Production and Characterization of Deoxynivalenol and Other Secondary Metabolites of *Fusarium culmorum* (CMI 14764, HLX 1503)

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The production of 3-acetyldeoxynivalenol (ADON) by *Fusarium culmorum* (CMI 14764) in liquid culture is discussed, together with the chemical hydrolysis of ADON to deoxynivalenol (DON). Two forms of DON, mp 153–155 and 176–178 °C, are reported; the ^{1}H NMR of the latter showed the presence of bound water. ADON was the major secondary metabolite produced by the fungi. In addition, three minor metabolites, i.e. culmorin, sambucinol, and 7,8-dihydroxycalonectrin, and trace amounts of 10 other metabolites, i.e. calonectrin, culmorone, 3-deacetylcalonectrin, 15-deacetylcalonectrin, 8-keto-15-deacetylcalonectrin, dideacetylcalonectrin, 8-hydroxycalonectrin, 8-ketocalonectrin, isotrichodermin, and sambucoin, were isolated. These compounds were characterized by MS and ^{1}H NMR. Oxidation of the C-3 position in the trichothecene molecule was common to all the compounds isolated in either the form of an hydroxy or acetoxy moiety.

INTRODUCTION

The identification of 4-deoxynivalenol (DON) in grains as the causative agent responsible for feed refusal by swine (Vesonder et al., 1973) created a demand for material to perform animal feeding trials and toxicology studies. Several procedures have been used to achieve this goal including the infection of field corn with *Fusarium graminearum* (Bennett et al., 1981; Scott et al., 1984) and the use of both solid (Vesonder et al., 1981; Greenhalgh et al., 1983; Ehrlich and Lillehoj, 1984) and liquid (Miller et al., 1983a; Greenhalgh et al., 1984a) cultures of the fungi. Field procedures produced predominantly DON and only small amounts of 3-acetyldeoxynivalenol (ADON), while the in vitro experiments produce ADON, which can be converted chemically to DON (Blight and Grove, 1974). The fact that DON is one of the main products isolated from *Fusarium*-infected crops is due in part to the hydrolysis of any ADON formed by plant enzymes (Miller et al., 1983b).

Isolates of *Fusarium culmorum* have been reported to produce a variety of secondary metabolites, including the trichothecenes, DON (Mirocha et al., 1976) and ADON (Blight and Grove, 1974), diacetoxyscirpenol (DAS), neosolaniol, T-2 and HT-2 toxins (Ueno et al., 1973), 15-diacetylnivalenol (Blight and Grove, 1974), the sesquiterpene culmorin (Ashley et al., 1937; Barton and Werstuk, 1968; Blight and Grove, 1974), the polyketide zearalenone (Caldwell and Tuite, 1970; Chelkowski et al., 1984), and 2-acetylquinazolin-4(3*H*)-one (Blight and Grove, 1974). It is now thought that the formation of DAS, neosolaniol, T-2 and HT-2 reputedly by *F. culmorum* is in error, due to an incorrect classification of the isolate (Marasas et al., 1984).

The large-scale production of ADON in liquid culture by *Fusarium roseum* (ATCC 28114) and *F. culmorum* (CMI 14764) has been reported (Greenhalgh et al., 1984a). This paper elaborates on other metabolites produced by *F. culmorum* (CMI 14764) that have been isolated during the production of ADON. The hydrolytic conversion of ADON to DON is also discussed, and some of the physical characteristics of DON are described.

EXPERIMENTAL SECTION

Materials. Samples of culmorin, 15-deacyl-8-ketocalonectrin (DEAKECAL), ADON, 7,8-dihydroxycalonectrin (DHCAL), and isotrichodermin (Greenhalgh et al., 1984b), culmorone, calonectrin, 8-hydroxycalonectrin (8OHCAL), and sambucoin (Greenhalgh et al., 1985) have been isolated previously from *F. roseum* (ATCC 28114) and characterized by mass spectrometry (MS) and ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy (NMR). Silica gel Lichroprep Si 60 (25–40 μm) was obtained from Merck, Darmstadt, Germany. The developing solvent used for all TLC experiments was 10% acetone in chloroform (0.75% alcohol). Although ADON and DON are not as toxic as HT-2 and T-2 toxins, standard laboratory practices for toxic compounds were observed when handling the crude fungal extracts and pure materials.

