Deoxynivalenol Fatty Acid and Glucoside Conjugates†

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The synthesis of 10 conjugates of the mycotoxin 4-deoxynivalenol (DON) is described. The compounds synthesized include eight fatty acid esters and two glucosides. Fatty acid esters of DON were synthesized at positions 3 and 15 by reaction of DON with the appropriate acid chlorides in the presence of pyridine, while the glucosides were obtained by a Koenigs–Knorr reaction with acetobromoglucose. HPLC methods for the analysis of these compounds are also described.

INTRODUCTION

4-Deoxynivalenol (DON) is one of the most important mycotoxins produced by *Fusarium* species (Ueno, 1983). This compound has been found to contaminate a number of cereals grown in temperate climates (Tanaka et al., 1988; Scott et al., 1981) and to cause severe toxicoses in both humans and animals (Rousseaux, 1988). One study has shown that, in the case of a yeast-raised product made from contaminated wheat flour, the concentration of DON increased by over 100%, something not observed in other products investigated (Young et al., 1984). The most plausible reason for this apparent increase was that the toxin had been metabolized by the wheat to a compound other than DON, which, under certain conditions, could be transformed back to DON. In addition, partial resistance of wheat to Fusarium head blight has been suspected of being mediated in part by the formation of a DON conjugate (Miller and Greenhalgh, 1988).

The metabolism of mycotoxins by plants and animals falls into two categories: transformation and conjugation. The transformation products include those which are deepoxidized (Yoshizawa et al., 1983; King et al., 1984; Chatterjee et al., 1986; Côté et al., 1986), deacylated (Sakamoto et al., 1986; Abbas et al., 1988; Johnsen et al., 1988), and isomerized from 9-enes to 8-enes (Greenhalgh et al., 1984). The reported conjugates include glycosides (Kamimura, 1986; Engelhardt et al., 1988; Gorst-Allman et al., 1985) and glucuronides (Roush et al., 1985; Corley et al., 1985).

Ethers and esters are the most plausible DON metabolites as they can be hydrolyzed to DON. Acetylation of DON is unlikely as the resulting acetates are well characterized and have not been found in sufficient quantities in contaminated grain. While maize cell cultures have been reported to produce zearalenone glucosides (Engelhardt et al., 1988), 15-Ac-scirpenol 4-glucoside is the only trichothecene glucoside reported to date and was obtained from *Fusarium sulphureum* cultures (Gorst-Allman et al., 1985). Glucuronides have been reported, but these are normally associated with animal metabolism. Fatty acid esters of trichothecenes have been reported in the literature (Chakrabarti and Ghosal, 1986), but the validity of the evidence has been questioned (Mirocha et al., 1990).

DON fatty acid esters and glucosides were thus considered to be the most likely metabolites to be found in grain. This paper reports on the synthesis and characterization of eight fatty acid esters and two glucosides of DON.

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EXPERIMENTAL PROCEDURES

Equipment. ^{1}H NMR spectra were recorded at 250 MHz on a Bruker WM250 spectrometer with 16K data points, a 2200-Hz spectral window, 60° pulses, and an 8-s repetition rate. ^{1}H chemical shifts are referenced either to residual $CHCl_3$ at 7.24 ppm or to CD_2HOD at 3.30 ppm. ^{13}C NMR spectra were recorded at 62.9 MHz with 16K data points, a 15-kHz spectral window, and a 3-s repetition rate. Chemical shifts are referenced either to $CDCl_3$ at 77.0 ppm or to CD_3OD at 49.0 ppm and reported relative to TMS.

Mass spectra were obtained on a Finnigan MAT 4500 GC/MS system. Fast atom bombardment (FAB) mass spectra were obtained on a Finnigan MAT 312 spectrometer mounted with a saddle field atom gun (Ion Tech) and coupled to a Super INCOS data system. High-purity xenon (99.995%), from Matheson, was used as the bombardment gas (8 kV), and the resulting positive ions were extracted (3 kV) into the mass analyzer. A mass range of 100–1300 amu was scanned exponentially in 10 s. A nominal resolution of 1000 was maintained, and the electron multiplier was kept at a value of 2.5 kV.

