similar to that reported for VII*a* and VII*b*; however, there is no definite spectroscopic evidence of its existence.

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Antitumor Agents XXVIII: Structural Elucidation of the Novel Antitumor Sesquiterpene Lactone, Microlenin, from *Helenium microcephalum*

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Received July 29, 1977, from the *Division of Medicinal Chemistry, School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, NC 27514.* Accepted for publication December 28, 1977.

Abstract □ The structure and stereochemistry of the novel dimeric antitumor sesquiterpene lactone, microlenin, were determined on the basis of ^{1}H-NMR, ^{13}C-NMR, and mass spectral evidence.

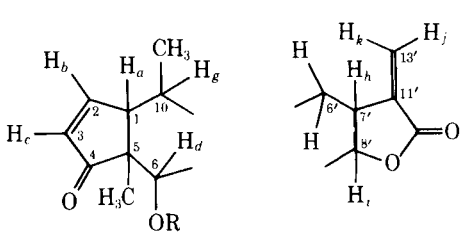
Keyphrases □ Microlenin—isolated from *Helenium microcephalum* whole plant extract, structure and stereochemistry determined □ *Helenium microcephalum*—whole plant extract, microlenin isolated, structure and stereochemistry determined □ Antineoplastic agents, potential—microlenin, isolated from *Helenium microcephalum* whole plant extract, structure and stereochemistry determined

A search for ample supplies of helenalin for the purpose of elucidating the structure–activity relationships between sesquiterpene lactones and antitumor activity led to the investigation of the plant *Helenium microcephalum*. Previously, it was reported (1) that this species contains helenalin in good yield. Examination of the whole plant extract revealed that, after the removal of helenalin, the mother liquor had constituents possessing significant inhibitory activity against the Walker 256 carcinosarcoma in rats. Further work with the chloroform extract resulted in the isolation and structure determination of mexicanin-E; microhelenins-A, B, and C (2, 3); and microlenin, a novel antineoplastic principle which resulted in a T/C value of 172 at 2.5 mg/kg/day in Walker 256 carcinosarcoma in Sprague–Dawley rats. The detailed structural determination of microlenin by physical methods is now reported.

RESULTS AND DISCUSSION

Microlenin (1*a*) was isolated by elution with chloroform–ethyl acetate from a silica gel column. Although the structure of microlenin was described previously (4), the stereochemistry and conformational assignments for microlenin require further comments. Microlenin contains an α,β-unsaturated cyclopentenone (IR bands at 1707 and 1580 cm^{-1}; ^{1}H-NMR: doublet of doublets of H_b and H_c at δ 7.84 and 6.02 ppm) and an α,β-unsaturated lactone of the type shown in partial structure A (IR bands at 1756 and 1664 cm^{-1}; narrowly split ^{1}H-NMR doublet of H_j and H_k at δ 6.13 and 5.73 ppm).

The presence of a secondary hydroxyl group (IR band at 3521 cm^{-1};



Partial Structure A

0022-3549/78/0900-1228$01.00/0

Table I—^{1}H-NMR Spectra of II*b*, III, and I*b* at 100 MHz in Deuterochloroform [a]

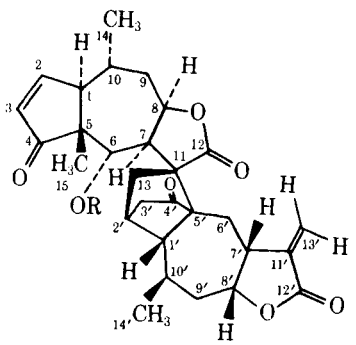
Assignment	II*b*	III[b]	I*b*
H-1	3.05 (ddd, 2.0, 3.0, 11.0)	—[d]	3.10 (ddd, 2.0, 3.0, 11.0)
H-2	7.69 (dd, 2.0, 6.0)	7.83 (dd, 2.25, 6.0)	7.67 (dd, 2.0, 6.0)
H-3	6.09 (dd, 3.0, 6.0)	6.28 (dd, 2.25, 6.0)	6.06 (dd, 3.0, 6.0)
H-6	5.41 (br)	—[d]	5.62 (br)
H-7	3.53 (m)	—[d]	2.48 (d, 8.0)
H-8	4.92 (m)	4.64 (m)	4.95 (m)
H-13a	6.46 (d, 3.0)	6.23 (d, 1.5)	
H-13b	6.15 (d, 2.25)	5.70 (d, 1.5)	
H-14[c]	1.27 (d, 7.0)	1.19 (d, 6.0)	1.23 (d, 7.0)
H-15[c]	1.00[c]		0.94
H-7′			3.29 (m)
H-8′			4.71 (m)
H-13′a			6.28 (d, 3.0)
H-13′b			5.52 (d, 3.0)
H-14′			1.18 (d, 7.0)

