Antitumor Agents XXIII: Helenalin, an Antitumor Principle from *Anaphalis morrisonicola* HAY.

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Abstract □ A chloroform extract of the whole plant of *Anaphalis morrisonicola* HAY. showed significant antitumor activity against Ehrlich ascites carcinoma, Walker 256 carcinosarcoma, and P-388 lymphocytic leukemia. Systematic fractionation of the extract led to isolation and characterization of helenalin, a pseudoguaianolide, as the major active principle.

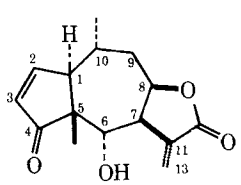
Keyphrases □ Helenalin—isolated from whole plant chloroform extract of *Anaphalis morrisonicola* □ *Anaphalis morrisonicola*—whole plant chloroform extract, antitumor activity evaluated, helenalin isolated □ Antitumor activity—whole plant chloroform extract of *Anaphalis morrisonicola* evaluated

In the continuing search among Formosan plants for new potential antitumor agents (1), the chloroform extract of the whole plant of *Anaphalis morrisonicola* HAY. (2) (Compositae), a hitherto uninvestigated species, showed significant inhibitory activity. This paper reports the isolation and characterization of the major active constituent of this extract, which was identified as helenalin (I). *In vivo* antitumor activity was assayed by using literature methods (3, 4). Previously, helenalin at 33.3 mg/kg/day caused 99% inhibition of Ehrlich ascites cell growth[1]; at 2.5 mg/kg/day, it produced significant (T/C ≥ 125%) inhibitory activity against the growth of Walker 256 ascites carcinosarcoma in rats (T/C = 316%); and at 25 mg/kg/day, it inhibited P-388 lymphocytic leukemia[1] (T/C = 127%).

DISCUSSION

The extraction of the active principles was carried out according to the procedure described previously (5). The final chloroform extract of the active crude preparation was separated further by silica gel column chromatography. Each fraction (C–H) was subsequently tested *in vivo* for antitumor activity (Ehrlich ascites carcinoma) and led to the isolation of the active principle, I, in 0.00032% yield. Compound I was obtained as colorless needles by preparative TLC followed by recrystallization from benzene (mp 170–173° and empirical formula $C_{15}H_{18}O_4$).

Compound I exhibited IR bands at 3450 (OH), 1765 (γ-lactone), and 1710 (cyclopentenone) cm^{-1} and NMR signals at δ 0.98 (3H, s, C_5-CH_3), 1.28 (3H, d, J = 6, C_{10}-CH_3), 4.45 (1H, d, J = 4.5, H_6), 5.00 (1H, t, J = 6, H_8), 5.85 (1H, d, J = 3, H_{13}), 6.09 (1H, dd, J = 6, 3, H_3), and 7.77 (1H, dd, J = 6, 1.5, H_2) ppm. The correspondence of the properties of this compound with those described in the literature (6) and its mixed melting-point and TLC determinations, as well as superimposable IR and NMR spectra, established its identity as helenalin, a pseudoguaianolide isolated previously from *Helium autumnale* (7), *H. microcephalum* (8), and *Balduina angustifolia* (6).



I

Table I—Effects of Fractions from *A. morrisonicola* on Inhibition of Ehrlich Ascites Tumor Growth

Fraction	Survival at Day 7	Volume, ml	Ascitocrit	Inhibition, %
0.05% Polysorbate control	6/6	2.33[a]	37.0[a]	—
A	5/6	1.54	12.8	77.0
B	6/6	1.92	37.0	18.0
C	6/6	1.18	39.0	47.0
D	6/6	1.67	45.0	13.0
E	6/6	1.00	26.0	70.0
F	5/6	0.04	10.0	99.5
G	6/6	1.82	36.0	24.0
H	6/6	2.51	36.0	0.0

[a] Standard deviation on the control ascitocrit was 7.8; on the volume, it was 1.2.

The structural requirement for the significant antitumor activity of helenalin (I) is the presence of an O=CC=CH_2 system as part of a ketone, such as a β-unsubstituted cyclopentenone, or as part of a lactone, such as an α-methylene-γ-lactone, as previously postulated by extensive structure–activity relationship studies (9–11). Further studies on the mechanisms of action of helenalin are in progress[1].

