Damsin, the Cytotoxic Principle of *Ambrosia ambrosioides* (Cav.) Payne

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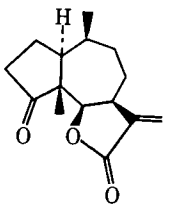
Abstract □ An alcoholic extract of the above ground parts of *A. ambrosioides* which showed significant activity against the KB cell culture was fractionated by solvent partitioning and chromatography on silicic acid. The active constituent was the pseudoguaianolide, damsin. A new sesquiterpene, damsinic acid, inactive against KB cells, was also isolated.

Keyphrases □ Cytotoxic principle—*Ambrosia ambrosioides* □ Damsin—isolated, identified □ Chromatography, column, paper—separation □ IR spectrophotometry—identity, structure □ UV spectrophotometry—identity, structure □ NMR spectroscopy—identity, structure □ Mass spectroscopy—identity, structure

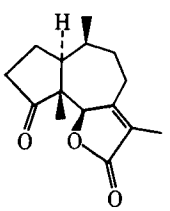
A random screening of botanical sources for anticancer activity showed that the alcoholic extract of the leaves and stems of *Ambrosia ambrosioides* (Cav.) Payne (*Franseria ambrosioides* Cav.)[1] family *Compositae* gave reproducible inhibitory activity against the cell culture (Eagle's KB) of a human carcinoma of the nasopharynx.[2] This report records the fractionation procedure developed for the isolation of the active substance damsin (I) and the isolation and partial characterization of a new sesquiterpene, damsinic acid.

The fractionation steps for a typical run are summarized in Fig. 1 and the cytotoxicities of the fractions are given in Table I. The powdered dried plant material was extracted by percolation with ethanol at room temperature and the residue remaining after evaporation of the solvent at reduced pressure was partitioned between chloroform and water. The active chloroform residue was partitioned between petroleum ether[3] and 10% aqueous methanol. Chromatography of the active 10% aqueous methanol residue on silicic acid separated the mixture into a number of fractions, one of which possessed the cytotoxic activity. Elution of the column was by a continuous gradient technique beginning with chloroform and ending when the effluent was about 10% methanol in chloroform. A final wash of the column with methanol eluted the remainder of the adsorbed material.

The active column fraction (*G*) yielded a crystalline substance responsible for the cytotoxicity and was characterized as damsin (I) (1) by melting point and examination of the IR, UV, NMR,[4] and mass spectra.[5] Direct comparison with an authentic sample[6] gave identical IR and UV spectra, and an undepressed mixture melting point. In addition damsin was isomerized on treatment with hydrogen and palladium on charcoal catalyst to yield dihydroisoambrosin (isodamsin) (II) which was identified by comparison with a known sample. This reaction is characteristic for damsin (2) and other such compounds (3).



I



II

During development of the isolation scheme it was found useful and convenient to monitor the separation by paper chromatography employing benzene–petroleum ether (1:1) as solvent on paper impregnated with formamide. Zimmerman's reagent was chosen as the most sensitive detecting spray reagent as it was observed that all of the cytotoxic fractions showed the same spray-sensitive zone (R_f 0.45). Another material reacting with the detecting reagent and exhibiting a higher mobility (R_f 0.70) in the paper system was present in the tailing end of the damsin fraction. By dividing the absorption column peak on the basis of the paper chromatographic results, Fraction H containing the compound with R_f 0.70 was separated from the damsin fraction. It was necessary to further purify Fraction H by partition column chromatography employing the paper chromatographic solvent system before the material could be crystallized. This substance devoid of cytotoxic activity is a new sesquiterpene for which the authors have chosen the name damsinic acid because of its close relationship to damsin.

The mass spectrum of damsinic acid exhibited a parent ion peak at *m/e* 250.1549 corresponding to $C_{15}H_{22}O_3$ (calculated *m/e* 250.1569) supported also by elemental analysis. The IR absorption peaks at $\nu_{max.}$ 1,695 and 1,625 cm.$^{-1}$ were assigned to an α,β-unsaturated acid function and that at 1,735 cm.$^{-1}$ to a cyclopentanone. A broad band between 2,500 and 3,300 cm.$^{-1}$ was consistent with a bonded —OH vibration of a carboxylic acid. The UV spectrum showed a weak peak at $\lambda_{max.}$ 287 mμ (ϵ 46) for the ketone and end absorption, 204 mμ (ϵ, 9,000). Damsin gives a spectrum of close similarity with $\lambda_{max.}$ 295 mμ (ϵ 31), 211 (8,900).

[1] The inclusion of the genus *Franseria* in *Ambrosia* was proposed by W. W. Payne, *J. Arnold Arboretum*, **45**, 401(1964).