HPLC equipment consisted of a Varian 5000 system and either a 5-μm Chrosorb RP-18 column (25 × 0.3 cm), a 5-μm silica gel column (30 × 0.3 cm), or a 10-μm Partisil ODS-2 column (25 × 0.94 cm).

Synthesis of Fatty Acid Esters. The following procedure was used to prepare all the DON fatty acid esters. A solution of 4-deoxynivalenol (50 mg, 0.17 mmol), the appropriate fatty acid chloride (50 mg, 0.17 mmol), and pyridine (80 μL, 1 mmol) in CH_2Cl_2 was stirred at room temperature. After 24 h, the solution was washed with saturated $NaHCO_3$, dried over Na_2SO_4, filtered, and concentrated. Chromatography on silica gel (25 g) with 1:9 EtOAc/hexane (50 mL), 1:3 EtOAc/hexane (200 mL), and 1:1 EtOAc/hexane (100 mL) separated the acylated DON from pyridine and unreacted DON. However, the separation of the 3-acylated DON, 15-acylated DON, and excess fatty acid was unsatisfactory. This separation was achieved by preparative TLC on silica gel in 1:3 EtOAc/hexane. The ratio of 3-acyl-DON to 15-acyl-DON was roughly 3:1. Overall isolated yields ranged from 27% to 46%, the remainder being unreacted DON with a small amount of 3,15-diacyl-DON. HPLC analysis was performed on a 5-μm silica gel column (25 × 0.94 cm) and elution with 7% iPrOH in hexane at 4 mL/min with detection at 205 nm.

Mass spectral data for these esters may be found in Table I and NMR data in Table II. All compounds showed almost identical IR spectra with bands at 3400, 2900, 1740, 1680, 1160, and 960 cm^{-1}. High-resolution mass spectrometry: DON-3-stearate (DON-3-18,0) (DON-3-18,0: 3 refers to the position of the acid on DON, 18 to the number of carbons in the fatty acid, and 0 to the number of double bonds in the acid.) calcd mass for $C_{33}H_{54}O_7$ 562.3872, found 562.3711; DON-15-18,0 calcd mass 562.3872, found 562.3796; DON-3-18,1 calcd mass for $C_{33}H_{52}O_7$ 560.3715, found 560.3530; DON-15-18,1 calcd mass 560.3715, found 560.3596; DON-3-18,2 calcd mass for $C_{33}H_{50}O_7$ 558.3559, found 558.3411; DON-15-18,2 calcd mass 558.3559, found 558.3472; DON-3-18,3 calcd mass for $C_{33}H_{48}O_7$ 556.3402, found 556.3188; DON-15-18,3 calcd mass 556.3402, found 556.3235.

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Table I. Relative Intensities of EIMS Peaks for DON Fatty Acid Esters

mass	3-AcDON MW 338	15-AcDON MW 338	3-18,0 MW 562	15-18,0 MW 562	3-18,1 MW 560	15-18,1 MW 560	3-18,2 MW 558	15-18,2 MW 558	3-18,3 MW 556	15-18,3 MW 556
M^+	8%	1	5	3	4	1	2			
297			2	3	2	8	2	5	1	5
296	1		7	6	5	4	5	4	4	4
279	1	1	2	6	12	28	10	13	5	13
278	4	7	8	22	8	20	8	23	4	21
249	7	60	15	39	15	30	13	32	10	38
163	80	50	26	17	15	16	20	18	17	17
137	38	75	10	21	22	72	23	52	15	57
55		52	44	38	100	100	98	80	84	84
43			100	100	90	97	100	100	100	100

