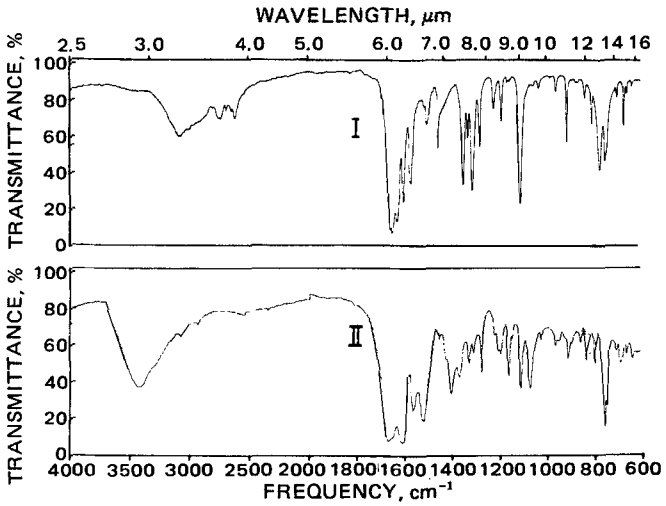

Figure 3—*IR spectra of I and II.*

ppm due to the hydrogen-bonded (enolic) protons at C-4 and/or C-4′ (13).

The poor solubility of II in deuterated chloroform necessitated the use of an NMR spectrometer equipped with a Fourier transform data system. The sample was analyzed overnight; all peaks were identifiable and appeared at their expected chemical shifts, but the spectrum could not be quantitated. Therefore, nonaqueous titration was used to quantitate the enolic protons. As expected, titration curves of II yielded two sharp breaks corresponding to two protons. One proton is believed to be at C-4 or C-4′ on each ligand molecule.

The IR spectra of I and II are shown in Fig. 3. Due to intramolecular hydrogen bonding, the carbonyl stretching frequency of I appeared at 1660 cm^{-1} (13). When I was *O*-methylated and no intramolecular hydrogen bonding could occur, the carbonyl peak was shifted to 1725 cm^{-1} (13). Since there was no shift to longer wave numbers in the carbonyl stretching frequency of II, both carbonyl groups in each ligand probably were bonded. Based on the presence of two titratable protons on II, it seems reasonable that one carbonyl group in each ligand was still intramolecularly hydrogen bonded and that the other carbonyl was bonded with magnesium.

Intramolecular hydrogen bonding also affected the position of the hydroxyl peak in the IR region. This peak appeared at 3100 cm^{-1} for I; however, II exhibited a large, broad water band between 3700 and 2800 cm^{-1}. Since II is believed to retain one intramolecular hydrogen bond on each ligand, the hydroxyl band at 3100 cm^{-1} was probably concealed beneath the water band.

In summary, elemental analyses conclusively established a 2:1 ligand–metal stoichiometry for II and the IR and other analytical data are consistent with Structure II in Scheme I. X-ray studies on the chelate are currently underway to substantiate further the structure.

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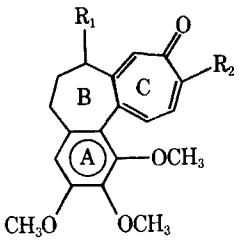
X-Ray Analysis of Sulfur-Containing Colchicine Derivatives

CATHERINE KOERNTGEN and T. N. MARGULIS ˣ

Abstract □ The crystal and molecular structures of deacetylthiocolchicine hydrochloride dihydrate and thiocolchicine hexahydrate were determined by X-ray diffraction. The replacement of oxygen by sulfur on the C ring methoxy group causes greater puckering of the troponoid ring. The conformation of one A ring methoxy group differs from that of colchicine derivatives that do not contain sulfur.

