Isolation and Characterization of a Secondary Metabolite Produced by Fusarium graminearum: 2,6,6,9-Tetramethyltricyclo[5.4.0.0]undecane-5,8,11-triol (5-Hydroxyculmorin)

Huguette Cohen, Claude Charrier, Louis Ricard, and Michel Perreau

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ISOLATION AND CHARACTERIZATION OF A SECONDARY METABOLITE PRODUCED BY *FUSARIUM GRAMINEARUM*: 2,6,6,9-TETRAMETHYLTRICYCLO[5.4.0.0]UNDECANE-5,8,11-TRIOL (5-HYDROXYCULMORIN)

HUGUETTE COHEN,*

Laboratory Services Division, Food Production and Inspection Branch, Agriculture Canada, Building No. 22, C.E.F., Ottawa, Ontario K1A 0C6 Canada

CLAUDE CHARRIER, LOUIS RICARD,

Laboratoire DCPH, Ecole Polytechnique, Palaiseau 91128, France

and MICHEL PERREAU

Laboratoire d'Etude des Microstructures, CNRS/ONERA (OM), 29 avenue de la Division Leclerc B.P. 72, 92322 Chatillon Cedex, France

ABSTRACT.—5-Hydroxyculmorin [**1**], a sesquiterpene, was isolated from corn contaminated by *Fusarium graminearum*, and its structure was determined by spectroscopic and single-crystal X-ray crystallography procedures. High resolution ^{1}H-nmr (400 MHz) spectroscopy allowed the assignment of all protons. The latter, combined with ^{13}C nmr and the data obtained from X-ray crystallography, enabled a complete elucidation and confirmation of structure for the isolated compound.

In recent years, toxins from *Fusarium graminearum* Schwabe have been isolated from wheat, corn (1), and other cereals in north temperate climates. Their presence causes a reduction in the quality of grains, primarily because this fungus produces mycotoxins such as the trichothecene deoxynivalenol (DON) and analogues, zearalenone (ZEA), and estrogenic compounds (2). These compounds when present in grains are known to cause feed refusal, lowered weight gains (3), vomiting (3), and immunological problems in animals (4). In the present study, corn was inoculated with *F. graminearum* by the toothpick technique (5), and during large scale extraction of DON for toxicological studies, we isolated a new compound, namely 5-hydroxyculmorin [**1**].

Culmorin was first isolated from *Fusarium culmorum* as a secondary metabolite (6–8). Preliminary nmr analysis of this compound by Barton and Werstuik (6) did not fully assign the chemical shifts required to determine the structure by nmr alone. Later this compound was identified at trace levels in other strains, for example in *Fusarium sporotrichoides* and *Fusarium crockwellensi*, in laboratories using solid or liquid cultures as a medium (9,10). This, however, is the first time 5-hydroxyculmorin has been extracted from corn growing outdoors artificially inoculated with *F. graminearum*. As indicated previously, *F. graminearum* is known to produce zearalenone, DON, and analogues but was not known to produce either culmorin or 5-hydroxyculmorin. The 5-hydroxyculmorin was extracted from ground contaminated corn previously defatted with hexane using EtOH-$CHCl_3$ (80:20). The extract obtained was fractionated by a series of chromatography columns (Si gel 400).

In this paper, the structure of **1** was deduced by spectral analysis and unequivocally established by X-ray crystallographic analysis.