Table II. DON Fatty Acid Ester NMR Data

	3-substituted		15-substituted	
	^1H	^{13}C	^1H	^{13}C
2	3.90 (d, 4.4 Hz, 1 H)	79.0	3.63 (d, 4.4 Hz, 1 H)	80.8
3	5.20 (dt, 11, 4.4, 1 H)	71.0	4.52 (dt, 10.5, 4.4, 1 H)	70.1
4α	2.34 (dd, 15, 4.4, 1 H)		2.24 (dd, 14.8, 4.4, 1 H)	
		40.3		43.3
4β	2.13 (dd, 15, 11, 1H)		2.13 (dd, 14.8, 10.5, 1 H)	
5		45.7		46.3
6		51.9		51.3
7	4.80 (s, 1 H)	74.4	4.83 (s, 1 H)	73.6
8		200.0		199.6
9		135.9		135.6
10	6.54 (dq, 5.9, 1.5, 1 H)	138.5	6.59 (dq, 5.8, 1.5, 1 H)	138.8
11	4.66 (d, 5.9, 1 H)	70.1	4.88 (d, 5.8, 1 H)	69.0
12		65.1		65.4
13A	3.08 (d, 4.3, 1 H)		3.07 (d, 4.2, 1 H)	
		47.4		47.3
13B	3.15 (d, 4.3, 1 H)		3.13 (d, 4.2, 1 H)	
14	1.13 (s, 3 H)	14.0	1.07 (s, 3 H)	13.7
15A	3.74 (d, 11.5, 1 H)		4.20 (d, 11.5, 1 H)	
		62.2		62.0
15B	3.85 (d, 11.5, 1 H)		4.24 (d, 11.5, 1 H)	
16	1.86 (bs, 3 H)	15.1	1.88 (bs, 3 H)	15.3
1′		173.3		173.2
2′	2.35 (t, 7, 2 H)	34.2	2.35 (t, 7, 2 H)	34.0
3′	1.62 (m, 2 H)	25.0	1.63 (m, 2 H)	24.5

[a] The remaining chemical shifts matched those reported in the literature for fatty acids and their esters (Wenkert et al., 1976).

Synthesis of 3,15-Diacetyl-DON (1) and 15-Acetyl-DON (2). A solution containing 3-acetyl-DON (500 mg), pyridine (750 μL), and acetyl chloride (750 μL) in CH_2Cl_2 (50 mL) was stirred at room temperature for 15 min. The solution was then washed with saturated $NaHCO_3$ and brine, dried over Na_2SO_4, and concentrated. The residue was chromatographed on silica gel (25 g) with 1:9 EtOAc/hexane (50 mL) followed by 1:3 EtOAc/hexane (300 mL). A yield of 490 mg (87.2%) of 3,15-diacetyl-DON (1) was obtained.

A solution of 1 (490 mg) and concentrated HCl (400 μL) in methanol (50 mL) was stirred overnight at room temperature, neutralized with sodium methoxide (264 mg), and concentrated. The residue was partitioned between water and ethyl acetate. The organic solution was dried over Na_2SO_4, concentrated, and flash chromatographed on silica gel (150 g) with 1:1 EtOAc/hexane yielding 210 mg of 15-acetyl-DON (2) and 94 mg of recovered 3,15-diAcDON (1).

The physical characteristics of both compounds matched those previously published (Yoshizawa et al., 1978; Savard et al., 1987). For the synthesis of 7,15-diAcDON see Savard et al. (1987).