[a] Values are in parts per million; multiplicities are indicated by the usual symbols: d, doublet; m, multiplet whose center is given; and br, slightly broadened singlet. Unmarked signals are singlets. Figures in parentheses are coupling constants in hertz. [b] The 60-MHz NMR spectrum of III was reported previously (6). [c] Three-proton intensity. [d] Obscured signal.

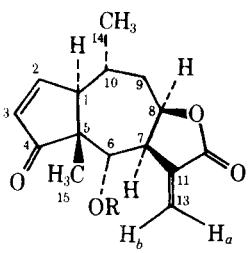
^{1}H-NMR doublet at δ 5.51 ppm) that disappeared upon addition of deuterated water was confirmed by acetylation of microlenin with acetic anhydride and pyridine. This reaction gave the monoacetate (I*b*) ($C_{31}H_{36}O_8$, high-resolution mass spectrum, mp 278°), which showed the loss of a free hydroxyl in its IR spectrum (carbon tetrachloride). This loss was accompanied by a paramagnetic shift of a signal in its ^{1}H-NMR spectrum from δ 4.20 to 5.62 ppm[1].

A comparison of the chemical shifts and splitting patterns of the ^{1}H-NMR spectra of helenalin acetate (II*b*) (5), mexicanin-E (III) (6–10), and microlenin acetate (I*b*) provided strong evidence for the presence of the partial structure A in I*a* and I*b* (Table I). The ^{1}H-NMR spectrum of I*b* showed the low field doublet of doublets at δ 7.67 ($J_{1,2}$ = 2.0 and $J_{2,3}$ = 6.0 Hz) and 6.06 ($J_{1,3}$ = 3.0 and $J_{2,3}$ = 6.0 Hz) ppm, characteristic of the unsubstituted α,β-unsaturated cyclopentenone found in helenalin (II*a*) and III. The usual narrowly split doublets at δ 6.28 (H_k, J = 3.0 Hz) and 5.52 (H_j, J = 3.0 Hz) ppm due to the α-methylene-γ-lactone moiety of III were also present.

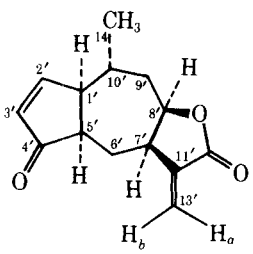
The presence in the ^{1}H-NMR spectrum of one methyl singlet and two methyl doublets led to the postulation of the novel dimeric sesquiterpene lactone structure, I*b*, for microlenin acetate (devoid of stereochemistry). The conclusions that the acetyl side chain was attached to C-6 (with H_d being accounted for by a somewhat broadened singlet at δ 5.62 ppm) and that the lactone rings were closed at C-8 (with multiplet at δ 4.95 ppm) and C-8′ (with multiplet at δ 4.71 ppm) were based on analogous substitution patterns found in other pseudoguaianolides (*i.e.*, II*b* and III).



I*a*: R = H
I*b*: R = $COCH_3$



II*a*: R = H
II*b*: R = $COCH_3$



III

Extensive double resonance experiments (Table I) on I*b* led to the assignment of protons that confirmed the carbon skeleton and oxygenation pattern postulated for microlenin. For example, identification of H_a (H-1) as a doublet of doublets of doublets ($J_{1,2}$ = 2.0 Hz, $J_{1,3}$ = 3.0 Hz, and $J_{1,10}$ = 11.0 Hz) at δ 3.10 ppm was achieved by double irradiation at the frequency of H_k and H_c. The location of H_h (H-7′) as a multiplet at δ 3.29 ppm was established by double irradiation at the frequency of H_j and H_k.

