Changes in Mold-Damaged Wheat Flours Stored at Various Temperatures

Fluorescent Compounds: 4,6,8(14),22-Ergostatetraen-3-one and Its C_{24}-Ethyl Analog

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A fluorescent fraction isolated from the petroleum ether extract of moldy wheat flour was purified by column chromatography and crystallized from petroleum ether in a yield of 60 mg per 6000 g of moldy flour. Mass spectrometry indicated that the chromatographically homogeneous fraction contained two compounds, $C_{28}H_{40}O$ (75%) and the higher homolog $C_{29}H_{42}O$ (25%). Elemental analysis confirmed the assigned compositions. Spectroscopic examination (mass, UV, IR, and nmr) and comparison with an authentic sample showed the major component, molecular weight 392, to be 4,6,-8(14),22-ergostatetraen-3-one. It is shown that the minor component is the C_{24}-ethyl analog, a finding of biosynthetic interest. It is suggested that the detection of the strongly fluorescent compounds may constitute a simple method for the early detection of storage damage in wheat flour.

The recent discovery of mycotoxins has stimulated considerable interest (Wogan, 1965). The structures of autofluorescent aflatoxins with molecular formulae $C_{17}H_{12}O_6$ and $C_{17}H_{12}O_7$ (and their dihydroderivatives) were described by Asao *et al.* (1963, 1965). Yokotsuka *et al.* (1967) found that about one-third of 73 industrial strains of *Aspergillia* produced autofluorescent compounds of the general formula $C_{12}H_{20}N_2O$ and $C_{11}H_{18}N_2O_2$ that had R_f values by thin-layer chromatography (tlc) comparable to those of aflatoxins. Bassir and Adekunle (1968) isolated two fluorescent compounds that had the molecular formula of $C_{24}H_{44}O_3$ and were identified as nonaromatic spatial isomers of hydroxy-conjugated fatty acids.

Cereals stored at elevated temperatures and high moisture levels show rapid increases in mold count. Increase in mold count from about 1000 to 3,000,000 is accompanied by extensive breakdown of lipids (Pomeranz *et al.*, 1956, 1968). Deterioration is also accompanied by formation of at least four compounds that show autofluorescence under ultraviolet light (Daftary and Pomeranz, 1965). We report here the characterization of two new petroleum-ether-soluble compounds isolated from moldy wheat flour (Daftary *et al.*, 1970). The compounds were absent in sound flour.

EXPERIMENTAL

Materials. Four wheat flours with a moisture content of about 18% were stored for 16 weeks at 23°, 30°, or 37° C. Petroleum ether (b.p. 35–60° C) extractable lipids decreased from 0.75 to 0.89 to 0.17 to 0.36%. The decrease in lipids was accompanied by an increase in mold count from about 100 to 2,700,000 per g of wheat flour. The most abundant molds were *Asperigillus niger*, *A. candidus*, *A. versicolor*, and *A. flavus*. From 6000 g of mold damaged flour, 12 g of petroleum-ether-extractable lipids were obtained.

Isolation of Autofluorescent Compounds. Isolation and purification of the autofluorescent compounds was made by column chromatography. Seven columns in series were used. The preparation of columns and elutions were based on the general procedures described by Barron and Hanahan (1958) and Distler and Baur (1966). Details of the procedure used in our final isolation are given in Table I; doubling the amounts of separated lipids (in earlier fractionations) yielded slightly impure preparations. Separations by tlc were described by Daftary and Pomeranz (1964). All organic solvents, analytical reagent grade, were redistilled before use. The elution from the columns was monitored by tlc or followed by UV light (long wave 3360 A). The columns were prepared by making a slurry of adsorbent with the first eluant (except in column C in which the slurry was made with petroleum ether) and allowing it to stand for 2 hr. All eluates containing fluorescent material were evaporated under vacuum (below 40° C), and the dried material was redissolved in 2 to 3 ml of solvent used in the subsequent column. The fractionation and purification were quite reproducible, provided specified solvents and adsorbents were used; change in source of materials required minor modifications in the procedure. Tlc of some fractions from column chromatographic separations are shown in Figures 1 and 2.