Synthesis of Glucosides. *(A) Synthesis of Glucoside Acetate [Based on the Methods of Goto et al. (1979) and Conrow and Bernstein (1971)].* THe following procedure was also used with 7,15-diacetyl-DON and 3-acetyl-DON. 15-Acetyl-DON (2) (100 mg) and cadmium carbonate (65 mg) were suspended in freshly distilled toluene (40 mL). This mixture was refluxed, and 15 mL of solvent was distilled off. 1β-Bromo-1-deoxy-2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranose (acetobromoglucose) (210 mg) was added in 5 mL of dry toluene, and the resulting solution was refluxed with a Dean-Stark trap. After 1.5 h, more acetobromoglucose (100 mg) and $CdCO_3$ (30 mg) were added, and the reflux was continued for a further 1.5 h. At this time, the mixture was cooled and filtered through Celite. Flash chromatography of the concentrated filtrate on silica gel (150 g) with ethyl acetate/hexane (1:2) yielded a mixture of the acetyl-DON and the DON glucoside acetate. These were separated by preparative TLC on silica gel with 5% MeOH in chloroform as eluent, to yield the DON glucoside acetate (3) as a white solid (76 mg): MS (FAB/glycerol), 3 761 (0.3%, M + Gly + 1), 669 (1.5%, M + 1); DON 3-glucoside hexaacetate, 825 (0.2%, M + Gly + Na), 803 (0.2%, M + Gly + 1), 733 (0.6%, M + Na), 711 (0.5%, M + 1).

(B) Hydrolysis of Acetates. Potassium cyanide (2 mg) was added to a solution of the DON glucoside acetate (3) (19 mg) in methanol (4 mL). This solution was stirred at room temperature for 4 h and then passed through a short silica gel column (Pasteur pipet) to remove the KCN. After concentration of the eluate, the product was chromatographed on silica gel (2 g) with 1:1 EtOAc/hexane followed by EtOAc to yield a mixture of the DON glucoside and DON (12.4 mg). Further purification by HPLC (Partisil 10 μm, ODS-2 column, 30 × 0.97 cm, 17% MeOH in H_2O) yielded the pure DON glucoside (4) (4 mg): MS (FAB/glycerol) 4, 917 (0.1%, 2M + 1), 573 (0.2%, M + Gly + Na), 551 (1.2%, M + Gly + 1), 481 (1.2%, M + Na), 459 (2.0%, M + 1). DON 3-glucoside, 939 (0.5%, 2M + Na), 917 (0.5%, 2M + 1), 573 (M + Gly + Na), 551 (3.0%, M + Gly + 1), 481 (28%, M + Na), 459 (12%, M + 1).

RESULTS AND DISCUSSION

Synthesis. 4-Deoxynivalenol has three hydroxyl groups, at positions 3, 7, and 15. While the 3- and 15-hydroxyl groups are readily acylated, the 7-OH is not, neither synthetically nor biosynthetically. For this reason, it was deemed reasonable to synthesize only conjugates of DON

Table III. NMR Data for DON Glucoside Acetates

	DON 3-glucoside hexaacetate (3)		DON 15-glucoside pentaacetate	
	^1H	^{13}C	^1H	^{13}C
2	3.76 (d, 4.5 Hz)	80.1	3.82 (d, 4.3 Hz)	79.1
3	4.43 (dt, 11, 4.5)	75.3	5.10 (dt, 11, 4.3)	71.1
4α	2.24 (dd, 14.8, 4.5)		2.66 (dd, 15, 4.3)	
		41.6		40.4
4β	2.08 (dd, 14.8, 11)		2.10 (dd, 15, 11)	
5		45.7		45.7
6		50.0		51.2
7	6.05 (s)	74.9	4.80 (s)	73.8
8		192.3		199.6
9		136.9		135.7
10	6.61 (dq, 5.9, 1.3)	137.5	6.56 (dq, 5.9, 1.4)	138.6
11	4.68 (d, 5.9)	72.7	4.79 (d, 5.9)	71.0
12		64.9		64.9
13A	2.79 (d, 4.0)		3.11 (s)	
		47.8		47.4
13B	3.11 (d, 4.0)		3.11 (s)	
14	0.88 (s)	13.7	1.10 (s)	13.5
15A	4.29 (s)		3.48 (d, 10.5)	
		62.4		69.5
15B	4.29 (s)		4.13 (d, 10.5)	
16	1.83 (s)	15.2	1.82 (s)	14.9
1′	4.61 (d, 8.1)	100.3	4.47 (d, 8.0)	100.5
2′	5.07 (dd, 9.5, 8.1)	70.2	4.90 (masked by OD)	67.9
3′	5.22 (t, 9.5)	71.4	5.20 (t, 9.4)	72.8
4′	5.11 (t, 9.7)	68.5	4.98 (t, 10)	68.4
5′	3.71 (m)	72.0	3.82 (m)	71.9
6′A	4.13 (dd, 12.2, 2.5)		4.11 (dd, 12.1, 2.4)	
		61.9		61.9
6′B	4.22 (dd, 12.2, 5.0)		4.29 (dd, 12.4, 4.9)	
Ac	1.90, 1.99, 2.00, 2.05, 2.06, 2.20	20.5,20.6,20.8,169.3,169.5, 169.8,170.1,170.3,170.6	1.97, 2.01, 2.07, 2.10, 2.18	20.4,20.6,20.8,169.3, 169.5,170.3,170.6