Conversely, irradiation at the frequency of H_h collapsed H_j and H_k into singlets and also slightly sharpened the signal of H_i (H-8′) at δ 4.71 ppm. Irradiation at the frequency of H_i converted H_h into a broad doublet of doublets. The foregoing evidence led to the assignment of the partial structure A for I*a*. The remaining three oxygen atoms of the empirical formula of I*a* were derived from a γ-lactone (IR band at 1763 cm^{-1}) and a cyclopentanone (IR band at 1744 cm^{-1}).

The ^{13}C-NMR spectrum (Table II) of I*a* indicated that microlenin contained 29 carbon atoms and possessed the following skeletal systems: seven >CH–; five $-CH_2-$; three each of –CHO–, >C<, and $-CH_3$; two each of –CH=, >C=O, and –C=O(O); and one each of CH_2= and >C=. The extreme similarities of the ^{1}H-NMR (Table I) and ^{13}C-NMR (Table II) parameters of I*a*, I*b*, II*a*, II*b*, and III further strengthened the chemical evidence that microlenin must be derived from helenalin and mexicanin-E. The major differences between the ^{1}H and ^{13}C-NMR spectra of I*a* (or I*b*), II*a* (or II*b*), and III were observed among the proton signals at C-7, C-13a,b, C-2′, and C-3′ and among the carbon signals at C-11, C-13, C-2′, and C-3′ (Tables I and II).

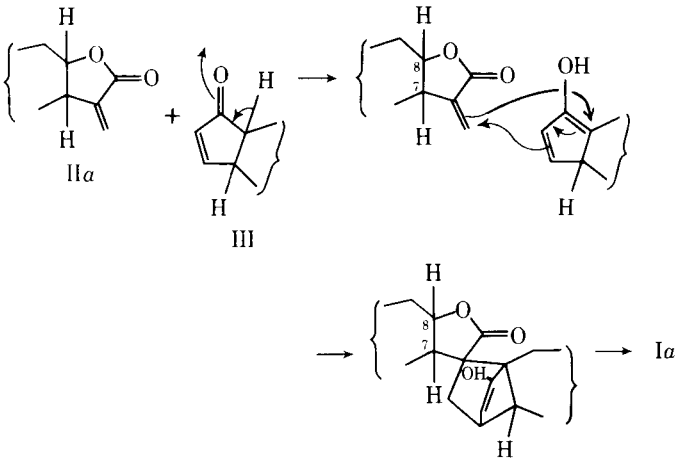
In the ^{13}C-NMR spectrum of microlenin, the carbon signals due to C-11 (singlet), C-13 (triplet), C-2′ (doublet), and C-3′ (triplet) at 67.4, 34.6, 35.5, and 38.2 ppm were observed, respectively. However, in the case of helenalin and mexicanin-E, these characteristic signals [C-11 (singlet), C-13 (triplet), C-2′ (doublet), and C-3′ (doublet)] were seen at 139.8, 129.8,

Table II—^{13}C-NMR Spectra of II*a*, III, and I*a* [a]

Assignment[b]		II*a*	III	I*a*	
C-1	(C-1′)	51.90 d	(45.0 d)	52.9 d	(38.8 d)
C-2	(C-2′)	163.98 d	(166.7 d)	163.9 d	(35.5 d)
C-3	(C-3′)	121.90 d	(132.9 d)	129.4 d	(38.2 t)[g]
C-4	(C-4′)	193.00	(194.3)	215.0[f]	(213.7)[f]
C-5	(C-5′)	57.70	(48.2 d)	57.6	(54.6)
C-6	(C-6′)	79.10 d	(28.0 t)	81.4 d	(26.7 t)
C-7	(C-7′)	52.40 d	(53.6 d)	53.6 d[e]	(53.1 d)[e]
C-8	(C-8′)	74.30 d	(78.9 d)	70.8 d	(76.5 d)
C-9	(C-9′)	40.40 t	(38.7 t)	41.2 t	(41.0 t)
C-10	(C-10′)	26.70 d	(31.7 d)	26.4 d[d]	(26.1 d)[d]
C-11	(C-11′)	139.80	(142.5)	67.4	(140.7)
C-12	(C-12′)	170.20	(170.2)	181.1	(169.1)
C-13	(C-13′)	129.80 t	(121.0 t)	34.6 t[g]	(122.5 t)
C-14	(C-14′)	20.30 q	(21.5 q)	20.1 q[e]	(21.5 q)[e]
C-15		18.80 q		19.7 q	

[a] Run in acetone-d_6–pyridine-d_5 (1:1) on a Varian XL-100 instrument. Unmarked signals are singlets. [b] Assignments based on predicted shifts and comparisons with data in the literature and on file. [c–g] These assignments may be interchanged.

