Trichothecenes Produced by *Fusarium crookwellense* DAOM 193611[1]

Denis R. Lauren,* Anne Ashley, Barbara A. Blackwell, Roy Greenhalgh, J. David Miller, and Gordon A. Neish

The trichothecene fraction produced by a liquid culture of *Fusarium crookwellense* DAOM 193611 was separated by open-column liquid chromatography on silica gel and by HPLC on a cyano bonded-phase column. The major trichothecene produced was 4,15-diacetoxynivalenol. Other secondary metabolites formed in appreciable amounts were the 7- and 8-hydroxy derivatives of isotrichodermin. Several unknown compounds were isolated and characterized by their MS and ^{1}H and ^{13}C NMR spectra. Among these compounds were 7,8-dihydroxyisotrichodermin, 8-ketoisotrichodermin, and 4,15-diacetoxy-7-deoxynivalenol.

The toxin-producing potential of various *Fusarium sp.* fungi associated with cereal crops have been studied extensively, but little work has been done on fusaria associated with forage crops or pastures.

A series of trials that commenced in New Zealand in 1984 has examined the possible impact of *Fusarium sp.* present in pastures on the health of livestock and their fertility. To date, this work has concentrated on the estrogenic mycotoxin, zearalenone (I). It is produced in cultures by some *Fusarium sp.* isolated from New Zealand pastures (Gallagher, 1985; di Menna et al., 1985) and has also been found on fresh pasture leaves (di Menna et al., 1985).

Recently, we conducted a study with 25 *Fusarium* isolates from a New Zealand pasture (Lauren et al., 1987). Their potential for the production of zearalenone when cultured on rice was examined together with the production of other secondary metabolites (e.g., trichothecenes) in liquid cultures. Two of these isolates were identified as *Fusarium crookwellense*, a species known to occur commonly in pasture soils of eastern Australia (Burgess et al., 1982). This species has been reported to produce zearalenone (Neish et al., 1985), but no information on the production of other secondary metabolites, such as trichothecenes, was available. We found that both *F. crookwellense* isolates produced small quantities of zearalenone on rice and gave butenolide (II) as well as several known and unknown trichothecenes in liquid culture.

In the present study, the isolation and characterization of the trichothecene component, which includes five new compounds, produced by *F. crookwellense* DAOM 193611 is described.

MATERIALS AND METHODS

Standard Materials. Isotrichodermin (ITD), 7-hydroxyisotrichodermin (7-OH-ITD), 8-hydroxyisotrichodermin (8-OH-ITD), culmorone, 3-deoxysambucinol, isotrichodermol, sambucoin, culmorin, sambucinol, and the mixed epimers of 3-hydroxyapotrichothecene have been isolated previously and characterized by mass spectrometry (MS) and ^{1}H and ^{13}C nuclear magnetic resonance spectroscopies (NMR) (Greenhalgh et al., 1984, 1986a–c).

Apparatus. Mass spectrometry and nuclear magnetic resonance instrumentation and basic methodology have been described elsewhere (Greenhalgh et al., 1986a,b). Packed-column gas chromatography with electron capture detection (GC/ECD) conditions for *N*-(heptafluorobutyryl)imidazole- (HFBI-) derivatized materials has also been described (Greenhalgh et al., 1986a). High-performance liquid chromatography (HPLC) was performed on a Varian Model 5500 system fitted with a UV-200 variable-wavelength detector. Gas chromatography/mass spectrometry (GC/MS) analyses used a Finnigan Model 4500 GC/MS system. Underivatized samples in ethyl acetate (1 μL) were injected on column at ambient temperature. Separation was achieved on a DB-5 fused silica column (20 m × 0.32 mm (i.d.), 0.25-μm film), temperature programmed from 140 to 260 °C at 15 °C/min and with helium carrier gas at 10 psi. Mass spectra were obtained in the electron impact (EI) mode and compared with the extended trichothecene data base library (Plant Research Centre, Ottawa). Preliminary GC screening of underivatized chromatographic fractions employed a Finnigan ion trap detection system (ITDS). Separation was achieved on a 20-m DB-5 column as above, using 2-μL injection at 230 °C with a split ratio 10:1.

F. crookwellense DAOM 193611 was cultured in MYRO medium as previously described (Lauren et al., 1987). The culture filtrate (850 mL) was extracted with ethyl acetate (4 × 300 mL). The combined extract was dried over anhydrous sodium sulfate and then evaporated under vacuum at 45 °C to give a yellow gum (170 mg). This material was chromatographed in a glass column (2-cm i.d.) dry packed with silica gel (25 g; Merck Kieselgel 60, 70–230 mesh; used directly from the bottle). The fractions were analyzed by GC and GC/MS and the major new components isolated by preparative HPLC using a cyano bonded-phase column (Vydac 501 TP 10 Nitrile, 25 cm × 10 mm (i.d.)). The solvents used were either HPLC or distilled in glass grade from Caledon Labs, Georgetown, Ontario, Canada. The chloroform contained 0.75% alcohol.

Silica Column Fractionation. The yellow gum was applied to the column in 10% ethyl acetate/chloroform (10 mL, plus two 10-mL rinses). Each application was allowed to percolate into the column before adding the next. A clear material (14 mg), which was insoluble in the application solvent, was shown by both GC/ITDS and GC/ECD analysis after HFBI derivatization to contain no compounds of interest.

Twelve fractions (S1–S12) were collected and evaporated under vacuum at 45 °C. The residues were dissolved in ethyl acetate (5 mL) for analysis by GC/ITDS, GC/ECD after HFBI derivatization, HPLC, and then GC/MS for positive identification of the constituents.

