Production and X-ray Crystal Structure of 3α-Acetoxy-7α,15-dihydroxy-12,13-epoxytrichothec-9-en-8-one

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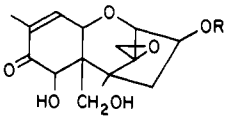
The trichothecene present in greatest concentration in cultures of *Fusarium graminearum* (HLX 1506) and *Fusarium culmorum* (HLX 1503) is shown to be 3α-acetoxy-7α,15-dihydroxy-12,13-epoxytrichothec-9-en-8-one on the basis of analysis of X-ray crystallographic diffraction data. The crystals of this metabolite incorporate continuous channels capable of accommodating small molecules such as water. Reliable procedures for production of experimental quantities of this metabolite and its parent alcohol are given.

3-Acetoxy-7,15-dihydroxy-12,13-epoxytrichothec-9-en-8-one (3-acetylvomitoxin, 3-acetyldeoxynivalenol, I, R =



I

Ac) has been reported as a metabolic product of *Fusarium roseum* (Yoshizawa and Morooka, 1973) and *Fusarium culmorum* (Blight and Grove, 1974). Its parent alcohol (I, R = H) has been obtained by hydrolysis of the ester (I, R = Ac; Blight and Grove, 1974) and by extraction of *Zea mays* infected with *Fusarium graminearum* (Bennett et al., 1981). The interconversion of these and analogous compounds by several biological systems has been reported (Yoshizawa and Morooka, 1975; Yoshizawa et al., 1980). The structures of the natural products (I, R = H, Ac) have been assigned on the basis of elemental analysis, ^{1}H NMR spectra, and ultraviolet spectra (Blight and Grove, 1974; Yoshizawa and Morooka, 1973) and on their mode of fragmentation in the mass spectrometer (Bennett et al., 1981). Neither of the metabolites has been synthesized, nor has either been converted by unambiguous chemistry to a product of established structure. In view of their potential importance as vectors in diseases of man and domestic animals, it seemed important to establish their structures by X-ray crystallography and this work is reported. Fermentation procedures and improvements in the isolation method reported in the literature (Blight and Grove, 1974) are presented.

MATERIALS AND METHODS

X-ray Crystallography. Crystal data were as follows (from single-crystal photographs and diffractometry, using Cu Kα radiation): λ_{α_1} = 1.54056 Å, T = 24 °C, FW = 338.4, tetragonal, I4 (optical activity of the dissolved crystals rules out $I\bar{4}$ and I4/m), a = 18.099 (3) Å, c = 10.631 (4) Å, V = 3482.4 Å^3, Z = 8, D_x = 1.290 g cm^{-3}, and μ = 8.03 cm^{-1}. The crystals were colorless, transparent needles (c). The selected specimen was a needle fragment 0.5 mm long and of cross section 0.13 × 0.17 mm. This was mounted with the needle axis parallel to the mounting fiber. The cell dimensions were obtained by least-squares analysis of the diffractometer settings (Picker four circle) of 17 well-centered reflections with 2θ angles 120–130°. The intensities of the independent reflections with 2θ < 130° were measured by using Ni-filtered Cu Kα radiation. The θ–2θ scan method was used, and individual reflection profiles were analyzed as described by Grant and Gabe (1978). The standard deviations of the measured intensities were evaluated from the counts, and only those for which $I_{net} > 3\sigma(I_{net})$ were used in the analysis. These numbered 1269, of a possible 1581. Absorption corrections were regarded as unnecessary and were not applied. The structure was solved by direct methods (MULTAN; Main et al., 1980). The refinement was by block-diagonal least squares, minimizing $\sum w\Delta F^2$, where $1/w = \sigma^2(F_0) + 0.001F_0^2$. The hydrogen atoms were placed in chemically reasonable positions (those attached to carbon, other than methyl) or located in a ΔF synthesis (both hydroxyls and one methyl group) or omitted altogether for lack of sufficient indication (two methyl groups); their parameters were not refined. The refinement converged at R = 0.055 (for observed reflections only; including unobserved, 0.071; R_w = 0.080). The final difference–Fourier synthesis disclosed two features indicative of scattering matter not included in the model: (1) ill-resolved peaks as high as 0.25 (6) e/Å^3 in positions appropriate for the omitted methyl hydrogen atoms; (2) residual density of as much as 0.55 (6) e/Å^3 along the 4-fold axis. The total scattering matter involved is about 7 e, and most of this is concentrated within a sphere of 2-Å radius, centered at 0, 0, 0.55. Because this feature was not represented in the phasing of the structure factors, its magnitude is probably underestimated. Its chemical identity cannot be demonstrated, but a possible interpretation is given below. The final coordinates of the non-hydrogen atoms are given in Table I, and selected interatomic distances and angles in Table II. The coordinates of the hydrogen atoms, the thermal parameters of all atoms, and the observed and calculated structure factors are given in Tables III–V (supplementary material; see paragraph at end of paper regarding supplementary material).