Keyphrases □ Colchicine derivatives—X-ray diffraction determination of crystal and molecular structures □ Thiocolchicines, various—X-ray diffraction determination of crystal and molecular structures □ X-ray diffraction—determination of crystal and molecular structures of various thiocolchicines □ Crystal and molecular structures—various thiocolchicines, X-ray diffraction determination

Colchicine (I), an ancient drug, is used primarily in the treatment of gout. Attempts to use this powerful mitotic inhibitor in the treatment of human malignancies have been largely unsuccessful. Other mitotic poisons, however, are being used successfully with human tumors. In particular, vinblastine and vincristine are used for the treat-



I: R_1 = $NHCOCH_3$, R_2 = OCH_3
II: R_1 = NH_3^+, R_2 = SCH_3
III: R_1 = $NHCOCH_3$, R_2 = SCH_3
IV: R_1 = $NHCOCH_3$, R_2 = SC_2H_5
V: R_1 = $NHCOCH_3$, R_2 = S-*n*-C_4H_9
VI: R_1 = $NHCH_3$, R_2 = OCH_3

ment of Hodgkin's disease, leukemia, and some solid cancers (1).

Thiocolchicines, in which sulfur replaces oxygen in the troponoid methoxy group of a colchicine derivative, were first prepared in 1954 (2–5). Recently, a series of new thiocolchicines was prepared (6) with groups larger than methyl on the sulfur atom. In general, substitution of sulfur for oxygen in itself does not inhibit, and in some cases may increase, colchicine activity.

Deacetylthiocolchicine hydrochloride (II) inhibited the binding of colchicine to microtubule protein and had nearly equivalent antimitotic and anti-inflammatory activities (7). Compound II also had about the same activity against mouse leukemia as colchicine (6). In the *allium* test, II and thiocolchicine (III) were somewhat more active than colchicine in their ability to arrest mitosis (8). With groups larger than methyl attached to the sulfur atom, activity was diminished; ethylthiocolchicine (IV) was slightly active and *n*-butylthiocolchicine (V) was inactive (6).

Colchicine is believed to bind to tubulin, preventing assembly into microtubules. Microtubules are involved in several important biological processes including chromosome movement, development of asymmetric cell shape, cell motility, and, possibly, nerve transmission. To gain a detailed understanding of the colchicine–tubulin interaction, X-ray diffraction studies of inactive, as well as active, colchicine derivatives and other antimitotic drugs are being performed. This report describes the crystal and molecular structures of II and III.

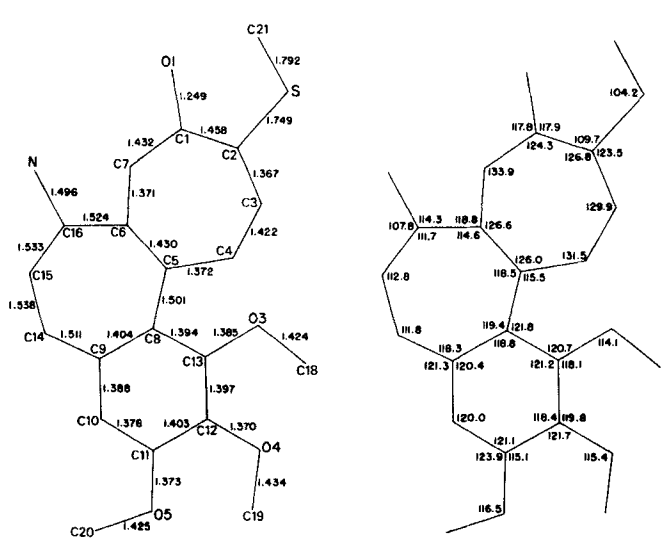


Figure 1—*Bond lengths (Å) and bond angles (°) for atoms other than hydrogen in II. Standard deviations are approximately 0.005 Å and 0.4°, respectively.*

Table I—Coordinates of Atoms Other than Hydrogen in II [a]