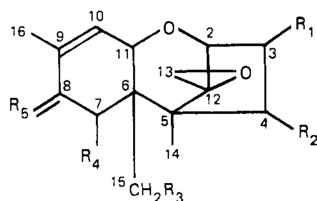
Fraction S1. A residue (11 mg) was eluted with 10% ethyl acetate/chloroform (60 mL) and consisted of phthalates.

Fraction S2. An oil (1 mg) that eluted with a further 50 mL of 10% ethyl acetate/chloroform was identified as

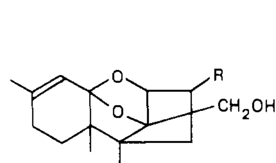
Ruakura Agricultural Research Centre, Ministry of Agriculture and Fisheries, Private Bag, Hamilton, New Zealand (D.R.L.), and Research Branch, Agriculture Canda, Ottawa, Ontario K1A 0C6, Canada (A.A., B.A.B., R.G., J.D.M., G.A.N.).

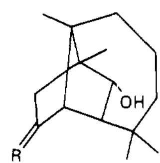
[1]PRC Contribution No. 1043.

0021-8561/87/1435-0884$01.50/0 © 1987 American Chemical Society

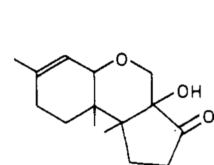


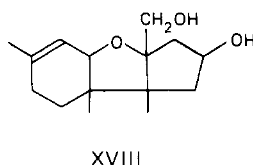
	R_1	R_2	R_3	R_4	R_5
III	OAc	H	H	H	H_2
IV	OAc	H	H	H	O
V	OAc	H	H	OH	H_2
VI	OAc	H	H	H	H,OH
IX	OH	H	H	H	H_2
XI	OH	OAc	OAc	OH	O
XIII	OH	OAc	OAc	H	O
XIV	OAc	H	H	OH	H,OH
XVI	OH	H	H	OH	H_2
XVII	OH	H	H	H	H,OH



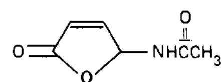


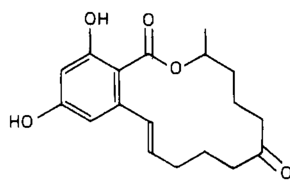
VIII R=H XV R=OH VII R=O XII R=H,OH





X XVIII





II I

Figure 1. Secondary metabolites isolated from *F. crookwellense* DAOM 193611.

isotrichodermin (ITD) (III) by comparison of its MS with that of an authentic sample.

Fraction S3. An oil (1.7 mg) obtained with 20% ethyl acetate/chloroform (50 mL) consisted mainly of a new compound, IV (ca. 85%), together with a small amount of isotrichodermin.

Fraction S4. An oil (7.5 mg) eluted with a further 50 mL of 20% ethyl acetate/chloroform was a mixture of 7-hydroxyisotrichodermin (7-OH-ITD, V) (ca. 60%) [MS, m/z 109 (base), 308 (8, M^+)], 8-hydroxyisotrichodermin (8-OH-ITD, VI) (ca. 25%) [MS, m/z 123 (base), 308 (3, M^+)], and the unknown compound IV (ca. 10%), with smaller amounts of culmorone (VII) [MS, m/z 110 (base), 236 (25, M^+)], 3-deoxysambucinol (VIII) [MS, m/z 250 (base, M^+)], isotrichodermol (IX) [MS, m/z 93 (base), 250 (29, M^+)], and sambucoin (X) [MS, m/z 93 (base), 250 (64, M^+)].

Fraction S5. An oil (13.5 mg) obtained with 50% ethyl acetate/chloroform (50 mL) contained 8-OH-ITD (ca. 70%), 7-OH-ITD (ca. 20%), the unknown compound IV, and a compound later identified as 4,15-diacetoxynivalenol (4,15-DANIV, XI).

Fraction S6. An oil (15 mg) obtained with a further 50 mL of 50% ethyl acetate/chloroform consisted of 4,15-DANIV (ca. 90%) with minor quantities of culmorin (XII) [MS, m/z 95 (base), 238 (8, M^+)], 8-OH-ITD, and another new compound XIII.

Fraction S7. An oil (7 mg) eluted with ethyl acetate (50 mL) contained a third new compound, XIV (ca. 70%), together with culmorin (ca. 20%), sambucinol (XV) [MS, m/z 124 (base), 266 (24, M^+)], and 4,15-DANIV.

Fraction S8. An oil (7 mg) eluted with a further 50 mL of ethyl acetate contained sambucinol (ca. 50%), the new compound XIV (ca. 20%), 4,15-DANIV, and two unknown compounds characterized as 7-hydroxyisotrichodermol (7-OH-ITDOL, XVI) [MS, m/z 109 (base), 266 (1, M^+)] and 8-hydroxyisotrichodermol (8-OH-ITDOL, XVII) [MS, m/z 123 (base), 266 (1, M^+)], by deacetylation of 7-OH- and 8-OH-ITD.

Fraction S9. An oil (1 mg) obtained with a third 50-mL aliquot of ethyl acetate was mainly 8-OH-ITDOL, with some sambucinol, 4,15-DANIV, and traces of butenolide.

Fraction S10. A solid (2.6 mg) obtained with 5% methanol/chloroform (50 mL) was mainly butenolide (II) by HPLC and MS analysis.

Fraction S11. A solid (25 mg) obtained with 10% methanol/chloroform (100 mL) was mainly butenolide combined with the two epimers of 3-hydroxyapotrichothecene (HAPO, XVIII) [MS, m/z 107 (base), 252 (24, M^+)].