at positions 3 and 15. Synthesis of the fatty acid esters was performed by reaction of DON with the corresponding acid chlorides in the presence of pyridine, yielding the 3- and 15-acyl-DON in a ratio of 3:1. These two compounds were easily separated by preparative TLC. Four pairs of fatty acid esters were synthesized, the stearates (18:0), oleates (18:1), linoleates (18:2), and linolenates (18:3).

The electron impact mass spectra (EIMS) of these esters showed some interesting characteristics (Table I). The molecular ion peaks were very small, and their intensities decreased as the extent of unsaturation increased. Also, molecular ion peaks for the 15-esters were always smaller than those of the 3-esters. Some other ions (297, 279, 278, 249, 137) were always more abundant for the 15-esters than for the 3-esters, while the rest of the spectra were very similar. Due to the low abundance of the molecular ions and the high mass of the compounds, it was difficult to obtain a good match for their expected molecular mass by high-resolution MS.

The NMR spectra of these esters (Table II) showed the DON moiety resonances of the 3-acyl- and 15-acyl-DON to be identical with those of 3-AcDON and 15-AcDON, respectively, while the fatty acid moieties showed spectral characteristics typical of such esters (Wenkert et al., 1976).

The DON 3-glucoside and the DON 15-glucoside had to be synthesized separately since they could not be separated by chromatography. The 3-glucoside 4 was made by a Koenigs–Knorr reaction of either 7,15-DiAcDON or 15-AcDON (2) with acetobromoglucose in the presence of cadmium carbonate in anhydrous toluene (Goto et al., 1979; Conrow and Bernstein, 1971), followed by hydrolysis of the protecting acetate groups with KCN in methanol (Herzig et al., 1986) (Figure 1). The same procedure with 3-AcDON yielded the DON 15-glucoside.

NMR showed that all the acetates on the glucose moiety were hydrolyzed at approximately the same rate while those on the DON moiety were hydrolyzed more slowly.

