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ACETYLATED MYCOTOXINS FROM *FUSARIUM GRAMINEARUM*

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Abstract—In addition to zearalenone, deoxynivalenol and 15-acetyl deoxynivalenol, three new acetylated mycotoxins: 4-acetyl zearalenone, 4-acetyl *cis*-zearalenone and 3,15-diacetyl deoxynivalenol, were isolated from a *Fusarium graminearum* cultured on corn. Chemical correlations and relevant spectroscopic data on zearalenone derivatives are presented.

INTRODUCTION

In connection with a survey we are conducting to determine the origin of food rejection by farm animals in Galicia (N.W. Spain), we observed that *Fusarium graminearum* infected corn was frequently found in fields as well as in stored grain. TLC and HPLC analysis of the toxic corn (*Artemia salina* and rabbit skin test) proved the presence of zearalenone (**1**) and of deoxynivalenol (**2**) as the major products, but the recent isolation from the same fungus of 15-acetyl deoxynivalenol, associated with feed refusal by swine [1], prompted us to investigate the production of toxins in a *F. graminearum* laboratory culture.

In this paper we describe the isolation from *F. graminearum* grown on corn of 4-acetyl zearalenone (**1a**), 4-acetyl *cis*-zearalenone (**1b**), 3,15-diacetyl deoxynivalenol (**2a**) and 15-acetyl deoxynivalenol (**2b**) [1] in addition to the aforementioned zearalenone (**1**) and deoxynivalenol (**2**). Compounds **1a**, **1b** and **2a** have not been isolated previously as natural products. The structures of **1a** and **1b** have been deduced on the basis of spectroscopic data and chemical correlations.

RESULTS AND DISCUSSION

Column chromatography on silica gel of extracts from the mycelium of *F. graminearum* grown on cracked corn gave three fractions.

Fraction C, the most polar one (eluted with hexane–ethyl acetate; 1:3) produced two compounds further separated by HPLC and identified by comparison [1] of their spectroscopic properties as deoxynivalenol (**2**) and its 15-acetyl derivative (**2b**). Fraction B (eluted with hexane–ethyl acetate; 1:1) produced a compound whose spectroscopic data correspond to 3,15-diacetyl deoxynivalenol (**2a**). Finally, fraction A, the less polar one (eluted with hexane–ethyl acetate; 2:1) showed the NMR spectral characteristics of a zearalenone skeleton. Semipreparative HPLC gave zearalenone (**1**) as the major product and a mixture of two other faster eluting compounds **1a** and **1b** which were further separated and purified by the same technique. Both **1a** and **1b** presented identical mass spectra and quite similar UV and ^{1}H NMR spectra indicating that both compounds were monoacetates possessing either the same zearalenone skeleton with a different acetylation pattern at the phenolic groups or a different geometry at the double bond.

As no reliable correlation could be established on the basis of existing spectroscopic data [2], we decided to carry out chemical correlations between **1**, and the methylated derivatives of **1a** and **1b**, i.e. **1c** and **1h**, respectively. Selective methylation of **1** produced **1d** which was acetylated to **1f**. The other isomer, **1e**, could not be prepared by direct methylation, which produced in all attempts the dimethylated derivative thus contradicting a previous report [3]. Nevertheless, **1e** was prepared by treatment of compound **1** with chloromethyl methyl ether (ClMOM) followed by methylation and deprotection. Acetylation of **1e** gave a compound identical to **1c**.

In order to deduce the substitution pattern of **1a** and **1b** a series of NOE experiments were performed on **1c**, **1f** and **1h**. Thus irradiation of the methoxy group in these three compounds gave NOE with H-3 in **1c**, with H-3 and H-5 in **1f** and with H-3 in **1h**. For its part, irradiation of H-2′ produced NOE with H-5, H-5, and H-1′, respectively. These data proved that **1c** was 4-acetyl-2-methyl zearalenone and so **1a** was 4-acetyl zearalenone. They also suggested a *cis*-skeleton for **1h**, further supported by the lack of NOE between H-2′ and H-5. In fact, photochemical isomerization [4] of **1**, **1c**, and **1f**, yielded the three *cis*-isomers **1g**, **1h** and **1i**, respectively, proving that **1b** was 4-acetyl-*cis*-zearalenone. Furthermore, submitting **1g** to the sequence of reactions described for the preparation of **1c** from **1** (protection, methylation, deprotection and acetylation) the *cis*-zearalenone derivative **1h** was obtained.