Instruments. Gas chromatography (GC) was performed with a Varian Model 6000 gas chromatograph equipped with a ^{63}Ni electron-capture detector. Separation was achieved on a glass column (2 m × 0.45 cm i.d.) packed with 3% OV-3 on 100–120-mesh Chromosorb W HP. The carrier gas flow was 25 mL/m argon/methane. The column temperature was programmed from 160 to 230 °C at 2°/min; the detector temperature was maintained at 300 °C and that of the injector at 230 °C. High-resolution electron-impact (EI) and fast atom bombardment (FAB) MS were recorded on a Finnigan MAT 312 mass spectrometer. Xenon (99.995%, Matheson) was used as the bombardment gas at 8 kV, and the resulting ions were extracted into the mass analyzer at an acceleration potential of 3 kV. Mass spectra were calibrated against perfluorokerosene; the relative peak intensity (%) is indicated in parentheses. ^{1}H NMR spectra were obtained on a Bruker WM 250-MHz spectrometer, in deuteriochloroform ($CDCl_3$). Chemical shifts are referenced to $CDCl_3$ at 7.24 ppm and are reported in ppm.

Production of 3-Acetyldeoxynivalenol. The preparation of the inoculum and production cultures, together with the fermentation procedure for the production of ADON from *F. culmorum* (CMI 14764, HLX 1503), has been described (Greenhalgh et al., 1984a). Minor modifications to that procedure have since been introduced. In a typical batch, an inoculum (1.5 L) in a medium (30 L) containing glucose malt extract, yeast extract peptone (60 g each), inorganic salts, and water was added to the production medium (glycerol, sucrose, salts; 180 L), which was stirred at 350 rpm at 28 °C. Air was sparged into the fermentor at 71 L^{-1} min. After inoculation, the pH was 5.8, but fell to 4.1 as the culture grew, where it was

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maintained. Prior to harvesting the culture after 8 days, the pH was increased to 7.0 by the addition of 20% K_2CO_3. The culture was filtered and the mycelium washed. The combined filtrate (160 L) and washings were treated with NaCl (15 kg) and extracted with methylene chloride (3 × 50 L). The combined extracts were evaporated in a cyclone evaporater (5 °C (100 torr)), to ca. 3 L, and then this concentrate was taken to dryness in vacuo. The residue (ca. 400 g) was dissolved in methanol (2 L) and water (100 mL), extracted with petroleum spirits (bp 30–60 °C, 3 × 2 L) and the methanol raffinate evaporated to dryness (325 g; 34 ± 4% ADON, 730 mg/L of culture).

An aliquot of the crude fungal extract (40 g) was dissolved in hot absolute ethanol (50 mL) and hexane (140 mL) slowly added. On cooling, the solution deposited crystals (A) that were filtered off, and the mother liquor was taken to dryness to yield a brown viscous oil (B). The crystals (A) were dissolved in methylene chloride (ca. 70 mL) and chromatographed on an activated Florisil column (25 cm × 5 cm i.d., 100–200 mesh, 100 g), and the ADON was eluted with 1% methanol in methylene chloride (2.4 mL). The product was recrystallized from ethanol/hexane to give ADON (I): 11.8 g; fine colorless needles; mp 180–183 °C; $[\theta]_{240} = -190°$, $[\theta]_{320} = +27°$ (c 1.57, MeOH).

Hydrolysis of 3-Acetyldeoxynivalenol. ADON (5.5 g) was dissolved in methanol (100 mL) and passed through a Rexyn 201 (OH^-) ion-exchange column (20 cm × 3 cm i.d., 50 g) previously conditioned with methanol. The column was washed with methanol (1.5 L), and the filtrate and washings were combined and evaporated to give a thick oil (4.6 g, 87% yield). Treatment of this material with ethanol–diethyl ether gave colorless prisms of DON (II, form A): mp 153–155 °C; Anal. Calcd for $C_{15}H_{20}O_6$: C, 60.78; H, 6.81; O, 32.4. Found: C, 60.91; H, 6.90; O, 32.2. M/z 296.12645, calcd 296.12606; $[\alpha] = +6.72$ (c 0.5, EtOH), $[\theta]_{240} = -185°$, $[\theta]_{320} = +29°$ (c 1.86, MeOH). The MS and NMR data were in agreement with those previously reported (Blackwell et al., 1984).