Fraction S12. A brown oil (55 mg) obtained by washing the column with methanol (100 mL) was found to be mostly insoluble in ethyl acetate but did contain traces of butenolide and HAPO.

Fractions S3–S7 contained minor unknown compounds whose MS suggested they were trichothecenes. The total trichothecene content of the original yellow gum (170 mg) was approximately 63 mg.

Preparative HPLC. Preliminary tests with the original crude culture extract using the cyano column showed that this bonded-phase material had a different selectivity than silica for several of the major components. This facilitated purification of the fractions, and the major unknown components were isolated by HPLC separation of fractions S3, S6, and S7 from the silica column.

With a mobile phase of 5% 2-propanol/hexane, most components eluted before 4,15-DANIV (k' = 5). Some minor components, including the unknown XIII, eluted between k'5 and 12.5, and butenolide had a k' of 19. With use of a mobile phase of 3% 2-propanol/hexane, the fraction k' 0–1.3 contained ITD; the fraction k' 1.3–3 contained culmorin, 3-deoxysambucinol, isotrichodermol, sambucoin, the unknown compound IV, and three minor components; the fraction k' 3–4.7 contained 7-OH-ITD, 8-OH-ITD, the α epimer of HAPO (α-HAPO, XVIII) [MS, m/z 107 (base), 252 (15, M^+)], and sambucinol; and the fraction k' 4.7–9 contained the β epimer of HAPO (β-HAPO, XVIII) [MS, m/z 107 (base), 252 (37, M^+)], 7-OH-ITD, 4,15-DANIV, and the unknown compound XIV.

For preparative HPLC the fractions S3, S6, and S7 were each dissolved in 500 μL of 60% ethyl acetate/hexane. Injections of 100–200 μL were used, and in all cases the flow rate was 4 mL/min and the detector was set at 220 nm.

Fraction S3, with a mobile phase of 2% 2-propanol/hexane, yielded one product, collected as a single peak between 7.0 and 8.5 min (k' = 1.6). Evaporation of the solvent gave a white solid, IV (1.6 mg), 90% pure (GC/

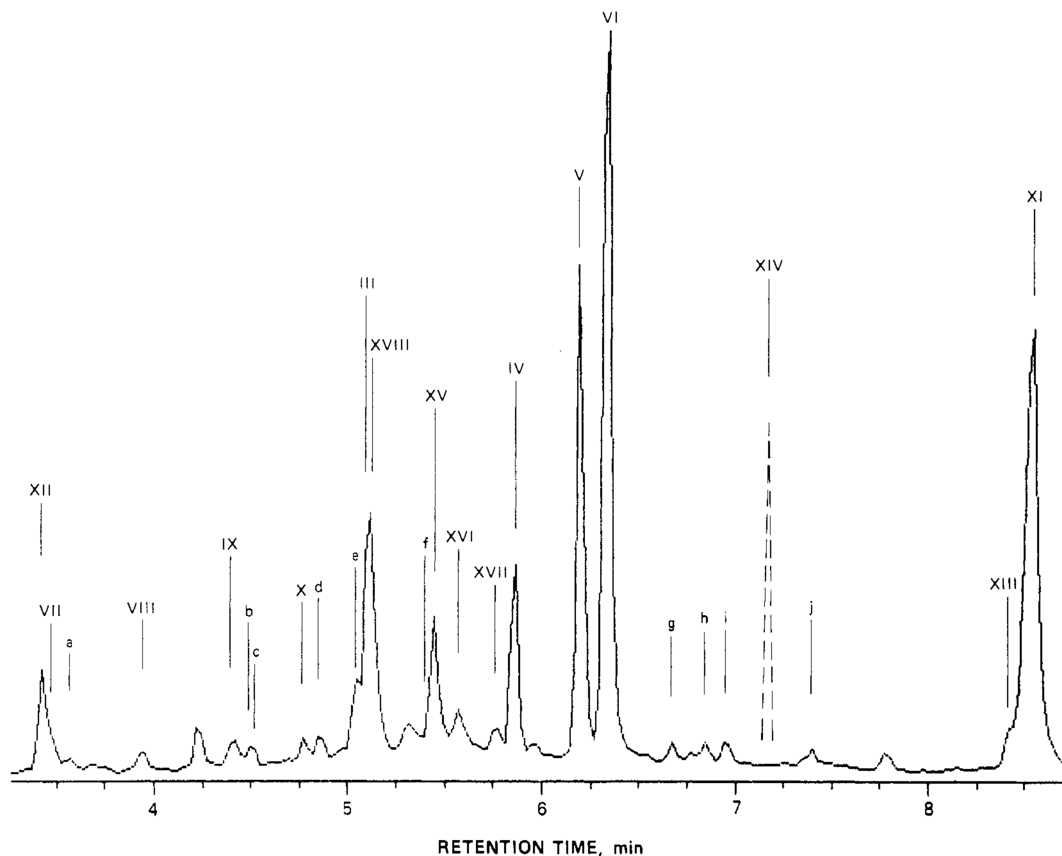


Figure 2. Total ion GC/MS (EI) of underivatized crude extract from liquid culture of *F. crookwellense* DAOM 193611 showing the major metabolites 7-OH-ITD (V), 8-OH-ITD (VI), and 4,15-DANIV (XI). Chromatographic conditions are as in text. Component identification relates to Figure 1 except for unknowns a–j.

