TABLE VI—R_f VALUES OF INTERFERING SUBSTANCES 250 μ SILICA GEL–TOLUENE

Substance	R_f
BHT	0.97
Anhydrovitamin A	0.97
Alpha tocopherol	0.49
BHA	0.38
Beta tocopherol	0.32
Gamma tocopherol	0.30
Delta tocopherol	0.20

Assays Other Than Multivitamins—Thin-layer separation should be applicable to most types of formulations in addition to multivitamins. One such application was to a liquid tonic formula labeled to contain 20 I.U. vitamin E, as *d*-alpha tocopheryl acetate/oz. Assay of the unsaponifiable fraction prior to chromatography indicated 440 I.U. apparent vitamin E/oz. TLC revealed the large amount of reducing substances. Assay of the eluted alpha tocopherol band gave a reasonable result of 20.6 I.U./oz.

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Keyphrases

Vitamin E analysis—multivitamin products
TLC—separation, analysis
UV light—α-tocopherol zone visualization
Colorimetric analysis—spectrophotometer

Alkaloids of *Ochrosia maculata* Jacq. (*Ochrosia borbonica* Gmel.)

Isolation of the Alkaloids and Study of the Antitumor Properties of 9-Methoxyellipticine

By GORDON H. SVOBODA, GERALD A. POORE, and MARILYN L. MONTFORT

Inclusion in the authors' phytochemical screening program of plants of the family *Apocynaceae* related to *Catharanthus roseus* G. Don seemed logical in view of the success realized with the Madagascan periwinkle. Screening of the appropriate extracts of *Ochrosia maculata* elicited both oncolytic and neurosedative activities. The former was found to be associated with 9-methoxyellipticine, the latter with reserpine. While 9-methoxyellipticine possesses experimental antitumor activity of a somewhat lesser order than some of the available clinically active agents, it does exhibit a broader spectrum than most of these compounds. Its moderate degree of potency as an antitumor agent is expressed by its activity against several of the solid mouse neoplasms maintained in these laboratories.

THE GENUS *Ochrosia*, family *Apocynaceae*, consists of approximately 36 species of trees or woody shrubs native to tropical Asia, Oceania, and the Mascarene and Seychelle Islands. *Ochrosia maculata* Jacq., a local name for which is "bois jaune" because of the bark and wood being yellow and bitter (1), is a tree 6–12 m. (20–40 ft.) in height, bearing 3 leaves (rarely 4) in a whorl, being oblong to oblong-lanceolate, 13.5–15.2 cm. (3–6 in.) long, obtuse or somewhat acute, glossy, and often spotted. The flowers are white, being

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about 0.8 cm. (1/3 in.) across, with calyx lobes ovate. The fruit is bright red and reputedly poisonous.

The various species of this genus have been rather severely handled by taxonomists, producing a confusing state of botanical nomenclature and classification. The following classification of Pichon (2, 3) is the one usually accepted: family, *Apocynaceae*, subfamily, *Plumerioideae;* tribe, *Rauvolfieae;* subtribe, *Ochrosiinae.*

The botanical name now usually considered as being correct for this plant is *Ochrosia maculata* Jacq., having a distribution restricted to the Mascarene and Seychelle Islands.[1] *O. borbonica* Gmel. is considered to be a direct synonym, as is *Cerbera undulata* (4). However, *O. borbonica* has in the past been considered as being a synonym for the Pacific *O. oppositifolia* (Lamk.) K. Schum., a distinct species which is stated to occur from the Mascarene Islands to Polynesia. Consequently, the early chemical investigations recorded as being performed on *O. borbonica* should probably be referred to as *O. maculata* rather than to *O. oppositifolia*, inasmuch as the early French workers were interested in the use of the Mascarene group species as a tonic and febrifuge.