On two occasions, the product of hydrolysis gave crystals that melted at 176–178 °C (DON, form B). A differential scanning calorimeter indicated this material first softened at 151–154 °C, crystallized, and then remelted at the higher temperature. Anal. Calcd for $C_{15}H_{20}O_6$: C, 60.78; H, 6.81; O, 32.4. Found: C, 60.7; H, 6.84; O, 32.8. M/z 296.12675, calcd 296.12606; $[\alpha] = 218$ nm, ϵ 6890 (c 0.054, EtOH); $[\theta]_{240} = -205°$, $[\theta]_{390} = +32°$ (c 2.19, MeOH); ^{1}H NMR δ 1.74 (1 H, t, 15-OH, $J_{15,OH} = 5.6, 4.7$ Hz), 2.05 (1 H, d, 3-OH, $J_{3,OH} = 4.0$ Hz), 3.73, 3.89 (2 H, H-15, quar, $J_{15,OH} = 5.6, 4.7$ Hz, $J_{AB} = 11.7$ Hz), 4.53 (1 H, d quar, H-3, $J_{3,OH} = 4.0$ Hz, $J_{2,3} = 4.5$ Hz, $J_{3,4} = 10.6, 4.6$ Hz). Heating DON form B at 90 °C in vacuo for 18 h converted it to DON form A.

Chromatography of Oil B. The brown viscous oil remaining after removal of ADON was analyzed by FAB/MS, and the spectrum showed ions m/z 267, 339, 383, and 477, suggesting the presence of sambucinol (M + H), ADON (M + H), DHCAL (M + H), and culmorin (2M + H), respectively, as minor metabolites (Paré et al., 1985). Their presence was confirmed by GC of the heptafluorobutryl-derivativatized oil, which showed peaks at R_r 10.29, 17.49, 24.40, and 4.94 min respectively, corresponding to these compounds.

A sample (10 g) of oil B was dissolved in methylene chloride and chromatographed on Silica gel (1 kg, activated overnight at 125 °C). It was slurried in methylene chloride and packed in a column (1.75 m × 5 cm i.d.). The eluting solvent was passed through the column at 0.8 mL/M by an Eldex pump (Model 1001/19-120-S) and fractions (ca. 17 mL) were collected on a fraction collector (Ultrorac 7000, LKB Produkter AB, Sweden). The initial solvent was 25% ethyl acetate in methylene chloride (5 L), changing to 2% methanol in methylene chloride (1.5 L), 5% methanol in methylene chloride (5 L), and methanol (3 L). Every third fraction was analyzed by TLC, and the fractions were subsequently bulked according to their contents. Metabolites were obtained pure by further chromatography of the bulked samples on Whatman Prep-TLC plates (20 cm × 20 cm × 250 cm).

Fractions 122–197 were combined and after TLC gave a compound with the following analysis: R_f 0.62; MS, m/z 91 (base), 105 (72), 123 (70), 95 (44), 81 (30), 202 (27), 262 (21), 290 (20) 350 (M^+); ^{1}H NMR, indicated it to be calonectrin (III), first isolated by Gardner et al. (1972). Fractions 130–141 contained small amounts of a second compound with the following analysis: R_r 0.28; MS, m/z 91 (base), 220 (82), 123 (82), 248 (80), 106 (70), 308 (M^+); NMR δ 0.79 (3 H, s, CH_3-14), 1.70 (3 H, CH_3-16), 2.02 (3 H, CH_3-15Ac), 1.9–2.3 (6 H, H-4,7,8, unresolved m), 2.82, 3.06 (2 H, H-13, $J_{AB} = 4.0$ Hz), 3.48 (1 H, H-2, $J_{2,3} = 4.6$ Hz), 3.82, 4.06 (2 H, H-15, $J_{AB} = 12.2$ Hz), 4.13 (1 H, H-11, $J_{11,10} = 5.4$ Hz), 4.43 (1 H, H-3, $J_{3,2} = 4.6$, $J_{3,4} =$ ca. 6, 9.5 Hz), 5.48 (1 H, H-10, $J_{10,11} = 5.4$, $J_{10,16} = 1.4$ Hz). These data indicate the compound to be 3-deacetylcalonectrin (3DEACAL) (IV) first synthesized by Gardner et al. (1972). Another compound with an R_r similar to that of calonectrin was also isolated from this TLC band [MS, m/z 124 (base), 93 (94), 108 (78), 79 (38), 277 (26), 173 (23), 133 (23), 159 (18), 202 (17), 292 (15) (M^+)]; it had a ^{1}H NMR identical with that of isotrichodermin (V).