EXPERIMENTAL[2]

Extraction of *A. morrisonicola* HAY.—The *A. morrisonicola* was collected in Mt. Ho-Fan, Nan-Tau Shen, Taiwan[3], during September 1975. The ground, air-dried, whole plant material (2.5 kg) was extracted with chloroform at room temperature, yielding, after removal of the solvent, a thick green-black tar (Fraction A). This fraction showed 77% inhibition of Ehrlich ascites tumor growth and T/C = 193% inhibition against Walker 256 ascites (survival system) carcinosarcoma.

Fraction A was shaken with a mixture of methanol (1.5 liters), hexane (1.5 liters), and water (0.5 liter). The hexane layer was not investigated further since it caused only 18% inhibition of Ehrlich ascites tumor growth. The aqueous layer was concentrated *in vacuo* and extracted with chloroform. The chloroform extract, upon evaporation, yielded 14.7 g of a dark-brown syrup.

Isolation of Helenalin (I)—The syrup was chromatographed on silica gel (6 × 70 cm) with elution with 1 liter of hexane–benzene (1:1) (Fractions C and D, 500 ml each), 500 ml of chloroform (Fraction E), and 3.5 liters of 0.5% methanol–chloroform (Fractions F–H). As seen in Table I, the active principles were concentrated in Fraction F (99.5% inhibition),

[1] See I. H. Hall, K. H. Lee, E. C. Mar, C. O. Starnes, and T. G. Waddell, *J. Med. Chem.*, **20**, 333 (1977).

[2] Unless otherwise specified, melting points were determined on a Thomas-Hoover melting-point apparatus and are corrected. IR spectra were determined in chloroform with a Perkin-Elmer 257 grating IR spectrophotometer. NMR spectra were measured in $CDCl_3$ with a Jeolco C 60 HL NMR spectrometer, using tetramethylsilane as an internal standard. Silica gel for column chromatography refers to EM silica gel 60; silica gel for TLC and preparative TLC refers to Merck silica gel G and EM silica gel GF-254, respectively. The plates were developed with chloroform–acetone (3:1) and visualized by spraying with concentrated sulfuric acid and heating.

[3] Collected and identified by H.-C. Huang. A voucher specimen was deposited at the Herbarium of the Department of Botany, School of Pharmacy, Kaohsiung Medical College, Taiwan, Republic of China.

which gave a major single fluorescent spot on TLC under UV light together with traces of five nonfluorescent components. Subsequent isolation of this single fluorescent spot by preparative TLC led to a crystalline residue, which was recrystallized from benzene to yield colorless needles (8 mg), mp 170–173°. The identity of this compound with an authentic sample of helenalin (I) was established by TLC, IR, and NMR spectroscopic comparisons and by mixed melting-point determination.

REFERENCES

(1) K. H. Lee, T. Ibuka, H. C. Huang, and D. L. Harris, *J. Pharm. Sci.*, **64**, 1077 (1975), and references cited therein.

(2) A. T. Hsieh and T. I. Yang, "Nomenclature of Plants in Taiwan," National Taiwan University, Taiwan, Republic of China, 1969, p. 910.

(3) C. Piantadosi, C. S. Kim, and J. L. Irvin, *J. Pharm. Sci.*, **58**, 821 (1969).

(4) R. I. Geran, N. H. Greenberg, M. M. MacDonald, A. M. Schumacher, and B. J. Abbott, *Cancer Chemother. Rep. (Part 3)*, **3**, 1 (1972).

(5) K. H. Lee, H. C. Huang, E. S. Huang, and H. Furukawa, *J. Pharm. Sci.*, **61**, 629 (1972).

(6) K. H. Lee, D. C. Anuforo, E. S. Huang, and C. Piantadosi, *ibid.*, **61**, 626 (1972), and references cited therein.

(7) K. H. Lee, T. Ibuka, M. Kozuka, A. T. McPhail, and K. D. Onan, *Tetrahedron Lett.*, **1974**, 2287, and references cited therein.

(8) K. H. Lee, Y. Imakura, D. Sims, A. T. McPhail, and K. D. Onan, *Chem. Commun.*, **1976**, 341, and references cited therein.

(9) K. H. Lee, E. S. Huang, C. Piantadosi, J. S. Pagano, and T. A. Geissman, *Cancer Res.*, **31**, 1649 (1971).

(10) K. H. Lee, H. Furukawa, and E. S. Huang, *J. Med. Chem.*, **15**, 609 (1972).

