Toxigenic Potential of *Fusarium compactum* R8287 and R8293[†]

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Secondary metabolite production by two strains of *Fusarium compactum*, R8287 and R8293, previously implicated in Sandhill crane intoxication, was investigated. Fermentations were carried out both in liquid cultures and on rice. Under the conditions used, strain R8293 did not produce any trichothecenes. After 2 days of fermentation in inoculum medium, small amounts of enniatins B and B_1 were detected by GC/MS in the ethyl acetate extract. This is the first report of the production of enniatins by this species. Liquid cultures of strain R8287 produced 8-acetylneosolaniol as the major metabolite by 9 days. After 3 days of fermentation, enniatin B but not B_1 was also detected but was later metabolized. When grown on rice for 29 days/27 °C, this strain gave small amounts of neosolaniol and large amounts of 8,15-isoneosolaniol, also known as acuminatin. The 8,15-isoneosolaniol was characterized by comparison of its ^1H and ^{13}C NMR spectra with those of a synthetic sample. It had been previously incorrectly identified as 4,8-isoneosolaniol.

INTRODUCTION

The nomenclature associated with the taxonomy of the genus *Fusarium* and the chemistry of the secondary metabolites produced by this species is complex. In the case of taxonomy this is primarily due to the different systems of classification employed, whereas from a chemistry standpoint, different names for the same compound or for compounds differing by an acetyl moiety fail to indicate any relation between the compounds. This is particularly true for the trichothecenes. Two examples suffice to illustrate this point.

Visconti et al. (1989) isolated eight strains of *Fusarium acuminatum* Ell. & Ev. from corn, which were identified according to the taxonomic system of Nelson et al. (1983). This was in agreement with the taxonomic system of Booth (1971). Later these isolates were reclassified by Marasas and Nelson as *F. compactum* sensu Gordon, a species that is not found in the system of Nelson et al. (1983).

F. compactum is a plant pathogen, and the mycotoxins it produces were implicated in the intoxication of Sandhill cranes in the United States. This species was the most toxigenic of those isolated from waste peanuts, which formed part of the birds diet (Cole et al., 1988). When cultured on autoclaved peanuts, two strains, *F. compactum* R8287 and R8293, produced isoneosolaniol, which was reputed to be the 4,8-diacetyl isomer of neosolaniol (4,8-isoNEO). However, comparison of its ^1H and ^{13}C NMR data with that reported by Ilus et al. (1977) and Greenhalgh et al. (1990), respectively, for the naturally occurring and synthetic 4,8-diacetyl isomer of neosolaniol, suggested that the product formed by *F. compactum* was actually the 8,15-diacetyl isomer (8,15-isoNEO). This compound is also known as acuminatin and was isolated also from an *F. compactum* (Visconti et al., 1989). The ^1H NMR data had been reported previously (Savard and Greenhalgh, 1987).

To define more clearly the nature of the secondary metabolites produced by the two strains of *F. compactum* associated with the Sandhill crane intoxication, fermen-

tations were carried out in liquid culture and on rice at different moisture levels. This paper reports on the results, provides the NMR and MS data of neosolaniol, its isomers, and acetyl derivatives, and reports the detection of enniatins.

MATERIALS AND METHODS

Fermentation. The procedures previously reported for studying the toxigenic potential of fusaria cultured in liquid and on solid media (Miller et al., 1990) were employed. Lyophilized cultures of the two strains, *F. compactum* R8287 and R8293, were kindly supplied by Dr. P. Nelson. Both were grown in the two-stage fermentation of Miller and Blackwell (1986) as well as on rice at 20, 32, and 40% moisture contents. After fermentation periods of 2–3 and 9 days at 27 °C for liquid cultures and 29 days at 27 °C for the rice cultures, the samples were worked up. For the liquid cultures, this involved removal of the mycelia by filtration and transferring the broth (300 mL) to a Clin-Elut column, which was extracted with ethyl acetate (3 × 100 mL). The crude fungal extract resulting from evaporation of the solvent was redissolved in ethyl acetate and analyzed by GC/MS. The cultures on rice (50 g for each moisture level) were blended with hexane (2 × 75 mL) followed by ethyl acetate (2 × 75 mL), the extracts were filtered, and solvent was removed. The residues were redissolved in ethyl acetate and the crude extracts analyzed by GC/MS.