Planchon (5) has referred to the bark and leaves of *O. maculata* (described as *O. borbonica*) as having been used in Réunion, Mauritius, and the Mascarene Islands as a bitter tonic, stomachic, and febrifuge. Ochrosine, a substance isolated by Boissard, has been found by Vinson (5) to be tonic and analeptic. Carrieu [quoted by Barquissau (5)] found that when the bark was used medicinally, it was not toxic even in high doses and that it was no more effective in the treatment of intermittent fevers than were other bitter tonics. It appeared to be cardiotonic, acting as a diuretic and augmenting the impulsive force of the heart. Vinson considered it to be a good remedy for anemia.

A defatted ethanolic extract of the bark[2] showed moderate experimental antitumor activity against adenocarcinoma 755, as well as a CNS depressant and vasodilator activity in the mouse behavior test (6). Subsequent application of a sequential extraction technique using *n*-hexane, benzene, ether, and ethanol provided extracts which elicited other biological activities, *e.g.*, weak serotonin-like effects, anti-inflammatory, diuretic, and nonreserpine-like CNS depressant responses. It was, however, the antitumor activity and the CNS depressant effects which prompted the major phytochemical effort which ensued.

9-Methoxyellipticine has been isolated from this species by two other groups of investigators (7, 8) and its effect against the L-1210 leukemia has been described (7). Other species of this genus have also yielded this alkaloid as indicated by the following documentation: Section 1, *Lactaria: O. elliptica* Labill. (9, 10); *O. coccinea* (Teijsm. and Binnend.) Miq. [*Excavatia coccinea* (Tejsmann and Binnendijk) Mgf.] (10); *O. moorei* F. Muell. (11); Section 2, *Echinocaryon: O. glomerata* Valeton (11); *O. oppositifolia* (Lamk.) K. Schum. (12); Section 3, *Phragmochrosia:* contains only one species, *O. apoensis* Elmer, which has not yet been examined.

Reserpine has been obtained from the leaves of *O. poweri* Bailey (11) and from the bark of *O. coccinea* (13). Its isolation from *O. maculata* is herein reported for the first time.

A number of different extraction and purification techniques were investigated, including the selective extraction and gradient pH techniques which proved to be so useful in the investigation of the alkaloids of *Catharanthus roseus* G. Don (14, 15). However, neither of these proved to be of any special value in the present investigation. While a number of probe runs utilizing a variety of techniques were used, the following procedure was the one eventually considered to be the best for the authors' purposes.

Extraction of the bark with ammoniacal benzene yielded an extract from which relatively pure 9-methoxyellipticine deposited during concentration. Eventual extraction of the bark with ethanol yielded an extract which showed both antitumor and CNS depressant activities. Additional quantities of 9-methoxyellipticine were isolated therefrom using classical techniques. It was, however, necessary to utilize column chromatography to isolate reserpine. All fractions were monitored with TLC and biological activities were checked primarily with the X-5563 plasma cell myeloma and with mouse behavior patterns.

EXPERIMENTAL

Coarsely ground bark (100 kg.) was extracted by stirring with one 400-l. portion of benzene and 4 l. of concentrated ammonium hydroxide. Two subsequent benzene extractions of 400 l. each were run and combined with the first. Concentration *in vacuo* yielded two crops of yellow-gold needles, 7.612 g. and 3.150 g., at 4 l. and 800-ml. volumes, respectively. Interpretation of the physical data (X-ray, UV, and IR spectra) indicated that these two crops of crystals were mainly 9-methoxyellipticine. TLC using the system of ethyl acetate–ethanol (9:1) on Silica Gel G indicated 9-methoxy-

[1] Personal communication from Dr. Julian A. Steyermark, Botanical Institute, Caracas, Venezuela, based on information provided by the Royal Botanic Gardens at Kew.

[2] The drug used in this investigation was obtained from the Meer Corporation, New York, N. Y.

ellipticine to be the major component in both cases.

Concentration of the benzene crude mother liquor (CML) *in vacuo* yielded 279.3 g. of a dark brown syrup which was found to possess moderate anti-inflammatory activity (carageenin-induced edema), a weak reserpine-like CNS depression in mice, and antitumor activity against the adenocarcinoma 755.