RESULTS AND DISCUSSION

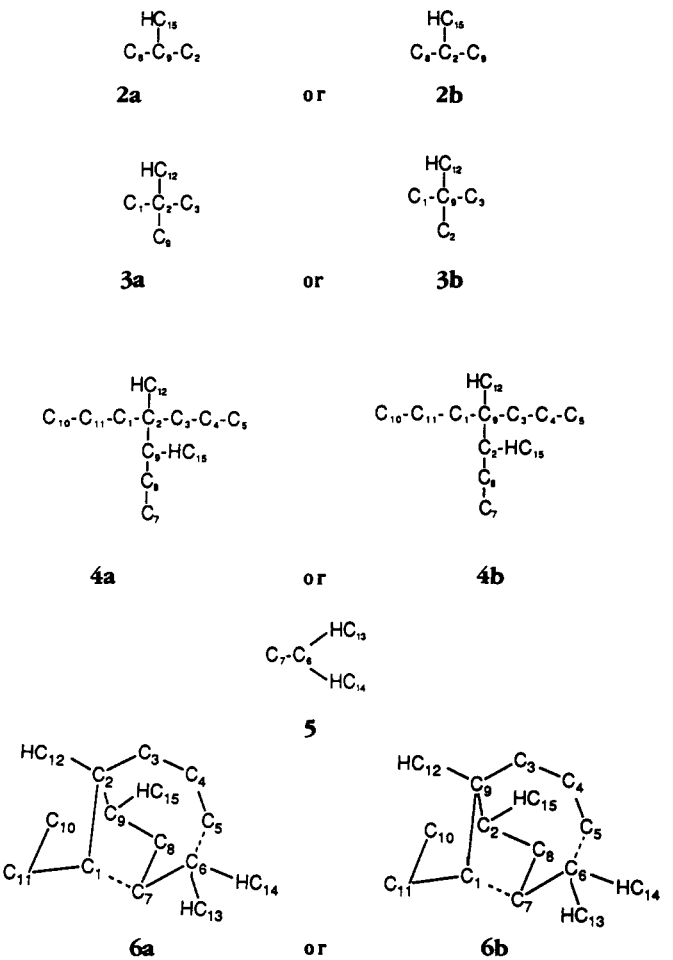
Elemental and ms analyses indicated the empirical formula $C_{15}H_{26}O_3$ (mol wt = 254). The ir spectrum of compound showed an absorption at 3400 cm^{-1} due to the hydroxyl groups. The ^{13}C-nmr spectrum showed 15 resonance lines which, after a DEPT experiment, were attributed to four Me, three CH_2, two CH, three quaternary

carbons, and three hydroxyl-bearing CH groups. As the resonances were between 13 and 80 ppm, the compound did not possess any unsaturation and was tricyclic.

A study of the ^{1}H-nmr spectrum obtained using selective decoupling led us to identify and to measure J_{HH} couplings, and at the same time to identify some of the sequences of the molecule from the three protons attached to the C*H*OH groups, which resonate in a region well detached from the overall spectrum (Table 1). The H-11 proton, the most deshielded at 4.37 ppm, is coupled to H-1 (J_{HH} = 5 Hz) and to the two methylenic protons H-10 (J_{HH} = 9.8 Hz) and H-10′ (J_{HH} = 4.3 Hz) with values corresponding to vicinal couplings. These data led to the first sequence C-1–C-11–C-10. In the same way, the coupling constant between H-7 and H-8 (J_{HH} = 5.6 Hz) led to the sequence C-7–C-8; couplings between H-4 and H-4′ with H-5 (J_{H4H5} = 11.6 Hz) and H-3 or H-3′ ($J_{H3H4'}$ = 6.8 Hz, $J_{H3'H4}$ = 12.6 Hz) gave a new linkage C-3–C-4–C-5.

The protons of the four methyl groups gave sharp singlets: H-12 = 1 ppm, H-13 = 1.21 ppm, H-14 = 0.94 ppm, and H-15 = 0.86 ppm, indicating that the four methyls are each bonded to a quaternary carbon and that at least one of the three quaternary carbons bears a *gem*-dimethyl group.