Mass Spectrometry. Mass spectra (MS) were determined by using a Finnigan GC/MS Model 4500 system, operating in the electron impact (EI) mode. Underivatized crude fungal extracts and trichothecene standards were separated on a DB-5 fused silica capillary column (20 m × 0.32 mm, 0.25-μm film). Samples were injected on-column, and the GC was temperature-programmed from 120 to 290 °C at 15 °C/min. Helium was used as the carrier gas at 10 psi.

NMR Spectroscopy. ^1H and ^{13}C spectra were run on a Bruker AM 250-MHz spectrometer in deuteriochloroform. The chemical shifts are referenced to chloroform at 7.24 ppm for ^1H and at 77.0 ppm for ^{13}C and reported relative to tetramethylsilane.

Standards. Neosolaniol (NEO) was purchased from Sigma Chemical Co., St. Louis, MO. 8-Acetylneosolaniol (8-ANEO) and 8,15-isoNEO had been synthesized previously (Savard and Greenhalgh, 1987). 4,8-isoNEO was isolated from *F. sporotrichioides*. Samples of enniatins B and B_1 were isolated from *F. avenaceum* DAOM 196490 and *F. tricinctum* NRRL 26430, respectively, by L. Blais at PRC, Ottawa. The structures of these compounds are shown in Figure 1.
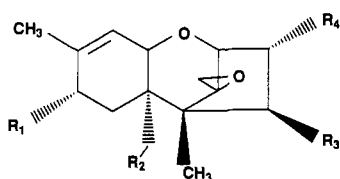
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A. TRICHOTHECENES

Compound	R₁	R₂	R₃	R₄
Neosolaniol	OH	OAc	OAc	OH
4,8-Isoneosolaniol	OAc	OH	OAc	OH
8,15-Isoneosolaniol	OAc	OAc	OH	OH

B. ENNIATINS

$B \quad R^1 = R^2 = R^3 = CH\begin{smallmatrix}CH_3\\CH_3\end{smallmatrix}$

$B_1 \quad R^1 = R^2 = -CH\begin{smallmatrix}CH_3\\CH_3\end{smallmatrix}$

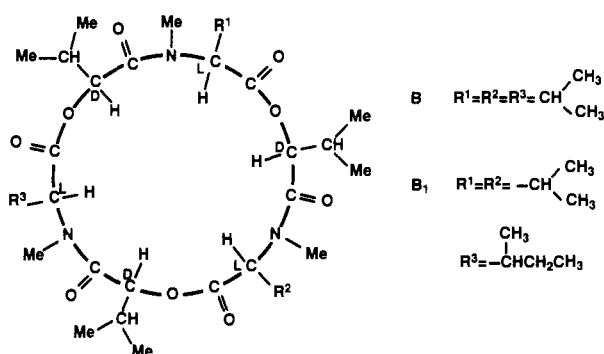$R^3 = -\overset{CH_3}{\underset{}{CHCH_2CH_3}}$

Figure 1. Structures of the trichothecenes, neosolaniol, 4,8-isoneosolaniol, and 8,15-isoneosolaniol, together with enniatins B and B₁.