A 10-g. aliquot of this crude mother liquor was dissolved in 500 ml. of $EtCl_2$, an equal volume of 1% H_2SO_4 was added, and the $EtCl_2$ was removed *in vacuo*. The acid H_2O-insoluble material was filtered off and held for further examination. The aqueous acid phase was extracted with 500-ml. portions of $EtCl_2$ at pH levels of 1.0, 3.5, and 8.2. Only the lower two showed weak reserpine-like depression. While TLC indicated the possible presence of reserpine, none could be isolated in crystalline form. The insoluble material cited above showed activity against the AC755 and TLC indicated that it was probably caused by the presence of 9-methoxyellipticine or a close relative. None was isolated in crystalline form.

Extraction of the bark with three 400-l. portions of ether gave 75.71 g. of a dark brown syrup which showed weak reserpine-like CNS depression and oncolytic activity against the X-5563 myeloma. A work-up of a 10-g. aliquot in a manner analogous to that just described did not yield any crystalline isolates.

Final extraction of the drug involved the use of three 400-l. portions of 95% ethanol. The combined extracts were concentrated *in vacuo* to approximately 11 l. A 50-ml. aliquot of this concentrate yielded 17.36 g. of dried residue which elicited a weak CNS depression in mice and a serotonin-like bluing of the skin. Some activity was also shown against the P-1534 leukemia.

A 2.2-l. aliquot of the ethanol extract concentrate, containing approximately 763.8 g. of extractive, was extracted with one 8.8-l. and two 4.4-l. portions of 1% H_2SO_4. The combined aqueous acid extracts were extracted at the existing pH of 0.7 with one 16-l. and two 8-l. portions of $EtCl_2$, yielding 15.0 g. of a dark brown gum. Although this extract elicited a weak reserpine-like depression in mice, the alkaloid could not be isolated therefrom directly. A 10-g. aliquot, when chromatographed on 300 g. of partly deactivated Alcoa F-20 alumina (Table I), yielded 0.099 g. of off-white, rod-like crystals from methanol which proved to be reserpine.

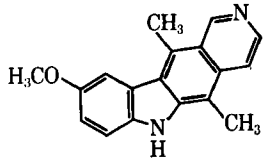
Two subsequent $EtCl_2$ extractions were performed at pH levels of 3.2 and 8.6 as previously described. The residue from the pH 3.2 extract (13.78 g.) proved to be of no interest and was eventually discarded. Concentration *in vacuo* of the $EtCl_2$ at pH 8.6 to an approximate volume of 1.2 l. yielded 16.30 g. of insoluble material which was shown to be mainly 9-methoxyellipticine. Concentration of the crude mother liquor yielded 24.9 g. of dried material which was eventually discarded.

A flow diagram for the extraction scheme is presented in Scheme I.

Both the roots and leaves (1.5 kg. each) were extracted in an analogous manner. The yields of ammoniacal benzene, ether, and ethanol extracts from the roots were 1.33, 1.0, and 13.36 g., respectively. A weak diuretic activity was observed for the ethanol extract, the other two extracts being relatively inactive in all tests. The yields of the corresponding extracts from the leaves were 47.67, 26.99, and 47.95 g., respectively. Both the ammoniacal benzene and ether extracts showed slight anti-inflammatory activity and the latter showed slight antibacterial activity as well. The ethanol extract displayed slight antibacterial activity. No other biological responses were noted. The benzene extract was subjected to column chromatography on Al_2O_3. No crystalline entities were obtained. Details of the above work are not warranted.

9-Methoxyellipticine—Recrystallization of 26.94 g. of crude alkaloid from hot methanol (600 ml./g.) yielded 13.36 g. of first crop material as fine blades showing both parallel extinction and oblique extinction with negative elongation. A second crop of 2.36 g. was obtained from the mother liquor. The physical data for this recrystallized material agreed in all respects with those for the authentic alkaloid.[3] Such data have been sufficiently well documented so as to preclude repetitive presentation here.