A comparison between the ^{1}H NMR spectral data obtained for the *cis*- and *trans*-zearalenone derivatives showed that the chemical shift for the olefinic proton H-2′ was consistently upfield (*ca* 0.4 ppm) on the *cis*-compounds and this can be used as a diagnostic signal to distinguish both types of skeletons.

The results we present here constitute the first report on the isolation of those acetylated mycotoxins from a

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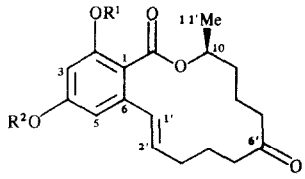
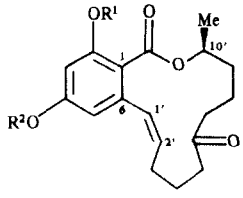
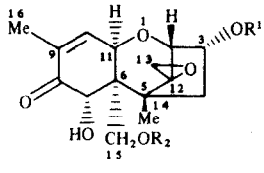


	R^1	R^2
1	H	H
1a	H	Ac
1c	Me	Ac
1d	H	Me
1e	Me	H
1f	Ac	Me



	R^1	R^2
1g	H	H
1b	H	Ac
1h	Me	Ac
1i	Ac	Me



	R^1	R^2
2	H	H
2a	Ac	Ac
2b	H	Ac

natural source. It is also worth noting that **1b** is the first *cis*-zearalenone isolated from a fungus. A survey to determine the presence of these compounds on corn samples is under way.

EXPERIMENTAL

General. UV were recorded in MeOH soln. NMR spectra were determined on a Bruker WM-250 apparatus in $CDCl_3$ soln containing TMS as the int. standard. Mass spectra were obtained on a Kratos MS-25 mass spectrometer by E.I. at 70 eV. HPLC was performed on a Perkin Elmer series 10 chromatograph with a normal phase microporasil column (25 cm × 4.6 mm) eluting with hexane–EtOAc (4:1). For the toxicity analysis the *Artemia salina* [5] and the rabbit skin test [6] were used.

Extraction and isolation. To six 1 l erlenmeyer flasks containing 200 g each of sterilized cracked corn (40% humidity) were transferred *Fusarium graminearum* inoculums isolated from toxic corn. The flasks were maintained at 27° in the dark for 15 days and then another 2 weeks at 6° in the daylight. After that period the mycelium was extracted with Me_2CO and the solid residue dried and extracted once with MeCN–4% KCl (9:1) and twice with $CHCl_3$. The combined extracts were dried, concd and defatted with hexane–EtOAc (4:1) finally giving 27 g of an oil which was adsorbed in 15 g of silica gel and submitted to CC on 110 g of the same adsorbent.

Elution with hexane–EtOAc (2:1) produced 2.6 g of fraction A which was separated by prep. TLC on silica gel (hexane–Et_2O–HOAc; 35:15:1). The band coincident with a sample of zearalenone (**1**) was isolated and further submitted to semi-prep. HPLC (hexane–EtOAc; 8:2) giving 30 mg of 4-acetyl zearalenone (**1a**) ($RR_t = 0.67$); 15 mg of 4-acetyl-*cis*-zearalenone **1b** ($RR_t = 0.86$) and 35 mg of zearalenone **1** ($RR_t = 1$).

Elution of the column with hexane–EtOAc (1:1) gave 1.2 g of a mixture (fraction B) from which was isolated by prep. TLC on silica gel (hexane–EtOAc; 1:1) a band (blue fluorescence after spraying with $AlCl_3$) positive in the rabbit skin test which gave 70 mg of 3,15-diacetyl-deoxynivalenol (**2a**).

Finally, elution with hexane–EtOAc (1:3) produced Fraction C which after purification by prep. TLC on silica gel (hexane–EtOAc; 1:3) gave 40 mg of deoxynivalenol (**2**) and 20 mg of 15-acetyl deoxynivalenol (**2b**).

Preparation of zearalenone derivatives **1c–1h**. Compounds **1c** and **1h** were obtained by direct methylation of **1a** and **1b** with Me_2SO_4 and K_2CO_3 at reflux in dry Me_2CO.

Compound **1d** was prepared by selective methylation of **1** either by overnight treatment with CH_2N_2 in an ethereal soln or by reaction with Me_2SO_4 and K_2CO_3 in dry Me_2CO (20 min at room temp).