[1] Part of this unusually large shift was attributed to a change of solvent from dimethyl sulfoxide-d_6 to deuterochloroform.



Scheme I

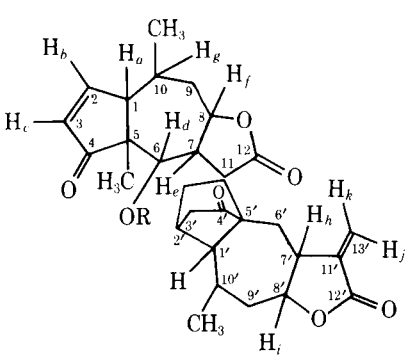
166.7, and 132.9 ppm, respectively. In the ^{1}H-NMR spectrum of I*b*, identification of H_e (H-7) as a broad doublet at 2.48 ppm was achieved by irradiating at frequencies corresponding to those of H_d and H_f. Conversely, irradiation at the frequency of H_e converted a broad singlet at 5.62 ppm (H_d) into a sharp singlet and also sharpened H_f.

These observations allowed expansion of partial structure A to Structure B, which required attachment of the quaternary methyl group to C-5 as in I*a* (devoid of stereochemistry).

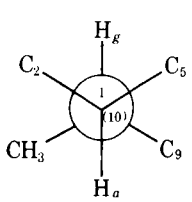
The cooccurrence of I*a* with II*a* and III, together with the foregoing evidence, suggested that it had a dimeric sesquiterpene lactone structure resulting from a Diels–Alder-type condensation that involved the 11,13-double bond of helenalin and the enol form of the cyclopentenone ring of a norpseudoguaianolide (mexicanin-E) (Scheme I).

The high-resolution mass spectral data of I*b* (Schemes II–IV) further corroborated these structural assignments. Diagnostically important peaks at *m/e* 95, 96, 123, 124, 232, 305, and 476 indicated the presence of a pseudoguaianolide sesquiterpene lactone bearing oxygen substituents at the 4-, 6-, and 8-positions (11, 12) and a saturated (exocyclic double) bond at C-11 as found in I*b*.

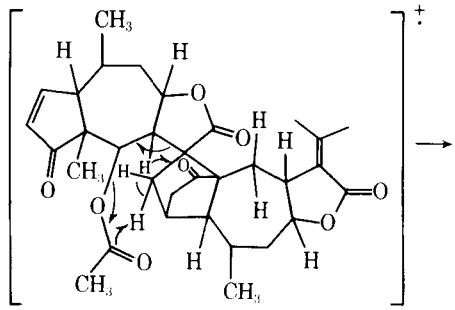
Assignment of the stereochemistry of microlenin was based on two considerations. First, there was the similarity of chemical shifts and coupling constants among microlenin acetate (I*b*), helenalin acetate (II*b*), and mexicanin-E (III). This fact led to the conclusion that microlenin possessed the stereochemistry of helenalin acetate at C-1, C-5, C-6, C-7, and C-8 and that of mexicanin-E at C-1′, C-7′, C-8′, and C-10′. The stereochemistry at C-10 and C-5′ remained undefined. The magnitude of $J_{1,10}$ determined the relative configuration at C-1 and C-10; therefore, if the configuration of H_a was established, the configuration at C-10 would be known. Since $J_{1,10}$ = 11.0 Hz, H_a and H_g must be diaxial as shown in Structure C.



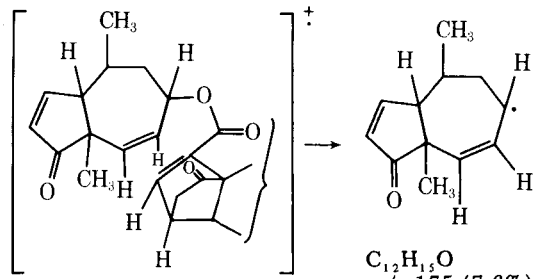
Structure B



Structure C



$C_{31}H_{36}O_8$
m/e 536 (1.8%)
Found: 536.2407
Calc.: 536.2408



$C_{29}H_{32}O_6$
m/e 476 (10.5%)
Found: 476.2195
Calc.: 476.2199

$C_{12}H_{15}O$
m/e 175 (7.6%)