MS): mp 122–124 °C; MS, m/z 123 (base), 138 (92), 79 (49), 107 (42), 253 (35), 216 (27), 306 (3) (M^+); ^{1}H NMR δ 0.75 (3 H, CH_3-14), 0.92 (3 H, CH_3-15, $J_{15,7}$ = 1.1 Hz), 1.81 (3 H, CH_3-16, $J_{16,10}$ = 1.5 Hz), 2.03 and 2.17 (2 H, H-4, J_{AB} = 14.8, $J_{4,3}$ = 10.9, 4.6 Hz), 2.14 (3 H, Ac-CH_3), 2.26 and 2.87 (2 H, H-7, J_{AB} = 15.3, $J_{7,15}$ = 1.1 Hz), 2.86 and 3.10 (2 H, H-13, J_{AB} = 3.9 Hz), 3.83 (1 H, H-2, $J_{2,3}$ = 4.6 Hz), 4.32 (1 H, H-11, $J_{11,10}$ = 5.7 Hz), 5.22 (1 H, H-3, $J_{3,2}$ = 4.6, $J_{3,4}$ = 10.9, 4.6 Hz), 6.51 (1 H, H-10, $J_{10,11}$ = 5.7, $J_{10,16}$ = 1.5 Hz). These data are consistent with the structural assignment of 8-ketoisotrichodermin (8-keto-ITD).

Fraction S6 with a mobile phase of 3% 2-propanol/hexane gave a compound with a strong UV response between 23 and 28 min (k' = 7.6). Evaporation of the solvent gave a white solid (9.3 mg) that was at least 90% 4,15-DANIV (XI) by GC/MS: mp 136–138 °C; MS, m/z 179 (base), 123 (27), 189 (27), 247 (23), 336 (16), 396 (4) (M^+); ^{1}H NMR δ 1.09 (3 H, CH_3-14), 1.88 (2 H, CH_3-16, $J_{16,10}$ = 1.6 Hz), 1.90 (3 H, CH_3-15 Ac), 2.14 (3 H, CH_3-4 Ac), 3.07 and 3.08 (2 H, H-13, J_{AB} = 4.3 Hz), 3.25 (1 H, 3-OH, $J_{3,OH}$ = 2.7 Hz), 3.80 (1 H, 7-OH, $J_{7,OH}$ = 2.0 Hz), 3.82 (1 H, H-2, $J_{2,3}$ = 4.7 Hz), 4.19 and 4.32 (2 H, H-15, J_{AB} = 12.34 Hz), 4.22 (1 H, H-3, $J_{3,2}$ = 4.8 Hz, $J_{3,4}$ = 3.1, $J_{3,OH}$ = 2.7 Hz), 4.72 (1 H, H-11, $J_{11,10}$ = 5.9 Hz), 4.86 (1 H, H-7, $J_{7,OH}$ = 2.0 Hz), 5.16 (1 H, H-4, $J_{4,3}$ = 3.1 Hz), 6.63 (1 H, H-10, $J_{10,11}$ = 5.9, $J_{10,16}$ = 1.6 Hz). These spectral data confirmed that the compound was 4,15-DANIV. In addition, a minor component (compound XIII) with strong UV response was collected at k' 9.6 and identified as 4,15-diacetoxy-7-deoxynivalenol (4,15-DA-7-DON): MS, m/z 121 (base), 109 (96), 173 (63), 91 (62), 189 (59), 201 (37), 278 (29), 218 (26), 380 (25) (M^+); ^{1}H NMR δ 0.80 (3 H, CH_3-14), 1.82 (3 H, CH_3-16, $J_{16,10}$ = 1.5 Hz), 1.97 (3 H, CH_3-15 Ac), 2.14 (3 H, CH_3-4 Ac), 2.46 and 2.92 (2 H, H-7, $J_{7,15}$ = 1.6, J_{AB} = 15.9 Hz), 2.79 and 3.07 (2 H, H-13, J_{AB} = 3.9 Hz), 3.21 (1 H, 3-OH, $J_{OH,3}$ = 2.6 Hz), 3.77 (1 H, H-2, $J_{2,3}$ = 4.9 Hz), 4.10 and 4.17 (2 H, H-15, J_{AB} = 12.4 Hz), 4.21 (1 H, H-3, $J_{3,2}$ = 4.9, $J_{3,4}$ = 2.9, $J_{3,OH}$ = 2.6 Hz), 4.51 (1 H, H-11, $J_{11,10}$ = 5.9 Hz), 5.06 (1 H, H-4, $J_{4,3}$ = 2.9 Hz), 6.59 (1 H, H-10, $J_{10,11}$ = 5.9, $J_{10,16}$ = 1.5 Hz).

A mobile phase of 3% 2-propanol/hexane was also used for fraction S7. A product was collected between 13 and 15 min (k' = 3.7) that had little UV absorbance at 220 nm. Evaporation of the solvent gave a white solid (2.5 mg) that GC/MS showed to be a single product (compound XIV): mp 80–84 °C; MS, m/z 100 (base), 107 (75), 135 (61), 95 (51), 123 (49), 148 (47), 324 (27) (M^+), 165 (24); ^{1}H NMR δ 0.89 (3 H, CH_3-15), 1.06 (3 H, CH_3-14), 1.87 (3 H, CH_3-16, $J_{16,10}$ = 1.6 Hz), 1.91 (1 H, 8-OH, $J_{OH,8}$ = 6.9 Hz), 2.07 (2 H, H-4, $J_{4,3}$ = 8.8, 6.9, J_{AB} = 7.8 Hz), 2.10 (3 H, Ac-CH_3), 2.56 (1 H, 7-OH, $J_{OH,7}$ = 9.7 Hz), 3.10 and 3.17 (2 H, H-13, J_{AB} = 4.3 Hz), 3.76 (1 H, H-2, $J_{2,3}$ = 4.5 Hz), 3.98 (1 H, H-8, $J_{8,7}$ = 5.8 Hz), 4.16 (1 H, H-11, $J_{11,10}$ = 5.9 Hz), 4.41 (1 H, H-7, $J_{7,8}$ = 5.5, $J_{7,OH}$ = 9.7 Hz), 5.17 (1 H, H-3, $J_{3,2}$ = 4.5, $J_{3,4}$ = 8.8, 6.9 Hz), 5.59 (1 H, H-10, $J_{10,11}$ = 5.9, $J_{10,16}$ = 1.6 Hz). The data are consistent with the structure 7,8-dihydroxyisotrichodermin (7,8-DHITD).