Production of Vomitoxin 3-Acetate (I, R = Ac). *Inoculum.* A slant of *F. roseum* [ATCC 28114, HLX 1506 (accession number to the culture collection held at the Atlantic Research Laboratory)] or *F. culmorum* (CMI 14764, HLX 1503) was macerated with water (50 mL) for 20 s in a Waring blender and the homogenate used to inoculate medium (100 mL) containing black strap molasses (2 g), dextran (3 g), fish meal (1.5 g), pharmamedia (Traders Oil Mill Co., Fort Worth, TX 76101), and water. These cultures (10) were incubated at 25 °C for 48 h on a shaker. Each culture described a circle of 3.81-cm diameter in a horizontal plane at 220 rpm. Such cultures of *F. graminearum* were used directly for production of I (R = Ac) in surface culture. Similar cultures of *F. cul-*

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morum were used for the second-stage tank fermentation described in a later section.

Production Cultures. (a) Glucose (8 kg), yeast extract (Difco, 80 g), and peptone (80 g) were dissolved in tap water treated as described (Brewer et al., 1982), and the solution made up to 80 L. This medium (1 L) was dispensed into earthenware fermentation vessels (available from Blue Mountain Pottery, Ltd., Collingwood, Ontario L9Y 3Z8, Canada), which were then plugged with cotton, heated in an autoclave for 2 h at 121 °C, cooled, and inoculated with the seed culture (2 mL) described in the previous paragraph. The vessels were incubated at 28 °C for 8 days. (b) The inoculum (1.5 L) prepared as described was added to medium (30 L) containing glucose (600 g), malt extract (60 g), yeast extract (60 g), peptone (60 g), KH_2PO_4 (60 g), $MgSO_4 \cdot 7H_2O$ (60 g), $FeSO_4 \cdot xH_2O$ (6 g), ammonium chloride (90 g), and water (Brewer et al., 1982), which had been sterilized at 121 °C for 2 h. The culture was stirred at 150 rpm at 28 °C and sparged with air at 20 L min^{-1}. When the culture was 72 h old, it was pumped into production medium (180 L) consisting of sucrose (7.2 kg), glycerol (1.8 kg), NaCl (0.9 kg), KH_2PO_4 (0.64 kg), $(NH_4)_2HPO_4$ (0.18 kg), $MgSO_4 \cdot 7H_2O$ (36 g), and water (Brewer et al., 1982), which had been sterilized at 122 °C for 2.5 h. The culture was treated with sterile rapeseed oil (0.9 L) and was stirred at 350 rpm at 28 °C. Air was sparged into the fermenter at 71 L min^{-1}. The initial pH of the fermentation broth was 6.3, and it declined to 4 over 24 h at which it was maintained by addition of sterile potassium carbonate solution (10% w/v) during the remainder of the fermentation. After 8 days of growth, the culture was harvested.