Analytical Methods. Elemental analyses for C, H, O, and N were made by Galbraith Laboratories, Inc., Knoxville, Tenn. Melting points were determined on a Kofler hot stage microscope. Mass spectra were determined on an MS9 mass spectrometer (Associated Electrical Industries) by the direct insertion technique, at 150° C source temperature. Exact mass measurements were made at a resolution of at least 12,000 (10% valley) using heptacosafluorotributylamine to provide reference masses. Nmr spectra were measured with a 60 Mc spectrometer (Model A-60, Varian Associates, Calif.) in spectropure carbon tetrachloride containing TMS as an internal standard. Infrared spectra were obtained by the KBr-pellet method on a Perkin-Elmer Grating Infracord Spectrophotometer, Model 457. UV spectra were measured in 95% ethanol using a Cary Recording Spectrophotometer Model 11 (Applied Physics, Corp., Pasadena, Calif.).

RESULTS AND DISCUSSION

The fluorescent material from the earlier separations was crystallized from petroleum ether to yield slightly yellow needles, m.p. 105–107° (uncorrected). The second, purer preparation of fluorescent material, prepared by chromatography of smaller quantities, melted at 107–109.5° C. The crystals, as such, or their solutions in organic solvents showed no fluorescence in ultraviolet light. The material showed a

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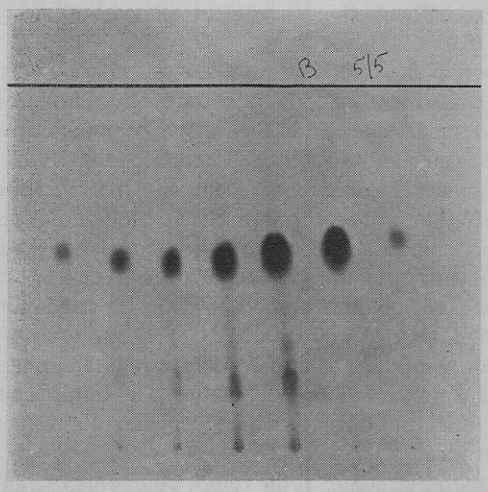

Figure 1. Tlc of the column chromatographic fractions containing the fluorescent compounds

Spots 1 and 7 represent total free lipids from the damaged flours; spots 2, 3, 4, 5, and 6 represent the eluates from columns B, C, D, E, and G, respectively (Table I). Spots, 120 γ each, were developed with chloroform:methanol (199:1); picture taken under ultraviolet light

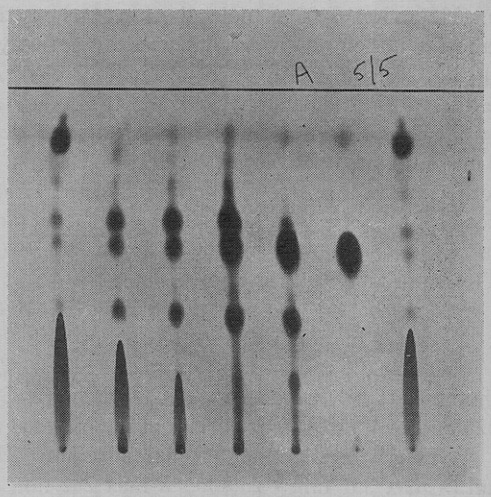

Figure 2. Tlc of the column chromatographic fractions containing the fluorescent compounds

Description of spots as in Figure 1. Picture taken under ultraviolet light after spraying with the sulfuric acid reagent and charring

strong blue fluorescence when adsorbed on silicic acid columns or on silica gel tlc plates, but not on Sephadex LH-20 columns. Spots of the compound turned greenish-yellow when sprayed lightly with a saturated solution of potassium dichromate in 70% (by volume) aqueous sulfuric acid; and turned black on heating the sprayed plates for 30 min at 180° C (after spraying). The R_f values in chloroform and in chloroform:methanol (199:1) as developing systems in tlc were 0.30 and 0.55, respectively. No systematic studies were made on the stability of the isolated material, but visual observation indicated some degradation of solutions exposed to light or heat, and of spots separated by tlc on silica gel G and stored in air. The material had a moderate solubility in carbon tetrachloride, but after being kept for several days in ethanol solution, a white carbon tetrachloride insoluble substance formed. This compound was presumably a dimer or polymer of the original material as it exhibited an R_f of 0.0 on silica gel/chloroform tlc and it was not fluorescent.