RESULTS AND DISCUSSION

The GC/MS chromatogram of the underivatized crude culture extract from *F. crookwellense* is shown in Figure 2. This extract was obtained by solvent extraction of the liquid culture medium. Virtually all compounds isolated in the silica column fractions can be accounted for by their peaks in the chromatogram of the original extract. This indicates that little degradation had occurred during fractionation. The major exception was 7,8-DHITD (XIV), which is absent from this chromatogram. However, it was present in chromatograms of small-scale culture extracts and is therefore believed to be a true metabolite of *F.*

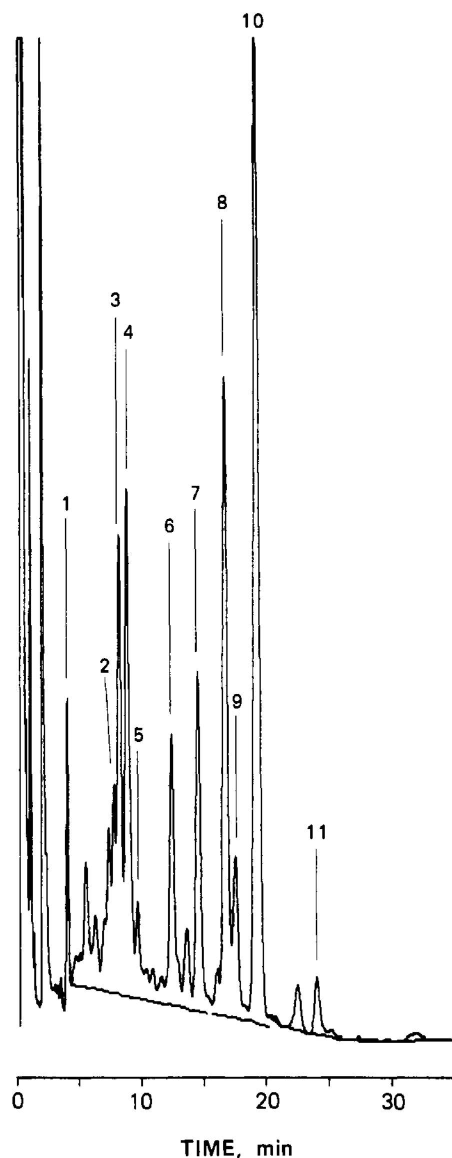


Figure 3. Gas chromatogram (GC/ECD) of HFBI derivatized sample of the crude extract from liquid cultures of *F. crookwellense* DAOM 193611. Peak identification for the HFBI derivatives: (1) culmorin; (2) 7-OH-ITDOL; (3) β-HAPO; (4) sambucinol plus α-HAPO; (5) 8-OH-ITDOL; (6) unknown; (7) 7-OH-ITD; (8) 8-OH-ITD plus 7,8-DHITD; (9) 7,8-DHITD; (10) 4,15-DANIV; (11) 4,15-DA-7-DON.

crookwellense rather than a degradation product. The absence of 7,8-DHITD in the GC/MS chromatograms of more concentrated solutions is thought to be due to interference from coextracted impurities in the solution. GC/ECD chromatograms of HFBI derivatized crude culture extract did show peaks consistent with the presence of 7,8-DHITD. The chromatograms (Figure 3) of samples stored either in neat form or in ethyl acetate solution at −5 °C for several weeks remained consistent, indicating that the samples were stable. Again most of the prominent peaks can be accounted for, except for the one at 12.5 min (6). The compound that gave rise to this peak was a minor component in silica column fraction S8 and was not identified by GC/MS. It also eluted after 4,5-DANIV on HPLC.

The ratio of the peaks at 16.7 min (8) and 17.6 min (9) in Figure 3, which are due to 8-OH-ITD and 7,8-DHITD, varied with different analytical runs. This was attributed to the degree of derivatization of the latter compound. Under mild reaction conditions (50 °C/30 min), pure

Table I. ^{13}C NMR Chemical Shift Assignments for Novel Trichothecenes from *F. crookwellense*

C no.	8-keto-ITD (IV)	7,8-DHITD (XIV)	4,15-DANIV (XI)	4,15-DA-7-DON (XIII)
2	78.5	78.9	79.8	79.0
3	71.4	71.5	78.4	78.5
4	42.1	40.6	83.9	83.8
5	45.4	46.4	49.4	47.5
6	43.4	44.2	52.5	48.8
7	37.8	73.8	73.3	38.2
8	189.0	71.1	199.1	196.7
9	137.7	138.8	136.0	138.9
10	137.6	122.6	138.4	136.9
11	71.6	69.8	69.3	68.4
12	57.5	65.0	64.3	64.4
13	48.1	47.8	46.1	46.7
14	10.6	13.2	7.5	6.1
15	18.3	14.7	61.8	64.5
16	15.3	20.3	15.1	15.3
CH_3	20.8	20.9	20.9	20.8
(Ac)				20.5
C=O	172.5	170.6	170.1	170.2
(Ac)			172.7	172.7

7,8-DHITD gave two peaks with the peak at 17.6 min predominating, while under stronger derivatizing conditions (60–65 °C/120 min), it gave mainly the peak at 16.5 min. Pure 8-OH-ITD gave a single peak at 16.5 min.