Scheme II

If the usual assumption is made that the C-7 (C-7′) side chain is equatorial and beta as in all sesquiterpene lactones of known stereochemistry, the value (J = 8.0) of $J_{7,8}$ requires that H_f (C-8) be *cis* to H_e (H-7) and alpha (4); thus, the lactone ring was *cis*-fused. The *cis*-fused α-methylene-γ-lactone ring was confirmed by the magnitude of $J_{7',13a}$ and $J_{7',13'b}$ (<Hz) according to the Samek (13) rule. The stereochemistry at H_d (H-6) was deduced by comparison of the observed coupling constant with that obtained from the dihedral angle in a Dreiding model. The small value ($w_{1/2}$ = 3.0) of $J_{6,7}$ could be explained only by a β-orientation of H_d (C-6).

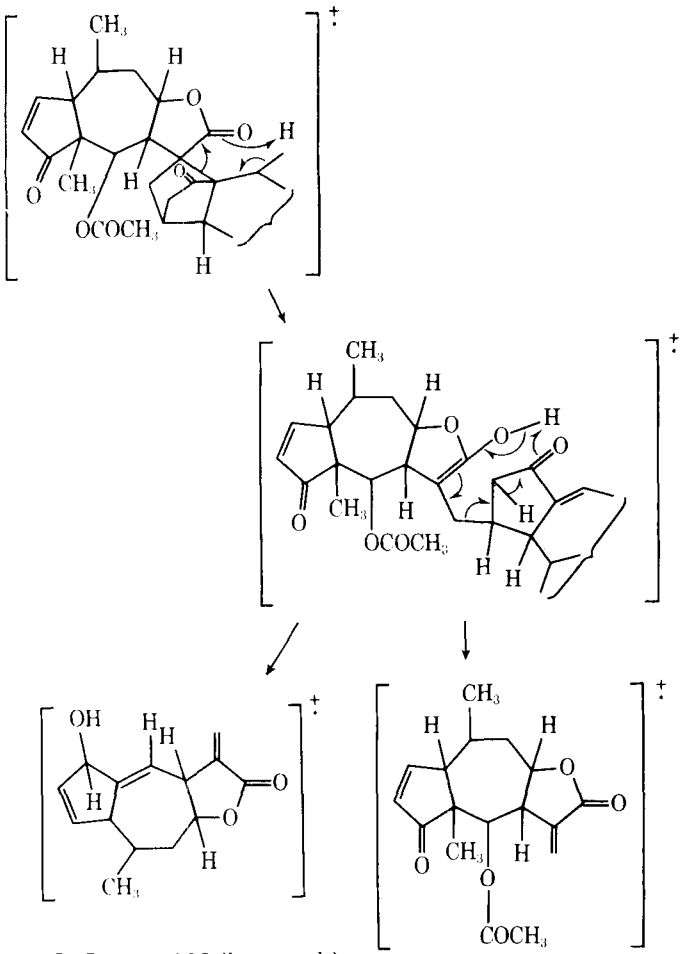
Second, the stereochemical assignments were based on the biogenetic consideration of the cooccurrence of helenalin and mexicanin-E (which have known absolute configurations) with microlenin. Since helenalin has a conformation closer to a twist-chair rather than a chair form (14), the presence of a *cis*-fused α-methylene-γ-lactone ring in addition to a β-oriented 5-methyl group would produce a severe steric hindrance on the β-face of the molecule. On the other hand, the cycloheptane ring in mexicanin-E adopted a conformation approximately midway between a chair and a twist-chair form (3). Furthermore, it contained one *cis*-fused α-methylene-γ-lactone ring and one *cis*-fused cyclopentenone ring. Consequently, microlenin would be produced by a Diels–Alder-type condensation involving the 11,13-double bond of helenalin, which approached from the α-face of the enolic form of the cyclopentenone ring of mexicanin-E.

This evidence led to the establishment of Structure I*a* for microlenin. Further confirmation of this assignment was achieved by a single-crystal X-ray analysis of microlenin (4).