Molecular weight determination on the 107–109.5° C sample by mass spectroscopy showed that the chromatographically homogeneous fluorescent substance in fact consisted of a mixture of two compounds with molecular weights of 392 and 406. As estimated by relative molecular ion abundances, the higher homolog made up 25% of the total. The characterization of

Table I. Details of the Column-Chromatographic Procedure Used in the Isolation of Fluorescent Metabolites

Column	Column Dimensions, cm	Adsorbent, g	Material Introduced on Column	Eluant	Remarks
A	4.5 × 60	200, silicic acid,[a] 100 mesh	6 g, petroleum-ether extract	chloroform	Elution was followed by UV light. The fluorescent band (1.45 g) was collected
B	2.2 × 35	30, as in A	1.45 g from A	chloroform	As in A; 730 mg collected
C	2.2 × 35	30, as in A	730 mg from B	(i) benzene (ii) 10% ether in benzene (v/v) (iii) ether	As in A; collected 290 mg in (ii)
D	2.2 × 35	20, Sephadex LH-20[b]	290 mg from C	chloroform	Elution followed by fluorescence of tlc spots, 190 mg
E	2.2 × 35	30, silicic acid, minus 325 mesh[c]	190 mg from D	benzene	As in A; 70 mg collected
F	1.5 × 30	20, as in E	70 mg from E	(i) *n*-hexane (ii) 50% benzene in *n*-hexane (v/v)	As in A; 40 mg collected in (ii)
G	1.5 × 30	20, as in F	40 mg from F	10% ether in *n*-hexane	As in A; 29 mg of pure compound collected, and recrystallized

[a] Mallinckrodt Chemicals, N.Y.
[b] Pharmacia, Piscataway, N.J.; Sephadex LH-20 in chloroform was allowed to stand overnight. Superbrite glass beads (Minnesota Mining and Mfg. Co., St. Paul, Minn.) were used to form about 1'' thick bed on top of the Sephadex bed in the column.
[c] Bio-Rad; Richmond, Calif.

both *m/e* 392 and *m/e* 406 as molecular ions was based on the observation that their relative abundances did not vary on decreasing the electron energy to a nominal 12 eV. At that voltage, the two molecular ions were still of moderate abundance while all other ions in the spectrum had decreased rapidly in abundance on lowering the electron energy; even at 14 eV other ions were of negligible abundance. Exact mass measurements on *m/e* 392 and 406 yielded the molecular formulae $C_{28}H_{40}O$ [required 392.3079, found 392.307(1)] and $C_{29}H_{42}O$ [required 406.3236, found 406.322(0)]. The elemental analysis of the mixture (C, 85.58; H, 10.61; O, 4.07%; N, traces or none) was in agreement with the mass spectra assuming the presence of approximately 25% of the higher homolog ($C_{28}H_{40}O$ plus 25% $C_{29}H_{42}O$ requires C, 85.65; H, 10.32; and O, 4.05%). The origin of the material and the C_{28}/C_{29} formula suggested a steroidal structure. The UV spectrum (λ max 351 nm, ε 25,800; λ max 282 nm, ε 5800; λ max 239, ε 4100) is of considerable interest. Intense UV bands at long wavelengths are unusual among nonaromatic naturally occurring steroids. No examples of nonaromatic steroids having λ max above 310 nm appear in the comprehensive tables of steroid spectra of Neudent and Ropke (1965).

The presence of a polyenone chromophore was indicated by the observed spectrum. Woodward's rules (see for instance Pasto and Johnson, 1969) yield a calculated absorption maximum of 351 nm for the chromophore I (Figure 3); this value is in excellent agreement with experiment.

