Tumor Inhibitors I: Preliminary Investigation of Antitumor Activity of *Sarracenia flava*

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Abstract □ The chloroform and aqueous extracts of the roots of *Sarracenia flava* showed antitumor activity against human epidermoid carcinoma of the nasopharynx (KB). Betulin was identified as one constituent responsible for this activity. Testing data are reported for betulinic acid and derivatives.

Keyphrases □ *Sarracenia flava*—chloroform and aqueous extracts examined for antitumor activity □ Betulin and betulinic acid—isolated from *Sarracenia flava*, examined for antitumor activity □ Antitumor agents, potential—isolation and screening of active constituents from *Sarracenia flava*

Reports[1] that the ethanol (moonshine) extracts of the roots of *Sarracenia flava*[2] (golden trumpet) have been used as a folk remedy by residents of the Okefenokee swamp region of southeastern Georgia prompted the examination of this and related species. The genus *Sarracenia,* one of three genera in the Sarraceniaceae, has been the subject of many investigations since the mid-19th century. The insectivorous habit, the conspicuous leaves and flowers, and the apparent ease of hybridization have led to interest in the group by botanists, biochemists, and horticulturists. Various aspects of the genus associated with its insectivorous habit were summarized (1). More recently, it was proved conclusively that nutrients from entrapped insects are absorbed into foliar tissue (2). The historical development of the genus has been discussed (1, 3, 4), and recently a chromatographic review was published (5). In spite of this great interest in *Sarracenia,* there is no evidence in the literature of a detailed examination of its chemical constituents.

The authors wish to report confirmation of antitumor activity in the roots of *S. flava* and also to comment on the activity of compounds and derivatives from this plant.

RESULTS AND DISCUSSION

Preliminary studies involving methanol extraction of the plant roots[3] yielded two fractions, a methanol-soluble oil and a methanol-insoluble, beige-colored solid. Testing of these crude fractions[4] showed that against human epidermoid carcinoma of the nasopharynx (KB) a 27-μg/ml dose was required to inhibit 50% growth of the test cells *versus* the control (ED_{50}); in the same system the solid showed an ED_{50} of 11 μg/ml. The solid was judged to warrant further study and showed in the later tests ED_{50}'s of 3.0, 2.6, and 2.6 μg/ml. These tests confirmed KB activity and further investigations were justified. Neither sample was active against L-1210 lymphoid leukemia in BDF_1 mice or Walker carcinosarcoma 256 (intramuscular) in random-bred albino rats.

Further extraction of the finely ground roots[5] was carried out

Table I—Activity of Fractionated Plant Material

Fraction[a]	Plant Material, %	ED_{50}[b]	Slope[c]
A	7.9	>100	0.0
B	13.6	26	−1.09
C	2.3	>100	0.0
I	2.0	>100	0.0
J	0.3	24	−0.51

[a] See Scheme I. [b] Activity against human epidermoid carcinoma of the nasopharynx *in vitro*, expressed as micrograms per milliliter that inhibited 50% of control dose. [c] Slope is the change of response for each one-log change of dose.

Table II—Activity of Betulinic Acid and Some Related Compounds[a]

Compound	Dose, mg/kg	T/C
Betulinic acid[b]	400	76
	200	90
	100	101
Betulinic acid[c]	200	91
	100	91
	50	101
Methyl betulinate[d]	400	91
	200	100
	100	103
Acetobetulinic acid	400	91
	200	93
	100	93
Betulonic acid	400	94
	200	96
	100	102
Methyl betulonate	400	94
	200	96
	100	91

[a] All tests on L-1210 lymphoid leukemia in BDF_1 strain of mice using intraperitoneal injections, with hydroxypropyl cellulose (HPC, Klucel) as a vehicle unless otherwise noted. T/C is the ratio of the mean survival time of the test animals to the control animals. [b] Test on Walker carcinosarcoma 256 (subcutaneous) in random-bred albino rats using intramuscular injections, with carboxymethylcellulose as a vehicle. [c] Test on P-388 lympholytic leukemia in BDF_1 strain of mice. Injections were intraperitoneal, using saline with polysorbate 80 as a vehicle. Activity against KB cell culture showed an ED_{50} of 26 and a slope of −1.16. [d] Alcohol used as a vehicle.