Fractions 198–206 yielded one principal band, R_f ca. 0.40, that on further TLC purification resulted in the isolation of two compounds. The more polar, R_f 0.36, was identified as sambucoin (VI) by comparison of its MS [m/z 93 (base), 109 (67), 124 (53), 69(40), 81 (38), 235 (27), and 250 (25) (M^+)] and ^{1}H NMR spectra (Greenhalgh et al., submitted). The other compound [R_r 0.40, mp 118–119.5 °C] had mass [m/z 110 (base), 123 (48), 218 (42), 69 (36), 95 (34), 236 (17) (M^+)] and ^{1}H NMR spectra identical with those of culmorone (VII). Further investigations have revealed that culmorone eluted in fractions 232–280 and sambucoin in 198–239.

TLC of fractions 198–280 also showed a minor band, R_r 0.53, that on elution gave an oil: MS, m/z 43 (base), 121 (139), 109 (19), 262 (18), 203 (13), 364 (11) (M^+); ^{1}H NMR δ 0.81 (3 H, s, CH_3-14), 1.82 (3 H, H-16, $J_{16,10} = 1.6$ Hz), 1.96 (3 H, CH_3-15, Ac), 2.14 (3 H, CH_3-3Ac), 1.95–2.23 (2 H, H-4, m), 2.51, 2.85 (2 H, H-7, $J_{AB} = 16.0$, $J_{7\alpha,15} = 1.5$ Hz), 2.88, 3.12 (2 H, H-13, $J_{AB} = 3.8$ Hz), 3.84 (2 H, H-2, $J_{2,3} = 4.6$ Hz), 4.07 (2 H, H-15, m), 4.44 (1 H, H-11, $J_{11,10} = 5.1$ Hz), 5.23 (1 H, H-3, $J_{3,2} = 4.6$, $J_{3,4} = 5.5, 10.4$ Hz), 6.55 (1 H, H-10, $J_{10,11} = 5.1$, $J_{11,16} = 1.6$ Hz). These data are consistent with the structure 3,15-diacetoxy-12,13-epoxytrichec-9-en-8-one (8-ketocalonectrin, KECAL, VIII).

Fractions 236–259 yielded a compound with the following analysis: R_r 0.11; MS, m/z 91 (base), 109 (92), 81 (80), 140 (79), 123 (61), 266 (7) (M^+); ^{1}H NMR δ 0.89 (3 H, H-14), 1.73 (3 H, H-16), 2.84, 3.06 (2 H, H-13, $J_{AB} = 3.9$ Hz), 3.45, 3.69 (2 H, H-15, $J_{AB} = 12.0$, $J_{\alpha,OH}$,$J_{\beta,OH} = 5.2, 5.7$ Hz), 3.49 (1 H, H-2, $J_{2,3} = 4.6$ Hz), 4.04 (1 H, H-11, $J_{11,10} = 5.8$ Hz), 4.43 (1 H, H-3, $J_{3,2} = 4.5$, $J_{3,4} = 3.4, 7.5$ Hz), 5.48 (1 H, H-10, $J_{10,11} = 5.8$, $J_{10,16} = 1.2$ Hz). These data are consistent with the structure 3,15-dihydroxy-12,13-epoxytrichothec-9-ene (3,15-dideacetylcalonectrin, DIDEACAL, IX).

Fractions 260–445 contained a compound, R_r 0.25, that crystallined from ethyl acetate, mp 180–183 °C, and was identified as 3-ADON; it was present only in trace amounts

due to incomplete removal from the crude fungal extract. A second compound, R_f 0.21, was isolated and characterized as 15-deacetyl-8-ketocalonectrin (DEAKECAL, X): mp 201–202.5 °C; MS, m/z 109 (base), 91 (53), 79 (38), 121 (32), 203 (28), 292 (22), 231 (18), 161 (18), 175 (16), 322 (12, M^+).