RESULTS AND DISCUSSION

By GC/MS, the ethyl acetate extract of the liquid culture broths from *F. compactum* R8293 after 2 and 9 days of fermentation contained a variety of fatty acids such as hexadecanoic, octadecanoic, and octadecadienoic, but no trichothecenes. After 2 days of fermentation, the GC/MS showed two small peaks in the ratio 2:1 which had disappeared by 9 days. The EI/MS of these peaks corresponded to enniatin B [m/z 640 (M⁺ + 1), 510, 409, 296 (30), 282 (26), 214 (18), 196 (100), 182 (32), 169 (54), 154 (21), 141 (26), and 86 (95)] and enniatin B₁ [m/z 653 (M⁺), 552, 423, 383, 339, 310 (28), 296 (30), 282 (26), 210 (40), 196 (100), 182 (44), 169 (56), 154 (28), 142 (24), 100 (48), and 86(92)], respectively. Enniatin B contains three valine moieties (m/z 86), whereas B₁ has two valine (m/z 86) and one isoleucine moiety (m/z 100). The enniatins are cyclohexadepsipeptides that are known to be produced by several *Fusarium* species, including *F. avenaceum* (Strongman et al., 1988), *F. culmorum* (Bosch et al., 1989), *F. lateritium*, *F. sambucinum* (Cook et al., 1948; Russell, 1966), and *F. tricinctum* (Burmeister and Plattner, 1987). This is the first report of their production by *F. compactum*. No trichothecenes were detected in the rice culture of this strain.

The major metabolite produced by *F. compactum* R8287 in liquid culture and extracted from the broth by ethyl acetate at days 3 and 9 (Figure 2) had the same GC retention time (scan 391) and EI/MS [m/z 382 (M⁺ − 42), 364 (14), 304 (9), 278 (27), 203 (22), 185 (32), 180 (70), 163 (16), 138 (11), 121 (41), 105 (23), 91 (13), 79 (10), 55 (14), and 43 (100) as a synthetic sample of 8-ANEO (4,8,15-triacetyl T-2 tetraol). Another trichothecene produced in liquid culture was diacetoxyscirpenol (DAS) (scan 347),
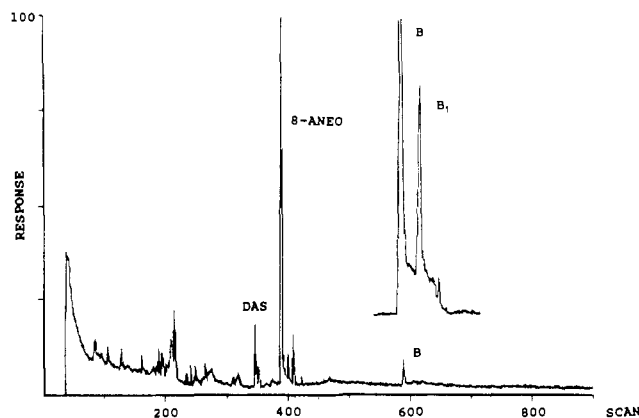

Figure 2. Reconstructed ion chromatograms of ethyl acetate extract of the broth from liquid culture of *F. compactum* R8287 at day 3 and enniatins B and B₁ standards. DAS, diacetoxyscirpenol; 8-ANEO, 8-acetylneosolaniol; B, enniatin B. GC conditions are described in the text.
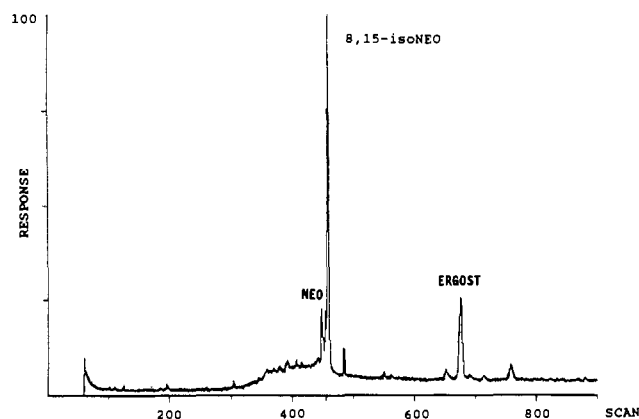

Figure 3. Reconstructed ion chromatogram of ethyl acetate extract of *F. compactum* R8287 cultured for 29 days on rice at 40% moisture level. NEO, neosolaniol; 8,15-isoNEO, 8,15-diacetyl T-2 tetraol; ERGOST, ergosterol. GC conditions are described in the text.

which was identified also by EI/MS. Again, traces of enniatin B (scan 590) were detected after 3 days of fermentation, but no B₁. No enniatins were present in the broth by day 9.