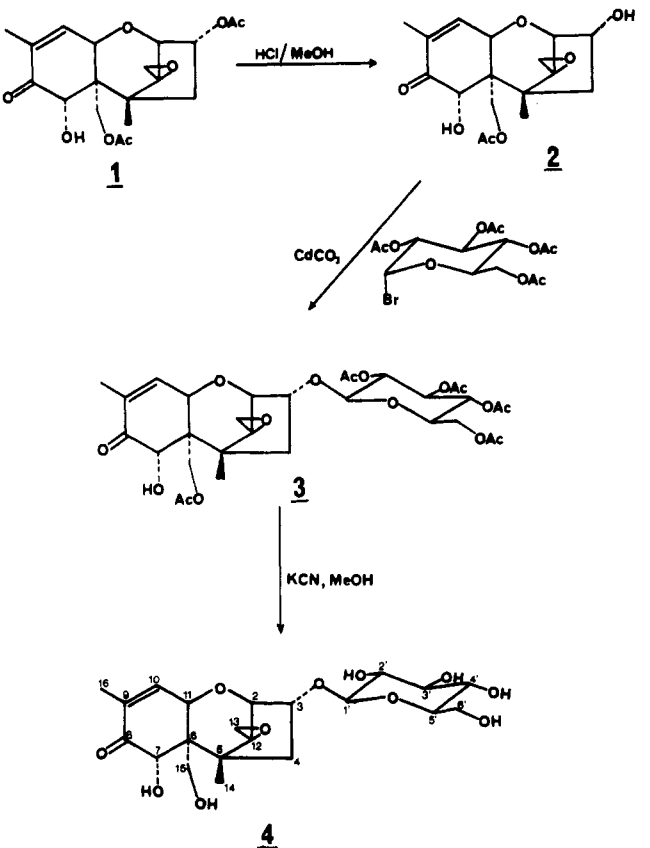

Figure 1. Synthesis of DON 3-glucoside from 3,15-diacetyl-DON.

This confirmed the mechanism proposed by Herzig et al. whereby a hydroxyl group catalyzed the hydrolysis of neighboring acetates. The rate-limiting step is thus hydrolysis of the first acetate on the glucose moiety, while

Table IV. NMR Data for DON Glucosides[a]

	DON 3-glucoside (4)		DON 15-glucoside (CD_3OD)	
	^1H	^{13}C	^1H	^{13}C
2	3.63 (d, 4.1)	82.2	3.53 (d, 4.1)	82.3
3	4.48 (dt, 11.2, 4.5)	75.0**	4.36 (dt, 11.3, 4.6)	69.8
4α	2.62 (dd, 14.7, 4.4)		2.43 (dd, 14.6, 4.5)	
		42.1		44.5
4β	1.98 (dd, 14.7, 11.0)		1.95 (dd, 14.8, 11)	
5		46.7		47.4
6		53.7		53.4
7	4.81 (s)	75.9**	4.84 (s)	75.7**
8		201.6		202.4
9		137.2		136.9
10	6.63 (dq, 5.9, 1.5)	139.3	6.59 (dq, 5.9, 1.6)	140.3
11	4.97 (d, 5.9)	71.7*	4.97 (d, 5.9)	71.6
12		66.6		66.8
13A	3.07 (d, 4.3)		3.06 (d, 4.3)	
		48.3		under CD_3OD ≈48–49
13B	3.11 (d, 4.3)		3.09 (d, 4.5)	
14	1.13 (s)	14.7	1.11 (s)	14.75
15A	3.69 (dd, 12.4, 3.4 (to OH))		3.55 (d, 10.5)	
		61.9		69.8
15B	3.74 (dd, 12.3, 2.2 (to OH))		4.17 (d, 10.5)	
16	1.83 (s)	15.4	1.82 (s)	15.3
1′	4.40 (d, 7.7)	104.3	4.02 (d, 7.5)	104.9
2′	3.25–3.4 (m)	77.2**	3.04 (dd, 8.8, 7.8)	75.2**
3′	3.25–3.4 (m)	78.1	3.27 (m)	77.9*
4′	3.25–3.4 (m)	71.4*	3.20 (m)	71.6
5′	3.25–3.4 (m)	78.1	3.6 (m)	78.1*
6′A	3.69 (m)		3.6 (m)	
		62.6		62.8
6′B	3.84 (dd, 12, 2)		3.84 (dd, 11.8, 1.8)	

[a] Data having matching asterisks may be interchanged.

the more isolated acetates on the DON part cannot benefit from neighboring group participation.

Although the glucosides were relatively stable, their thermal stability was too low for their mass spectra to show a molecular ion even under chemical ionization conditions. The largest ion found corresponded to a molecular ion for DON. However, FAB-MS gave the appropriate information including an M + 1 ion at m/z 459, M + Na at 481, and even the 2M + 1 ion at 917.