The ^{13}C NMR chemical shift assignments of the major new compounds isolated from *F. crookwellense* are given in Table I. Assignments for 7-OH- and 8-OH-ITD have been previously reported (Greenhalgh et al., 1986b). The assignment of C-7 and C-8 resonances in 7,8-DHITD (XIV) is based on the observation that the C-7 chemical shifts of hydroxylated compounds are generally at lower field than those of C-8 (Greenhalgh et al., 1986b,c). This assignment of C-7 and C-8 in 7,8-DHITD is reversed from that in dihydroxycalonectrin (DHCAL), a C-15 acetoxy analogue, which has the same substitution pattern in the "A" ring. However, comparison of the C-7 chemical shifts in related compounds 7-OH-ITD and 7-OH-CAL shows an upfield shift of 3 ppm at C-7, associated with substitution of an acetoxy group at C-15. The C-10 chemical shifts in all the compounds in Table I are consistent with the previously observed correlation (Greenhalgh et al., 1986c) that hydroxylation at C-8 produces a downfield shift of 3 ppm (from 119 to 122 ppm), while oxidation to a keto group effects a greater downfield shift of 19 ppm (to 138 ppm).

The most abundant trichothecenes produced by *F. crookwellense* were, in order, 4,15-DANIV, 8-OH-ITD, 7-OH-ITD, 7,8-DHITD, and HAPO. Both 7-OH-ITD and 8-OH-ITD have been reported previously, being produced in minor quantities by both *Fusarium culmorum* and *Fusarium graminearum*, which are known to produce 3-acetoxydeoxynivalenol (ADON) as the major product (Greenhalgh et al., 1986a,b). 4,15-DANIV was first isolated by Tatsuno et al. (1970) from a species originally classified as *Fusarium nivale* isolate Fn-2B but now known to be an isolate of *Fusarium sporotrichioides* (Marasas et al., 1984). It has since been reported as produced by several other *Fusarium* species, although not previously by *F. crookwellense*. The ^{1}H NMR and mass spectra of 4,15-DANIV are consistent with the structure previously determined (Cole and Cox, 1981). The downfield shift of H-10 (6.63 ppm) is compatible with the presence of a keto group at C-8, as observed in various 3-ADON derivatives (Greenhalgh et al., 1986c) and confirmed by ^{13}C NMR (Table I). Also confirmatory is the H-7 resonance, which appears downfield as a doublet, coupled only to the 7-OH proton (J = 2.0 Hz). The epoxide methylene protons have the