F. compactum R8287 was also grown on rice at different moisture levels. This had an effect on the total mass extracted; for hexane the amounts were 30, 166, and 257 mg and for ethyl acetate, 62, 116, and 235 mg, respectively, for 28, 32, and 40% moisture contents. The major metabolite present in the ethyl acetate extract had a GC retention time (scan 461); the EI/MS [m/z 323, 263 (12), 249 (6), 221 (5), 203 (21), 185 (11), 173 (14), 157 (10), 145 (11), 121 (20), 105 (18), 91 (14), 55 (8), 43 (100) suggested it to be the 8,15-isomer of NEO. This metabolite was the dominant one for all three moisture levels. Chromatography of the combined extracts on a silica gel column gave an oil, the ¹H and ¹³C NMR spectra of which were identical with those of a synthetic sample of 8,15-diacetyl T-2 tetraol (Savard and Greenhalgh, 1987) and identical with those of a sample of acuminatin isolated from *F. compactum* ITEM 484 (Visconti et al., 1989), confirming that it was 8,15-isoNEO. The GC/MS of the crude extract (Figure 3) also showed trace amounts of NEO as a shoulder (scan 456) and several sterols, including ergosterol (scan 677); no enniatins were detected.

The ¹H NMR data reported by Cole et al. (1988) for the major metabolite obtained by culturing R8287 on peanuts are similar to those of the 8,15-isoNEO that we isolated from culturing the same strain on rice. The ¹H resonances

Table I. **¹H NMR Spectral Assignments for Isoneosolaniols, Neosolaniol, and Acetylneosolaniol**

	4,8-isoNEO[a]	NEO(4,15)	8,15-isoNEO[b]	ANEO(4,8,15)
H-2	3.64; $J_{2,3}$ = 4.9	3.67; $J_{2,3}$ = 4.9	3.62; $J_{2,3}$ = 5.0	3.67; $J_{2,3}$ = 4.9
3	4.21; m	4.15; J = 4.9, 2.8	4.25; $J_{3,2}$ = 5.0, 3.0	4.14; $J_{3,2}$ = 4.9, 3.0
4α	5.37; $J_{4,3}$ = 3.1	5.25; $J_{4,3}$ = 2.8	4.34; $J_{4,3}$ = 3.0	5.21; $J_{4,3}$ = 3.0
7α	1.80; J_{AB} = 15.3	1.88; J_{AB} = 14.5	2.15; J_{AB} = 15.0	1.96; J_{AB} = 15
7β	2.30; $J_{7,8}$ = 5.9	2.32; $J_{7,8}$ = 5.5	2.28; $J_{7,8}$ = 5.5	2.28; $J_{7,8}$ = 5.5
8β	5.35; $J_{8,7}$ = 5.9	4.11; $J_{8,7}$ = 5.5	5.24; $J_{8,7}$ = 5.0	5.24; $J_{8,7}$ = 5.5
10	5.79; $J_{10,11}$ = 6.0	5.67; $J_{10,11}$ = 6.1	5.76; $J_{10,11}$ = 5.5	5.78; $J_{10,11}$ = 5.9
11	4.21; m	4.25; J = 5.7	4.07; J = 5.5	4.29; J = 5.9
13α	2.77; J_{AB} = 4.0	2.80; J_{AB} = 4.0	2.77; J_{AB} = 4.0	2.79; J_{AB} = 4.0
13β	3.01	3.05	3.03	3.03
14	0.82	0.85	0.78	0.80
15α	3.59; J_{AB} = 13	4.11; J_{AB} = 12.5	3.97; J_{AB} = 12.5	4.05; J_{AB} = 12.5
15β	4.25	4.32	4.32	4.26
16	1.72	1.86	1.74	1.73
Ac	2.07	2.04	2.02	2.00
	2.13	2.04	2.04	2.01
				2.12

[a] Isolated from *F. sporotrichioides* (Greenhalgh et al., 1990). [b] Visconti et al. (1989).