The structure was completed by heteronuclear multiple bond connectivity



SCHEME 1

TABLE 1. ^{1}H- and ^{13}C-nmr Data of 5-Hydroxyculmorine [1].[a]

Carbon[b]	δ	Proton	δ	Coupling constants nmr								Connective C					
												C-CH			C-C-CH		
C-1	50.38	H-1	2.01			$^{3}J_{H1,11}$	5.0	$^{3}J_{H1,7}$	0.0			53.22 (C-2)	71.47 (C-11			37.79 (C-6)	
C-2[c]	53.22																
C-3	32.79	H-3	1.38	$^{2}J_{H3,3'}$	15.5	$^{3}J_{H3,4'}$	1.9	$^{3}J_{H3,4'}$	6.8								
		H-3′	1.63	$^{2}J_{H3,3'}$	15.5	$^{3}J_{H3',4}$	12.6	$^{3}J_{H3',4'}$	2.0								
C-4	32.49	H-4	1.81	$^{2}J_{H4,4'}$	13.2	$^{3}J_{H4,3}$	1.9	$^{3}J_{H4,5'}$	12.6	$^{3}J_{H4,5}$	11.6						
		H-4′	1.60	$^{2}J_{H4',4}$	13.2	$^{3}J_{H4',3}$	6.8	$^{3}J_{H4',3}$	2.0	$^{3}J_{H4',5}$	2.7						
C-5	80.47	H-5	3.48			$^{3}J_{H5,4}$	11.6	$^{3}J_{H5,4'}$	2.7								
C-6	37.79																
C-7	52.24	H-7	2.02			$^{3}J_{H7,8}$	5.6					37.79 (C-6)	50.38 (C-1)	78.70 (C-8)	53.22 (C-2)	51.30 (C-9)	71.47 (C-11)
C-8	78.70	H-8	3.79			$^{3}J_{H8,7}$	5.6			$^{4}J_{H8,10}$	1.8						
C-9	51.30																
C-10	36.31	H-10	1.65	$^{2}J_{H10,10'}$	13.7	$^{3}J_{H10,11}$	9.8			$^{4}J_{H10,8}$	1.8						
		H-10′	1.76	$^{2}J_{H10',10}$	13.7	$^{3}J_{H10',11}$	4.3										
C-11	71.47	H-11	4.37			$^{3}J_{H11,10}$	9.8	$^{3}J_{H11,10'}$	4.3	$^{3}J_{H11,1}$	5.0						
C-12	22.33	H-12	1.00										53.22 (C-2)		51.30 (C-9)	50.38 (C-1)	32.79 (C-3)
C-13	27.42	H-13	1.21										37.79 (C-6)		52.24 (C-7)	80.47 (C-5)	
C-14	21.80	H-14	0.94										37.79 (C-6)		52.24 (C-7)		
C-15	13.43	H-15	0.86										51.30 (C-9)		53.22 (C-2)	78.70 (C-8)	

[a] J values are reported in Hz and chemical shifts are given in δ units (downfield of TMS).
[b] According to ^{13}C-^{1}H COSY experiments.
[c] Values for C_2 and C_9 may be reversed.

(HMBC). This method was useful for revealing the connectivities between methyl protons and carbons separated by two or three bonds. The signal intensity of these proton resonances has a magnitude greater than that of the other signals, and their cross peaks can be easily detected in the 2D spectra. Also, both the two-bond and three-bond J_{CH} couplings to methyl protons are usually rather large (4–5 Hz) (11, 12), sufficient to provide an efficient transfer mechanism.

Taking into account the direct ^{13}C and ^{1}H connectivities summarized in Table 1, the following partial connectivities were revealed: the protons of the Me-15 are correlated with the carbons C-2, C-8, and C-9. Since C-2 and C-9 are quaternary carbons, these data could lead to two possible arrangements **2a** or **2b** (Scheme 1). The protons of the Me-12 showed cross peaks with the carbons C-1, C-2, C-3, and C-9, suggesting **3a** and **3b** (Scheme 1) as two other possibilities. The association of these four fragments led to only two possible configurations, which, completed with the three previous sequences, gave **4a** or **4b** (Scheme 1). The protons of Me-13 and Me-14 are correlated with the same carbons, C-6 and C-7, that gave the *gem*-dimethyl chain as shown in **5** (Scheme 1). As the proton H-13 is also coupled to C-5, this implied a bond between C-5 and C-6 and, since the proton H-7 showed connectivities with the carbons C-1, C-2, C-6, C-8, C-9, and C-11, this implied a C-1–C-7 bond, leading to **6a** or **6b** (Scheme 1).