The infrared spectra of both samples of fluorescent material were identical; that of the sample m.p. 105–107° C, (Figure 4) showed major absorbance bands at 2940, 2860, 1650, 1562, 1445, 1347, 1320, 1265, 1217, 1191, 964, 946, 872, and 758 cm^{-1}. The strong carbonyl band at 1650 cm^{-1} indicates that the oxygen function is present as an α,β-unsaturated ketone. The infrared spectrum was of little further direct assistance in the structural assignment, although the presence in the natural product of virtually every band observed in the fingerprint region of the infrared spectra of steroids of type II (Jones *et al.*, 1955) strengthens the assignment of partial structure II (Figure 3).

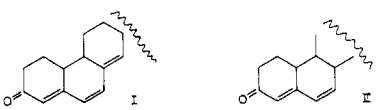

Figure 3. Steroid structure types

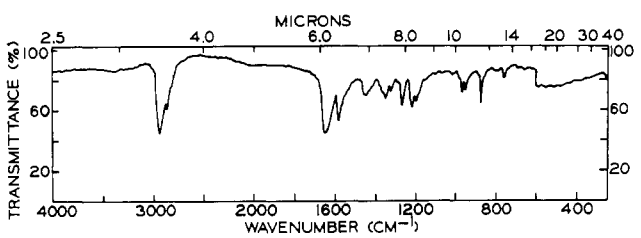

Figure 4. Infrared spectrum of the fluorescent natural product, m.p. 105–107° C

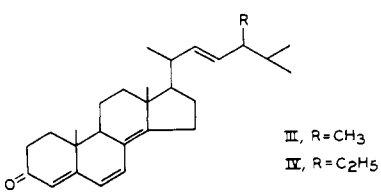

Figure 5. Structural formulas of 4,6,-8(14),22-ergostatetraen-3-one (III) and its C_{24}-ethyl homolog (IV)

In view of the foregoing results it was considered that the major component of our mixture might be 4,6,8(14),22-ergostatetraen-3-one (III, in Figure 5) a compound previously isolated from a natural source (Morimoto *et al.*, 1967). That this was indeed the case was evident from a comparison of the mass spectrum of the natural product with that of a sample of III; those spectra are reproduced from our preliminary communication (Daftary *et al.*, 1970) in Figure 6. Furthermore this assignment is supported by the reported m.p. of III, 114–115° C (Barton and Bruun, 1951) and by its reported UV spectrum: λ max 350 nm, ε 27,100; λ max 282 nm, ε 7100; λ max 237 nm, ε 4700 (Elks, 1954). The infrared and nmr spectra of III and the natural product were found to be essentially identical.

A probable structure of the minor component $C_{29}H_{42}O$ can be deduced from the spectroscopic properties of the natural product compared with those of an authentic sample of compound III. Exact mass measurements, low electron energy measurements, and the detection of metastable ions (m*) allow construction of Figure 7 for the fragmentation of the natural product upon electron impact. Most of the major daughter ions arise after side chain cleavage and hydrogen rearrangement to give *m/e* 268. But since *m/e* 268 is formed from both molecular ions, as is also shown by the observed metastable transitions, the additional CH_2 group of the minor

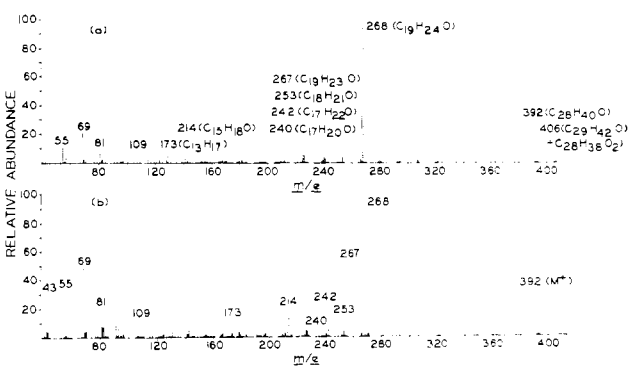

Figure 6. Mass spectra (70eV) of (a) the fluorescent material, m.p. 105–107° C, and (b)4,6,8(14), 22-ergostatetraen-3-one