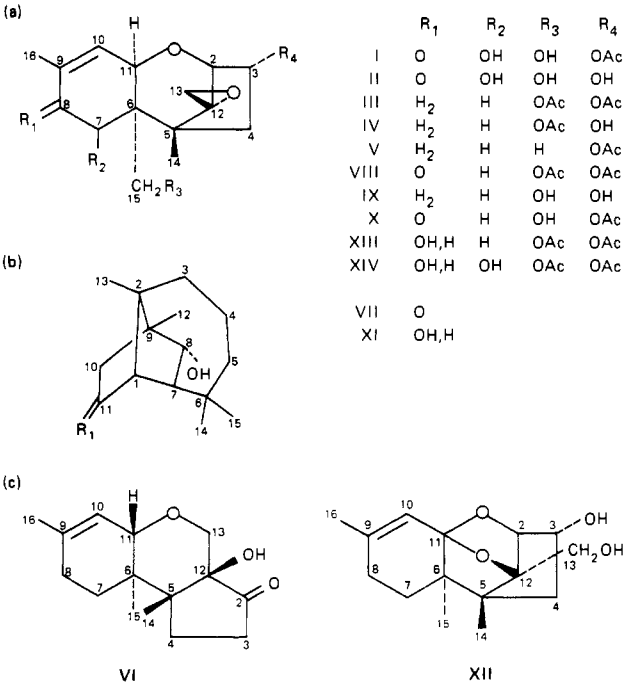
Fractions 569–604 on TLC gave four TLC bands at R_f 0.38, 0.33, 0.30, and 0.27 (1% ethanol in ether). One compound was identified as culmorin (XI): mp 178–180 °C; MS, m/z 95 (base), 109 (90), 121 (87), 135 (60), 149 (54), 177 (48), 220 (48), 205 (20), 191 (18), 238 (10, M^+); confirmed by ^{1}H NMR. The second compound was recrystallized from methanol to give white prisms: mp 234–236 °C; MS, m/z 124 (base), 82 (78), 109 (29), 95 (24), 161 (22), 67 (21), 251 (20), 266 (22) (M^+); ^{1}H NMR δ 0.81 (3 H, H-15, $J_{15,7}$ = 0.5 Hz), 1.04 (3 H, s, H-14), 1.48 (1 H, H-4α, J_{AB} = 15.1, $J_{4\alpha,2}$ = 0.9, $J_{4\alpha,3}$ = 4.3 Hz), 1.73 (3 H, br s, H-16), 1.34, 1.93 (2 H, dqu, m, H-7, J_{AB} = 13.0, $J_{7,8}$ = 5.3, 1.7 Hz), 1.80, 2.02 (2 H, H-8, m, coupling unresolved), 2.21 (1 H, 13-OH, $J_{13,OH}$ = 6.9, 3.5 Hz), 2.40 (1 H, 3-OH, $J_{3,OH}$ = 6.5 Hz), 2.57 (1 H, H-4β, J_{AB} = 15.1, $J_{4\beta,3}$ = 8.1 Hz), 3.90 (1 H, br s, H-2), 4.03, 4.10 (2 H, H-13, J_{AB} = 11.2, $J_{13,OH}$ = 6.9, 3.5 Hz), 4.22 (1 H, H-3, $J_{3,OH}$ = 6.5, $J_{3,4}$ = 8.1, 4.3 Hz), 5.42 (1 H, H-10, mult). It was thought to be sambucinol (XII) (lit. mp 227 °C; Mohr et al., 1984). This was confirmed by X-ray crystallography: orthorhombic, space group $P2_12_12_1$, a = 6.587 (1) Å, b = 13.072 (1) Å, c = 15.521 (1) Å, T = 24 °C. The third compound was identified as 8-hydroxycalonectrin (80HCAL, XIII) by its mass spectrum [m/z 121 (base), 105 (93), 111 (80), 306 (80), 288 (78), 91 (64), 137 (54), 187 (43), 159 (41), 145 (38), 215 (38), 262 (34), 275 (30), 202 (33), 266 (20, M^+)] and ^{1}H NMR. The fourth band was 7,8-dihydroxycalonectrin (DHCAL, XIV); its structure was confirmed by comparison of its mass spectrum [m/z 82 (base), 100 (27), 109 (20), 121 (18), 136 (14), 291 (10), 382 (5) (M^+)] and ^{1}H NMR.

RESULTS AND DISCUSSION

In general, liquid cultures are the preferred method of biosynthesis for large amounts of material because of the ease of product workup. In the case of the production of DON, liquid cultures of *F. culmorum* or *F. roseum* offer an additional advantage in that the 3-ADON produced is much easier to purify than DON. Recently, Ehrlich and Lillehoj (1984) utilized the triacetyl derivative of DON to facilitate the isolation of DON from a corn matrix. In the original procedure ADON was hydrolyzed with NaOH (1.1 equiv) in ethanol (95%) at room temperature for 24 min. The sodium acetate formed was removed by an ion-exchange resin to give DON (93% yield). Currently the hydrolytic step is achieved by using an OH^- ion-exchange resin in methanol, which gives a product more easily crystallized although with a slightly lower yield of DON (87%).