In order to determine the last bond C-9–C-10 (**6a**) or C-2–C-10 (**6b**), the coupling $J_{H8H10} = 1.8$ Hz was important since the carbons C-8 and C-10 are not bonded to each other. This coupling was across four bonds in a W shape, which is specific of $^{4}J_{HexoHexo}$ in a norbornane structure (13), implying C-9–C-10 bonding in **6a** or C-2–C-10 in **6b**. The structure of the 5-hydroxyculmorin was consistent with both cases. Only the assignments of C-2 and C-9 (quaternary carbons) are still uncertain.

The coupling of 9.8 Hz between H-10 and H-11 indicated that they are both in an exo position (14), implying that 8-OH and 11-OH were in endo positions.

As the coupling $^{3}J_{H1H7}$ was zero, H-7 was in an endo position (14). This result confirmed the fact that the chain C-2–C-3–C-4–C-5–C-6 is bonded to C-7 in the exo position.

Finally, the carbon of the Me-12 ($\delta = 22.3$ ppm) is more deshielded than C-15 ($\delta =$ 13.5 ppm). This was typical of norbornane derivatives where the methyl of the bridge resonated at about 20 ppm while the methyl of the bridge head was at 15 ppm (15). The structure as established by nmr (Figure 1) was confirmed by a single crystal X-ray structure determination (Figure 2).

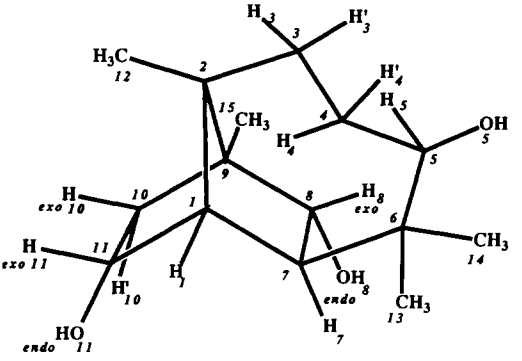


FIGURE 1. 5-hydroxyculmorin [**1**]. Carbons and hydrogens are numbered for identification by ^{1}H and ^{13}C nmr.

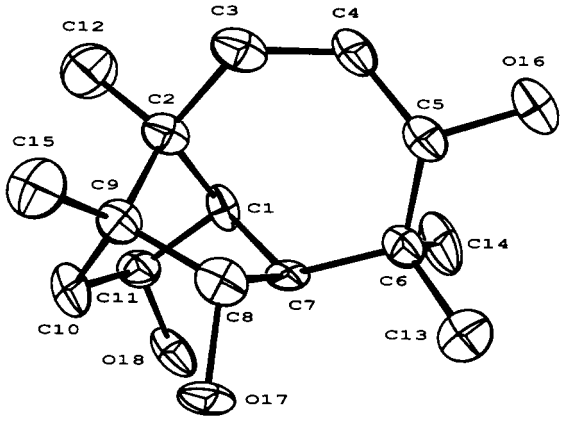


FIGURE 2. ORTEP representation of 5-hydroxyculmorin [**1**]. Ellipsoids are scaled to enclose 40% of the overall electronic density. Hydrogen atoms are omitted for clarity.

EXPERIMENTAL

GENERAL INSTRUMENTATION.—^{1}H- and ^{13}C-nmr spectra were taken on a Bruker AM-400 spectrometer in CD_3OD with TMS an internal standard. The 70 eV ei mass spectrum was obtained on a VG ZAB-IF mass spectrometer. Ir spectra were recorded on a Perkin-Elmer 257 spectrometer. The melting point (uncorrected) was taken with a Mel-Temp II (sold by Aldrich). Flash Si gel chromatography employed Kiesel gel 60, 230–400 mesh (Merck, Nogent, France) under pressure of 0.4 atm. Tlc was performed on Si gel 60 plates (250 μm thick), and the spots were visualized with *p*-anisaldehyde–MeOH–H_2SO_4 (5:90:5).