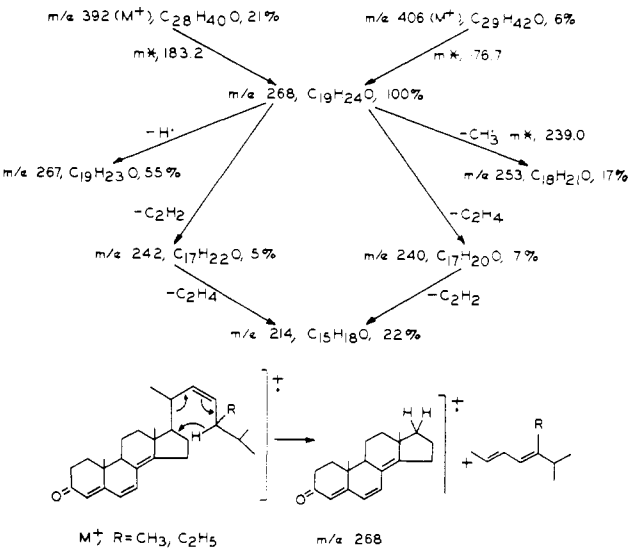

Figure 7. Mass spectrometric fragmentation of the natural product, m.p. 107–109.5° C

component must be present in the side chain. In agreement with this, none of the abundant daughter ions have homolog ions of 14 units greater mass, as has the molecular ion. Since the ultraviolet spectra of III and the fluorescent material are virtually identical, both the major and the minor component must possess the same chromophore. It follows that the structure of the minor component must be identical with III except that a hydrogen in the side chain must be replaced by a methyl group. This also accounts for the absence of discernible differences between the infrared spectra of III and the natural product. The nmr spectrum of the fluorescent material (Figure 8) provides some information on the position of this extra methyl group. While the methyl, methylene, and methine regions of the spectrum are complex, the olefinic protons give easily interpreted resonance signals. The hydrogen on C_4 appears in its expected low field position (4.4 τ) as a singlet, while the hydrogens on C_6 and C_7 give rise to the AB system centered at 3.7 τ, J = 9 Hz. Those results are in accord with expectation (see for instance, Neudent and Ropke, 1965) and are also found in the nmr spectrum of III. The olefinic protons of the C_{22}–C_{23} double bond in III give rise to a complex signal at 4.75 τ. An identical signal occurs in the nmr spectrum of the fluorescent material, showing that the methyl group is not at C_{22} or C_{23}. With the exception of IV, the proposed structure, none of the remaining possibilities is reasonable since unknown steroidal skeletons would be involved.

We commented in our preliminary communication (Daftary *et al.*, 1970) on the biosynthetic significance of formation of the C_{24}-methyl and C_{24}-ethyl homologs in the same biological system. The fact that C_{29} steroids are almost exclusively plant biosynthesized makes our findings of additional interest.

The two crystalline samples isolated from the wheat flour by the slightly different techniques were almost identical in most respects except in their UV spectra and in the composition of the ion *m/e* 406 in their mass spectra. The less pure sample, m.p. 105–107° C, apparently contained a third component in low abundance which was responsible for a strong UV absorption at 328 nm and for an ion $C_{28}H_{38}O_2$ which contributed to *m/e* 406. From the mass spectral data this component is estimated to be present in 5 to 10% amounts, and besides noting its highly unsaturated nature, no characterization has been possible.

In a completely different field of endeavor our results suggest a simple test for deterioration of cereals during storage under adverse conditions. The characteristic fluorescence of the ergostatetraenes can be utilized in detecting early stages of mold damage. Preliminary investigations have shown that the fluorescence is apparently mold-species-dependent and could be useful in mold classification.

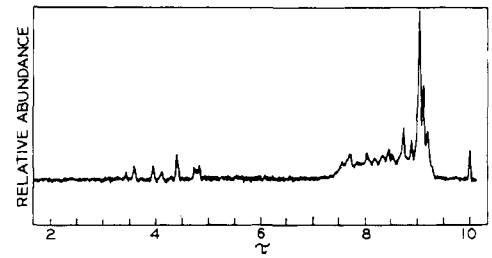

Figure 8. Nmr spectrum of the natural product, m.p. 105–107° C

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