Harvest and Extraction. Surface and tank cultures were harvested and extracted in the same way, except that the culture filtrate of the tank cultures was adjusted to pH 7. The procedure described is for the surface cultures; quantities must be increased by a factor of 2.5 for the submerged cultures. The culture was filtered through a pad of Celite 545 2 cm thick, supported on polyester cloth in a large, glazed earthenware nutsche filter of diameter 1 m. The mycelium in the filter was washed with water (15 L); the filtrate was treated with sodium chloride (8 kg) and then basified to pH 9.3 with sodium hydroxide solution (10%, ca. 70 mL). The solution was extracted with methylene chloride (3 × 25 L, 15 min for each extraction). The aqueous phase from the first extraction usually separated cleanly in ca. 1 h, and the lower emulsified phase was pumped into a holding tank where it was mixed with the second extract. After the mixture was allowed to stand at room temperature for 18 h, the clear methylene chloride phases were evaporated (5 °C, 100 mmHg) to ca. 3 L in a cyclone evaportor as has been recently described (Brewer et al., 1982). The concentrate was evaporated to dryness, the residue (ca. 30 g) was dissolved in methyl alcohol (1 L) and petroleum ether (bp 30–60 °C, 1 L) and then water (50 mL) was added. The methyl alcohol phase was separated and extracted with petroleum ether (3 × 1 L) and then evaporated (for surface cultures, mean residue weight 9.92 ± 2.25 g, 38 ± 4.7% I (R = Ac), 47 ± 9 mg L^{-1} I (R = Ac), n = 11; for tank cultures, mean residue weight 1.14 ± 0.6 g L^{-1}; 31 ± 15% I (R = Ac), 0.35 ± 0.2 g L^{-1} I (R = Ac), n = 9; ± = standard deviation, n = number of fermentations).

Purification. This residue (3 g) was dissolved in chloroform (5 mL) and the solution applied to a silica gel column prepared in the following way. A thin-walled glass jacket was sealed to the lower end of a length of industrial glass pipe (152 × 7.62 cm), its upper end was left open, and an overflow was constructed about 6 cm from the top. This permitted water at 20 ± 0.1 °C to be pumped through an inlet at the bottom to the overflow, thus maintaining the column at constant temperature. The lower seal of the jacket was as close to the backing flange as possible. To the lower end of the pipe was fitted a stopcock, which was sealed to a 7.62-cm standard flange so as to minimize the dead space between the bottom of the column and the fraction collector. This dead space was filled sequentially with glass wool, glass spheres (diameter 0.5 cm, ca. 50), Fenski rings (0.5-cm depth), and silica gel (Clarkson, 100–200 mesh) to bring the packing level to the Teflon gasket between the glass flange joints. The column was then filled to a depth of 20 cm with chloroform (EtOH, 0.8%) and silica gel (Merck, 10–50 μm, 450 g) poured in. The mixture was agitated with a packing tool (Howard and Martin, 1950) to remove air and the silica allowed to settle under gravity for 18 h. The excess chloroform was then allowed to run through the column until a layer of 1–2 mm was left on the top. The sample was applied, and when it had run into the column, the sides of the glass pipe were washed with several 5-mL lots of chloroform. The column was developed first with chloroform–ethyl alcohol (50:1, 650 mL) and then with chloroform–ethyl alcohol (400:13, 50 mL), after which fractions (~25 mL) were collected using the same solvent, keeping a constant head on the column and changing the collector every 7 min. The acetate (I, R = Ac) was usually found in fractions 45–60, but fractions 45–47 contained additional yellow pigments and fractions 57–60 contained material of lower R_f than I (R = Ac), which did not absorb at 254 nm but did react with the cerric sulfate reagent. Fraction 47–56 were combined (0.732 g) and dissolved in boiling diethyl ether (50 mL), the solution was evaporated until crystallization started (25 mL) and kept at 4 °C for 18 h, and the colorless needles were collected: 0.376 g; mp 185–187 °C; $[\alpha]^{20}_D$ +35 ± 4° (c 0.35, $CHCl_3$); ^1H NMR δ 1.166 (3 H, H-14), 1.907 (3 H, H-16, $^4J_{10,16}$ = 1.4 Hz), 2.145 (3 H, Ac-CH_3), 2.179, 2.371, 5.234 (3 H, ABX, H-4, H-3, $^2J_{AB}$ = −15.0 Hz, $^3J_{AX}$ = 11.31 Hz, $^3J_{BX}$ = 4.15 Hz), 3.919 (H, H-2, $^3J_{2,3}$ = 4.40 Hz), 3.126, 3.186 (2 H, H-13, $^2J_{AB}$ = 4.36 Hz), 3.774, 3.869, (2 H, H-15, $^2J_{AB}$ = −11.69 Hz), 4.689 (H, H-11, $^2J_{10,11}$ = 5.90 Hz), 4.834 (H, H-7), 6. 613 (H, H-10). Anal. Calcd for $C_{17}H_{22}O_7$: C, 60.3; H, 6.6; O, 33.1. Found: C, 60.1; H, 6.9; O, 32.9. This material (100 mg) was dissolved in 95% ethyl alcohol (2 mL), cooled to 4 °C, seeded with two or three crystals, and kept at 4 °C for 48 h. The mother liquors were aspirated and the crystals washed with ethyl alcohol–water, (9:1) precooled to 0°, 2 or 3 times (the crystals dissolve rapidly in 1 mL of 90% ethyl alcohol at 10 °C). The crystals were dried (20 °C/0.01 mmHg), and a suitable specimen was selected for X-ray crystallography. As reported by Blight and Grove (1974), this compound often crystallizes as fine needles, mp 136–137 °C; we were unable to obtain such a specimen suitable for X-ray crystallography. Both crystal habits on hydrolysis (Blight and Grove, 1974) gave vomitoxin (I, R = H).