Atom	x	y	z
S	0.75908 (14)	−0.34348 (9)	0.39608 (4)
Cl	−0.03668 (15)	0.38244 (10)	0.44701 (6)
N	0.1248 (4)	0.0095 (2)	0.4998 (1)
O-1	0.5744 (4)	−0.2684 (2)	0.47997 (9)
O-3	0.5451 (3)	0.1921 (2)	0.33360 (9)
O-4	0.3745 (4)	0.3709 (2)	0.2862 (1)
O-5	0.0204 (3)	0.3636 (2)	0.2689 (2)
Water O-1	−0.2595 (6)	−0.0944 (4)	0.5601 (1)
Water O-2	−0.1420 (4)	0.1675 (3)	0.5137 (1)
C-1	0.5251 (5)	−0.2092 (3)	0.4417 (1)
C-2	0.6118 (5)	−0.2281 (3)	0.3910 (1)
C-3	0.5887 (5)	−0.1655 (3)	0.3460 (1)
C-4	0.4834 (5)	−0.0664 (3)	0.3369 (1)
C-5	0.3597 (4)	−0.0120 (3)	0.3677 (1)
C-6	0.3073 (4)	−0.0454 (3)	0.4197 (1)
C-7	0.3846 (5)	−0.1285 (3)	0.4502 (1)
C-8	0.2691 (4)	0.0889 (3)	0.3428 (1)
C-9	0.0848 (4)	0.0858 (3)	0.3348 (1)
C-10	−0.0011 (5)	0.1763 (3)	0.3101 (1)
C-11	0.0940 (5)	0.2711 (3)	0.2942 (1)
C-12	0.2777 (5)	0.2781 (3)	0.3026 (1)
C-13	0.3633 (4)	0.1854 (3)	0.3264 (1)
C-14	−0.0166 (5)	−0.0172 (3)	0.3548 (1)
C-15	−0.0256 (5)	−0.0196 (3)	0.4153 (1)
C-16	0.1477 (4)	0.0194 (3)	0.4414 (1)
C-18	0.5971 (6)	0.2217 (5)	0.3858 (2)
C-19	0.3480 (8)	0.4740 (4)	0.3161 (2)
C-20	−0.1682 (6)	0.3653 (4)	0.2651 (2)
C-21	0.8542 (7)	−0.3551 (4)	0.3316 (2)

[a] Estimated standard deviations are given in parentheses as deviations in the last figure. The numbering system omits O-2 and C-17 to make it compatible with the numbering used in demecolcine (10).

EXPERIMENTAL

All X-ray data for cell dimensions and intensities were collected on an automatic diffractometer with monochromatized CuKα (λ = 1.54178 Å) radiation. Cell dimensions were calculated by least-squares fitting of the diffractometer angles for 15 reflections. Intensities were measured with $2\theta - \theta$ scans and converted to structure factors without correction for absorption. Densities were measured by flotation. Refinement of each structure was accomplished by full-matrix least squares with atomic scattering factors from the literature (9).

The structure of II was solved by the conventional heavy atom method. The crystal[1], obtained by slow evaporation from aqueous solution, had approximate dimensions of 0.2 × 0.2 × 0.4 mm. The final value of $R = \Sigma||F_{obs}| - |F_{cal}||/\Sigma|F_{obs}|$ was 0.035 for 1911 observed reflections[2]. In

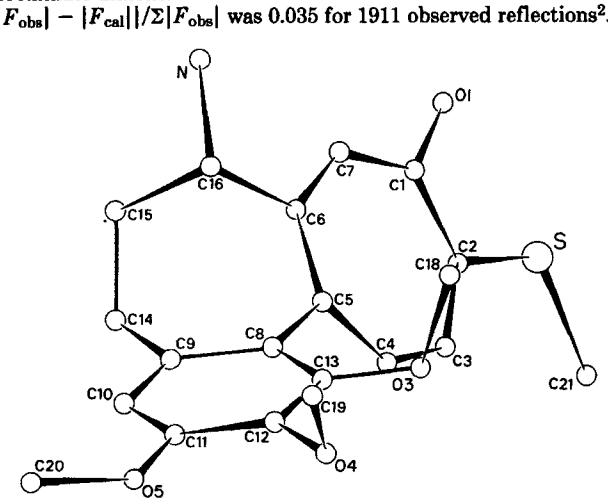


Figure 2—*One molecule of II, with hydrogen atoms omitted.*

[1] Obtained from Roussel-Uclaf.

[2] Observed and calculated structure factors and thermal parameters are available from the authors.