The structure for 9-methoxyellipticine (I) has been reported as 9-methoxy-5,11-dimethyl-6*H*-pyrido-[4,3-*b*]-carbazole (10).



I

Reserpine—Methanol recrystallization of the reserpine obtained from the column previously described yielded 0.051 g. of colorless rods. Physical measurements, as well as biological effects and potency, were identical in all respects to those for authentic reserpine previously isolated in these laboratories.

BIOLOGICAL PROPERTIES

The experimental antitumor properties exhibited by 9-methoxyellipticine are of a somewhat lesser order than some of the presently available clinically active compounds and are certainly less than those of acronycine (17). It does, however, exhibit a broader spectrum than most clinically active agents in use today. Its moderate degree of potency is expressed in its activity against a number of solid mouse neoplasms maintained in these laboratories.

Methodology—Procedures for animal tumor testing in these laboratories have been previously described (18) and consist essentially of subcutaneous trocar implantation of solid tumors in the axillary region and intraperitoneal inoculation of ascitic and leukemic cells with standard cell inocula. Treatment is normally initiated 24 hr. later, exceptions being made for the X-5563 myeloma, S-91 melanoma, Ridgeway osteogenic sarcoma, and the Shionogi carcinoma 115. In the case of the X-5563 myeloma treatment was initiated 3 days postimplantation, whereas with the other three systems it was initiated

[3] Kindly supplied by Dr. C. C. G. Culvenor, C.S.I.R.O., Melbourne, Australia. Also see *Reference 16*.

