The structure of fusarochromanone: new mycotoxin from *Fusarium roseum*, "Graminearum"

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An isolate of *Fusarium roseum* "Graminearum" obtained from overwintered oats in Alaska produced toxic cultures when grown on rice. The toxic principle called fusarochromanone isolated from these cultures reproduced the signs of tibial dyschondroplasia in poultry and also reduced hatchability of fertile eggs. Spectroscopic data (nmr, ir, and ms) indicated the mycotoxin to be a chromanone derivative. The chromanone ring structure was also confirmed by single crystal X-ray.

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Un produit, isolé du *Fusarium roseum* "Graminearum" et qui a été obtenu à partir d'avoine de l'Alaska gardée pour l'hiver, a donné lieu à des cultures toxiques lorsqu'on l'a fait croître sur du riz. Le principe toxique, qui se nomme fusarochromanone et qui a été isolé de ces cultures, permet de reproduire la dyschondroplasie tibiale chez les poules et a aussi réduit la possibilité d'éclosion des oeufs fertiles. Les données spectroscopiques (rmn, ir et sm) indiquent que la mycotoxine est un dérivé de la chromanone. La structure du cycle chromanone a aussi été confirmée par diffraction des rayons X par un cristal unique.

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Introduction

Species of *Fusarium* are worldwide in distribution and are capable of causing plant disease such as wilt, scab, and rot in the field (1). They also produce a wide variety of mycotoxins such as zearalenone (2), an estrogen, and the trichothecenes. The latter cause complications to farm animals when ingested in animal feeds and have been also found in "Yellow rain" (3).

Since *Fusarium* fungi infect important agricultural plants and products, they have been associated with human and animal intoxications (4). Notable among these reports are bone malformation (tibial dyschondroplasia) in poultry and the incidence of low hatchability of fertile eggs when birds were fed diets containing *Fusarium* infected feed (5, 6). Tibial dyschondroplasia (TDP) is characterized by the presence of a cone of cartilage which extends distally from the proximal tibiotarsal physis (7, 8). Similar lesions occur in swine, horses, bulls, and dogs (9).

Recently we isolated a mycotoxin (herein called fusarochromanone) from rice cultures of *Fusarium* grown in the laboratory. Purified fusarochromanone reproduced the signs of TDP in chicks and also reduced hatchability of fertile eggs (10). This report describes the elucidation of the structure of this mycotoxin.

Results and discussion

Several isolates of *Fusarium roseum* "Graminearum" were collected from the overwintered oats in Fairbanks, Alaska, during 1978. All these isolates were screened for the toxicity by growing them on rice. Rice cultures of one of the isolates were found to be toxic to chickens and elaborated lesions of TDP. Several kilograms of rice cultures were extracted with chloroform-methanol-ammonium hydroxide (90:10:1, vol/vol/vol). The extract was subsequently chromatographed to give pure toxin as shown in Scheme 1.

Mass spectral analyses of fusarochromanone (1) yielded an

SCHEME 1. Isolation and purification of fusarochromanone.

ion at nominal mass of 292 (measured at 292.1421 to fit $C_{15}H_{20}N_2O_4$ within 1 ppm). Fast atom bombardment (FAB) mass spectrometry indicated a protonated species at m/z 293, which was peak matched to 293.1524 to fit a composition of $C_{15}H_{21}N_2O_4$ within 7.8 ppm. The FAB also showed an ion at m/z 558.2960, which fitted to within 6.3 ppm for a composition of $C_{30}H_{41}N_4O_8$, presumably due to a dimer.

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TABLE 1. ^1H nuclear magnetic resonance assignment for fusarochromanone and its derivatives

	Chemical shifts (ppm)				Multiplicity	Coupling constants (Hz)			
	1	2	3	4		1	2	3	4
H-3	2.7	2.7	2.7	2.7					
H-7	7.86	7.94	7.85	7.87	d	8	8	8	8
H-8	6.08	6.08	6.09	6.06	d	8	8	8	8
H-2'	3.03	3.08	3.04	3.01	ddd	15,7.5,4	6,6,16	16,4,6	dd,16,8
	3.88	3.27	3.26	2.92					
H-3'	3.50	4.30	4.50	3.93	m				quint, 6
H-4'	3.60	3.76	4.18	4.16	m		d, 6	11,6,6	t, 6
			4.33	3.62					
CMe ₂	1.26	1.46	1.46	1.46					
ArNH ₂	9.46	9.48	9.47	9.46					
	9.60								
OAc		1.98	2.06						
			1.98						
NHAc		6.42	6.40				d, 6	d, 6	

