Antitumor Agents II: Eupatolide, a New Cytotoxic Principle from *Eupatorium formosanum* HAY.

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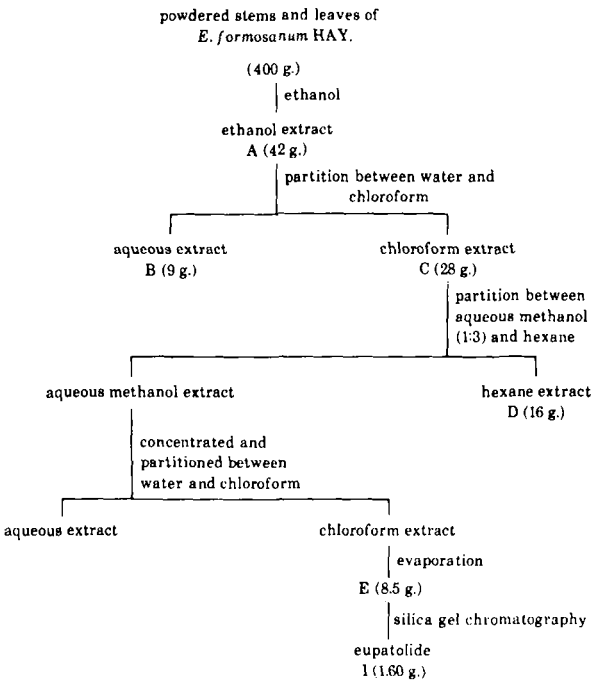
Abstract □ An alcoholic extract of the aboveground parts of *Eupatorium formosanum*, which showed significant inhibitory activity of the *in vitro* growth of tissue culture cells derived from human epidermoid carcinoma of larynx (H.Ep.-2), was examined and provided a new cytotoxic principle, eupatolide, a germacranolide sesquiterpene lactone.

Keyphrases □ *Eupatorium formosanum* HAY.—isolation, structure identification of eupatolide, cytotoxic activity □ Eupatolide—isolation, structure identification, cytotoxic activity □ Antitumor agents, potential—eupatolide, major constituent of *Eupatorium formosanum* HAY. □ Cytotoxicity—eupatolide, major constituent of *Eupatorium formosanum* HAY. □ Structure–activity relationships—sesquiterpene lactones as antitumor agents

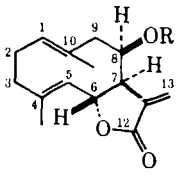
Interest in naturally occurring agents with potential antitumor or cytotoxic activity (1) led the authors to consider those Formosan plants used as folk medicine for the treatment of cancerous disease. An examination of the active antitumor principles of *Eupatorium formosanum* was undertaken since this particular species was previously reported (2, 3) to have antileukemic as well as antipyretic and anti-inflammatory activities. Of further interest was the fact that the ethanolic extract of this plant was found to show reproducible inhibitory activity against the cell culture of a human epidermoid carcinoma of larynx (H.Ep.-2)[1]. The purpose of this paper is to report the systematic fractionation of the active extract of *E. formosanum* and the isolation and structural elucidation of eupatolide, the major active principle.

DISCUSSION

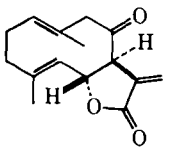
The alcoholic extract of the aboveground parts was concentrated and partitioned between water and chloroform. Guided by the assay in H.Ep.-2 cells, as shown in Scheme I and Table I, the active principles were concentrated in the chloroform extract (Fraction C). Partition of this extract between 25% aqueous methanol and hexane concentrated the activity in the aqueous methanol layer. The aqueous methanol extract was further concentrated and extracted with chloroform. Chromatography of the active chloroform extract (Fraction E) over silica gel led to the isolation of the active principle, eupatolide, in 0.4% yield. The cytotoxicity test of eupatolide against three different cell lines originating from human laryngeal carcinoma, human cells transformed with simian virus 40, and normal human fibroblasts is given in Table I.