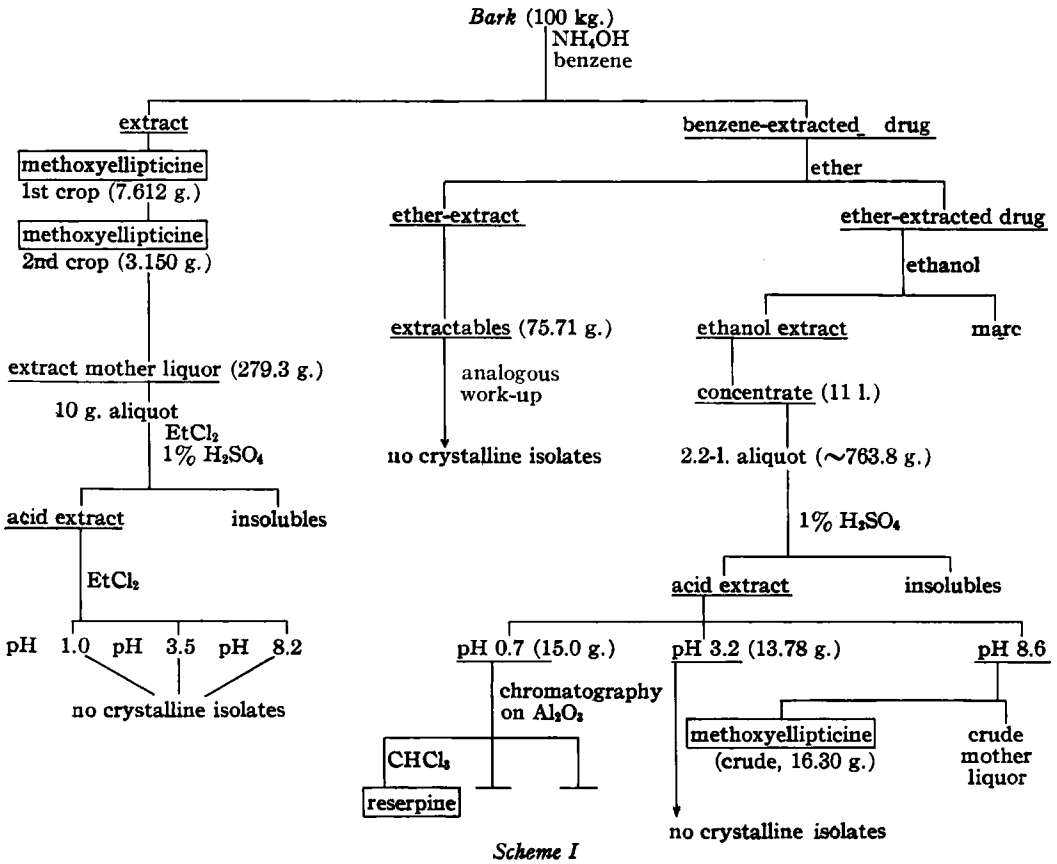
TABLE I—CHROMATOGRAPHY OF WEAK BASE FRACTION FROM EtOH EXTRACT

Fraction	Eluting Solvent	Volume, ml.	Wt., g.	Compound	Wt., g.	Crystallizing Solvent
1	Chloroform	205	0.25	Oil	—	—
2	Chloroform	90	1.17	Reserpine	0.051	Methanol
3	Chloroform	90	1.27	Reserpine	0.048	Methanol
4–19	Chloroform	6,645	2.29	Amorphous residues	—	—
20–30	Chloroform–methanol (99:1)	2,685	0.29	Amorphous residues	—	—
31–38	Chloroform–methanol (19:1)	3,480	0.31	Amorphous residues	—	—
39	Methanol	1,050	0.91	Amorphous residues	—	—

5 days postimplantation, this delay being occasioned because of the very slow rate of initial growth.

Activity of the alkaloid is expressed in terms of tumor inhibition of the solid tumor treated *versus* the control (T/C). When evaluating results for the leukemias and ascites forms, activity is expressed in terms of percent prolongation of life of the treated animals *versus* the controls (T/C).

Preparation of 9-Methoxyellipticine for Testing—This alkaloid was relatively insoluble in almost all of the standard testing vehicles, but this circumstance did not prevent its being tested. A suitable suspension was prepared by grinding the compound with small volumes of a nonionic dispersant[4] to obtain a suitable uniform suspension and diluting with additional diluent to the desired dosage volume. (The dispersant is a polyoxyethylated fatty acid that is H_2O-miscible and nontoxic when diluted to the proper concentration of 1:10 with either sterile distilled H_2O or sterile physiological saline solution.) 9-Methoxyellipticine was insoluble in saline, sesame oil, olive oil, gum acacia, and carboxymethylcellulose and no satisfactory suspensions therein could be obtained.



Scheme I

ANTITUMOR ACTIVITY OF 9-METHOXYELLIPTICINE

The authors' experience with this alkaloid has shown it to possess activity against a relatively broad spectrum of mouse neoplasms, 10 of 17 systems having responded to a somewhat narrow range of dose levels. Furthermore, it was demonstrated to be active in certain cases by both the

[4] Marketed as Emulphor by General Aniline and Film Corp., Melrose Park, Ill.