Attempts to assay the purity of DON by capillary column GC/MS, with on-column injection of underivatized DON, led to the observation that both the solvent and the amount injected affected the result. In methanol, approximately 8% degradation of DON was observed as opposed to less than approximately 1% with ethyl acetate. The principal product was isoDON. Injection of 250 ng or less gave reproducible results, the amount coincidental with the maximum load capacity of the capillary column.

Although Blight and Grove (1974) reported that ADON was dimorphic, this phenomenum was not found in our work. However, two forms of DON were isolated, one that melted at 153–155 °C (form A) and the other at 176–178 °C (form B), which differed only in the ^{1}H NMR spectra. In form A, all the OH protons were clearly resolved, the



	R_1	R_2	R_3	R_4
I	O	OH	OH	OAc
II	O	OH	OH	OH
III	H_2	H	OAc	OAc
IV	H_2	H	OAc	OH
V	H_2	H	H	OAc
VIII	O	H	OAc	OAc
IX	H_2	H	OH	OH
X	O	H	OH	OAc
XIII	OH,H	H	OAc	OAc
XIV	OH,H	OH	OAc	OAc
VII	O			
XI	OH,H			

Figure 1. Structures of the secondary metabolites of *F. culmorum* (CMI 14764, HLX 1503): (a) trichothecenes; (b) culmorins; (c) modified trichothecenes.

3-OH appeared as a doublet (2.06 ppm), 15-OH as a triplet (1.74 ppm), and the 7-OH as a doublet (3.86 ppm), indicating that none of the hydroxyl protons had exchanged. Coupling with adjacent methylene protons resulted in the appearance of the C-15 AB system as a double quartet at 3.73 and 3.89 ppm. In form B, the C-15 AB resonance collapsed to a simple quartet, and the 3-OH and 15-OH resonances are broad, suggesting that these OH protons, but not the 7-OH, exchange with trace amounts of water in the sample. Free water was detected in small amounts in form A and showed as a sharp resonance at 1.63 ppm. On heating form B to 160 and 180 °C, the NMR spectra of the two products were identical with that of form A and showed resonances for free water. The two forms of DON thus appear to differ only in the type and extent of hydration of the crystal lattice, which in form B appears bound in a polar ratio of 1:4. This interpretation is reinforced by the observation that the circular dichroic dispersion of the two forms were identical. Thus, the molecular environment of the carbonyl functionality in the two forms is the same and does not involve a hemiketal form as suggested by Blight and Grove (1972).

Gardner et al. (1972) reported the isolation of calonectrin and its 15-deacetyl derivative (15-DEACAL) from *Calonetria nivalis* (=*F. culmorum*; Marasas et al., 1984). The latter compound was isolated from *F. roseum* (Greenhalgh et al., submitted), but in the case of *F. culmorum*, 3-DEACAL is isolated. In the NMR spectrum, resonances attributed to H-2 (3.46 ppm) and H-3 (4.43 ppm) are upfield of those of calonectrin (3.72 and 4.14 ppm, respectively); these chemical shifts are typical of 3-deacetylated trichothecenes (Blackwell et al., 1984). Deacetylation of the C-15 position in CAL results in both an upfield shift of the AB system (3.45, 3.68 ppm) and the formation of a double quartet due to coupling with the 15-OH proton. The magnitude of the coupling constants differs slightly ($J_{15\alpha,OH}$ = 5.4 Hz, $J_{15\beta,OH}$ = 5.7 Hz), which suggests restricted rotation about the C–O bond. The acetyl moieties of calonectrin do not appear to be too stable. The occurrence of 3-DEACAL and CAL in fractions 122–197