Table II—Hydrogen Atom Coordinates and Bond Lengths for II [a]

Hydrogen Atom Bonded to	x	y	z	D[b], Å
Water O-1	0.648	−0.033	0.564	1.0
Water O-1′	0.676	−0.143	0.543	0.9
Water O-2	−0.178	0.227	0.485	1.0
Water O-2′	−0.249	0.155	0.523	0.9
N	0.022	0.067	0.509	1.1
N′	0.094	−0.055	0.510	0.8
N″	0.248	0.044	0.519	1.1
C-3	0.658	−0.189	0.311	1.1
C-4	0.514	−0.039	0.299	1.0
C-7	0.345	−0.141	0.487	1.0
C-10	−0.132	0.166	0.302	1.0
C-14	−0.137	−0.011	0.340	1.0
C-14′	0.043	−0.104	0.342	1.2
C-15	−0.063	−0.097	0.428	1.0
C-15′	−0.129	0.038	0.428	1.1
C-16	0.152	0.103	0.435	1.0
C-18	0.726	0.233	0.386	1.0
C-18′	0.559	0.290	0.396	0.9
C-18″	0.557	0.168	0.415	1.0
C-19	0.395	0.469	0.348	0.9
C-19′	0.450	0.533	0.298	1.1
C-19″	0.217	0.471	0.341	1.2
C-20	0.782	0.298	0.246	1.0
C-20′	0.801	0.431	0.247	0.9
C-20″	0.770	0.366	0.300	1.0
C-21	0.944	−0.267	0.333	1.2
C-21′	0.912	−0.425	0.327	0.9
C-21″	0.760	−0.356	0.307	1.0

[a] Estimated standard deviations are approximately 0.005, 0.003, and 0.001 for x, y, and z, respectively, and 0.1 Å for bond lengths. [b] CH, OH, or NH bond length.

the final cycles, hydrogen atoms, located in a difference map, were refined with isotropic temperature factors while all other atoms were refined anisotropically.

Thiocolchicine[1] yields crystals easily from aqueous solution, but they lose water rapidly. Therefore, data were collected with a crystal, approximately 0.3-mm cube, in a capillary surrounded by mother liquor[3]. A trial structure was obtained by direct methods using the computer program MULTAN. A difference map gave the location of one full water and two partially occupied water sites but showed evidence of considerable disorder in the water structure. This map also revealed about half of the hydrogen atoms, but they were not included in the refinement. The final R factor, with all atoms anisotropic, with one ordered and two half water oxygen atoms, was 0.12 for 1613 nonzero reflections[2].

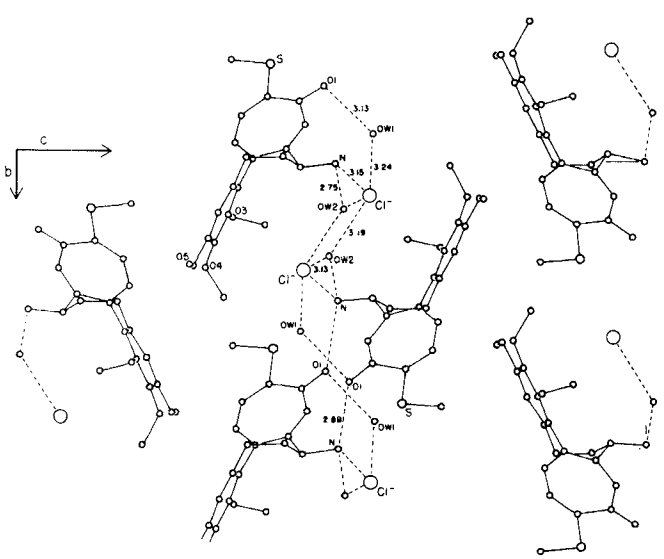


Figure 3—*Crystal structure of II. Dashed lines are hydrogen bonds. Hydrogen atoms are omitted.*

[3] Thiocolchicine also forms crystals from ethyl acetate. These are monoclinic, space group $P2_1$ with a = 21.23 ± 0.03, b = 7.188 ± 0.004, and c = 18.59 ± 0.05 Å, and β = 110.9 ± 0.2°, Z = 4.