TABLE II—EXPERIMENTAL TUMOR SPECTRUM OF 9-METHOXYELLIPTICINE

Tumor	Host, 10 Animals	I.p. Dosage, mg./kg./day	Av. Wt. Change, g., T/C	Av. Tumor Size, mm., T/C	Av. Life, T/C	Activity,[a] %
L-1210 leukemia	DBA/2	12 × 1 × 10	−0.6/−0.2	—	16.7/15.5	0
L-1210 leukemia III	DBA/2	12 × 1 × 10	−0.5/+2.8	—	13.9/11.0	26
C-1498 leukemia	C57B1/6	18 × 1 × 10	+0.1/+0.3	—	18.8/14.0	34
P-1534 leukemia	DBA/2	24 × 1 × 10	−1.7/+1.0	—	20.0/15.8	26
AKR leukemia	AKR	18 × 1 × 10	−3.6/+1.4	—	11.2/13.2	0
B-82 leukemia, solid	C58B1/6	18 × 1 × 10	−0.3/+1.6	20.8/14.9	—	0
Freund ascites	CAF[1]	18 × 1 × 10	+3.5/+8.8	—	17.6/17.1	0
S-180 ascites	CAF[1]	24 × 1 × 10	−3.1/−0.3	—	10.0/11.3	0
Taper hepatoma, ascites	C3H	18 × 1 × 10	+3.0/+3.6	—	13.7/10.6	29
Adenocarcinoma 755	C57B1/6	60 × 1 × 10	−4.4/+2.8	5.6/18.0	—	69 (7)
Mecca lymphosarcoma	AKR	12 × 1 × 10	+1.0/+2.6	14.9/22.0	—	32 (10)
X-5563 myeloma	C3H	12 × 1 × 10	−3.8/−2.2	4.2/9.4	—	55 (7)
Gardner lymphosarcoma	C3H	18 × 1 × 10	−1.5/+6.0	15.3/31.8	—	52 (4)
C3H mammary	C3H	24 × 1 × 10	−2.8/+3.6	18.8/30.7	—	39 (7)
High malignancy clone	C3H	18 × 1 × 10	−4.6/−0.1	3.9/9.2	—	58 (9)
S-91 melanoma	DBA/1	24 × 1 × 10	−5.2/+0.6	0/5.5	—	100 (7)
Ridgeway osteogenic sarcoma	AKR	24 × 1 × 14	−2.5/+1.3	8.9/7.9	—	0
Walker 256, carcinosarcoma	Rat	30 × 1 × 10	−2.0/+4.1	8.9/14.3	—	38 (5)[b]
Walker 256, ascites	Rat	30 × 1 × 10	+11.6/51.4	—	21.3/11.6	84 (2)[b,c]

[a] The number in parentheses indicates survivors on solid tumors, indefinite survivors at 45 days on leukemia or ascitic tests. [b] Five rats used. [c] Two indefinite survivors at 29 days postinoculation. Inoculated with ascites form.

intraperitoneal and oral routes. The corresponding dose ranges were from 12–24 mg./kg. and 24–36 mg./kg., respectively.

This alkaloid is active against a lymphocytic leukemia and several solid tumors which are not responsive to the oncolytic alkaloids from *Catharanthus roseus* G. Don. Its inability to show significant activity against most of the ascites systems lends credence to the assumption that its antitumor activity may not be due to direct cytotoxic action.

Activity Against the L-1210 III Leukemia—Unlike many other antitumor agents there does not appear to be a system of choice in determining its efficacy. It may be noteworthy, however, to cite its activity against the L-1210 III leukemia. This particular tumor was made resistant to 6-mercaptopurine by workers at the Sloan-Kettering Institute of Cancer Research. 9-Methoxyellipticine is one of the few compounds tested in these laboratories which has shown any activity against this neoplasm, both intraperitoneally and orally.

Activity Against the Walker Rat Tumor 256—This test was conducted in young Sprague-Dawley rats, the tumor being maintained in both the solid and ascites form. Significant activity was demonstrated against both forms, indefinite survivors being obtained in the case of the ascites form, 4 of 10 rats having survived for 29 days since the time of implantation. At the time of sacrifice no ascites fluid could be detected in any of the animals.

Lack of Activity Against 7,12-Dimethylbenz(a)-anthracene-induced Tumors—Huggins *et al.* (19) have shown that a single oral dose of this carcinogen, dissolved in sesame oil, administered to virgin