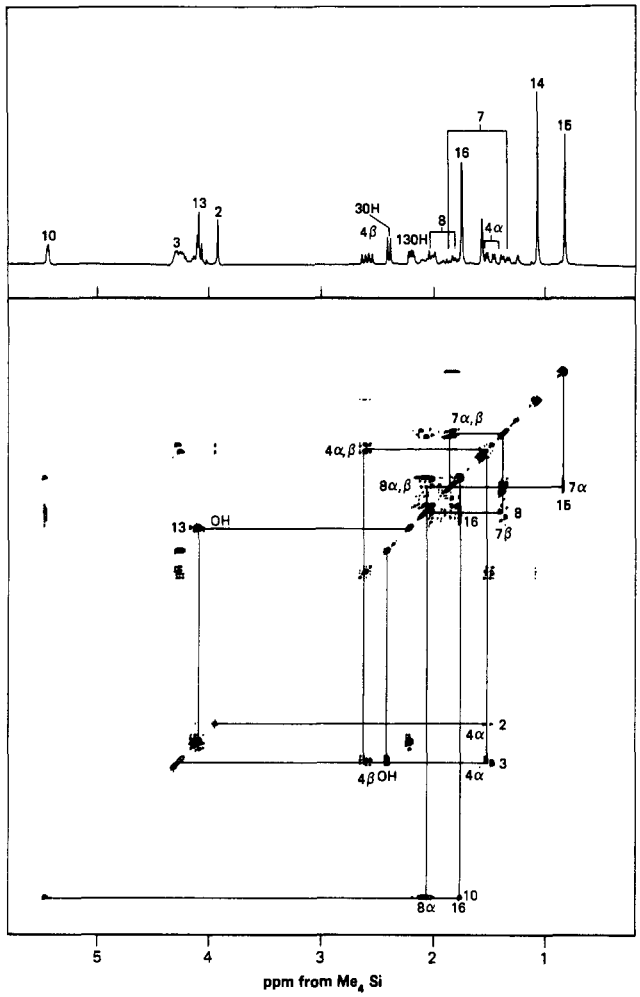


Figure 2. 250-MHz $^1H/^1H$ homonuclear correlation NMR spectrum (COSY-45) of sambucinol in $CDCl_3$. 512 × 1K FIDs were acquired with 16 transients each. Sine bell windows (shifted $\pi/4$ in f_1) were used for a 1K × 2K transform for a final resolution of 1.4 Hz/pt. The 16K dp 1D spectrum is shown above the plot, and resonances are labeled according to the numbering system in Figure 1.

suggests some degradation on workup since their respective R_f (0.28, 0.62) differ appreciably, and they should be readily resolved on the column. Similarly, the isolation of DIDEACAL (fractions 236–259) could indicate that some degradation of calonectrin occurred prior to chromatography.

Sambucinol has recently been isolated from *Fusarium sambucinum* and characterized (Mohr et al., 1984). X-ray crystallographic data collected on the sample isolated in our work were in complete accord with those described by the Swiss workers. The ^1H NMR assignments were further elaborated by a $^1H/^1H$ homonuclear correlation spectrum (Figure 2). The multiplet (5.42 ppm) due to H-10 shows long-range coupling with H-16 and H-8α. The H-2 resonance is a broad singlet, showing only long-range coupling with H-4α, $J_{2,3}$ being negligible. The COSY spectrum indicated that the fine splitting observed at H-4α (0.9 Hz) but not H-4β is due to long-range coupling with H-2. The AB systems of the C-7 and C-8 methylene protons are complicated by long-range coupling with H-8α and H-16 and overlap of the H-8 and H-7α protons. The coupling constants, $J_{7,8}$ = 5.3, 1.7 Hz, were measured at the resolved H-7α resonance. Coupling with H-10 conformed assignment of H-16 resonance (1.73 ppm). The distinction between H-14 (1.05 ppm) and H-15 (0.81 ppm) was made on the basis of long-range coupling between H-15 and H-7α. The assignments of the 3-OH and 15-OH protons at 2.40 and 2.21 ppm, respectively, were confirmed by the ^1H NMR spectrum in CH_3OD.

In addition to ADON, the other minor metabolites isolated were culmorin, culmorone, 3- or 15-DEACAL, DHCAL, DIDEACAL, CAL, sambucoin, and sambucinol, together with traces of other trichothecenes. All the trichothecenes isolated to date that are produced by this *F. culmorum* isolate are oxygenated at the C-3, not the C-4 position, with varying states of oxidation at positions C-7, C-8, and C-15. This is an analogous situation to that previously observed with *F. roseum* (Greenhalgh et al., 1984b and submitted). The two species differ in that *F. culmorum* appears to have enzymes present capable of deacylating both the 3- and 15-acetoxy moieties, whereas *F. roseum* predominantly deacylates the 15-acetoxy moiety.

Trichothecenes and related compounds, i.e. sambucinol, are postulated to be biosynthesized by the mevalonate pathway. The isolation of culmorin, which is also mevalonate derived (Hanson and Nyfeler, 1976), indicates that competing biosynthetic pathways are operating in this organism. Studies to determine the factors that influence the various biosynthetic pathways with a view to defining the processes involved are currently under way.

