chromium(VI) complex in 2 mL of dry methylene chloride at room temperature. The mixture was stirred for 66 h, diluted with 5 mL of methylene chloride, and washed with water, followed by 0.1 N hydrochloric acid. The methylene chloride layer was dried over anhydrous sodium sulfate. Gas chromatographic analysis of this layer indicated a single product. Evaporation of the solvent yielded 2.5 mg (80%) of the ketone 3. Calcd for $C_{24}H_{32}O_9$; mass spectrum, $m/e^+$ 464.

*Dimethyl Sulfide–N-Chlorosuccinimide.* Dimethyl sulfide (0.14 mL, 1.4 mmol) was added to a stirred suspension of 133 mg (1.0 mmol) of *N*-chlorosuccinimide in 10 mL of dry toluene at 0 °C under dry nitrogen. A white precipitate appeared immediately after the addition of dimethyl sulfide. The mixture was cooled to −25 °C ($CCl_4$–dry ice) and a solution of 300 mg (0.64 mmol) of T-2 toxin in 8 mL of toluene was added dropwise. The stirring was continued for 2 h and then a solution of 101 mg (1.4 mmol) of $Et_3N$ in 0.5 mL of toluene was added dropwise. The cooling bath was removed and after 5 min, 20 mL of diethyl ether was added. The organic layer was washed with 10 mL of 0.1 N HCl and twice with 15 mL of water. Evaporation of the dried organic layer yielded 285 mg of gum (96% yield). Analysis by GLC and mass spectra and NMR indicated the product was 3-dehydro-T-2 toxin. Attempts to crystallize the ketone were unsuccessful. The compound deteriorated slowly during storage.

*O-Methyloxime.* The methyloxime of **3** was prepared as described by Mirocha et al. (1974) for zearalenone. The GLC analysis indicated almost quantitative conversion to the oxime. Calcd for $C_{25}H_{35}O_9N_1$; mass spectrum, $m/e^+$ 493.

**Reduction of 3-Dehydro-T-2 Toxin.** *A.* $NaB^3H_3CN$. The tritiated sodium cyanoborohydride was prepared from $^3H_2O$ as described by Borch et al. (1971). The sp act. was 0.35 μCi/mg. Reduction of 15.3 mg (33.6 mol) of **3** with 2.17 mg (34.4 μmol) of $NaB^3H_3CN$ gave tritiated T-2 toxin **6** (α epimer) in 11% yield with a sp act. of 21 μCi/mmol (0.046 μCi/mg). The product had a mass spectrum and $R_f$ value by TLC identical with those of an authentic sample of T-2 toxin.

*B.* $NaB^3H_4$. Tritiated sodium borohydride (14 μmol, 100 mCi) was weighed into a one dram vial containing a stirring bar and 27 mg (58 μmol) of the ketone **3** in 1.2 mL of isopropanol. The reaction mixture was diluted with 4 mL of chloroform and washed with 3 mL of 0.3 N HCl. The aqueous layer was extracted with 3 × 4 mL of chloroform, and the chloroform layers were dried and evaporated to dryness. The naturally occurring α epimer was separated from the β epimer by successive thin-layer chromatography in 95:5 $CHCl_3$–ethanol. Nonradioactive T-2 toxin (13.1 mg) was added after the initial chromatographic step to aid in complete recovery of the tritiated T-2 toxin. The 3-[$^3$H]T-2 toxin was obtained in 23% yield with a specific activity of 790 mCi/mmol.

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Received for review January 10, 1977. Accepted March 17, 1977. Scientific Journal Series Paper No. 9840, Minnesota Agricultural Experiment Station. This research was supported by U. S. Public Health Service Contract FDA 223-74-7229. Part of this paper was presented at the 170th National Meeting of the American Chemical Society Chicago, Ill. Aug 24–29, 1975.

# Metabolites of *Alternaria alternata*: Ergosterol and Ergosta-4,6,8(14),22-tetraen-3-one

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Ergosterol and ergosta-4,6,8(14),22-tetraen-3-one (ETO) were identified as metabolites of *Alternaria alternata* isolates from sorghum grain and wheat. Ergosterol contents ranged from 40 to 330 μg/g and ETO from 1.8 to 6.6 μg/g in 21-day cultures from 4 isolates of *A. alternata* as determined by high pressure liquid chromatography. The compounds were identified by thin-layer chromatography, and by ultraviolet, infrared, mass, and nuclear magnetic resonance (proton and carbon-13) spectroscopy. Carbon-13 magnetic resonance spectra of these metabolites are presented.