All solvents used in this work were of analytical grade (Prolabo, Paris, France). The chemicals were obtained from Aldrich (Strasbourg, France).

FIELD AND GROWTH CONDITIONS.—Corn (*Zea mays* L.) was grown in an isolated field on the Animal Research Centre Greenbelt Farm (Ottawa, Ontario, Canada). The plot was disked to provide a seed bed and cleaned of weeds, and no herbicide control was used. Approximately 0.4 ha (1 acre) was planted in mid-May with the corn hybrid Pride 1169 in 0.9 m row spacing at a rate of 6400 seeds/ha with 110 kg/ha 10-20-0 fertilizer. Seventy-nine days after planting (mid-tassel stage), 5000 ears were inoculated with *F. graminearum* (DOAM 180377-Canada) by the toothpick method described previously (7). Fifty-three days later, the corn was harvested. The kernels were ground and stored in 2-kg plastic bags at −18°.

Extraction.—All extractions were performed using an ultrasonic (15 × 2 min) H_2O bath. Samples of contaminated corn (500 g) were first moistened with 30–50 ml of H_2O and were then defatted with 3 × 1 liter of hexane in a sonic H_2O bath. The defatted corn was then extracted successively with $CHCl_3$-EtOH (80:20) 3 × 500 ml, $CHCl_3$-EtOH (60:40) 3 × 600 ml, and $CHCl_3$-EtOH (60:40) 3700 ml. The $CHCl_3$/EtOH solutions were combined and evaporated.

Purification.—Each extract from 500 g of contaminated corn underwent a series of flash chromatography separations. The first separation as a pre-screening used Si gel cc (120 cm × 7.5 cm i.d., 230–400 mesh, 300 g). The elution solvents were: CH_2Cl_2 (500 ml); hexane-EtOAc (70:30) (1 liter); hexane-Et_2O (20:80) (1.5 liters); Et_2O-MeOH (90:10) (1 liter). The eluate was collected in 100-ml fractions which were monitored by tlc, using $CHCl_3$-Et_2O-MeOH (60:30:10). After migration, the tlc plates were heated at 80–110° for 3–5 min. DON gave a bright yellow color and the 5-hydroxyculmorin a rusty color, R_f= 0.40. The fractions collected were a mixture of DON, 5-hydroxyculmorin, and impurities.

The extract obtained from the first chromatography was re-chromatographed using Si gel cc (100 cm × 3 cm i.d., 230–400 mesh, 100 g). The elution solvents used were: hexane (500 ml); CH_2Cl_2-EtOAc (95:5) (1 liter); CH_2Cl_2-EtOAc (90:10) (1 liter); CH_2Cl_2-EtOAc (80:20) (500 ml); EtOAc (500 ml); EtOAc-MeOH (90:10) (1 liter). The elution solvents from CH_2Cl_2-EtOAc (90:10) to the EtOAc/MeOH phase were combined and evaporated. The fractions collected (10 ml) were screened using tlc plates and consisted of the 5-hydroxyculmorin and traces of DON. The final chromatographic purification was by Si gel cc (30 cm × 1 cm i.d., 230–400 mesh, 12 g). The elution solvents used were: hexane (50 ml), hexane-

EtOAc (80:20) (200 ml), hexane-Et_2O (70:30) (200 ml); hexane-Et_2O-MeOH (70:30:1) (200 ml). The fractions collected with hexane/Et_2O, hexane, and Et_2O/MeOH (2 ml) contained the 5-hydroxyculmorin.

The 5-hydroxyculmorin crystallized from MeOH in white fine needles: mp 217–218°; ms, *m/z* 100 (base), 107 (95), 121 (48), 135 (100), 93 (33), 147 (35), 175 (42), 218 (19), 236 (30), $[M]^+$ 254 (2). Empirical formula $C_{15}H_{26}O_3$ analysis: calcd C 70.83, H 10.30, O 18.87; found C 70.64, H 10.25 O 19.07. From 4 kg of contaminated corn, we obtained 120 mg of pure **1** and 60 mg of impure product.