EXPERIMENTAL[2]

Isolation of Microlenin from *H. microcephalum*—Microlenin was

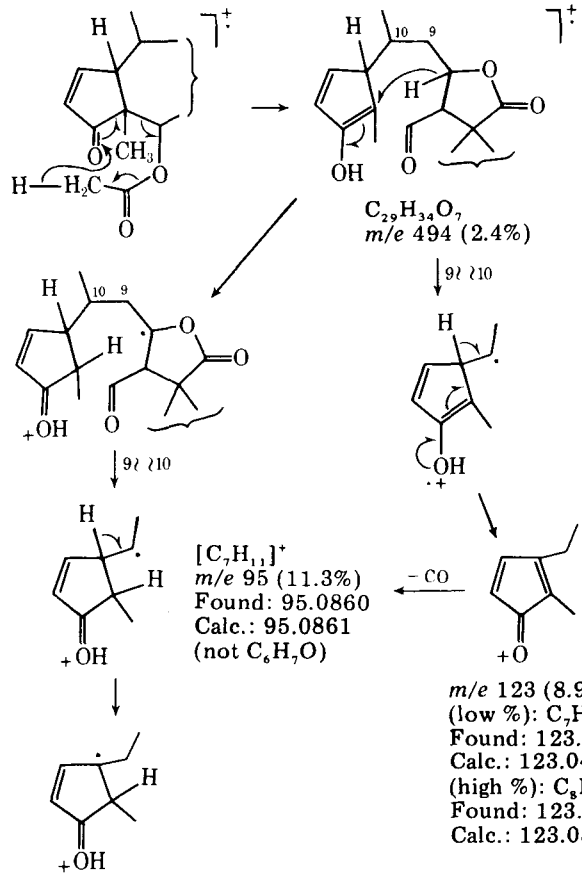
[2] Unless otherwise specified, melting points were determined utilizing a Thomas-Hoover melting-point apparatus and are uncorrected. Other instruments used were: for specific rotations, Perkin-Elmer model 141 polarimeter (1 = 1 dm); for IR spectra, Perkin-Elmer 257 grating IR spectrometer; for ^{1}H-NMR and ^{13}C-NMR spectra (tetramethylsilane as internal standard), Jeolco (60 MHz) or Varian XL-100 NMR spectrometer; for mass spectra, A.E.I. MS-902 spectrometer (direct inlet at 70 ev); and for optical rotatory dispersion and circular dichroism spectra: Cary model 60 spectrometer. Silica gel (Mallinckrodt CC-7, 200–325 mesh) was used for column chromatography and Merck silica gel G was used for TLC. Detection of components was made by spraying with a 1% $Ce(SO_4)_2$–10% H_2SO_4 solution followed by heating.



$C_{14}H_{16}O_3$, *m/e* 232 (base peak)
Found: 232.1094
Calc.: 232.1099

$C_{17}H_{21}O_5$, *m/e* 305 (13.9%)
Found: 305.1385
Calc.: 305.1389

Scheme III



$C_{29}H_{34}O_7$
m/e 494 (2.4%)

$[C_7H_{11}]^+$
m/e 95 (11.3%)
Found: 95.0860
Calc.: 95.0861
(not C_6H_7O)

m/e 123 (8.9%)
(low %): $C_7H_7O_2$
Found: 123.0442
Calc.: 123.0446
(high %): $C_8H_{11}O$
Found: 123.0808
Calc.: 123.0810

$C_8H_{12}O$ *m/e* 124 (7.6%)
(not $C_7H_8O_2$)
Found: 124.0886
Calc.: 124.0888

Scheme IV

isolated by column chromatography and preparative TLC from the chloroform extract of the whole plant of *H. microcephalum*, mp 280° (dec.), $[\alpha]_D^{22}$ +10.0° (c = 1.00, pyridine); IR (potassium bromide pellet): 3521 (OH), 1763 (γ-lactone), 1756, 1664 (α-methylene-γ-lactone), 1744 (cyclopentanone), 1707, and 1582 (cyclopentenone) cm^{-1}; ^{1}H-NMR (100 MHz, dimethyl sulfoxide-d_6): δ 0.75 (3H, s, CH_3-5), 1.07, 1.14 (3H each, d, J = 7.0 Hz, CH_3-10 and CH_3-10′), 5.74 (1H, d, J = 2.0 Hz, H-13′b), 6.03 (1H, dd, J = 3.0 and 6.0 Hz, H-3), 6.14 (1H, d, J = 3.0, H-13′a), and 7.84 (1H, dd, J = 2.0 and 6.0 Hz, H-2) ppm; ^{13}C-NMR [100 MHz, acetone-d_6–pyridine-d_5 (1:1)]: see Table II.