Table III—Selected Torsion Angles, τ, in Biologically Active Colchicine Derivatives

Atom	τ°, VI[a]	τ°, II[b]	τ°, III[b]
Troponoid Ring			
C-7–C-1–C-2–C-3	−3.0	7.7	3
C-1–C-2–C-3–C-4	0.9	2.5	1
C-2–C-3–C-4–C-5	−0.8	−8.6	−2
C-3–C-4–C-5–C-6	3.1	0.6	3
C-4–C-5–C-6–C-7	−3.7	8.2	−7
C-5–C-6–C-7–C-1	0.0	−3.8	10
C-6–C-7–C-1–C-2	3.1	−7.1	−8
B Ring			
C-8–C-5–C-6–C-16	−4.5	6.5	−3
C-5–C-6–C-16–C-15	80.8	73.8	82
C-6–C-16–C-15–C-14	−48.3	−51.7	−50
C-16–C-15–C-14–C-9	−41.3	−38.9	−39
C-15–C-14–C-9–C-8	71.1	70.3	68
C-14–C-9–C-8–C-5	4.2	3.5	7
C-9–C-8–C-5–C-6	−53.9	−60.1	−55
OCH_3 and SCH_3			
C-12–C-13–O-3–C-18	82	101	104
C-11–C-12–O-4–C-19	−82	72	78
C-10–C-11–O-5–C-20	8	8	1
C-3–C-2–(S or O-2)–C-21	0	0	−2

[a] See Ref. 10. [b] This work.

RESULTS AND DISCUSSION

Compound II dihydrate, $C_{20}H_{24}NO_4S^+Cl^-\cdot 2\,H_2O$, forms orthorhombic crystals, space group $P2_12_12_1$, with a = 7.538, b = 11.663, and c = 25.375 Å and four molecules per unit cell. All atoms are in general positions given by x, y, and z: ½ −x, −y, and ½ +z; ½ +x, ½ −y, and −z; and −x, ½ +y, and ½ −z. Atomic parameters are listed in Tables I and II. These parameters are used to calculate the bond lengths and angles given in Fig. 1. A picture of a single molecule is given in Fig. 2.

The crystal structure of II is illustrated in Fig. 3. The dashed lines show hydrogen bonds that link the molecules in sheets running perpendicular to the c axis. The ions are linked directly by an NH···O hydrogen bond in the same manner as is found in the crystal structure (10) of demecolcine (VI). In addition, there are hydrogen bonds from N to Cl^- and to water, from O-1 to water, from water to water, and from water to Cl^-. There are no intramolecular hydrogen bonds and no hydrogen bonding between sheets.

The substitution of sulfur for oxygen causes small, but significant, changes in the troponoid ring. While tropolone had been considered to be aromatic, recent results indicate that there is not complete delocalization of the π-electrons. The best diffraction work shows a clear alternation in bond lengths about the ring, whereas an aromatic ring ought to have equal lengths. Hamor and Derry (11) found that the tropolone ring is not planar but may be described as a boat with angles of about 2° between its planes.

In II, some of these effects are exaggerated because the sulfur provides less opportunity for electron delocalization than oxygen. The bond length alternation is apparent with the formally double bonds averaging 1.370

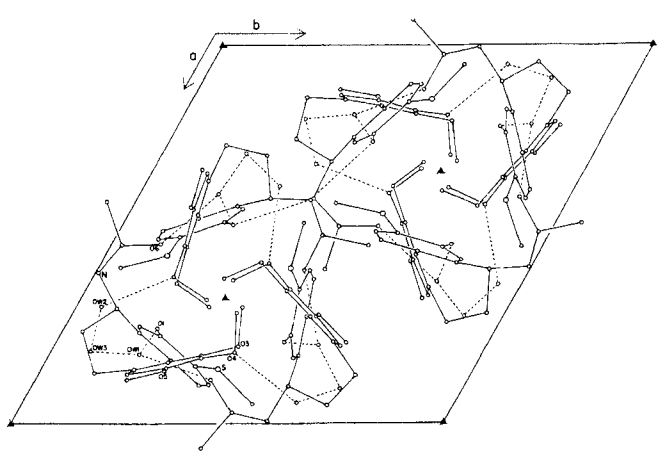


Figure 4—*Crystal structure of hydrated III. Dashed lines are hydrogen bonds. The region around the origin and corners of the unit cell contain disordered water of hydration. Some atoms in the C ring, the troponoid ring, are displaced for clarity.*