[2] The cytotoxicity was assayed under the auspices of the Cancer Chemotherapy National Service Center. (CCNSC) according to the procedures described in *Cancer Chemotherapy Rept.*, **25**, 1(1962).

[3] Skellysolve B, Skelly Oil Co., Kansas City, Mo.

[4] The authors thank Prof. David R. Dalton of Temple University for some of the NMR data reported here.

[5] The authors thank Dr. Rodger L. Foltz of Battelle Memorial Institute for the mass spectral data which were obtained on an AEI MS-9 double-focusing mass spectrometer.

[6] The authors are grateful to Prof. T. O. Soine, College of Pharmacy, University of Minnesota, for kindly providing the collection of his sesquiterpene compounds.

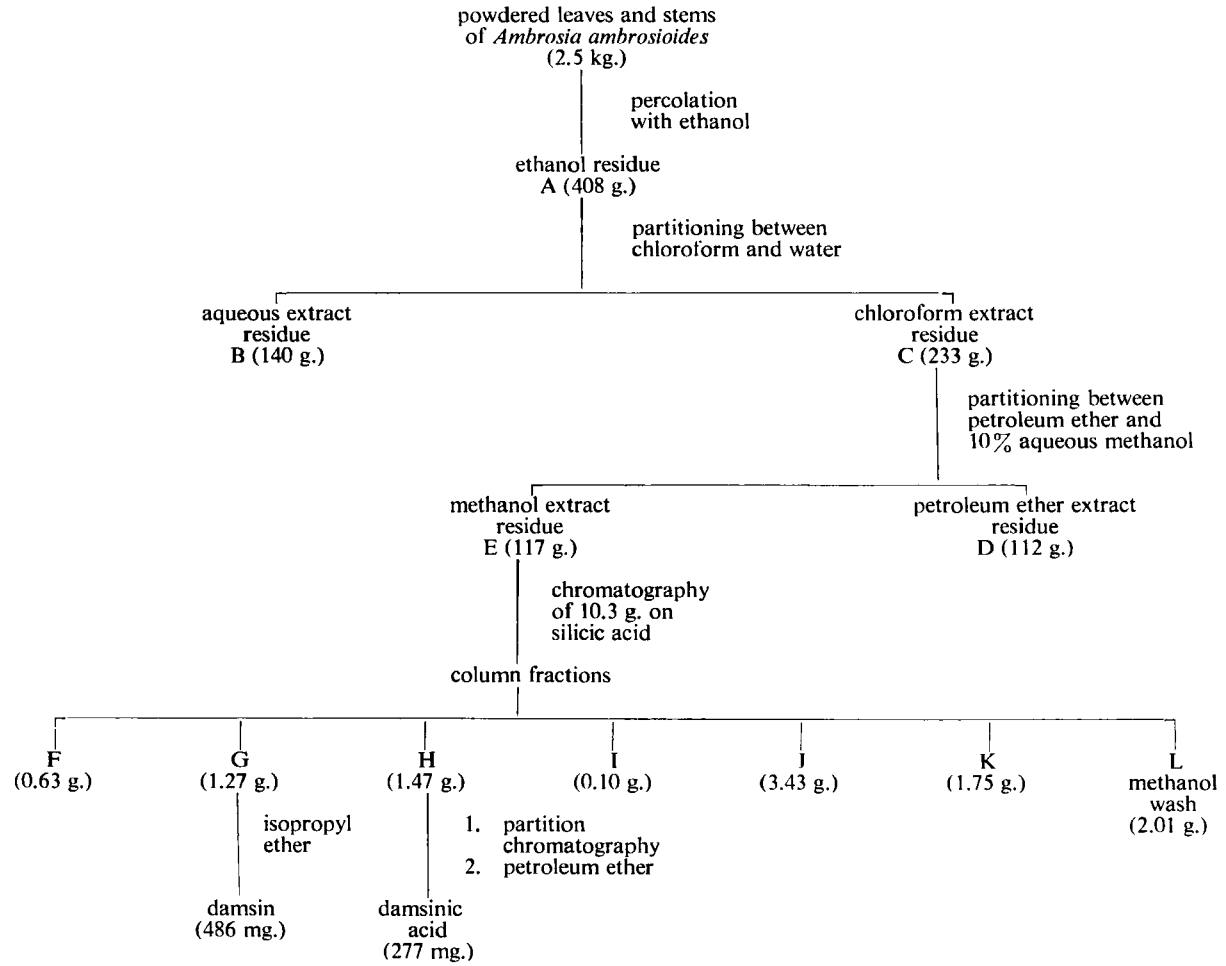


Figure 1—*Flow sheet for fractionation of extract from A. ambrosioides.*

The NMR spectrum of damsinic acid was similar to that of damsin (see *Experimental*) showing two methyl peaks at 1.06δ (3H, singlet) and 1.05δ (3H, doublet, J = 7 c.p.s.), and two exocyclic methylene protons as somewhat broadened singlets at 5.66 and 6.27δ. The proton on C-6 of damsin which appears as a doublet at 4.55δ (J + 9 c.p.s.) is lacking in the acid. A broad peak at 10.76δ (lost on D_2O exchange) is in support of the carboxylic acid function as is the solubility of the compound in dilute aqueous sodium bicarbonate.