TABLE III—DOSE RESPONSE, ORAL THERAPY OF 9-METHOXYELLIPTICINE

Tumor	Dosage mg./kg./day	Av. Weight Change, g., T/C	Av. Tumor Size, mm., T/C	Av. Life, T/C	Activity,[a] %
L-1210 leukemia III	18 × 1 × 10	+0.7/+2.0	—	14.1/10.4	36
	24 × 1 × 10	−0.5/+2.0	—	13.9/10.4	34
	36 × 1 × 10	−2.4/+2.0	—	13.1/10.4	26
P-1534 leukemia	18 × 1 × 10	−0.5/+1.1	—	17.2/15.0	0
	24 × 1 × 10	−0.8/+1.1	—	15.9/15.0	0
	36 × 1 × 10	+0.8/+1.1	—	19.8/15.0	32
X-5563 myeloma	6 × 1 × 10	−1.6/−2.0	5.9/10.7	—	45 (8)
	12 × 1 × 10	−5.0/−2.0	6.8/10.7	—	37 (10)
	18 × 1 × 10	−5.2/−2.0	6.9/10.7	—	36 (10)
Gardner lymphosarcoma	18 × 1 × 10	+1.3/+6.3	25.6/31.8	—	0
	24 × 1 × 10	−3.7/+6.3	18.0/31.8	—	43 (7)
	36 × 1 × 10	−2.9/+6.3	15.7/31.8	—	51 (6)
C3H mammary	18 × 1 × 10	−1.8/+6.3	20.1/29.9	—	33 (6)
	24 × 1 × 10	−6.4/+6.3	6.5/29.9	—	78 (1)
	36 × 1 × 10	−/+6.3	−/29.9	—	N.S.[b]
High malignancy clone	18 × 1 × 10	−3.5/−3.6	2.6/7.2	—	64 (9)
	24 × 1 × 10	−4.8/−3.6	4.2/7.2		42 (8)
	36 × 1 × 10	−5.8/−3.6	0.9/7.2	—	88 (10)

[a] The number in parentheses indicates survivors on solid tumors, indefinite survivors at 45 days on leukemia or ascitic tests. [b] No survivors at end of treatment period.

Sprague-Dawley rats 50 days old induced breast tumors within 21–120 days of feeding. When testing against these tumors, 9-methoxyellipticine was found to be inactive at 30 mg./kg. i.p. and 60 mg./kg. *per os*. Furthermore, some weight loss and toxicity were evidenced in both regimens.

The biological data are summarized in Tables II and III.

SUMMARY

The oncolytic and CNS depressant activities first encountered in the authors' screening program with certain extracts from *Ochrosia maculata* Jacq. (*O. borbonica* Gmel.) have been found to be associated with 9-methoxyellipticine and reserpine, respectively. 9-Methoxyellipticine possesses a relatively broad spectrum of antitumor activity, 10 of 17 mouse neoplasms tested having responded. Both intraperitoneal and oral activity have been noted. Significant activity was seen against both the ascites and solid forms of the Walker rat carcinosarcoma 256.

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Keyphrases

Alkaloids—*Ochrosia maculata*
9-Methoxyellipticine—isolation, identity
Antitumor activity—9-methoxyellipticine
TLC—separation
X-ray crystallography—identity
UV spectrophotometry—identity
IR spectrophotometry—identity

Synthesis and Preliminary Evaluation of a New Quinuclidine Derivative as a Radioprotective Agent

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7,10-Ethano-1-thia-4,7-diazaspiro [4.5] decane dihydrochloride was prepared by the condensation of 3-quinuclidone hydrochloride with 2-aminoethanethiol hydrochloride. The compound was shown to have potential radioprotective properties against a lethal dose of X-radiation in mice. Optimum protection was obtained in a group of mice pretreated intraperitoneally with 0.20 mg./g. of 7,10-ethano-1-thia-4,7-diazaspiro [4.5] decane dihydrochloride 15 min. prior to exposure, where 58.3 percent survived. A toxicity study showed the i.p. LD_{50} of the compound to be 295 mg./kg. with a dose range of 257–339 mg./kg. ($p < 0.05$).

INTEREST IN quinuclidine, 1-azabicyclo[2.2.2]-octane, and its derivatives has been fairly recent. Although this molecule was first synthesized in 1909 (1), it received little attention until World War II. At that time, because of the shortage of quinine and the need for substitutes, quinuclidine became an important tool in the chemistry of synthetic antimalarials.

The greatest amount of synthetic work has been done on 3-substituted quinuclidines. Pharmacologic activity reported to date has included spasmolytic (2, 3), central nervous system stimulation (4), and ganglionic blockade (5).

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