Analytical Methods. The acetate (I, R = Ac) and related compounds were detected on thin-layer (SiO_2, 0.1 mm) chromatograms by spraying the plates with 1% cerric sulfate in 50% sulfuric acid. On heating at 100 °C for 2 min, the acetate appeared as a characteristic olive green spot (detection limit 0.1 μg). Quantitative analysis for trichothecins was carried out on the equipment described (Feicht and Taylor, 1982) with 28% methyl alcohol as a solvent and a Merck C_{18} reversed-phase column (particle size 10 μm, 4.5 × 20 cm, T = 25 °C, flow rate 1 mL min^{-1}). Under these conditions the retention time of I, R = H, was

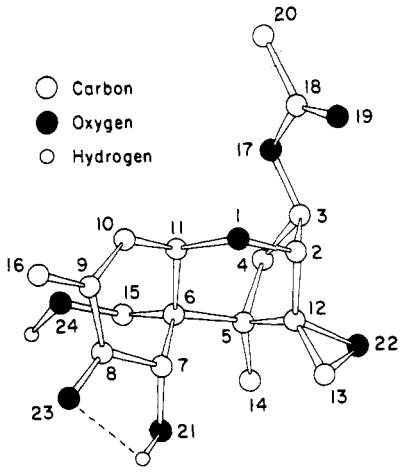

Figure 1. Structure of vomitoxin 3-*O*-acetate.

Table I. Fractional Coordinates (ESD's) of the Non-Hydrogen Atoms (the Molecule Defined Here Is the Enantiomer of That Depicted in Figure 1)

atom	*X*	*Y*	*Z*
O(1)	0.3652 (2)	0.1012 (2)	0.4067[a]
C(2)	0.3467 (3)	0.0266 (3)	0.3851 (6)
C(3)	0.2798 (3)	0.0004 (3)	0.4656 (6)
C(4)	0.2128 (3)	0.0260 (3)	0.3901 (6)
C(5)	0.2418 (3)	0.0571 (3)	0.2619 (6)
C(6)	0.2596 (2)	0.1423 (3)	0.2751 (5)
C(7)	0.3071 (3)	0.1710 (3)	0.1637 (6)
C(8)	0.3334 (3)	0.2508 (3)	0.1844 (6)
C(9)	0.3587 (3)	0.2719 (3)	0.3106 (6)
C(10)	0.3446 (3)	0.2260 (3)	0.4062 (6)
C(11)	0.3056 (3)	0.1531 (3)	0.3953 (6)
C(12)	0.3150 (3)	0.0173 (2)	0.2564 (6)
C(13)	0.3569 (4)	−0.0032 (3)	0.1452 (6)
C(14)	0.1893 (4)	0.0363 (3)	0.1588 (7)
C(15)	0.1856 (3)	0.1841 (3)	0.2810 (6)
C(16)	0.3981 (4)	0.3452 (3)	0.3248 (10)
O(17)	0.2766 (2)	0.0325 (2)	0.5899 (4)
C(18)	0.3321 (4)	0.0178 (3)	0.6689 (7)
O(19)	0.3860 (3)	−0.0173 (2)	0.6387 (5)
C(20)	0.3178 (5)	0.0512 (4)	0.7974 (7)
O(21)	0.2708 (2)	0.1638 (2)	0.0476 (4)
O(22)	0.3126 (2)	−0.0592 (2)	0.2107 (4)
O(23)	0.3371 (2)	0.2920 (2)	0.0938 (5)
O(24)	0.1959 (2)	0.2607 (2)	0.3002 (4)