Scheme I—Fractionation of the Cytotoxic Alcoholic Extract of E. formosanum



I: R = H
II: R = $COCH_3$
III: R = $COC(CH_2OH)=CHCH_2OH$



IV

Eupatolide (I), the new cytotoxic principle, was isolated from the chloroform eluate. Eupatolide, m.p. 188–190°, shows a molecular ion peak at *m/e* 248 and other prominent peaks in the mass spectrum[2] at *m/e* 230 (M-18) and 215 (M-18-15). Its elemental analysis agrees with a molecular formula of $C_{15}H_{20}O_3$. Eupatolide revealed IR bands in chloroform[3] at 1760 and 1660 cm.$^{-1}$ and a pair of low field doublets in the NMR spectrum (in acetone-d_6) at 6.22 (1H, J = 3) and 5.68 (1H, J = 3), characteristic of a γ-lactone conjugated with an exocyclic methylene grouping, a feature common to many sesquiterpene lactones of Compositae (5). The presence of a secondary hydroxyl group was indicated by an IR band in mineral oil at 3435 cm.$^{-1}$, a signal at 4.30 (1H, d, J = 4.5) in the NMR spectrum (in acetone-d_6) which disappeared on addition of D_2O, an ion of *m/e* 230 (M-18) in the mass spectrum, and the formation of an acetate (the monoacetoxy compound, II), m.p. 91–92°. The NMR spectrum of II showed the acetyl methyl group as a sharp singlet at 2.06 (3H). The presence of two vinyl methyl groups at C_{10} and C_4 in eupatolide was seen as two singlets, each slightly split by allylic coupling at 1.67 (J = 1.0) and 1.72 (J = 1.5), respectively. The *trans*-axial relationship between the protons at C_6 and C_7, with H_6 β, H_7 α, is seen in the signal for the lactonic H_6, which was a well-defined one-proton quartet at 5.30 (J = 7.5, 10.5), a feature common to this class of compounds. The proton at C_7 appeared as a

[1] The H.Ep.-2 cell culture assay was performed according to test procedures described in the literature (4).

[2] The authors thank Dr. Tanekazu Nadai and Mr. Hideo Yoshizumi, Faculty of Pharmacy, Meijo University, Nagoya, Japan, for their kindness in determining the mass spectra.

[3] This IR absorption band shifted to 1730 cm.$^{-1}$ in mineral oil and to 1725 cm.$^{-1}$ in KBr (private communication with Professor R. W. Doskotch, Sept. 10, 1971).

Table I—Cytotoxicity of Fractions from *E. formosanum*

Fraction	ED_{50}, mcg./ml.[a] H.Ep.-2[b]	W-18 Va-2[c]	W1-38[d]
A	8.35	—	—
B	>20	—	—
C	2.92	—	—
D	>20	—	—
E	0.41	—	—
I	0.469	0.034	0.044

[a] ED_{50} values were determined based upon the rapid microtiter method described previously (4). [b] H.Ep.-2 refers to human epidermoid carcinoma of larynx. [c] W-18 Va-2 refers to simian virus 40-transformed cells of human origin. [d] W1-38 refers to normal human diploid fibroblasts.

broad multiplet around 2.80. The remaining protons of the secondary hydroxyl group at H_8 and the two vinyl methyl hydrogens at H_1 and H_5 were seen as a one-proton broad singlet at 4.97 and a complex group (total 2H) between 4.60 and 4.85. That the lower of these (4.60) represents the proton of the hydroxyl grouping (H_8) was revealed by the NMR spectrum of acetyleupatolide (II), in which a one-proton multiplet at 4.70 and a broad one-proton singlet at 4.86 were distinct from the signal for the proton of the —CHOAc grouping, which had moved downfield at 5.72 as a one-proton multiplet.

The hydroxyl group at C_8 of eupatolide was readily oxidized by chromium trioxide in pyridine with the formation of 8-dehydroeupatolide (IV), which showed IR absorption bands in addition to the α-methylene-γ-lactone at 1770 and 1660 $cm.^{-1}$, a prominent 10-membered ring keto group at 1710 $cm.^{-1}$, and the absence of the hydroxyl group. The presence of a pair of doublets at 3.48 (1H, J = 10.5) and 3.00 (1H, J = 10.5) corresponding to the methylene protons at C_9 as shown in the NMR spectrum of IV, coupled with the fact that the downfield shift of the proton at C_7 (4.08, 1H, m) in IV compared with that in the NMR spectrum of I, in which the H_7 signal appeared at 2.80, further indicated that the H_7 is adjacent to the C_8 carbonyl group; *i.e.*, the original hydroxyl group in eupatolide has to be placed at C_8.

The evidence described here suggested that I was β-8-hydroxycostunolide, *i.e.*, eupatolide, a germacranolide which had been prepared previously (6–9) during the structural elucidation of the naturally occurring eupatoriopicrin (III) and epitulipinolide (II). Eupatoriopicrin was first isolated from *Eupatorium cannabinum* L. (Compositae) (6) and later was found to be the principal sesquiterpene lactone in several representatives of the tribe *Heleniae* (Compositae) such as the *Venegasia*, *Eriophyllum*, and *Chaenactis* genera (8). Epitulipinolide was isolated as a constituent of *Liriodendron tulipifera* L. (Magnoliaceae) (7). To confirm further the structure of the hydroxygermacranolide, the identity of I and II was established by direct comparison with authentic samples of eupatolide (deacetylepitulipinolide) and epitulipinolide[4].