Anal.—Calc. for $C_{29}H_{34}O_7$: C, 70.42; H, 6.93. Found: C, 70.41; H, 7.10.

Acetylation of Microlenin—Acetylation of microlenin (25 mg) with acetic anhydride (0.5 ml) and pyridine (1 ml) at room temperature for 18 hr, followed by usual workup, gave a product (*Ib*). This product was recrystallized from chloroform to give colorless crystals of a monoacetate (19 mg), mp 278° (dec.); IR (chloroform): no OH, 1757 (γ-lactone), 1741, 1662 (α-methylene-γ-lactone), 1715, and 1580 (cyclopentenone) cm^{-1}; mass spectrum: *m/e* (%) 536.2407 (M^+, 1.8), $C_{31}H_{36}O_8$ requires 536.2408; 476.2195 ($M^+ - CH_3COOH$, 10.5), $C_{29}H_{32}O_6$ requires 476.2199; 305.1385 (13.9), $C_{17}H_{21}O_5$ requires 305.1389; 232.1094 (100), $C_{14}H_{16}O_3$ requires 232.1099; 175 (7.6); 124.0886 (7.6), $C_8H_{12}O$ requires 124.0888; 123 [123.0442 (low %), $C_7H_7O_2$ requires 123.0446; 123.0808 (high %), $C_8H_{11}O$ requires 123.0810]; 95.0860 (11.3), C_7H_{11} requires 95.0861; ^{1}H-NMR (100 MHz, deuterochloroform): δ 0.94 (3H, s, CH_3-5), 1.18, 1.23 (3H each d, J = 7.0 Hz, CH_3-10 and CH_3-10′), 1.97 (3H, s, C-6-$OCOCH_3$), 2.48 (1H, d, J = 8.0 Hz, H-7), 3.29 (1H, m, H-7′), 4.71 (1H, m, H-8′), 4.95 (1H, m, H-8), 5.52, 6.28 (1H each, d, J = 3.0, H-13′), 5.62 (1H, s, H-6), 6.06 (1H, dd, J = 3.0 and 6.0 Hz, H-3), and 7.67 (1H, dd, J = 2.0 and 6.0 Hz, H-2) ppm.

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Antitumor Agents XXIX: Effects of Eupahyssopin on Nucleic Acid, Protein, Anaerobic, and Aerobic Metabolism of Ehrlich Ascites Tumor Cells

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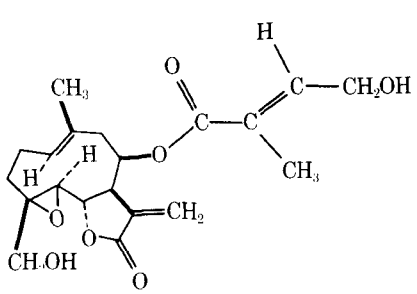
Abstract □ Evidence is presented that the antitumor agent eupahyssopin, a germacranolide, inhibits deoxyribonucleic acid, ribonucleic acid, protein, and cholesterol synthesis of Ehrlich ascites tumor cells in CF_1 male mice. Eupahyssopin appears to bind to nucleotide bases of deoxyribonucleic acid. The drug also inhibits deoxyribonucleic acid and messenger ribonucleic acid polymerase activities and, marginally, thymidylate synthetase activity. Template activity, as regulated by phosphorylation of histones and nonhistones, and cyclic 3′,5′-adenosine monophosphate levels also were affected by drug therapy. Lysosomal enzymes, *e.g.*, deoxyribonuclease and ribonuclease, and cathepsin activities were inhibited by eupahyssopin administration. A number of sulfhydryl-bearing enzymes of the glycolytic and Krebs cycles were inhibited by drug treatment, as were enzymes required for glycogen synthesis and breakdown and for phosphatase hydrolysis. Eupahyssopin also inhibited both basal respiration and coupled oxidative phosphorylation processes of the tumor cell. It was proposed that eupahyssopin, which contains the O=CC=CH_2 as an α-methylene-γ-lactone moiety within its allylic ester side chain, has the ability to undergo a rapid Michael-type addition with sulfhydryl groups of these enzymes and thus to inhibit their activities in a manner analogous to that described for other sesquiterpene antitumor agents containing the O=CC=CH_2 moiety.