Table IV—Positional Parameters in III [a]

Atom	x	y	z
S	0.5907 (5)	0.4963 (5)	0.7583 (2)
O-1	0.799 (1)	0.561 (1)	0.7407 (5)
O-3	0.574 (1)	0.372 (1)	0.4847 (5)
O-4	0.574 (1)	0.388 (1)	0.3684 (5)
O-5	0.712 (1)	0.571 (1)	0.3168 (5)
O-6	0.883 (1)	0.405 (1)	0.5748 (5)
N	0.994 (2)	0.589 (1)	0.5739 (7)
Water O-1	0.816 (2)	0.209 (1)	0.5273 (7)
Water O-2	0.314 (4)	0.368 (4)	0.601 (2)
Water O-3	0.283 (4)	0.192 (4)	0.303 (2)
C-1	0.763 (2)	0.553 (2)	0.6932 (7)
C-2	0.650 (2)	0.518 (2)	0.6901 (7)
C-3	0.589 (2)	0.502 (2)	0.6427 (7)
C-4	0.615 (2)	0.510 (2)	0.5836 (7)
C-5	0.714 (2)	0.543 (2)	0.5601 (7)
C-6	0.811 (2)	0.570 (1)	0.5887 (7)
C-7	0.836 (2)	0.582 (2)	0.6464 (7)
C-8	0.714 (2)	0.548 (2)	0.4948 (7)
C-9	0.786 (2)	0.641 (2)	0.4711 (8)
C-10	0.796 (2)	0.664 (2)	0.4079 (8)
C-11	0.714 (2)	0.565 (2)	0.3759 (8)
C-12	0.645 (2)	0.471 (2)	0.4011 (9)
C-13	0.646 (2)	0.465 (2)	0.4622 (7)
C-14	0.872 (2)	0.734 (2)	0.5061 (8)
C-15	0.955 (2)	0.709 (2)	0.5298 (8)
C-16	0.907 (2)	0.596 (2)	0.5492 (7)
C-17	0.976 (2)	0.499 (2)	0.5809 (8)
C-18	0.619 (2)	0.306 (2)	0.5041 (9)
C-19	0.620 (2)	0.329 (2)	0.3364 (9)
C-20	0.788 (2)	0.671 (2)	0.2891 (8)
C-21	0.456 (2)	0.458 (2)	0.7469 (8)
C-22	1.066 (2)	0.474 (2)	0.598 (1)

[a] See footnote *a*, Table I.

Å and the formally single bonds[4] averaging 1.428 Å. The ring may be described as a boat consisting of three planes: (C-3–C-4–C-5–C-6), (C-2–C-3–C-6–C-7), and (C-1–C-2–C-7–O-1). The dihedral angle is 6.7° between the first and second of these planes and 5.1° between the second and third, larger angles than are seen in other tropolones.

Table III lists the torsion angles around some selected bonds for II and VI. The larger torsion angles of the troponoid ring in II are a measure of the lack of planarity, since a planar ring would have all torsion angles zero. This buckling of the troponoid ring leads to some changes in distances between atoms on the A and C rings. In particular, the O-1–O-3 distance is 6.79 Å in VI and 6.53 Å in II. Torsion angles for the B ring (Table III) are similar to those in VI, the difference being due to differences in the troponoid ring buckling.