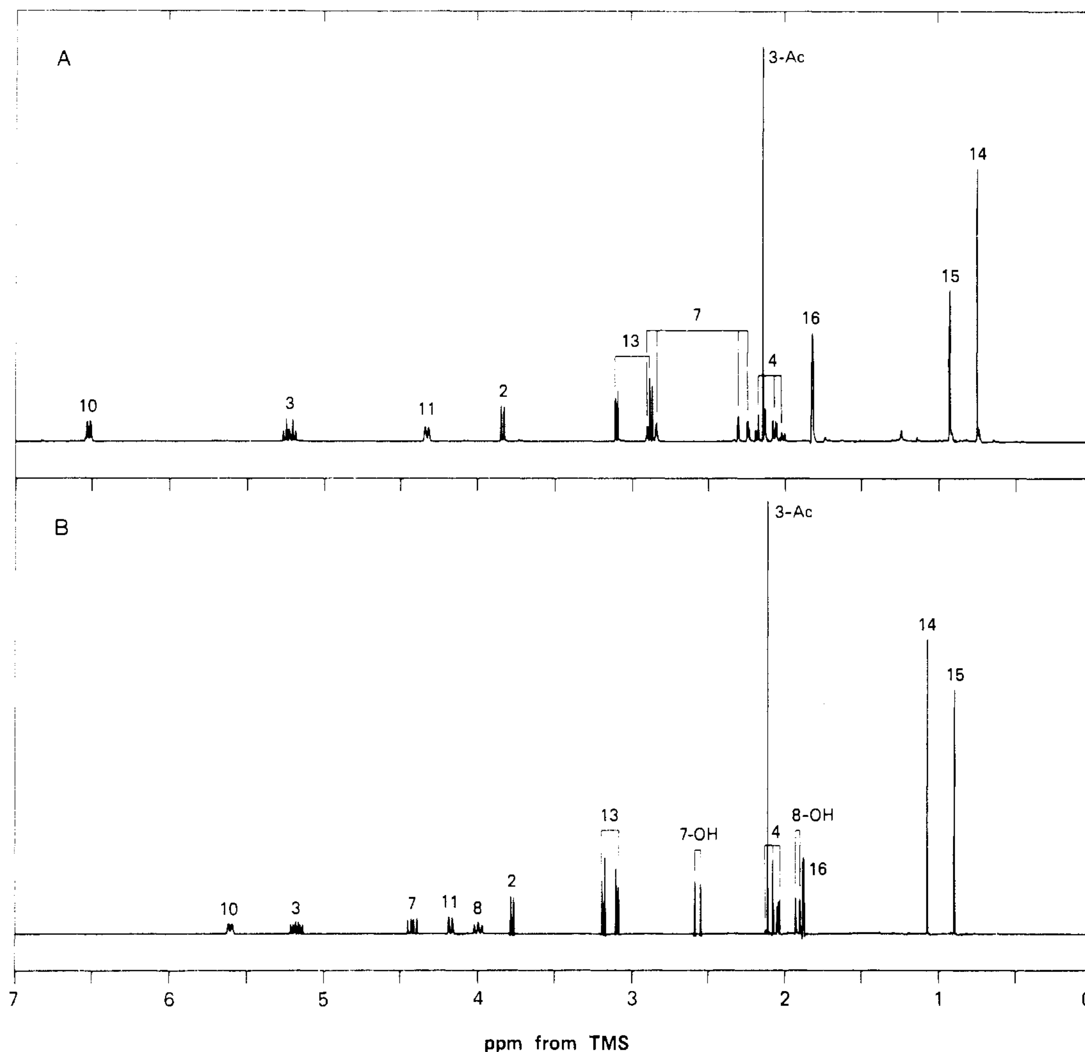


Figure 4. 250-MHz ^{1}H NMR spectra of (A) 8-ketoisotrichodermin (IV) and (B) 7,8-dihydroxyisotrichodermin (XIV). Spectra were assigned relative to those of isotrichodermin and 7-hydroxyisotrichodermin (Greenhalgh et al., 1986b).

characteristic chemical shift (ca. 3 ppm) and a J_{AB} of 4 Hz, but with an unusually small AB character.

The minor component, 4,15-diacetoxy-7-deoxynivalenol (XIII), has also been isolated and characterized as a minor component in *F. sporotrichioides* cultures (Greenhalgh, 1986). The ^{1}H NMR spectrum of this compound shows long-range coupling (1.6 Hz) between the H-7 and the protons of H-15. This coupling, which is not observed in 4,15-DANIV, is consistent with other trichothecences having a methylenic moiety at C-7 (Greenhalgh, 1986). This confirms the C-7 hydroxyl moiety in nivalenol-type trichothecenes as having an α configuration.

The ^{1}H NMR spectra of the new isotrichodermin derivatives, 8-keto-ITD (IV) and 7,8-DHITD (XIV), are shown in Figure 4. Assignments of the H-7 and H-8 protons in 7,8-DHITD follow from those of DHCAL (Greenhalgh et al., 1986c) in which both the 7- and 8-hydroxy groups have the α configuration. The coupling constants for H-7 and H-8 ($J_{7\beta,8\beta}$ = 5.5 Hz) as well as for their respective hydroxy protons ($J_{7,OH}$ = 9.2 Hz, $J_{8,OH}$ = 6.9 Hz) are virtually identical with those of DHCAL. The methylene protons at C-4 are almost an A_2 system (J_{AB} = 8 Hz), as was observed for ITD and both its 7- and 8-monohydroxy derivatives. The 1 ppm downfield shift of H-10 (Figure 4A) confirms the presence of a keto group in 8-keto-ITD. Consistent with other compounds having a keto group at C-8 and unsubstituted in C-7, a strong AB system is observed for the C-7 protons with coupling between protons to the methyl group of C-15 (J = 1.1 Hz).

F. crookwellense is probably closely related taxonomically to both *F. culmorum* and *F. graminearum* (Burgess et al., 1982). It is of interest, therefore, that the major trichothecene produced by this New Zealand strain of *F. crookwellense* is 4,15-DANIV, a 3,4-oxygenated trichothecene, in combination with significant amounts of several 3-oxygenated trichothecenes, such as ITD, 7-OH-ITD, 8-OH-ITD, 7,8-DHITD, and 8-keto-ITD. Canadian strains of both *F. culmorum* and *F. graminearum* produced only the 3-oxygenated trichothecenes, e.g. ADON (Greenhalgh et al., 1986a,b), while different Japanese strains of *F. graminearum* have been shown to produce either 3,4-oxygenated trichothecenes or 3-oxygenated trichothecenes, but not both (Ichinoe et al., 1983). Oxidation of the trichothecene ring system has been shown to involve hydroxylase enzymes, with the oxygen being derived from air rather than water (Desjardins et al., 1986). The sequence of the primary oxidation steps involving the 3- and 4-positions of the trichothecene nucleus by different species of *Fusarium* can be regarded as being governed by different enzyme systems and as such represents a possible genetic difference between the species.

Registry No. II, 16275-44-8; III, 91423-90-4; IV, 109802-15-5; V, 99571-97-8; VI, 99571-99-0; VII, 18374-95-4; VIII, 104148-46-1; IX, 104155-10-4; X, 90044-34-1; XI, 14287-82-2; XII, 18374-83-9; XIII, 77620-47-4; XIV, 109802-16-6; XVI, 109802-13-3; XVII, 109802-14-4; XVIII, 104148-45-0.

LITERATURE CITED

Burgess, L. W.; Nelson, P. E.; Toussoun, T. A. *Trans. Br. Mycol. Soc.* **1982**, *79*, 479–505.

Cole, R. J.; Cox, R. H. *Handbook of Toxic Fungal Metabolites*; Academic: New York, 1981.

Desjardins, A. E.; Plattner, R. D.; Vanmiddlesworth, F. *Appl. Environ. Microbiol.* **1986**, *51*, 493–497.

di Menna, M. E.; Lauren, D. R.; Holland, P. T. *N.Z. Vet. J.* **1985**, *33*, 193.

Gallagher, R. T. *N.Z. Vet. J.* **1985**, *33*, 37–38.

Greenhalgh, R. Plant Research Centre, Agriculture Canada, Ottawa, Ontario K1A 0C6, unpublished data, 1986.