EXPERIMENTAL[5]

Plant Material[6]—*E. formosanum* HAY. is a species belonging to the Compositae family of plants. It is also known as "Lark-gwei shieh" (the snow in June) and "Shan-teh Lan" (the mountain orchid) in folklore (2, 3). The present investigation was confined entirely to the stems and leaves of the plant which had been collected and identified in early Spring 1971 in Da-Tung Shan, Tainan, Taiwan.

Extraction of *E. formosanum* HAY.—The air-dried, milled plant (400 g.) was extracted in a soxhlet apparatus with alcohol USP until the extract was almost colorless. The residue (A) (42 g.), after removal of the solvent, was partitioned between water and chloroform. The combined chloroform extracts yielded a thick green-black tar (C) (28 g.), whereas the aqueous extract yielded a residue (B) (9 g.). The active chloroform extract (C) was shaken with a mixture of methanol (350 ml.), hexane (1 l.), and water (100 ml.), and the aqueous layer was separated and washed with hexane. The hexane layers were combined and concentrated to give D (16 g.). Concentration of the combined aqueous extracts followed by extraction with chloroform yielded a dark-brown syrup (E) (8.5 g.). TLC showed that this crude extract (E) was a mixture of mainly two components.

Isolation of Eupatolide—The crude residue (E) (8.2 g.) was chromatographed on silica gel (4 × 25 cm.), with elution with chloroform, chloroform–ethyl acetate, ethyl acetate with increasing amounts of acetone, and acetone. Thirty-two fractions of about 150 ml. each were collected and examined by TLC. The initial chloroform eluate (Fractions 1–4) contained only traces of low melting waxes. The subsequent chloroform eluate (Fractions 5–14) contained mainly material giving a single, fast moving spot on TLC. The chloroform–ethyl acetate (1:1) (15–19), ethyl acetate (20–25), ethyl acetate–acetone (26–27), and acetone (28–32) eluates contained only traces of an additional polar substance, whose structure is now under investigation.

Eupatolide (I)—The light-brown syrup obtained from Fractions 5–14 yielded colorless crystals when triturated with anhydrous ether. One recrystallization of this compound (I) from acetone afforded fine colorless tufts (1.60 g.), m.p. 188–190°. The NMR spectrum in pyridine-d_5 showed signals at 6.80 (1H, d, J = 4.5, OH)[7], 6.52 (1H, d, J = 3, H_{13}), 5.71 (1H, d, J = 3, H_{13}), 5.69 (1H, q, J = 7.5; 10.5, H_6), 4.98 (1H, br. s), 4.85–4.70 (2H, m) (H_1, H_5, and H_8), 2.88 (1H, m, H_7), 1.86 (3H, s), and 1.70 (3H, d, J = 1.5) (C_4—CH_3 and C_{10}—CH_3). The identity of this compound with an authentic sample of eupatolide (I) obtained from the hydrolysate of epitulipinolide[4] was established by TLC, IR spectroscopic comparison, and mixed melting-point determination.

Anal.—Calc. for $C_{15}H_{20}O_3$; C, 72.55; H, 8.12. Found: C, 73.01; H, 8.07.

Eupatolide Acetate—Acetylation of eupatolide with acetic anhydride in pyridine in the usual way gave the acetate (II), colorless needles from ether–hexane, m.p. 91–92°. The IR spectrum showed absorption at 1756, 1660 (α-methylene-γ-lactone), and 1740 $cm.^{-1}$ (acetyl carbonyl), and the NMR spectrum was in complete accord with the assigned structure and agreed in all details with that of epitulipinolide (7). Eupatolide acetate failed to depress the melting point of an authentic sample of epitulipinolide[4] on admixture, and the IR spectra were identical.

Oxidation of Eupatolide: Dehydroeupatolide (IV)—A solution of eupatolide (25 mg.) in pyridine (3 ml.) was added dropwise at 5° to a stirred solution of chromium trioxide (250 mg.) in pyridine (2.5 ml.). After 5 hr., a few drops of ethanol were added to the reaction mixture, which was diluted with chloroform and filtered. The chloroform solution was washed with 5% aqueous hydrochloric acid and water, dried, and evaporated to furnish the dehydroeupatolide (IV) as a syrup (20 mg.). The NMR spectrum of IV was also in complete agreement with the indicated structure and identical in all details with that of the oxidation product of deacetylepitulipinolide reported (7).

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[4] The authors are grateful to Professor R. W. Doskotch for providing the specimens of eupatolide and epitulipinolide for comparison.

[5] 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. Chemical shifts are reported in δ (p.p.m.) units; s refers to singlet, d to doublet, and t to triplet, and the J values are in hertz. Mass spectra were determined on a Hitachi RMU-7 instrument at 70 ev. using a direct inlet system. Silica gel for column chromatography refers to Baker A.R. No. 3405, and silica gel for TLC refers to Merck silica gel G developed with chloroform–acetone (3:1) and visualized with iodine vapor. Elemental analyses were performed by Atlantic Microlab, Inc., Atlanta, Ga.