Although ergosterol is considered to be a metabolite common to all fungi (Weete, 1974), its production by *Alternaria brassicicola*, *A. kikuchiana*, and *A. alternata* (Fries) Keissler (formerly *A. tenuis* Auct.) was reported only recently (Aizina et al., 1974; Starratt, 1976). We add further evidence for production of ergosterol by *Alternaria*, specifically *A. alternata* isolated from sorghum grain and wheat.

We also report the first evidence for production of ergosta-4,6,8(14),22-tetraen-3-one (ETO) by *Alternaria*

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*alternata*. ETO has been isolated previously from *Candida utilis* (Morimoto et al., 1967), *Fomes officinalis* (Schulte et al., 1968), *Lampteromyces japonicus* (Endo et al., 1970), *Penicillium rubrum* (White and Taylor, 1970; White et al., 1973), *Balansia epichloe* (Porter et al., 1975), and *Penicillium citrinum* (Price and Worth, 1974). In addition, Price and Worth (1974) found ETO in extracts of *Aspergillus flavus*, *A. fumigatus*, *A. ochraceus*, *Fusarium moniliforme*, *Penicillium islandicum*, and *P. patulum*. Cooks et al. (1970) isolated ETO and its C-24 analogue from wheat flour molded by several species of *Aspergillus*. Porter et al. (1975) found ETO in grasses associated with toxicity to cattle.

Carbon-13 magnetic resonance spectra and application of high-pressure liquid chromatography for analyses of ergosterol and ETO in culture media are reported.

## EXPERIMENTAL SECTION

**Fungal Isolates and Culture Conditions.** Isolates of *Alternaria alternata* were from sorghum grain (isolates RL-671-2, RL-8442-3, and FN-8442-5) and from hard red spring wheat (isolates HRS-5, HRS-8) harvested in Kansas and Minnesota, respectively. Each isolate was grown at 25 °C in the dark for 21 days on yellow corn–milled rice (1:1 by weight, whole grains). The substrate contained 35% moisture at the start of growth.

**Spectroscopic Methods.** Ultraviolet spectra of the compounds in absolute ethanol were recorded with Cary Model 118 and Perkin-Elmer Model 350 spectrophotometers. Infrared spectra (KBr pellets) were obtained with a Perkin-Elmer Model 467 spectrophotometer. An AEI MS-902 mass spectrometer was used to obtain mass spectra at 70 and 18 eV. Proton and carbon-13 magnetic resonance spectra of the compounds in deuteriochloroform (100% D, Aldrich Chemical Co.) were recorded by a Varian Associates XL-100-15 spectrometer equipped with a Nicolet Model TT-100 Fourier transform accessory. The spectrometer was locked on the deuterium signal from deuteriochloroform, and chemical shifts were measured relative to tetramethylsilane ($Me_4Si$). When carbon-13 spectra were recorded, protons were noise decoupled from carbon-13.

**Extraction and Cleanup.** Fifty grams of fungal invaded substrate was blended for 2 min with 100 mL of methanol and then centrifuged. The supernatant was poured off and another 100 mL of methanol was added. The residue was resuspended, shaken vigorously for about 1 min, and recentrifuged. The two methanol supernatants were combined, mixed with 25 g of KOH and 100 mL of ethanol, and refluxed for 30–45 min. The saponified mixture was diluted with 50 mL of water and extracted with 100- and 50-mL portions of hexane. Both hexane extracts were combined and reduced in volume (over a steam bath with aid of a gentle flow of nitrogen) for further cleanup by column chromatography or analyses by TLC or HPLC. Recoveries of ergosterol and ETO from spiked, fungus-free substrate were 93 ± 5 and 66 ± 10%, respectively. The procedure was scaled up proportionately when larger amounts of substrate were extracted for isolations of ergosterol and ETO.

**Thin-Layer Chromatography (TLC).** We used Brinkman SIL G-HR-25 precoated TLC plates activated 1 h at 110 °C. Solvent systems and $R_f$ values for ETO and ergosterol, respectively, were: chloroform–acetone (88:12 v/v), 0.87, 0.63; and benzene–acetone (90:10, v/v), 0.68, 0.45. Fluorescence of ETO was observed by use of a cabinet equipped with short- (254 nm) and long-wave (366 nm) ultraviolet lights. Chromagenic sprays were 50% sulfuric acid or 20% antimony trichloride in chloroform.