[a] Coordinate held constant during refinement.

Table II. Selected Interatomic Distances (Å) and Bond Angles (deg) and Their ESD's

Distances

O(1)–C(2)	1.409 (6)	C(9)–C(16)	1.514 (8)
O(1)–C(11)	1.436 (6)	C(10)–C(11)	1.501 (7)
C(2)–C(3)	1.556 (8)	C(12)–C(13)	1.454 (8)
C(2)–C(12)	1.493 (9)	C(12)–O(22)	1.469 (6)
C(3)–C(4)	1.526 (9)	C(13)–O(22)	1.468 (7)
C(3)–O(17)	1.445 (8)	C(15)–O(24)	1.414 (6)
C(4)–C(5)	1.564 (9)	O(17)–C(18)	1.337 (8)
C(5)–C(6)	1.581 (7)	C(18)–O(19)	1.207 (8)
C(5)–C(12)	1.508 (7)	C(18)–C(20)	1.515 (10)
C(5)–C(14)	1.498 (9)	O(21)–H(21)	0.97
C(6)–C(7)	1.553 (8)	H(21)···O(23)	2.26
C(6)–C(11)	1.537 (8)	O(21)···O(23)	2.657 (6)
C(6)–C(15)	1.539 (7)	H(21)···O(24)[a]	2.11
C(7)–C(8)	1.537 (7)	O(21)···O(24)[a]	3.025 (6)
C(7)–O(21)	1.404 (7)	O(24)–H(24)	0.92
C(8)–C(9)	1.468 (9)	H(24)···O(22)[b]	1.94
C(8)–O(23)	1.219 (7)	O(24)···O(22)[b]	2.812 (5)
C(9)–C(10)	1.337 (9)		