[6] Collected and identified by H.-C. Huang, School of Pharmacy, Kaohsiung Medical College. A voucher specimen has been placed in the herbarium of the Botany Department, Kaohsiung Medical College, Taiwan, Republic of China.

[7] Disappeared after addition of D_2O.

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ACKNOWLEDGMENTS AND ADDRESSES

Received October 8, 1971, from the *Department of Medicinal Chemistry, School of Pharmacy, and the †Department of Bacteriology and Immunology, School of Medicine, University of North Carolina, Chapel Hill, NC 27514, and from the ‡School of Pharmacy, Kaohsiung Medical College, Taiwan, Republic of China.

Accepted for publication December 29, 1971.

Supported by University Research Council Grant VF 170-1511 of the University of North Carolina at Chapel Hill.

The authors thank Professors G. P. Hager, C. Piantadosi, and G. H. Cocolas for their encouragement and interest in this work.

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Structure–Activity Relationships in Reactivators of Organophosphorus-Inhibited Acetylcholinesterase V: Quaternary Salts of Hydroxyiminomethylimidazoles

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Abstract □ The methiodides of (*E*)-1-methyl-2-hydroxyiminomethylimidazole, (*E*)-1-benzyl-2-hydroxyiminomethylimidazole, and (*Z*)-1-methyl-5-hydroxyiminomethylimidazole were tested *in vitro* as reactivators of phosphorylated acetylcholinesterase. From the hydrolysis rate measurements, it was ascertained that only the compounds derived from 2-hydroxyiminomethylimidazole had an activity comparable with that of methiodide of 2-hydroxyiminomethylpyridine, whereas the methiodide of (*Z*)-1-methyl-5-hydroxyiminomethylimidazole was inactive. The structure–activity relationships of these reactivators are briefly discussed.

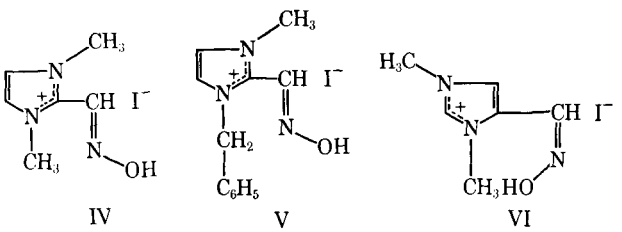
Keyphrases □ Acetylcholinesterase, organophosphorus inhibited—structure–activity relationships of reactivators (hydroxyiminomethylimidazole quaternary salts) □ Structure–activity relationships, quaternary salts of hydroxyiminomethylimidazoles—reactivators of organophosphorus-inhibited acetylcholinesterase □ Hydroxyiminomethylimidazoles, quaternary salts—structure–activity relationships

The quaternary salts of 2-hydroxyiminomethylpyridine and 4-hydroxyiminomethylpyridine are effective reactivators of organophosphorus-inhibited acetylcholinesterase (1, 2). On the basis of isosteric correlations, some authors studied the antidotic ability of quaternary salts of other heterocyclic aldoximes. Ashani *et al.* (3) described the synthesis and the pharmacological properties of 4-hydroxyiminomethyl-1-methylpyrimidinium iodide with *E*-configuration[1]; recently, Benschop *et al.* (5) studied the antidotic properties of some hydroxyiminomethyl-2-methylisothiazolium salts and those of related compounds. The tosylate of 3-hydroxyiminomethyl-2-methylisothiazolium restores the enzyme activity almost as rapidly and to the same extent as the isosteric 2-hydroxyiminomethyl-1-methylpyridinium methanesulfonate. (*Z*)-Isothiazole-5-carboxaldoxime reactivates the enzyme slowly but to a significant extent, whereas pyrazole-3(5)-carboxaldoxime, isoxazole-5-carboxaldoxime, and 5-hydroxyiminomethyl-2-methylisothiazolium tosylate are scarcely active.

In this note, quaternary salts derived from hydroxyiminomethylimidazoles (IV, V, and VI) were considered. The delocalization of the positive charge between the nitrogen atoms is a peculiar characteristic of these compounds, which can conceivably affect their ability to bind the enzyme anionic site.

In a previous study (6), the 2-hydroxyiminomethyl-1-hydroxyimidazole 3-oxide was prepared and found to be scarcely effective as a reactivator. The study of Compound IV has, therefore, the additional object of ascertaining the effect of the presence of a true quaternary ammonium group instead of an *N*-oxide.

For comparison with Compound V, the 2-hydroxyiminomethyl-1-benzylpyridinium iodide (VII) was prepared.



IV V VI

[1] The (*E*-*Z*) system of nomenclature for double-bond stereoisomers is used in this paper (4).