X-ray structure determination of **1**[1].—Crystals of the compound $C_{15}H_{26}O_3 \cdot MeOH$ were grown at room temperature from an MeOH solution. The sample used for data collection was sealed in a glass capillary and surrounded with Apiezon grease to reduce loss of solvent. Data were collected at 18°± 1° on an Enraf Nonius CAD4 diffractometer. The crystal structure was solved and refined using the Enraf Nonius supplied SDP package. The compound crystallized in space group $P2_12_12_1$, $a = 8.486$ (1) Å, $b = 11.962$ (1) Å, $c = 16.392$ (2) Å; $V = 1663.96$ (53) Å^3; $Z = 4$, $D_{calc} = 1.143$ g/cm^3; Mo Kα radiation ($\lambda = 0.71073$ Å) graphite monochromator; $\mu = 0.7$ cm^{-1}; $F(000) = 624$. A total of 1686 unique reflections were recorded in the range $2° \leq 2\theta \leq 50.0°$, of which 1096 were considered as unobserved [$F_2 < 3.0\,\sigma(F^2)$], leaving 590 for solution and refinement. The structure was solved by direct methods. The hydrogen atoms were included as fixed contribution in the final stages of least-squares refinement while using anisotropic temperature factors for all other atoms (Table 2). A non-Poisson weighting scheme was applied with a p factor equal to 0.08. The final R factors were $R = 0.051$, $Rw = 0.062$, goodness of fit = 1.34.

TABLE 2. Positional Parameters and Their ESDs.[a]

Atom	*x*	*y*	*z*	B(A2)
O-16	1.1350(6)	−0.2103(5)	0.7754(4)	4.9(2)
O-17	0.5615(6)	−0.0031(4)	0.7892(3)	3.6(1)
O-18	0.4029(6)	−0.2805(4)	0.7080(4)	4.4(1)
C-1	0.6616(9)	−0.2215(6)	0.6575(5)	2.5(2)
C-2	0.7100(9)	−0.1253(7)	0.5988(5)	3.2(2)
C-3	0.891(1)	−0.1004(8)	0.6011(5)	4.0(2)
C-4	0.997(1)	−0.1784(9)	0.6519(6)	5.5(3)
C-5	0.993(1)	−0.1608(7)	0.7409(6)	3.9(2)
C-6	0.8474(9)	−0.2080(7)	0.7854(5)	3.3(2)
C-7	0.6904(9)	−0.1745(6)	0.7426(4)	2.4(2)
C-8	0.666(1)	−0.0490(6)	0.7284(5)	2.9(2)
C-9	0.6062(9)	−0.0407(6)	0.6413(5)	2.8(2)
C-10	0.4436(9)	−0.0947(6)	0.6408(5)	3.2(2)
C-11	0.481(1)	−0.2218(7)	0.6434(5)	3.3(2)
C-12	0.670(1)	−0.1469(9)	0.5092(6)	5.8(3)
C-13	0.846(1)	−0.1574(9)	0.8724(6)	5.8(3)
C-14	0.856(1)	−0.3362(7)	0.7924(7)	5.4(3)
C-15	0.609(1)	0.0788(7)	0.6062(6)	4.8(3)
O-19	0.2000(8)	0.0686(6)	0.3721(4)	7.3(2)
C-20	0.166(2)	0.082(1)	0.4542(7)	10.9(4)

[a]Anisotropically refined atoms are given in the form of the isotropic equivalent displacement parameter defined as: (4/3) * [a2*B (1,1) + b2* (2,2) + c2*B (3,3) + ab(cos gamma)*B (1,2) + ac(cos beta)*B (1,3) + bc(cos alpha)*B (2,3)].

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[1]Atomic coordinates for this structure have been deposited with the Cambridge Crystallographic Data Centre and can be obtained on request from Dr. Olga Kennard, University Chemical Laboratory, Lensfield Road, Cambridge, CB 2 1EW, UK.

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