Compound **1e** was prepared by protection of **1** with chloromethyl methyl ether (1.1 equivalents of NaH and 1 equivalent of ClMOM in dry THF at room temp), followed by methylation with Me_2SO_4 and deprotection (stirring with 1 M HCl). The same procedure was used in the preparation of **1h** from **1g**.

Acetylation of **1d** and **1e** (Ac_2O–pyridine at room temp overnight) gave **1f** and **1c**, respectively.

Compounds **1g–1i** were obtained by isomerization of the double bond of **1**, **1c** and **1f** by a photochemical procedure. In a typical experiment 0.1 mmol of product in 10 ml of MeOH were degassed and irradiated with a 400 W mercury lamp for 70 hr. The reaction mixture was concentrated and the *cis*-isomers separated by prep. TLC.

4-Acetyl zearalenone (**1a**). ^{1}H NMR (250 MHz, $CDCl_3$): δ 1.40 (*d*, $J = 6.2$ Hz, 3H, H-11'), 1.42–1.85 and 2.04–2.42 (*m*, 10H), 2.30 (*s*, 3H, OAc), 2.60 (*m*, 1H, H-7'), 2.82 (*m*, 1H, H-5'), 5.03 (*m*, 1H, H-10'), 5.72 (*m*, 1H, H-2'), 6.66 (*t*, $J = 2.7$ Hz, 2H, H-3 and H-5), 7.02 (*dd*, $J = 1.6$ Hz and $J = 15.3$ Hz, 1H, H-1'), 11.91 (*s*, 1H, OH). ^{13}C NMR (62.83 MHz, $CDCl_3$): δ 210.93 (C-6'), 171.12 (C-12'), 168.70 (OAc), 164.61 (C-2), 155.01 (C-4), 143.36 (C-6), 133.48 (C-1'), 132.64 (C-2'), 113.65 (C-3), 109.57 (C-5), 108.30 (C-1), 74.03 (C-10'), 42.87 (C-7'), 36.64 (C-5'), 34.64 (C-9'), 30.99 (C-3'), 22.04 (C-8'), 21.04 (OAc), 20.95 (C-4'), 20.63 (C-11'). UV λ_{max}^{MeOH} nm: 230, 264, 322. MS *m/z* (rel. int.): 360 $[M]^+$ (38), 300 (14), 249 (13), 246 (8), 231 (14), 206 (33), 188 (100), 151 (24), 125 (24), 112 (35).

4-Acetyl-2-methyl zearalenone (**1c**). ^{1}H NMR (250 MHz, $CDCl_3$): δ 1.33 (*d*, $J = 6.3$ Hz, 3H, H-11'), 1.43–2.79 (*m*, 12H), 2.31 (*s*, 3H, OAc), 3.81 (*s*, 3H, OMe), 5.33 (*m*, 1H, H-10'), 6.02 (*m*, 1H, H-2'), 6.33 (*d*, $J = 15.6$ Hz, 1H, H-1'), 6.56 (*d*, $J = 1.8$ Hz, 1H, H-3), 6.85 (*d*, $J = 1.8$ Hz, 1H, H-5). ^{13}C NMR (62.83 MHz, $CDCl_3$): δ 211.40 (C-6'), 169.18 (C-12'), 167.10 (OAc), 157.31 (C-2), 152.23 (C-4), 136.77 (C-6), 134.24 (C-1'), 128.16 (C-2'), 110.49 (C-3), 105.43 (C-1), 103.88 (C-5), 71.49 (C-10'), 56.14 (OMe) 44.03 (C-7'), 37.55 (C-5'), 35.07 (C-9'), 31.16 (C-3'), 21.66 (C-8'), 21.20 (C-4'), 21.04 (OAc), 19.93 (C-11').

4-Methyl zearalenone (**1d**). ^{1}H NMR (250 MHz, $CDCl_3$): δ 1.39 (*d*, $J = 6.2$ Hz, 3H, H-11'), 1.51 (*m*, 1H, H-4'), 1.61–1.83 (*m*, 4H, H-8' and H-9'), 2.06–2.24 (*m*, 4H), 2.39 (*m*, 1H, H-3'), 2.62 (*m*, 1H, H-7'), 2.85 (*m*, 1H, H-5'), 3.82 (*s*, 3H, OMe), 5.02 (*m*, 1H, H-10'), 5.68 (*m*, 1H, H-2'), 6.39 (*d*, $J = 2.7$ Hz, 1H, ArH), 6.45 (*d*, $J = 2.7$ Hz, 1H, ArH), 7.02 (*dd*, $J = 15.3$ Hz and $J = 2.0$ Hz, 1H, H-1').