Table II. **¹³C NMR Spectral Assignments for Isoneosolaniol, Neosolaniol, and Acetylneosolaniol Isomers**

	4,8-isoNEO[a]	NEO(4,15)	8,15-isoNEO[b]	ANEO(4,8,15)
C-2	79.0	78.7	78.6	78.7
C-3	77.8	78.3	80.6	78.4
C-4	84.4	84.5	81.7	84.6
C-5	48.9	48.8	46.8	48.5
C-6	44.6	43.6	42.4	43.0
C-7	27.6	30.6	27.1	27.3
C-8	68.5	66.7	68.5	68.4
C-9	135.6	139.3	136.2	136.2
C-10	124.3	120.9	123.7	123.8
C-11	67.6	67.7	67.3	67.4
C-12	64.5	64.4	64.6	64.3
C-13	47.3	47.3	49.0	47.1
C-14	7.0	7.0	7.0	7.0
C-15	63.3	64.9	64.3	64.4
C-16	20.5	20.7	20.3	21.3
AcCH₃	21.4	21.2	21.1	20.3
	21.2	21.2	21.1	20.9
			21.0	20.8
				21.0
AcCO	169.6	169.8	170.4	170.0
	172.4	172.0	170.6	170.5
				172.6

[a] As in Table I.

for H-15 at 3.95 and 4.25 ppm are consistent for an acylated 15-OH moiety (Table I); free primary hydroxyls are found upfield by 0.4 ppm [cf. compounds 3, 13, and 14 in Savard et al. (1987)]. The ¹³C spectra also support this since when C-15 is acylated, the resonance occurs in the range 64–65 ppm as opposed to 63.2–63.5 ppm for a free OH. Cole et al. reported 65.3 ppm (¹³C) and 4.0 and 4.3 ppm (¹H). The chemical shift data for H-4, CH₃-14, and C-4 suggest a free hydroxyl group at C-4 with ¹³C resonances in the 80 ppm range. The resonance assigned to H-4 should be 4.3 ppm and not 5.3 ppm, and the resonances of H-3 and H-11 are reversed. All derivatives with an acetoxy moiety at C-4 show a ¹H resonance for CH₃-14 at 0.82–0.85 ppm, while for a free OH at this position, the resonance is 0.75 (HT-2) and 0.78 ppm (Savard et al., 1987). This evidence indicates that the acetyl moiety is at C-8 and not C-4. The structure previously assigned to the isoneosolaniol isolated from *F. compactum* therefore needs to be corrected. It has recently been demonstrated that NEO (4,15-diacetyl T-2 tetraol) will slowly isomerize to 4,8-isoNEO in CDCl₃, due to acid-catalyzed transesterification (Mesilaakso et al., 1989). Such a reaction, however, is slow and is not thought to have occurred in the work reported by Cole et al. (1988).

The 4,8-isoNEO was first isolated by Ilus et al. (1977)

from *F. tricinctum* 72187, which was later reclassified as *F. sporotrichioides*. Later, Ishii and Ueno (1981) designated 4,8-isoNEO as NT-1, which they isolated from the M-1-1 strain of *F. sporotrichioides* erroneously classified as *F. solani* (Marasas et al., 1983). We recently confirmed the production of 4,8-isoNEO by Canadian strains of *F. sporotrichioides* (Greenhalgh et al., 1990). The 4,8-isoNEO isomer was also produced by eight strains of *F. acuminatum* (actually *F. compactum*) isolated in Italy (Visconti et al., 1989).

In summary, the major metabolite produced when *F. compactum* R8287 was cultured on rice was 8,15-isoNEO and not 4,8-isoNEO as previously reported. Liquid cultures of this strain gave 8-ANEO as the main metabolite, together with some DAS. In our hands, strain R8293 did not make any trichothecenes. Small amounts of enniatins B and B₁ were detected in the broth of the R8293 strains after 2 days of fermentation, but only enniatin B was found for the R8287 strain after 3 days. Neither enniatin B nor B₁ was present after 9 days of fermentation. This is the first time that this species has been shown to produce these compounds.

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