The ^1H nmr spectrum of **1** (Table 1) shows two aromatic *ortho* protons ($J = 8$ Hz), a *gem*-dimethyl, an isolated methylene, and five exchangeable protons, three of which were readily exchanged with D_2O in CDCl_3 . The remaining two are those observed near 9 ppm as broad singlets. In addition, the spectrum displayed several multiplets between 2.8 and 3.8 ppm representing five protons. This five-proton multiplicity pattern

was assigned to a three-carbon unit **A** consisting of a methine flanked by two methylenes.

This assignment was deduced by acetylation of **1** with *N*-acetyl-imidazole, which afforded two major products identified as mono (**2**) and di (**3**) acetates of **1** by ^1H nmr (Table 1).

The ^{13}C nmr spectrum of fusarochromanone (Table 2) exhibits 14 carbon signals including a single resonance for *gem*-dimethyl carbons, three methylenes, one aliphatic methine, two aromatic methines, and seven quaternaries. The two-dimensional carbon-proton correlation (HETCOR) spectrum of the toxin established the attachment of protons at 3.5 (isolated methylene singlet), 6.08, and 7.86 ppm to the carbons at 48.96, 104.10, and 140.16 ppm, respectively. The methylene (42.44 ppm) and methine (49.71 ppm) carbons were correlated to the proton multiplets near 3.0 and 3.5 ppm, respectively. Among the quaternaries, two of them were carbonyls. Chemical shifts and ir data indicate these carbonyls are conjugated. One of the carbonyls (193.70 ppm) gave a 3-Hz triplet in the proton-coupled carbon spectrum; this triplet was collapsed to a singlet when the isolated methylene proton singlet was selectively

TABLE 2. ^{13}C nuclear magnetic resonance assignment for fusarochromanone

Carbon	Chemical shift (ppm)	Multiplicity
2	79.32	s
3	48.96	t
4	193.70	s
5	154.71	s
6	111.71	s
7	140.16	d
8	104.10	d
9	165.99	s
10	104.10	s
1'	198.80	s
2'	42.44	t
3'	49.71	d
4'	65.82	t
11 and 12	26.28	q

decoupled. Since no other changes in the carbonyl triplet were observed during coherent low-power irradiation of other protons, this carbonyl should be adjacent to the isolated methylene carbon and well removed from the other protonated carbons. Single frequency low-power irradiation also permitted assignment of most of the quaternary carbons by virtue of their long range coupling with protons. For instance, the resonance at 165.99 ppm was observed as a doublet of doublets ($J = 10$ and 2.5 Hz) in the uncoupled spectrum. Irradiation of the 6.08-ppm proton resulted in the collapse of the smaller couplings to give a 10-Hz doublet, whereas the 7.86-ppm proton irradiation caused the 10-Hz doublet to collapse to yield a broad singlet. The magnitude of coupling constants indicates that the aromatic protons at 6.08 and 7.86 ppm must be situated *ortho* and *meta*, respectively, to this quaternary carbon. The aliphatic quaternary carbon at 79.32 ppm exhibited a complex multiplet, which reduced to a 2.5-Hz triplet upon irradiation of the *gem*-dimethyl proton singlet. This observation, together with the fact that the decoupling of the dimethyl resonance also caused removal of the long range couplings observed for the isolated methylene carbon, provided a contiguous arrangement of the isopropyl carbon, isolated methylene, and the 193.70-

FIG. 1. Labelled drawing of 4 (50% probability).

ppm carbonyl. The chemical shift of the isopropyl quaternary carbon indicated it to be an ether carbon. The other end of this ether substituent was readily deduced to be the 165.99-ppm quaternary carbon since there was only one oxygen left to be accounted for (*vide infra*); the arrangement also provided a clue to the chromanone (3,4-dihydro-4*H*-1-benzopyra-4-one) system. Bookkeeping of all hetero atoms considered so far showed that —NH_2 should be one of the substituents, which was assigned to the 154.71-ppm quaternary carbon. The remaining quaternary carbons at 111.71 and 104.16 ppm were identified with the 198.80-ppm carbonyl bearing the three-carbon unit A and the γ -pyrone carbonyl, respectively, as substituents.