**Column Chromatography.** We used a 25 mm o.d. × 35 cm column, with 300-mL reservoir, packed successively with 5 g of anhydrous sodium sulfate (bottom layer), 12 g of silica gel 60 (0.063–0.200 mm, EM Reagents), and 15 g of anhydrous sodium sulfate. The sample in hexane was placed on the column, which was then washed successively with 100 mL of hexane, 300 mL of ethyl acetate–hexane (5:95, v/v), and 200 mL of ethyl acetate–hexane (10:90, v/v). ETO was in the last 150 mL of the eluate containing 5% ethyl acetate; its elution was monitored by use of a long-wave ultraviolet light. Ergosterol was in the 10% ethyl acetate fraction.

**High-Pressure Liquid Chromatography (HPLC).** We used a Varian Associates 4200 solvent delivery system, a SIL-X-I 50 cm × 2.6 mm i.d. (6.35 mm o.d.) column from Perkin-Elmer, and a Schoeffel Model SF 770 detector. The detector was set at 282 nm (or sometimes 292 nm) for ergosterol and at 350 nm for ETO analyses. Mobile phase was isooctane–ethyl acetate (92.5:7.5, v/v) at 2.50 mL/min flow rate; ergosterol was eluted at 4.2 min and ETO at 2.5 min. Higher plant sterols such as sitosterol, campesterol, or stigmasterol were eluted along with ergosterol at 4.2 min; however, only ergosterol contributed significantly to absorption of 282 or 292 nm light.

**Reference Compounds.** Ergosterol from Eastman Kodak Company was recrystallized twice from absolute ethanol. Authentic ETO was prepared from ergosterol by the method of Elks (1954).

## RESULTS AND DISCUSSIONS

Ergosterol isolated from cultures of *Alternaria alternata*, isolates HRS-5 and HRS-8, and the reference ergosterol had identical spectroscopic and chromatographic properties. The mass spectra consisted of a molecular ion at 396 with fragment ions at 363, 337, 271, and 253, all characteristic for ergosterol (Brooks and Middleditch, 1973). Ultraviolet spectra had maxima at 293.5 nm ($\epsilon$ 6200), 281.7 ($\epsilon$ 10 850), 271.2 ($\epsilon$ 10 190), 262.5 shoulder ($\epsilon$ 7080), in good agreement with the values reported by Goulston and Mercer (1969). The isolated sample and reference standard had infrared spectra identical with those previously reported (Sadtler Standard Spectra). Proton magnetic resonance were also the same as previously reported (Lenton et al., 1973).

We isolated ETO from a culture of isolate HRS-8. The highly fluorescent substance we obtained had infrared, ultraviolet, proton and carbon-13 magnetic resonance, and mass spectra identical with those of the reference sample and to published spectra (Cooks et al., 1970; Porter et al., 1975; White et al., 1973). The two samples of ETO had identical TLC $R_f$ values in two solvent systems. When TLC plates were sprayed with 50% sulfuric acid, the ETO spots turned bright yellow under visible light, and the original blue-green fluorescence under long-wave ultraviolet light changed to a distinctive, bright green (Schulte et al., 1968).

We used carbon-13 magnetic resonance to strengthen the identification indicated by other spectroscopic and chromatographic methods. Since we could not find literature references to carbon-13 spectra of these compounds, we show the spectra in Figure 1 and list resonance positions in Table I. We did not attempt a complete assignment of all lines in each spectrum. Twenty-six and 27 lines were observed from ergosterol and ETO, respectively, whereas 28 lines are expected from each compound if all resonances are adequately resolved. The six resonances from unsaturated carbons in ergosterol were observed from 116.14 to 141.07 ppm, and the line at 12.05 ppm represents either the C-18 or C-19 methyl group

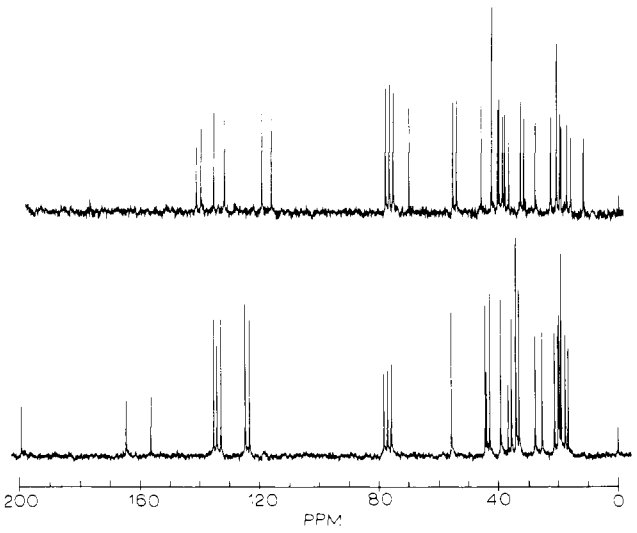


**Figure 1.** Carbon-13 magnetic resonance spectra of ergosterol (upper) and ergosta-4,6,8(14),22-tetraen-3-one (lower) in deuteriochloroform. Exact resonance positions are listed in Table I. The lines at 75.64, 76.91, and 78.18 ppm are from deuteriochloroform solvent. Spectral width was 2500 Hz with 16384 data points. Pulse duration was 35 $\mu$ s, repetition rate was 5 s, and number of accumulations was 256.