(11) K. H. Lee, T. Ibuka, and R. Y. Wu, *Chem. Pharm. Bull.*, **22**, 2206 (1974).

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Polyamine Metabolism II: *N*-(Monoaminoalkyl)- and *N*-(Polyaminoalkyl)acetamides in Human Urine

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Abstract □ TLC and high-pressure liquid chromatographic examination of the dansyl derivatives obtained from human urine indicated the presence of *N*-[3-[(4-aminobutyl)amino]propyl]acetamide (N^1-acetylspermidine), *N*-[4-[(3-aminopropyl)amino]butyl]acetamide (N^8-acetylspermidine), *N*-(4-aminobutyl)acetamide (*N*-acetylputrescine), and *N*-(5-aminopentyl)acetamide (*N*-acetylcadaverine). The ratio of N^1- to N^8-acetylspermidine ranged from 10.3 in the urine of a patient with hepatoma to 1.1 in the urine of a normal subject. The three cancer patients had a considerably higher ratio of N^1- to N^8-acetylspermidine than did the three normal subjects. These findings indicate that the ratio of N^1- to N^8-acetylspermidine in the 24-hr urine may serve as a biochemical marker for cancer.

Keyphrases □ Polyamines—*N*-(monoaminoalkyl)- and *N*-(polyaminoalkyl)acetamides, dansyl derivatives, TLC and high-pressure liquid chromatographic analysis, human urine □ *N*-(Aminoalkyl)acetamides—dansyl derivatives, TLC and high-pressure liquid chromatographic analysis, human urine □ Dansyl derivatives—*N*-(monoaminoalkyl)- and *N*-(polyaminoalkyl)acetamides, TLC and high-pressure liquid chromatographic analysis, human urine □ TLC—analysis, dansyl derivatives of *N*-(monoaminoalkyl)- and *N*-(polyaminoalkyl)acetamides, human urine □ High-pressure liquid chromatography—analysis, dansyl derivatives of *N*-(monoaminoalkyl)- and *N*-(polyaminoalkyl)acetamides, human urine

The diamine 1,4-butanediamine (putrescine) (I) and the polyamines *N*-(3-aminopropyl)-1,4-butanediamine (spermidine) (II) and *N,N'*-bis(3-aminopropyl)-1,4-butanediamine (spermine) (III) are excreted in human urine mainly in the form of conjugates which produce the free amines after hydrolysis (1). *N*-(4-Aminobutyl)acetamide (*N*-acetylputrescine) (IV) was identified in the urine of normal subjects (2), and *N*-(5-aminopentyl)acetamide (*N*-acetylcadaverine) (V) was identified in the urine of

$RHN(CH_2)_nNH_2$ $\qquad$ $RHN(CH_2)_3NH(CH_2)_4NH_2$

I: R = H, $n = 4$ $\qquad$ II: R = H

IV: R = CH_3CO, $n = 4$ $\qquad$ VI: R = CH_3CO

V: R = CH_3CO, $n = 5$

$H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$

III

$CH_3COHN(CH_2)_4NH(CH_2)_3NH_2$

VII

schizophrenic patients (3). *N*-[3-[(4-Aminobutyl)amino]propyl]acetamide (N^1-acetylspermidine) (VI), but not *N*-[4-[(3-aminopropyl)amino]butyl]acetamide (N^8-acetylspermidine) (VII), also was detected in urine of healthy children (4) and a patient with acute myelocytic leukemia (5). Recently, both VI and VII were identified in urine of normal subjects and cancer patients (6, 7).

The present paper reports the determination of the ratio of VI to VII in the urine of normal subjects and cancer patients who had not received therapy.

EXPERIMENTAL

Extraction of Polyamines and Conjugated Polyamines from Urine—Twenty-four hour urine samples were collected from three adults and one child as normal subjects and from three patients with diagnosed cancer (hepatoma, melanoma, and thyroid cancer) who had not received therapy. The urine was collected over toluene, kept refrigerated during collection, and stored at −20° until analysis.

An aliquot of urine (100 ml) was adjusted to pH 10–11 with 2 *N* NaOH (5 ml) and extracted with 2-methyl-1-butanol (4 × 50 ml). Nitrogen was bubbled through the organic solvent extract to remove ammonia. Concentrated hydrochloric acid (5 ml) was added to the extract, and the mixture was evaporated to dryness *in vacuo* at 45°.