The orientation of the glucoside linkage was determined by NMR to be β as $J_{1',2'}$ ranged from 7.5 to 8.0 Hz for the glucosides and their acetates. The chemical shifts of the 1′ carbons were also characteristic of β-glucosides, being about 100 ppm for the acetates and 104–105 ppm for the free glucosides [for α, one would expect $J_{1',2'}$ of 2–4 Hz and δ ^{13}C of 95 and 100 for the acetates and the free glucosides, respectively (Lemieux et al., 1957; Breitmaier and Voelter, 1978)].

The ^1H and ^{13}C NMR assignments of the glucoside acetates are given in Table III and those of the glucosides in Table IV. Comparison with the chemical shifts of 4-deoxynivalenol shows that the NMR spectra of the DON moiety of the glucosides are identical with those of DON except for the carbon attached to glucose.

HPLC Analysis. HPLC analysis of the fatty acid esters on a silica gel column showed that the degree of unsaturation of the side chain had only a small influence on the retention times of the compounds, while the site of esterification on DON had a very significant effect (Table V). While esterification at position 3 produced retention times on silica gel with 7% 2-propanol in hexane of 4.6–5.0 min, esterification at position 15 led to retention times of 7.5–8.0 min. The degree of unsaturation of the fatty acid chain only increased the retention time of the derivatives by 0.1–0.2 min per double bond.

HPLC analysis of the synthesized DON glucosides on a reverse-phase column showed that they both had the same retention time, which was shorter than that of DON.

Table V

ester	retention time,[a] min
DON 3-stearate (3-18,0)	4.6
DON 15-stearate (15-18,0)	7.5
DON 3-oleate (3-18,1)	4.8
DON 15-oleate (15-18,1)	7.6
DON 3-linoleate (3-18,2)	4.9
DON 15-linoleate (15-18,2)	7.9
DON 3-linolenate (3-18,3)	5.0
DON 15-linolenate (15-18,3)	8.0

[a] HPLC of DON fatty acid esters on silica gel (30 × 0.3 cm), with 7% iPrOH in hexane at 4 mL/min. Detection by UV absorbance at 224 nm.

Conclusion. Ten different DON conjugates, eight fatty acid esters and two glucosides, were synthesized and characterized. Although the DON 3-esters are easily differentiated from DON 15-esters by HPLC, the degree of unsaturation of the fatty ester functionality does not provide enough difference in retention time for an unambiguous assignment. The glucosides could not be differentiated by HPLC either. However, each compound could be differentiated by NMR or MS. The search for these compounds in DON-contaminated wheat is presently under way.

It has recently been suggested that the DON conjugate produced by wheat could be an acetylated glucoside with the acetate on the DON moiety (Schuster, 1988). This would mean that the usual deacetylation by the plant of the acetyl-DON produced by the fungus (Miller and Greenhalgh, 1988) would have been bypassed and glucosidation of AcDON would have taken place instead. This hypothesis requires further study.

Although glucoside conjugation is the most common xenobiotic conjugation reaction in plants, other likely conjugation agents include glutathione, hemicellulose, and even lignin (Lamoureux and Rusness, 1986). The possibility that one of these mechanisms could be a factor in DON conjugation also remains to be investigated.

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Registry No. 1, 56676-60-9; 1 15-*O*-desacetyl derivative, 50722-38-8; 2, 88337-96-6; 2 7-*O*-acetyl derivative, 54996-63-3; 3, 131180-20-6; 3 15-*O* isomer, 131193-32-3; 4, 131180-21-7; 4 15-*O* isomer, 131180-29-5; DON, 51481-10-8; DON-3-stearate, 131180-22-8; DON-15-stearate, 131180-23-9; DON-3-oleate, 131180-24-0; DON-15-oleate, 131180-25-1; DON-3-linoleate, 131180-26-2; DON-15-linoleate, 131180-27-3; DON-3-linolenate, 131193-54-9; DON-15-linolenate, 131180-28-4; α-D-acetobromoglucose, 572-09-8.