Bond Angles

C(2)–O(1)–C(11)	115.7 (4)	C(9)–C(8)–O(23)	123.1 (5)
O(1)–C(2)–C(3)	112.8 (4)	C(8)–C(9)–C(10)	118.2 (5)
O(1)–C(2)–C(12)	110.4 (4)	C(8)–C(9)–C(16)	117.8 (6)
C(3)–C(2)–C(12)	99.8 (5)	C(10)–C(9)–C(16)	124.0 (7)
C(2)–C(3)–C(4)	103.7 (5)	C(9)–C(10)–C(11)	125.3 (6)
C(2)–C(3)–O(17)	114.4 (5)	O(1)–C(11)–C(6)	113.2 (4)
C(4)–C(3)–O(17)	109.1 (5)	O(1)–C(11)–C(10)	102.4 (4)
C(3)–C(4)–C(5)	107.5 (5)	C(6)–C(11)–C(10)	115.5 (5)
C(4)–C(5)–C(6)	110.0 (5)	C(2)–C(12)–C(5)	104.4 (5)
C(4)–C(5)–C(12)	99.0 (4)	C(2)–C(12)–C(13)	125.0 (5)
C(4)–C(5)–C(14)	109.5 (5)	C(2)–C(12)–O(22)	114.9 (4)
C(6)–C(5)–C(12)	106.8 (4)	C(5)–C(12)–C(13)	127.8 (5)
C(6)–C(5)–C(14)	116.0 (5)	C(5)–C(12)–O(22)	115.9 (4)
C(12)–C(5)–C(14)	114.1 (5)	C(13)–C(12)–O(22)	60.3 (3)
C(5)–C(6)–C(7)	111.8 (4)	C(12)–C(13)–O(22)	60.4 (3)
C(5)–C(6)–C(11)	108.0 (4)	C(6)–C(15)–O(24)	111.9 (4)
C(5)–C(6)–C(15)	107.8 (4)	C(3)–O(17)–C(18)	117.7 (5)
C(7)–C(6)–C(11)	106.9 (4)	O(17)–C(18)–O(19)	123.0 (6)
C(7)–C(6)–C(15)	110.3 (4)	O(17)–C(18)–C(20)	111.0 (6)
C(11)–C(6)–C(15)	112.0 (4)	O(19)–C(18)–C(20)	126.0 (7)
C(6)–C(7)–C(8)	112.2 (4)	C(7)–O(21)–H(21)	115
C(6)–C(7)–O(21)	112.3 (4)	C(12)–O(22)–C(13)	59.3 (3)
C(8)–C(7)–O(21)	111.0 (4)	C(15)–O(24)–H(24)	105
C(7)–C(8)–C(9)	118.2 (5)	O(21)–H(21)···O(23)	104
C(7)–C(8)–O(23)	118.6 (5)	O(21)–H(21)···O(24)[a]	157
		O(24)–H(24)···O(22)[b]	157

[a] Atom generated by 2-fold screw axis ($^1/_2 - x$, $^1/_2 - y$, $^1/_2 + z$). [b] Atom generated by 4-fold rotation axis ($-y$, x, z).

~5 min and of I, R = Ac, ~11 min. Integrated areas were converted into μg of I (R = Ac) by the expression μg of I (R = Ac) = 0.056 × area × 9.168 × 10^{-4} derived as described (Feicht and Taylor, 1982) by chromatography of known weights of I, R = Ac, R = H. Mass spectra were determined with a Du Pont 21-110B mass spectrometer, and precise mass measurements were obtained by the peak matching method using an ion in the spectrum of perfluorokerosene as a standard. ^{1}H NMR spectra were obtained at 360 MHz with Nicolet instrumentation at the Maritime NMR center.

RESULTS AND DISCUSSION

The molecular structure of vomitoxin 3-acetate (I, R = Ac) is specified in Figure 1 and in Tables I and II. No attempt has been made to determine the absolute configuration; that depicted in the Figures 1 and 2 is consistent with that deduced for related compounds (McPhail and Sim, 1966).

The molecules are held together in the crystal by hydrogen bonds; the crystal contains channels, parallel to *c*, capable of accommodating small molecules. The structure can be visualized as having been assembled in the following way. Four molecules related by the 4-fold rotation axis are linked by hydrogen bonds to form a tetrameric ring (Figure 2) with a substantial hole at the center. The (projected) distance from the center to the nearest non-hydrogen atom [C(14)] is 3.49 Å; a small atom or molecule (e.g., water) could presumably pass through this hole without severe hindrance. Equivalent rings are packed in the *xy* plane on a square mesh with only van der Waals contact between them. There are further, interstitial, holes at the corners of the unit cell (assuming this to have been chosen so as to contain a ring); the distance from a corner to the nearest non-hydrogen atom [C(16)] is 3.35 Å. Additional layers are stacked at intervals of $c/2$ and laterally displaced by half the cell diagonal (the body-centering operation $^1/_2, ^1/_2, ^1/_2$). Therefore, the interstitial holes of one layer overlap the ring centers of the next (and vice versa), leaving a continuous channel. Adjacent layers are linked by hydrogen bonds between molecules related by the 2-fold screw axes. Each ring is linked to four different rings in each of the adjacent layers, and thus the hydrogen bonding network is space filling.