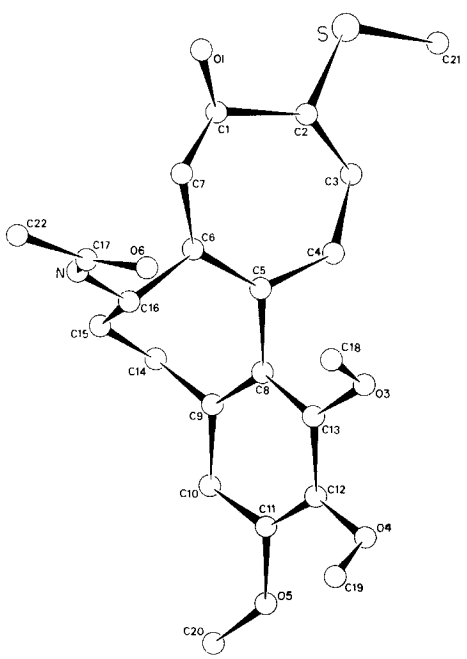
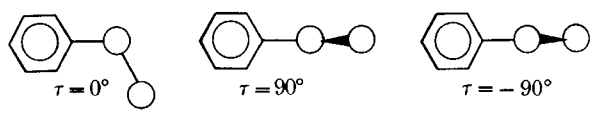


Figure 5—*One molecule of III.*

Compound III forms trigonal crystals, space group $P3_121$, with a = b = 14.532 ± 0.02 and c = 23.453 ± 0.03 Å. The observed density is 1.23 g/cm³. With six water molecules per molecule, the calculated density is 1.22 g/cm³. The compound is thus formulated as $C_{22}H_{25}NO_5S{\cdot}6H_2O$ with six formula weights per unit cell. All atoms are in general positions given by x, y, and z: −y, x − y, and ⅓ +z; y − x, −x, and ⅔ +z; y, x, and −z; −x, y − x, and ⅓ −z; and x − y, −y, and ⅔ −z. The thiocolchicine molecules are arranged around the 3_1 axis, leaving a large opening containing water of crystallization (Fig. 4). The methoxy groups point in, toward another 3_1 axis, giving a hydrophobic area in the crystal.

As in II and VI, there is an NH···O hydrogen bond to O-1. Most of the water is disordered. The oxygen of the one ordered water forms hydrogen bonds to carboxyls O-1 on one molecule and O-6 on another; two disordered waters, refined as half atoms, are hydrogen bonded to O-4 (a methoxy oxygen) and to water. A difference map shows numerous other small peaks due to disordered water in the hole at the origin. The disorder in the water structure accounts for the rather high *R* factor and leads to large standard deviations in bond lengths. For this reason, bond lengths and angles are not listed in detail. The molecule has the same structure as II within the limits of this analysis. Atomic parameters are listed in Table IV, and one molecule is shown in Fig. 5.

The conformation of the methoxy group is different in these two sulfur derivatives from that in VI. In general, methoxy groups on benzenoid rings adopt either a planar conformation (torsion angle $\tau = 0°$) with the methyl carbon in the plane of the benzenoid ring or a perpendicular conformation ($\tau = 90°$) with the methyl group sticking out as far as possible from this plane[5]. If the methyl group is on the opposite side of the ring from that of $\tau = 90°$, it would be described as $\tau = -90°$.



While the conformation of all three compounds is the same at O-3, O-5, and SCH_3 or OCH_3 on the troponoid ring, the O-4 methoxy is on the opposite side of the benzenoid ring in the sulfur-containing compounds (Table III).

Except for the large conformational changes possible in the methoxy groups, the colchicine molecule is rigid. All derivatives may be described as containing two nearly planar moieties twisted by about 54–60° from each other. Indeed, even inactive molecules such as colchicoside and isocolchicine (12) fit this basic description. The methoxy groups in such a wide variety of natural products are probably crucial to the interaction with tubulin (10, 12, 13). The structure of thiocolchicine serves as a reminder that an OCH_3 group may participate in hydrogen bonding and is not necessarily associated with hydrophobic interactions. In addition, methoxy can rather easily change conformation, which may play a role in the specificity of these molecules. For example, a change in the methoxy conformation may take place just before binding, with the methoxy group protecting the ring until the binding site is reached.

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[4] Excluding C-1–C-2, which is probably less involved in the π-electron delocalization (11).

[5] For colchicine derivatives, $\tau = 90°$ is defined as the methyl group on O-3, O-4, or O-5, pointing in the direction of C-16 and N (Fig. 5).