2-Methyl zearalenone (**1e**). ^{1}H NMR (250 MHz, $CDCl_3$): δ 1.34 (*d*, $J = 6.3$ Hz, 3H, H-11'), 1.45–2.50 (*m*, 11H), 2.73 (*m*, 1H, H-5'), 3.77 (*s*, 3H, OMe), 5.31 (*m*, 1H, H-10'), 5.95 (*m*, 1H, H-2'), 6.33 (*d*, $J = 15.2$ Hz, 1H, H-1'), 6.33 (*s*, 1H, ArH), 6.53 (*s*, 1H, ArH).

2-*Acetyl*-4-*methyl zearalenone* (**1f**). ^{1}H NMR (250 MHz, $CDCl_3$): δ1.31 (*d*, J = 6.3 Hz, 3H, H-11'), 1.55–2.42 (*m*, 11H), 2.27 (*s*, 3H, OAc), 2.64 (*m*, 1H, H-5'), 3.83 (*s*, 3H, OMe), 5.26 (*m*, 1H, H-10'), 5.95 (*m*, 1H, H-2'), 6.55 (*d*, J = 15.6 Hz, 1H, H-1'), 6.56 (*d*, J = 2.4 Hz, 1H, ArH), 6.89 (*d*, J = 2.4 Hz, 1H, ArH). ^{13}C NMR (62.83 MHz, $CDCl_3$): δ211.28 (C-6'), 168.99 (C-12'), 165.99 (OAc), 161.14 (C-4), 157.60 (C-2), 138.63 (C-6), 133.98 (C-1'), 129.33 (C-2'), 118.45 (C-1), 109.32 (C-3), 107.48 (C-5), 71.36 (C-10'), 55.55 (OMe) 43.65 (C-7'), 37.42 (C-5'), 35.06 (C-9'), 31.29 (C-3'), 21.48 (C-8'), 21.15 (C-4'), 20.76 (OAc), 19.75 (C-11').

cis-*Zearalenone* (**1g**). ^{1}H NMR (250 MHz, $CDCl_3$): δ1.34 (*d*, J = 6.3 Hz, 3H, H-11'), 1.44–2.31 (*m*, 11H), 2.53 (*m*, 1H, H-5'), 5.14 (*m*, 1H, H-10'), 5.41 (*m*, 1H, H-2'), 6.20 (*d*, J = 2.2 Hz, 1H, ArH), 6.39 (*d*, J = 2.2 Hz, 1H, ArH), 6.63 (*d*, J = 12.0 Hz, 1H, H-1'). ^{13}C NMR (62.83 MHz, $CDCl_3$): δ212.16 (C-6'), 171.04 (C-12'), 165.48 (C-2), 160.84 (C-4), 142.14 (C-6), 131.96 (C-1'), 129.98 (C-2'), 111.54 (C-3), 104.33 (C-1), 102.28 (C-5), 73.71 (C-10'), 41.63 (C-7'), 40.71 (C-5'), 33.01 (C-9'), 28.83 (C-3'), 21.25 (C-8', C-4', C-11').

4-*Acetyl*-cis-*zearalenone* (**1h**). ^{1}H NMR (250 MHz, $CDCl_3$): δ1.36 (*d*, J = 6.3 Hz, 3H, H-11'), 1.45–2.62 (*m*, 12H), 2.30 (*s*, 3H, OAc), 5.15 (*m*, 1H, H-10'), 5.46 (*m*, 1H, H-2'), 6.41 (*d*, J = 2.5 Hz, 1H, ArH), 6.63 (*dd*, J = 2.1 Hz, J = 12.1 Hz, 1H, H-1'), 6.68 (*d*, J = 2.5 Hz, 1H, ArH). ^{13}C NMR (62.83 MHz, $CDCl_3$): δ210.26 (C-6'), 170.74 (C-12'), 168.56 (OAc), 164.70 (C-2), 154.55 (C-4), 141.61 (C-6), 131.12 (C-1'), 131.00 (C-2'), 120.83 (C-3), 109.54 (C-1), 109.43 (C-5), 74.47 (C-10'), 41.52 (C-7'), 40.68 (C-5'), 30.02 (C-9'), 28.87 (C-3'), 21.29 (C-8'), 21.20 (OAc), 21.15 (C-4'), 21.04 (C-11'). UV λ_{max}^{MeOH} nm: 219, 253, 315. MS *m/z* (rel. int.): 360 $[M]^+$ (44), 300 (13), 249 (12), 246 (15), 231 (16), 206 (27), 188 (100), 151 (25), 125 (24), 112 (38).