Table I. Chemical Shifts (ppm) of Carbon-13 Magnetic Resonance Lines from Ergosterol and Ergosta-2,4,6,8(14),22-tetraen-3-one (ETO) in Deuteriochloroform Relative to Tetramethylsilane (0 ppm)

| Ergosterol | ETO |
|---|---|
| 12.05 | 16.64 |
| 16.27 | 17.62 |
| 17.59 | 18.97 |
| 19.63 | 19.44 |
| 19.92 | 19.64 |
| 21.10 | 19.96 |
| 22.98 | 21.21 |
| 28.24 | 25.34 |
| 31.96 | 27.65 |
| 33.06 | 33.05 |
| 37.00 | 33.91 |
| 38.34 | 34.09 |
| 39.07 | 35.59 |
| 40.34 | 36.70 |
| 40.76 | 39.19 |
| 42.78 | 42.82 |
| 46.21 | 43.93 |
| 54.49 | 44.31 |
| 55.70 | 55.66 |
| 70.33 | 122.83 |
| 116.14 | 124.29 |
| 119.41 | 132.35 |
| 131.79 | 133.72 |
| 135.35 | 134.78 |
| 139.58 | 155.73 |
| 141.07 | 163.97 |
| | 198.97 |

carbon. The two relatively high-intensity lines at 21.10 and 42.78 ppm from ergosterol may represent two carbons each. The line at 198.98 ppm in the spectrum of ETO is due to the conjugated carbonyl at C-3. Including C-3, there are nine unsaturated carbons in ETO, for which only eight resonances were observed. Apparently one of the lines from 134.78 to 122.83 ppm represents two carbons.

Ergosterol and ETO concentrations in 21-day cultures of four *A. alternata* isolates were determined by HPLC (Table II). Concentrations were much higher, and isolate-to-isolate variations were more pronounced with ergosterol than with ETO. Production of both ergosterol and

Table II. Ergosterol and Ergosta-4,6,8(14),22-tetraen-3-one (ETO) in 21-Day Cultures of *Alternaria alternata* Isolates on Corn-Rice (1:1)

| Isolate | Ergosterol, $\mu$g/g | ETO, $\mu$g/g |
|---|---|---|
| RL-671-2 | 300 | 6.6 |
| RL-8442-3 | 330 | 6.5 |
| FN-8442-5 | 40 | 1.8 |
| HRS-5 | 120 | 3.3 |

ETO was lowest with isolate FN-8442-5. Metabolic studies have indicated that ergosterol is a precursor of ETO (Morimoto et al., 1967; White et al., 1973). The greater ergosterol production by isolates RL-671-2 and RL-8442-3 than isolates FN-8442-5 and HRS-5 was of interest because we know from previous work (unpublished) that these pairs of isolates differ in their overall metabolite production patterns; i.e., all four produce alternariol, alternariol monomethyl ether, and altenuene, but only FN-8442-5 and HRS-5 also produce tenuazonic acid, altertoxin I, and several other metabolites not yet identified (Burroughs et al., 1976; Harvan et al., 1976; Seitz et al., 1976). However, the metabolic relationship between production of ergosterol and various other metabolites by *Alternaria* remains to be determined.

We established that ergosterol and ergosta-4,6,8(14),-22-tetraen-3-one are metabolites of *Alternaria alternata*. These findings are consistent with considerations that ergosterol is common to all fungi (Weete, 1974) and that ETO occurs widely among the fungi (Price and Worth, 1974). We are presently evaluating ergosterol as a potential indicator of the extent of fungal invasion in grain (Seitz, 1976). Since *A. alternata* is frequently present in a high percentage of the kernels of certain grains, especially kernels of freshly harvested sorghum and wheat, it was necessary for us to determine conclusively whether ergosterol is a metabolite of this fungus.

ACKNOWLEDGMENT

We thank D. B. Sauer and Rosemary Burroughs for mycological assistance and H. E. Mohr for chemical laboratory assistance.

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Received for review December 13, 1976. Accepted February 28, 1977. Mention of a trademark of proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture, and does not imply its approval to the exclusion of other products that may also be suitable.