Keyphrases □ Eupahyssopin—effects on nucleic acid, protein, anaerobic, and aerobic metabolism of Ehrlich ascites tumor cells □ Metabolism—nucleic acid, protein, anaerobic, and aerobic, in Ehrlich ascites tumor cells, effects of eupahyssopin □ Enzyme activity—Ehrlich ascites tumor cells, effects of eupahyssopin □ Antineoplastic agents, potential—eupahyssopin, effects on nucleic acid, protein, anaerobic, and aerobic metabolism of Ehrlich ascites tumor cells

A new germacranolide, eupahyssopin (I), was isolated from the chloroform extract of the whole plant *Eupatorium hyssopifolium* (1). Its stereochemistry and physical characteristics previously were reported (2). As an antitumor agent, eupahyssopin is active in the Walker 256 carcinosarcoma Sprague–Dawley rat survival system at 2.5 mg/kg/day (T/C = 330), in the P-388 lymphocytic leukemia screen in DBA/2 mice at 25 mg/kg/day (T/C = 132), and in the Lewis lung screen in $C_{57}B1_6$ mice at 25 mg/kg/day (T/C = 147) and caused 93% inhibition of Ehrlich ascites cell growth in CF_1 mice at 25 mg/kg/day (3). The effects of eupahyssopin on nucleic acid, chromatin protein, protein, anaerobic and aerobic glycolysis, and oxidative phosphorylation processes of Ehrlich ascites tumor cells are now reported.



I

EXPERIMENTAL

Male CF_1 mice, ~30 g, were implanted intraperitoneally on Day 0 with 10^6 Ehrlich ascites tumor cells. On Days 7, 8, and 9, the animals were treated with a subacute dose, 0.25 mg ip, of eupahyssopin suspended by homogenization in 0.05% polysorbate 80–water. Animals were sacrificed on the 10th day, and the ascites fluid was collected from the peritoneal cavity. *In vitro* oxidative phosphorylation studies were determined on untreated, harvested, Day 10 Ehrlich ascites cells. The number of tumor cells per milliliter and the 0.4% trypan blue uptake were determined with a hemocytometer.

Incorporation of thymidine into deoxyribonucleic acid was determined by the method of Chae *et al.* (4). One hour prior to animal sacrifice, 10 μCi of [^{14}C-methyl]-thymidine (54 mCi/mmole) was injected intraperitoneally. The deoxyribonucleic acid was isolated, and the carbon-14 content was determined in two parts of toluene, one part of octoxynol, 0.4% 2,5-diphenyloxazole, and 0.01% 1,4-bis[2-(5-oxazolyl)]benzene scintillation fluid and corrected for quenching.

The deoxyribonucleic acid concentration was determined by UV spectrophotometry at 260 nm, using calf thymus deoxyribonucleic acid as a standard. Uridine incorporation into ribonucleic acid was determined in an analogous manner, utilizing 10 μCi of 5-^{3}H-uridine (24.2 Ci/mmole). Ribonucleic acid was extracted by the method of Wilson *et al.* (5). Leucine incorporation into protein was determined by the method of Sartorelli (6), using 1 μCi of 1-^{14}C-leucine (56.9 mCi/mmole). The effects of eupahyssopin on 1-^{14}C-acetic acid (57.8 mCi/mmole) incorporation into cholesterol of Ehrlich ascites cells also were measured (7).

Nuclear deoxyribonucleic acid polymerase activity was determined on isolated nuclei (8). The incubation medium was that of Sawada *et al.* (9), except that 2-^{14}C-deoxyribothymidine triphosphate (45 mCi/mmole) was used and the insoluble nucleic acids were collected on glass fiber paper (GF/F) by vacuum suction. Deoxythymidylate monophosphate and diphosphate kinase activities were determined by the method of Maley and Ochoa (10), which is based on the disappearance of 0.1 μmole of reduced nicotinamide adenine dinucleotide at 340 nm in 20 min. Thymidylate synthetase activity was assayed by the method of Kampf *et al.* (11), utilizing a postmitochondrial (9000×*g* for 10 min) and 5 μCi of 5′-^{3}H-deoxyuridine monophosphate (11 Ci/mmole).

Phosphorylation of histones was determined by injecting intraperitoneally 10 μCi of γ-^{32}P-adenosine triphosphate (7.97 Ci/mmole) 1 hr prior to sacrifice. The nuclei were isolated (8), and the histone chromatin

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