Studies are currently in progress to determine the chemical and stereochemical structure of damsinic acid. Present evidence is compatible with the product obtained from reductive cleavage of the γ-lactone group of damsin.

Recently, Romo *et al.* (4) have reported the isolation of damsin from *Franseria ambrosioides* Cav., collected around Rio del Quelite, State of Sinaloa, Mexico. The authors had examined a collection from San Bernardo, Sonora, Mexico, and found it to give extracts inactive against KB cells and to lack damsin.

Table I—Cytotoxicity of Fractions from *A. ambrosioides*

Fraction	ED_{50}, mcg./ml.	Fraction	ED_{50}, mcg./ml.
A	1.9	H	15
B	>100	I	19
C	4.6	J	30
D	>100	K	25
E	0.55	L	>100
F	30	Damsin	0.32
G	2.8	Damsinic acid	>100

EXPERIMENTAL[7]

Plant Material—The above ground parts (leaves and stems) of *Ambrosia ambrosioides* (Cav.) Payne were collected in Pima County, Arizona, 1965.[8]

Extraction and Initial Separation—The powdered plant material (2.5 kg.) was extracted by percolation at room temperature with ethanol USP until the extract was nearly colorless. Removal of the solvent at reduced pressure and at 40° left a gummy residue of 408 g. which was partitioned between 2 l. each of chloroform and water by shaking in a separator. The chloroform phase was drawn off and the same volume of fresh chloroform was introduced and the extraction repeated. This was repeated once more for a total of three extractions. The combined chloroform layers were dried over anhydrous sodium sulfate and the solvent removed at reduced

[7] Melting points were determined with a Thomas-Hoover Uni-Melt capillary melting point apparatus and were uncorrected. The IR spectra were obtained in chloroform on a Perkin-Elmer, model 237 spectrophotometer. UV spectra were determined in methanol on a Cary model 15 recording spectrophotometer. NMR spectra were recorded on a Varian A-60A instrument in deuterochloroform with tetramethylsilane as internal standard and chemical shifts reported in δ units. Optical rotations were obtained in ethanol on a Cary model 60 optical rotatory dispersion instrument.

[8] Collected by R. J. Barr of Tucson, Arizona.

pressure and at 40°. The residue weighed 233 g. The water layer was concentrated at reduced pressure and then lyophilized to give a solid material weighing 140 g.

The total chloroform solubles were partitioned between 1 l. each of 10% aqueous methanol and petroleum ether by shaking in a separator. The petroleum ether layer was withdrawn and the extraction repeated with a fresh volume of the solvent. This extraction was repeated for a total of three times. Removal of solvents at reduced pressure from the separated extracts left residues of 112 g. from the petroleum ether phase and 117 g. from the aqueous methanol phase.

Adsorption Chromatography of the 10% Aqueous Methanol Fraction—A chromatographic column 5.0 × 45 cm. of 400 g. of silicic acid–diatomaceous earth[9] (4:1) previously activated at 100° for 10 hr. was poured as a slurry in chloroform. A 10.3-g. sample of the residue from the 10% aqueous methanol fraction was dissolved in about 10 ml. of chloroform and passed into the column. Elution was started with chloroform at a flow rate of 30 ml./hr. and 40-ml. fractions were collected on a mechanical fraction collector. After 2.5 l. of chloroform had passed into the column, a gradient elution apparatus giving a straight line gradient was attached containing 2 l. of chloroform in the mixing chamber and 2 l. of 12% methanol in chloroform in the reservoir. The chromatographic separation was stopped when the effluent had a composition of approximately 10% methanol in chloroform. A total of about 140 fractions was collected and the dry weights determined for each fraction by evaporation at reduced pressure in tared flasks. The column fractions were pooled on the basis of the dry weight analysis and paper chromatographic results. The column was finally washed with methanol to remove the most polar constituents. All pooled fractions and the final wash were biologically assayed.