4-*Acetyl*-2-*methyl* cis-*zearalenone* (**1h**). ^{1}H NMR (250 MHz, $CDCl_3$): δ1.37 (*d*, J = 6.3 Hz, 3H, H-11'), 1.42–2.75 (*m*, 12H), 2.36 (*s*, 3H, OAc), 3.82 (*s*, 3H, OMe), 5.21 (*m*, 1H, H-10'), 5.55 (*m*, 1H, H-2'), 6.36 (*dd*, J = 2.4 Hz, J = 10.2 Hz, 1H, H-1'), 6.57 (*d*, J = 2.0 Hz, 1H, ArH), 6.59 (*d*, J = 2.0 Hz, 1H, ArH).

2-*Acetyl*-4-*methyl* cis-*zearalenone* (**1i**). ^{1}H NMR (250 MHz, $CDCl_3$): δ1.33 (*d*, J = 6.3 Hz, 3H, H-11'), 1.56–2.42 (*m*, 12H), 2.30 (*s*, 3H, OAc), 3.82 (*s*, 3H, OMe), 5.17 (*m*, 1H, H-10'), 5.55 (*m*, 1H, H-2'), 6.56 (*d*, J = 11.6 Hz, 1H, H-1'), 6.57 (*d*, J = 2.1 Hz, 1H, ArH), 6.63 (*d*, J = 2.1 Hz, 1H, ArH).

15-*Acetyl deoxynivalenol* (**2b**). ^{1}H NMR (250 MHz, $CDCl_3$): δ1.10 (*s*, 3H, 14-Me), 1.90 (*s*, 3H, OAc), 1.91 (*dd*, J = 0.8 Hz, J = 1.5 Hz, 3H, 16-Me), 2.13 (*dd*, J = 10.6 Hz, J = 14.8 Hz, 1H, H-4), 2.25 (*dd*, J = 4.5 Hz, J = 14.8 Hz, 1H, H-4), 3.11 (*d*, J = 4.2 Hz, 1H, H-13), 3.16 (*d*, J = 4.2 Hz, 1H, H-13), 3.67 (*d*, J = 4.5 Hz, 1H, H-2), 3.77 (*d*, J = 2.0 Hz, 7-OH), 4.26 (*s*, 2H, H-15), 4.57 (*m*, 1H, H-3), 4.86 (*d*, J = 2.0 Hz, 1H, H-7), 4.91 (*d*, J = 5.9 Hz, 1H, H-11), 6.63 (*m*, J = 1.5 Hz, J = 5.9 Hz, 1H, H-10).

3,15-*Diacetyl-deoxynivalenol* (**2a**). ^{1}H NMR (250 MHz, $CDCl_3$): δ1.12 (*s*, 3H, 14-Me), 1.90 (*s*, 6H, 16-Me, 15-OAc), 2.15 (*s*, 3H, 3-OAc), 2.19 (*dd*, J = 11.0 Hz, J = 15.2 Hz, 1H, H-4), 2.33 (*dd*, J = 4.6 Hz, J = 15.2 Hz, 1H, H-4), 3.13 (*d*, J = 4.2 Hz, 1H, H-13), 3.17 (*d*, J = 4.2 Hz, 1H, H-13), 3.79 (*d*, J = 1.8 Hz, 1H, 7-OH), 3.93 (*d*, J = 4.4 Hz, 1H, H-2), 4.25 (*d*, J = 12.0 Hz, 1H, H-15), 4.29 (*d*, J = 12.0 Hz, 1H, H-15), 4.712 (*d*, J = 5.8 Hz, 1H, H-11), 4.83 (*d*, J = 1.8 Hz, 1H, H-7), 5.24 (*m*, J = 4.6 Hz, J = 10.9 Hz, 1H, H-3), 6.59 (*m*, J = 1.5 Hz, J = 5.8 Hz, 1H, H-10).

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