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Simultaneous Automated Determination of Spironolactone Metabolites in Serum

PETER NEUBERT [x] **and KLAUS KOCH**

Abstract □ An automated two-phase method for the simultaneous fluorometric determination of the spironolactone metabolites canrenone (II) and canrenoic acid (III) in serum is described. The determination is performed by two dichloroethane extractions of the same serum sample at different pH values. The fluorescence developed in 65% (v/v) sulfuric acid is measured in two separate fluorometers (one each for canrenone and canrenoic acid). Comparable specificity and sensitivity to the manual procedure are obtained, with sensitivity limits of 20 ng of II/ml and of 30 ng of III/ml in serum. This method is applicable to the automated determination of drugs and metabolites in biological material when several extraction steps are involved.

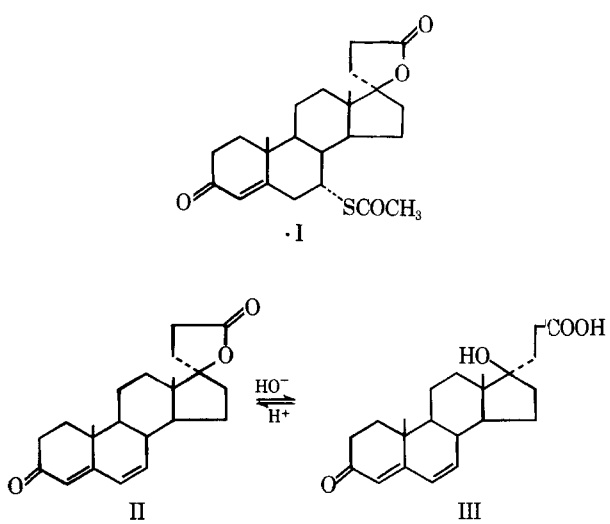
Keyphrases ◨ Canrenone and canrenoic acid—automated simultaneous fluorometric analyses, serum □ Fluorometry—automated simultaneous analyses, canrenone and canrenoic acid, serum □ Automated analyses—simultaneous fluorometric analyses of canrenone and canrenoic acid, serum □ Spironolactone metabolites—canrenone and canrenoic acid, automated simultaneous fluorometric analyses, serum ◨ Aldosterone antagonists—canrenone and canrenoic acid, automated simultaneous fluorometric analyses, serum

Spironolactone[1] (I) inhibits the aldosterone-initiated reabsorption of sodium ions from the distal portion of the renal tubule. The increased excretion of sodium ions is the underlying principle of the diuretic action in the treatment of edema.

Compound I is rapidly metabolized in the body after oral administration (1, 2). The elimination of the thioacetyl group on C-7 gives rise to canrenone[2] (II). Canrenone, as a γ-lactone, is in a pH-dependent equilibrium with the corresponding γ-hydroxycarbonic acid, canrenoic acid[2] (III) (Scheme I).

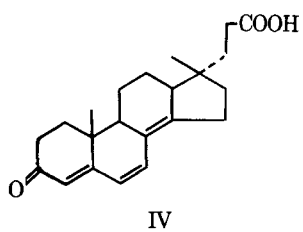
Compounds II and III are relatively stable in the physiological pH range of 5–9, but greater pH changes and enzymatic processes change the ratio of the two metabolites (3). For this reason, knowledge of the blood level curves for both metabolites is important for assessing the absorption, distribution, and excretion of spironolactone.

Gochmann and Gantt (4) first described the procedure for the fluorometric determination of canrenone by measurement of the fluorescence in 65% sulfuric acid. The structure of the fluorophore (IV) produced by the reaction of sulfuric acid with canrenone and canrenoic acid was elucidated (5).



Scheme I

The high intensity of the fluorescence of the trienone (IV) in 65% sulfuric acid permits the sensitive determination of the metabolites in both manual (6) and automated procedures. The manual procedure is time consuming since four extractions are necessary. The analysis is complicated further by the caustic nature of the reagents dichloroethane and 65% sulfuric acid. The automation of the analytical procedure was therefore desirable and is the subject of this report.



[1] Aldactone, Boehringer Mannheim GmbH.

[2] Compound II was formerly referred to as aldadiene, and III was referred to as aldadienic acid (1, 2).