Damsin (I)—The semicrystalline residue from pooled Fraction G was dissolved in hot isopropyl ether and on cooling deposited long colorless needles (486 mg.) m.p. 109–110.5°. The values in the literature are m.p. 102° (1) and m.p. 111° (5). The mass spectrum showed a parent ion at *m/e* 248.1414 and calculated at *m/e* 248.1412 for $C_{15}H_{20}O_3$. The IR spectrum exhibited peaks at $\nu_{max.}$ 1,765 (γ-lactone), 1,740 (cyclopentanone), and 1,665 cm.$^{-1}$ (double bond). The UV spectrum showed $\lambda_{max.}$ 211 mμ (ε, 8,900) and 295 (31), while the NMR showed a pair of doublets at 6.27 and 5.55δ (J = 3 c.p.s., exocyclic methylene), a doublet at 4.55δ (J = 8.7 c.p.s., C-6 proton), a singlet at 1.07δ (tertiary methyl), and a doublet at 1.08δ (J = 7.0 c.p.s., secondary methyl), as well as an envelope of peaks between 2.5 and 1.5δ. An authentic sample[6] of crude damsin purified by partition chromatography and crystallization gave an IR and UV spectrum indistinguishable from that of the authors' isolated material. A mixture melting point of the two samples was not depressed.

Dihydroisoambrosin (Isodamsin) (II)—Damsin (77 mg.) in 5 ml. of ethanol was added to 44 mg. of 5% Pd on charcoal catalyst in 10 ml. of ethanol previously saturated with hydrogen at room temperature and atmospheric pressure. After 24 hr. the reaction was stopped when only a fraction of 1 mole was consumed. Paper chromatographic examination using a solvent system of benzene and petroleum ether (2:1) on formamide-impregnated paper revealed three spots (Zimmerman's reagent): unreacted damsin (R_f 0.54) and two spots at R_f 0.68 and 0.26. A small partition column of 20 g. of diatomaceous earth containing 12 ml. of formamide and the same mobile solvent as used for paper chromatography separated the mixture. The small amount of crude material having R_f 0.68 did not crystallize but behaved chromatographically as authentic dihydrodamsin.[6] The material with R_f 0.26 crystallized from isopropyl ether to give 44 mg. of isodamsin (II) m.p. 163°, literature value m.p. 164–165° (4). The IR and UV spectra were identical with an authentic sample as was the mobility in the paper chromatographic system. A mixture melting point of the two samples was not depressed.

Damsinic Acid—A partition column for the isolation of damsinic acid was prepared by suspending 100 g. of diatomaceous earth in 1 l. of upper phase and adding 60 ml. of the lower phase while mixing vigorously. The two phases were prepared by mixing 1 l. of benzene, 1 l. of petroleum ether, and 80 ml. of formamide. After all the lumps were eliminated by shaking, a column with a 2.6-cm. i.d. was packed in 1–2-cm. segments by a cork plunger to a completed column height of 51 cm. Prior to use, the column was washed with 500 ml. of mobile phase and then 520 mg. of Fraction H dissolved in 4 ml. of mobile phase was added. Fifteen-milliliter fractions were collected at a flow rate of 20 ml./hr. The effluent fractions were examined by paper chromatography and those containing damsinic acid (R_f 0.70) were pooled and produced crystalline material from petroleum ether. After two recrystallizations and one clarification with charcoal 77 mg. of colorless crystals were obtained, m.p. 112–113°, $[\alpha]_D^{25}$ + 94° (c, 0.057% in ethanol). The chromatography of the remainder of Fraction H in about 0.5-g. amounts resulted in a total of 277 mg. of damsinic acid from 2.5 kg. of plant material or a yield of 0.011%.

Anal.—Calcd. for $C_{15}H_{22}O_3$: C, 71.97; H, 8.86. Found: C, 71.85; H, 8.79.

Paper Chromatography—Whatman No. 1 chromatographic paper spotted with material to be separated was dipped into a solution of 25% formamide in acetone in a manner such that the solution approached the spots from the two edges and did not appreciably spread the zones, allowed to air dry (10 min.), and then developed descendingly with the appropriate solvent. The solvent was benzene and petroleum ether saturated with formamide in proportions which result in a R_f 0.5 for the desired material. For damsin this ratio was 1:1. Detection was by spraying with Zimmerman's reagent (6) after the developed papers were dried at 60° to remove the stationary phase.

[9] Celite 545, Johns-Manville Corp., New York, N. Y., was purified if employed for partition columns by dispersing while heating for 1 hr. on the steam bath in 3 *N* HCl. The acid wash was repeated three times and then the material was collected by suction filtration and washed successively with distilled water (until the wash gave a negative chloride test with silver nitrate), methanol, benzene, skellysolve B, and finally methanol. Silicic acid, 100-mesh, is a product of Mallinckrodt Chemical Works, St. Louis, Mo. Skellysolve B is a petroleum ether fraction, b.p. 60–70°.

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