The channel is, of course, of noncircular cross section that varies with *z*. However, because of the 4-fold rotation

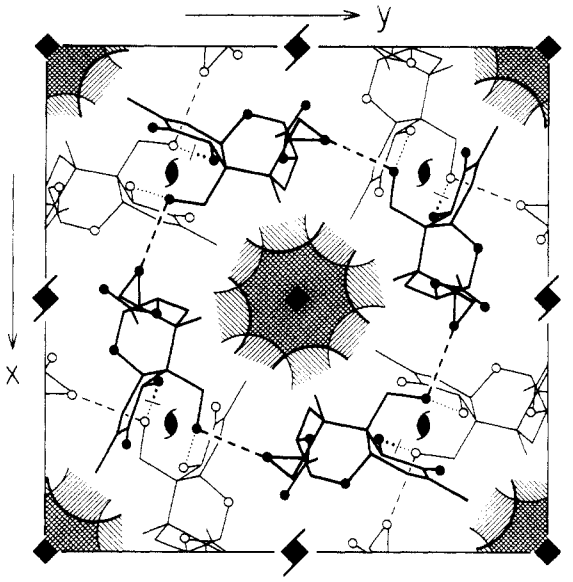

Figure 2. The unit cell contents projected along *c*. Of the two layers of molecules shown, the upper is depicted in bold outline. Oxygen atoms are represented as circles (solid or open). Intermolecular hydrogen bridges are shown as dashed lines if they join molecules in the same layer and as dotted lines otherwise. Interrupted H bridges join the upper layer to the one above it or the lower layer to the one below it. The holes in the layers (see the text) are bounded by arcs of (arbitrarily selected) radius 1.7 Å. "ring" holes are hatched one way, "interstitial" holes are hatched another, and the overlapped region (indicating the minimum channel dimensions) is crosshatched.

symmetry, an effective local radius can be recognized and could reasonably be defined as the distance from 0, 0, *z* to the nearest non-hydrogen atom. This radius ranges from the local minima (noted above) of 3.49 and 3.35 Å to local maxima of 3.83 and 4.25 Å. The greater of these maxima occurs at $z = 0.55$, and it is just here that the large concentration of residual electron density (described above) is centered. It is concluded that the concentration is a small molecule (perhaps H_2O), disordered, and possibly of partial occupancy. The mobility of the molecule along the channel is presumably restricted by the narrow parts.

If these channels accommodate small molecules (e.g., water and methyl alcohol), their occupancy by such solvents may explain the occurrence of different crystal habits of different melting point such as has been observed in this and closely related molecules (Blight and Grove, 1974). Presumably such occluded solvents are lost in the preparation of samples for elemental analysis. It is also clear that care should be exercised in preparation of samples in isotope dilution studies.

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Registry No. I (R = Ac), 50722-38-8; I (R = H), 51481-10-8.

Supplementary Material Available: Coordinates of Hydrogen atoms (Table III), thermal parameters of all atoms (Table IV), and observed and calculated structure factors (Table V) (13 pages). Ordering information is given on any current masthead page.

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Mutagen Formation by Nitrite–Spice Reactions

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Among several spices treated with sodium nitrite, pepper exhibited the strongest mutagenic activity by Ames method and nutmeg, chili pepper, and laurel the strong activity. No mutagenicity was observed for spices alone. The mutagen production was observed between pH 2 and pH 6, with the maximum between pH 3 and pH 3.5, and the reaction was very fast at 40 °C or above, even at the low levels of nitrite permitted by legal regulations. The mutagenicities of spice–nitrite reaction products were completely inactivated by S9 mix, but the activity of pepper–nitrite products toward TA 100 remained unchanged. By preparative TLC of spice–nitrite reaction mixtures, pepper provided two very active fractions, while nutmeg and chili pepper showed a different active fraction.

Studies on the possible formation of various mutagens by the reaction of food additives with food components or by that between coexistent food additives are no less important than those of the mutagens primarily contained in foods. An outstanding example has been the formation of *N*-nitrosamines by the reaction of secondary amines with added nitrites. Our group has also clarified the formation of a group of mutagenic *C*-nitro or *C*-nitroso products by the reaction between two commonly used food additives, sorbic acid and nitrite (Kada, 1974; Namiki and

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