# Chemical Transformation of 4-Chloroaniline to a Triazene in a Bacterial Culture Medium

Robert D. Minard, Stefan Russel, and Jean-Marc Bollag*

A *Paracoccus* species in an anaerobic medium causes the transformation of 4-chloroaniline into a product identified as 1,3-bis(*p*-chlorophenyl)triazene. The rate at which this process takes place parallels the rate at which the *Paracoccus* species converts nitrate to nitrite and the transformation appears to involve the relatively rapid chemical reactions of diazotization and coupling.

Numerous pesticides such as phenylurea, phenylcarbamate, and acylanilide herbicides and certain acaricides release variously substituted aniline moieties on breakdown in the environment. However, our knowledge of the further transformation or fate of these intermediary products is still fragmentary. It appears that complex oligomers like azobenzenes (Bartha and Pramer, 1967) and diphenylamines (Briggs and Ogilvie, 1971) predominate at high concentrations of anilines, and at lower concentrations simpler products like acylated compounds (Tweedy et al., 1970; Bollag and Russel, 1976; Russel and Bollag, 1977) and oxidation products of the aromatic amine group (Kaufman et al., 1973) can be found. However, there are also many unidentified products reported in the literature which indicate that the biological and nonbiological transformation products from the aniline moiety have not yet been sufficiently clarified and require further attention if one is concerned with the possible impact of such chemicals on the environment.

The present paper reports the chemical transformation of 4-chloroaniline in a culture medium due to a change of pH and ion composition brought about by the metabolic activity of bacteria.

## MATERIALS AND METHODS

The bacterium of the genus *Paracoccus* used in this study was isolated from soil by enrichment culture techniques with 4-chloroaniline in the growth medium (Bollag and Russel, 1976).

The bacteria were grown anaerobically in a Czapek–Dox medium containing: saccharose, 20 g; $NaNO_3$, 3 g; $K_2HPO_4$, 1 g; $MgSO_4$, 0.5 g; KCl, 0.5 g; $FeSO_4$, 0.01 g in 1 L of distilled water (final pH 7.4). After autoclaving sterilized 4-chloroaniline was added.

The 4-chloroaniline was purchased from Aldrich Chemical Co., Inc. (Milwaukee, Wis.) and recrystallized several times from hexane; its purity was established by TLC, melting point determination, and mass spectral analysis. Uniformly $^{14}C$-labeled 4-chloroaniline was purchased from California Bionuclear Corporation (Sun Valley, Calif.) and had a sp. act. of 4.5 $\mu$Ci/mmol. The labeled compound was diluted with sufficient unlabeled material to reach the desired concentration. The 4-chloroaniline was introduced into 50 mL of medium in 0.2 mL of ethanol solution after sterilization by membrane filtration (0.22-$\mu$m pore size Millipore filter) for a final concentration of 20, 50, 75, and 100 ppm and $10^{-3}$ $\mu$Ci of radioactivity per mL.

To obtain anaerobic conditions, the flasks were flushed with helium until all air was removed (Bollag and Nash, 1974) and incubated at 28 °C.

The total number of cells of the *Paracoccus* sp. during growth in the Czapek–Dox medium was determined by the dilution plate method using nutrient agar. Plates were incubated at 28 °C for 24 h.

For experiments designed to study the chemical transformation, a modified Czapek–Dox medium was used. Instead of nitrate, nitrite was introduced into the medium and pH was adjusted to 6.0.

Nitrite was measured colorimetrically by the $\alpha$-naphthylamine–sulfanilic acid method (American Public Health Association, 1971).

For all routine analyses, 5 mL of the growth medium or chemical reaction mixture (pH adjusted to 7.0) was extracted with an equal volume of diethyl ether. The organic phase was used for measurements of radioactivity and TLC analysis.

Radioactivity was determined with a Nuclear-Chicago Isocap-300 liquid scintillation counter. The samples were measured in a cocktail composed of 60 g of naphthalene, 100 mL of methanol, and 8 g of Omnifluor [(98% PPO (2,5-diphenyloxazole–2% Bis-MSB (*p*-bis(*O*-methylstyryl)benzene); New England Nuclear Corp., Boston, Mass.)] in 1 L of dioxane.

Precoated thin-layer plates (silica gel F-254) with fluorescent indicator (layer thickness, 0.25 mm) were purchased from Brinkman Instruments, Inc. (Westbury, N.Y.). The following solvent systems were employed in all analyses: ether–hexane (4:1, v/v) and benzene–dioxane–acetic acid (90:25:4, v/v).

For routine TLC analysis, 1.0 mL of ether extract was concentrated to 0.1 mL and spotted on plates. All com-

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