniliformin, beauvericin and enniatin B, a sample (20 g) of corn culture obtained as described above was extracted according to the method reported in ref. [13] to yield a crude extract of 11.8 mg. For the extraction of fumonisin B_1, a sample (10 g) of corn culture as obtained above was extracted as described in ref. [14] to give a crude extract of 8.8 mg.

Analysis of mycotoxins. The analysis of trichotecenes, zearalenones and moniliformin was performed by TLC according to the method described in ref. [15]. The analysis of beauvericin and fumonisin B_1 was performed as according to ref. [14]. The analysis of enniatin B was performed as according to ref. [16].

Biological methods. The phytotoxic activity of the culture filtrates and extracts as well as the chromatographic frs was tested using a tomato leaf-puncture assay. Otherwise, the phytotoxicity of the pure compounds was evaluated both by tomato leaf-puncture and tomato seedling assay.

Tomato leaf-puncture assay. Tomato plants (*Lycopersicon esculentum* Mill.) were grown in a chamber at 22° using a light/dark cycle of 10/14 hr. Fully expanded young leaves of 4-week-old plants were detached and 10 μl of the test soln were applied on a leaf which had previously been needle-punctured. The leaves were then kept in a moist-chamber. The effects were observed 2 days after the application of droplets. Compounds **1** and **2** and their methyl esters were assayed up to 2.7×10^{-3} M.

Tomato seedling assay. Tomato seeds (*L. esculentum*) were surface sterilized by rinsing in a soln of NaClO (1%) for 10 min, before being washed with H_2O and kept in Petri dishes on wet filter papers for 4 days to allow germination to take place. The seeds were then transferred to small Petri dishes (10 seedlings/dish) on filter papers wetted with 2 ml toxin soln. The dishes were kept in a growth chamber at 25° for 3 days and then the root length was measured. The toxic effect was expressed as reduction of root elongation with respect to the control. Three replications were made for each concn. The experiment was repeated twice. Compounds **1** and **2** and their methyl esters were assayed up to 2×10^{-4} M.

Mycotoxic activity. Each sample was dissolved in a minute amount of MeOH and brought to the final concn with sea water. Mycotoxic activity was tested using the assay on brine shrimps larvae according to the method described in ref. [17]. The toxins were tested up to 10^{-4} M.

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