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Toxicity of Methanethiol Produced by *Brevibacterium linens* toward *Penicillium expansum*

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Methanethiol production was studied in cultures of *Brevibacterium linens* ATCC 8377 grown upon three different media. *B. linens* did not produce methanethiol when grown on a medium lacking methionine but did produce methanethiol and methyl disulfide when grown on a defined medium containing methionine and an undefined medium containing Trypticase. Methanethiol concentrations produced by *B. linens* were capable of inhibiting germination of spores of *Penicillium expansum* NRRL 877 (toxic concentrations being above 0.33 ppm methanethiol).

The characteristic aroma and flavor of surface-ripened cheeses result in part from the proteolytic activity of microorganisms found in the surface smear of these cheeses. *Brevibacterium linens*, the dominant bacterium of the smear, produces proteolytic enzymes and is responsible for the pungent aroma associated with these cheeses (Boyavel and Desmazeaud, 1983; Parliment et al., 1982; Langhus et al., 1945). Grill et al. (1967) found that the surface smear of Trappist cheese was necessary for production of methanethiol from methionine. Methanethiol, hydrogen sulfide, and methyl thioacetate are important sulfur-containing compounds in the aroma of surface-ripened cheeses (Dumont et al., 1974, 1976; Parliment et al., 1982).

A notable aspect of Limburger, Trappist, and other surface-ripened cheeses is their inability to support mold growth (Grecz et al., 1959; Shih and Marth, 1972). Grecz et al. (1961, 1962) investigated the possibility that an antibiotic produced by *B. linens* was responsible for the resistance to molding; however, the antibiotic was not identified. More recent work conducted with brevibacteria from human skin determined these bacteria produced an antibiotic active against gram-positive and -negative bacteria (Ryall et al., 1981; Al-Admany and Noble, 1981). Ryall et al. (1981) postulated that methanethiol produced by brevibacteria may inhibit the growth of dermatophytic fungi. Whether a constituent of surface-ripened cheese aroma is responsible for the resistance to molding exhibited by surface-ripened cheeses has not been examined.

Volatile compounds produced by *B. linens* have been demonstrated to inhibit spore germination and mycelial growth of several mold species including several commonly reported on spoiled cheese (Beattie and Torrey, 1985), and inhibition of mold growth depended upon the presence of methionine in bacterial growth media. *Penicillium expansum*, the most resistant of molds tested, was selected as the test organism for this study. We report herein on the toxicity of methanethiol on spores of *P. expansum*, the rate of production of methanethiol by *B. linens*, and use of polyester/polyethylene laminate pouches as controlled atmosphere growth chambers.

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MATERIALS AND METHODS

Organisms and Culture Conditions. *B. linens* ATCC 8377 was obtained from the American Type Culture Collection, Rockville, MD, and *P. expansum* NRRL 973 was obtained from the Northern Regional Research Center, Peoria, IL. Malt agar (Difco Laboratories, Detroit, MI) supplemented with 3 g/L of yeast extract (MYE) was used for growth and maintenance of *P. expansum*. A medium (TYE) containing Trypticase (BBL Microbiology Systems, Cockeysville, MD; 10 g/L), yeast extract (Difco; 5 g/L), and agar (Difco; 5 g/L) was used for growth and maintenance of *B. linens*.

A defined medium (DMM), which was also used for bacterial growth, contained buffered mineral salts solution (Meynell and Meynell, 1970), amino acids (0.125 μg/mL each) alanine, aspartic acid, glutamic acid, lysine, methionine, and tryptophan (all Sigma Chemical Co., St. Louis, MO), and vitamins (per L) biotin (5 μg), folic acid (50 μg), and thiamine (1.0 mg) (all Sigma). Methionine could be excluded from the medium (DMA) without affecting bacterial growth. When required, media were solidified with 15 g of Noble agar (Difco) per liter. The pH of each medium was 7.0.

Bacterial inocula were prepared in DMA broth; these cultures were incubated aerobically at 30 °C for 12-18 h. Petri plates containing TYE, DMM, or DMA agar were inoculated by spreading 0.5 mL of the broth culture of *B. linens* on the agar surface. Inoculated plates were placed in flexible pouches (16.2 cm × 24.4 cm) composed of polyester (0.5 mil)/polyethylene (1.5 mil) laminate (Dazey Corp., Industrial Airport, KS). Oxygen-transmission rates for this laminate were in the range 11.9-27.1 cm^3/m^2 for 24 h as determined by the manufacturer using American Society for Testing and Materials Method D-3985-81

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