Greenhalgh, R.; Meier, R.-M.; Blackwell, B. A.; Miller, J. D.; Taylor, A.; ApSimon, J. W. *J. Agric. Food Chem.* **1984**, *32*, 1261–1264.

Greenhalgh, R.; Levandier, D.; Adams, W.; Miller, J. D.; Blackwell, B. A.; McAlees, A. J.; Taylor, A. *J. Agric. Food Chem.* **1986a**, *34*, 98–102.

Greenhalgh, R.; Meier, R.-M.; Blackwell, B. A.; Miller, J. D.; Taylor, A.; ApSimon, J. W. *J. Agric. Food Chem.* **1986b**, *34*, 115–118.

Greenhalgh, R.; Blackwell, B. A.; Paré, J. R. J.; Miller, J. D.; Levandier, D.; Meier, R.-M.; Taylor, A.; ApSimon, J. *Mycotoxins and Phycotoxins*; Steyn, P. S., Vleggar, R., Eds.; Elsevier: Amsterdam, 1986c; pp 137–152.

Ichinoe, M.; Kurata, H.; Sugiura, Y.; Ueno, Y. *Appl. Environ. Microbiol.* **1983**, *46*, 1364–1369.

Lauren, D. R.; di Menna, M. E.; Greenhalgh, R.; Miller, J. D.; Neish, G. A.; Burgess, L. W., submitted for publication in *Appl. Environ. Microbiol.* **1987**.

Marasas, N. F. O.; Nelson, P. E.; Toussoun, T. A. *Toxigenic Fusarium Species, Identity and Mycotoxicology*; Pennsylvania State University: University Park, PA, 1984.

Neish, G. A.; Cohen, H.; Nelson, P. E. *Fusarium Notes* **1985**, *4*, 2–4.

Tatsuno, T.; Morita, Y.; Tsunoda, H.; Umeda, M. *Chem. Pharm. Bull.* **1970**, *18*, 1485–1488.

Received for review September 15, 1986. Accepted May 11, 1987. This work was supported by grants from Ottawa Carleton University Institute, the Stapledon Memorial Trust, and the Trimble Agricultural Fellowships Fund.

Minor Components from Growing Buds of *Artemisia capillaris* That Act as Insect Antifeedants

Katsumi Yano

Capillin, capillarin, methyleugenol, *ar*-curcumene, and bornyl acetate were isolated as the minor component from the growing buds of *Artemisia capillaris*. These compounds showed an antifeeding activity to the larva of the cabbage butterfly. Relationships between the activity and the chemical structure of whole components are also discussed.

Isolation of 1-phenyl-2,4-pentadiyne and capillen from the growing buds of *Artemisia capillaris* and their antifeeding activity for larvae of the cabbage butterfly, *Pieris rapae crucivora*, were already reported (Yano, 1983). Relationships between the antifeeding activity for larvae and the chemical structure of phenylalkynes of the type $C_6H_5C{\equiv}CR$ were also previously studied (Yano, 1986). Miyazawa and Kameoka (1977) reported chemical components in the essential oil from aerial parts of *A. capillaris*. In this paper, isolation of the minor components in the viscous substance secreted from the growing buds of *A. capillaris* and their antifeeding activity for larvae of the cabbage butterfly are reported. Relationships between the activity and the chemical structure of whole components are also discussed.

EXPERIMENTAL SECTION

Isolation of Chemical Components. Four grams (0.23% yield) of the essential oil were obtained from the growing buds (1739 g, in June) of *A. capillaris* according to the previous paper (Yano, 1983). The oil was chromatographed on the silica gel column (100–200 mesh, 70 g, l = 63 cm, d = 1.8 cm) and divided into three fractions: terpene hydrocarbons (3% of the oil), phenylalkynes (83%), and polar components (14%).

Gas Chromatograph. A Shimadzu GC-3BT was operated to isolate the minor components under the following conditions. For γ-terpinene (**5**) and caryophyllene (**6**): 25% PEG 6000 column [3 mm × 3 m, temperature 155 °C]; carrier gas, He at 30 mL/min; range, 2 mV; filament current, 80 mA. For bornyl acetate (**7**) at 160 °C and methyleugenol at 170 °C: 10% silicone DC 560 [3 mm × 4.4 m]; carrier gas, He at 30 mL/min; range, 2 mV; filament current, 80 mA.

Biological Activity. A leaf disk (d = 2 cm) of cabbage, *Brassica oleracea* var. capitata, was punched out with a cork borer. The larvae of the cabbage butterfly were collected at cabbage field. After being held in a breeding box for 1 day, larvae in the 5th instar (weight 0.2000–0.2600 g) were used for a feeding test. In the previous paper (Yano, 1983), the sample disks were prepared by coating a liquid compound on the surface of a leaf disk. But it was difficult to coat a liquid compound equally on the surface of a disk and impossible to coat a crystalline compound in the state of solid. In these studies, sample disks were dipped into acetone solution (10^{-1} mol/L) of the compounds for 2–3 s according to the method of Hosozawa et al. (1974), and control disks were dipped into acetone. These treated disks were allowed to stand under a draft to evaporate the acetone. In the beaker (100 mL, d = 5 cm, h = 7 cm) a sample disk (left side), a control disk (right side), and larva (between both disks) were placed. The temperature was kept at 23–24 °C. After 2 h, two leaf disks were removed, the eaten area of a disk was measured, and its percentage was calculated. This leaf disk test was repeated about 10 times for every chemical components isolated from the essential oil. From the results of 10

Department of Chemistry, Fukuoka University of Education, Munakata, Fukuoka 811-41, Japan.

0021-8561/87/1435-0889$01.50/0 © 1987 American Chemical Society