The substitution pattern in fusarochromanone is unique. A naturally occurring chromanone with an amino substituent at C6 has not been reported in the literature. The placement of the side chain at C7 is also unique. Although the nmr data is quite convincing in proving the structure of fusarochromanone, it was desirable to obtain a single crystal X-ray analysis of fusarochromanone.

Many solvents were used in an attempt to grow crystals of 1 suitable for the analysis, without any success. Finally, an acetone solution of 1 yielded pale yellow needle-shaped crystals. Unfortunately, these crystals were very thin and the best crystal that could be obtained was $0.18 \times 0.26 \times 0.05$ mm. Data of sufficient quality to solve the structure could not be obtained except at low temperature. Further, the ^1H nmr spectrum of these crystals in CDCl_3 indicated incorporation of acetone; however, there was no evidence of modification of the ring structure. Since our objective was to confirm the chromanone ring system, no further attempts were made to produce a more accurate structure.

Crystal data

$\text{C}_{18}\text{H}_{24}\text{N}_2\text{O}_4$ fw = 332.39
Orthorhombic, $P2_12_12_1$ (#18), $a = 11.282(4)$, $b = 23.265(6)$, $c = 6.246(4)$ Å, $Z = 4$, $V = 1639.4$ Å³, $\mu = 0.89$ cm⁻¹, $\rho = 1.35$ g cm⁻³ (-100°C , Mo $\text{K}\alpha$, $\lambda = 0.71073$ Å, graphite monochromator). A labeled drawing of the molecule is given in Fig. 1. Atomic coordinates for non-hydrogen atoms are given in Table A, bond lengths and angles are summarized in Table B, and bond angles in Table C.²

Experimental

Infrared spectra were recorded on a Perkin Elmer 283 spectrophotometer. Proton nmr spectra were obtained on Varian XL-100 and

²A complete set of structure factor listings, thermal parameters, atomic coordinates including hydrogen atom positions, bond distances, bond angles, and torsional angles may be purchased from the Depository of Unpublished Data, CISTI, National Research Council of Canada, Ottawa, Ont., Canada K1A 0S2.

XL-200 nmr spectrometers operating at 100 MHz and 200 MHz, respectively, using chloroform-*d* solutions. Carbon-13 nmr spectra were recorded on the Varian XL-200 spectrometer operating at 50.306 MHz using standard acquisition parameters. To obtain un-decoupled carbon spectra the decoupler was kept on during a delay of 1 s and was gated off during acquisition to maintain maximum nOe (nuclear Overhauser enhancement). The 2D-nmr experiment was run using the pulse sequence HETCOR resident in the spectrometer system. Ultraviolet spectra were recorded on a Perkin Elmer 554 spectrophotometer in MeOH solutions. Mass spectra were obtained on a Kratos MS-50 high resolution mass spectrometer operating at 10 000 resolution. The standard Kratos FAB source were used. Thin-layer chromatographic analyses were carried out on precoated silica gel-254 (EM Reagents) and KC18 (Whatman) glass plates; components were visualized under uv light (either 254 or 356 nm).

For single crystal X-ray analysis, a crystal (obtained by slow evaporation of solvent from an acetone solution) was mounted on an Enraf-Nonius CAD4 diffractometer. Intensity data were collected at low temperature (-100°C) using an ω - 2θ scan. Data were collected in the $+h, k, l$ octant to a maximum 2θ value of 48.0° . Three standard reflections measured every two hours during data collection showed a total loss of intensity of 10.3%. A linear decay correction was applied. Lorentz and polarization corrections were applied to the data. Since the linear absorption coefficient is small, an absorption correction was not made.

A total of 1662 reflections were collected (1608 unique and not systematically absent) and the space group of the crystal was determined to be $P2_12_12_1$ (#18 International Tables) from the systematic absences $h00: h = 2n$, and $0k0: k = 2n$ and subsequent least-squares refinement. The structure (4) was solved by direct methods (11). A total of 19 atoms were located from an *E*-map and the remaining atoms located in succeeding difference Fourier syntheses. Because of a paucity of data, isotropic temperature factors were used for C, O, and N. It was not possible to locate a hydrogen on N2 in difference maps although N1 had two peaks of reasonable intensity ($0.4 \text{ e}/\text{\AA}^3$) and distances (0.6, 1.2 Å). Therefore, hydrogen atoms were included at calculated positions (C—H bond length = $0.95 \text{ \AA}$ and N—H = $0.90 \text{ \AA}$) but their positions were not refined. Hydrogen atoms were assigned fixed isotropic *B* values approximately equal to the *B* value of the atom to which they were attached. The structure was refined using full-matrix least-squares techniques where the function minimized was:

$$\sum w(|F_o| - |F_c|)^2$$

and the weight, *w*, is defined as:

$$w = 4F_o^2 / \sigma^2(F_o^2)$$

All calculations were carried out on Digital Equipment Corporation PDP 8A and 11/34 computers using the Enraf-Nonius CAD4-SDP programs (12). The values of the atomic scattering factors were taken from the International Tables and effects of anomalous scattering were included (13). The model converged for 912 reflections $F_o^2 > 3.0\sigma(F_o^2)$ and 97 variables with agreement factor:

$$R = \sum ||F_o| - |F_c|| / \sum |F_o| = 0.093$$

$$R_w = \{ \sum w(|F_o| - |F_c|)^2 / \sum w|F_o|^2 \}^{1/2} = 0.108$$

In the final cycle, the maximum shift/error was 0.01, the goodness of fit 2.45, and the largest peak in the difference map $0.5 \text{ e}/\text{\AA}^3$.

Isolation and purification of fusarochromanone (1)

Five hundred grams of crude cultures (10) was extracted 5 times with the solvent system described in the scheme. The extracts were pooled and concentrated *in vacuo* at 40°C to give an oily residue, which was dissolved in 50 mL methanol in 200 mL of distilled water. This mixture was further concentrated to approximately 150 mL of solution and applied to an XAD-2 column (3.5×60 cm), rinsed with 1 L of distilled water, and eluted with 1 L of 90% methanol. The methanol fraction was concentrated *in vacuo* at 40°C , and reconstituted in 50 mL CHCl_3 -MeOH (9:1, v/v). This solution was mixed with 50 g of

anhydrous sodium sulfate and the mixture was dried at room temperature. The dry yellow powder was then packed on the top of a column (3.5 × 60 cm) containing 150 g of Florisil, and eluted with 2 L of CHCl₃-MeOH (9:1, v/v) followed by 2 L of CHCl₃-MeOH-NH₄OH (90:10:1, v/v/v). Fusarochromanone was detected by irradiating the column with long-wave uv (365 nm) light since the toxin produces an intense blue fluorescence under the uv light. The fractions containing fusarochromanone were concentrated (40°C) and subsequently purified by tlc using CHCl₃-MeOH-NH₄OH (80:20:2, v/v/v).

Fusarochromanone (**1**): mp 132–134°C; uv $\lambda_{\max}$ (MeOH) (nm): 248 (ϵ 21 800), 277 (ϵ 8 800), 383 (ϵ 11 700); ir $\lambda_{\max}$ (KBr) (cm⁻¹): 3373, 3267, 3160 (NH₂, OH), 1655 (CO), 1562 (CO, -H bonded); nmr (CDCl₃) (see Tables 1 and 2); ms (M^+ 292.1421 calcd. for C₁₅H₂₀N₂O₄).

Acetylation of **1**

Fifty milligrams of 1-acetylimidazole was added to a solution of **1** (50 mg) in CHCl₃ (2 mL). The reaction mixture was kept at room temperature for 2 h. The mixture was then chromatographed on preparative tlc (CHCl₃-MeOH; 9:1). Two major bands corresponding to R_f of 0.7 and 0.4 were scraped and eluted with CHCl₃; removal of solvent gave gummy solids.

Monoacetate (**2**): ir $\lambda_{\max}$ (KBr) (cm⁻¹): 3380, 3280 (NH, OH), 1660 (CO), 1560 (amide II); nmr (CDCl₃) (see Table 1); ms (M^+ 334, $M - \text{AcO}$ 275).

Diacetate (**3**): ir $\lambda_{\max}$ (KBr) (cm⁻¹): 3382, 3322, 3275 (NH₂, NH), 1728 (CO ester), 1650 (CO), 1590, 1572, 1545 (CO -H bonded, amide II, Ar); nmr (CDCl₃) (see Table 1); ms (M^+ 376, $M - \text{AcO}$ 317).

(**4**): A solution of **1** (50 mg) in acetone (15 mL) was kept at room temperature in a test tube and acetone was allowed to evaporate slowly to give needle-shaped crystals of **4**, mp 118–119°C; ir $\lambda_{\max}$ (KBr)

(cm⁻¹): 3413, 3300, 3275 (NH₂, OH), 1650 (CO); nmr (CDCl₃) (see Table 1); ms ($M + 1$ 333).

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