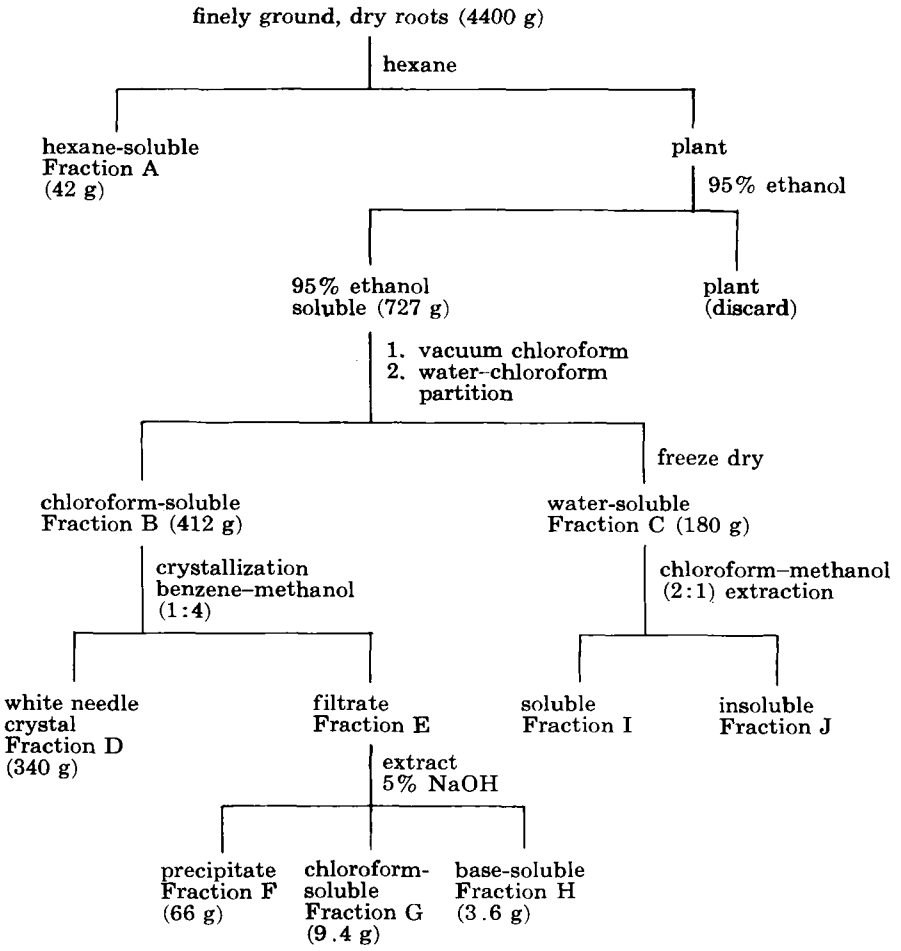
[1] Private conversation with local residents.

[2] The plant material used in this investigation was identified as *Sarracenia flava* L. (Sarraceniaceae) by Dr. Sidney McDaniel, Department of Botany, Mississippi State University. A voucher (preserved) specimen (SM-16,702) representing material collected for this investigation is available for inspection at the Herbarium of the Department of Botany, Mississippi State University.

[3] Collected in Okefenokee Swamp region during February 1968.

[4] Cancer Chemotherapy National Service Center, Bethesda, MD 20014, 1970.

[5] Collected in the Okefenokee Swamp region during May 1969.



Scheme I—Procedure for extraction of the roots of Sarracenia flava

using the Wall *et al.* (6) procedure for KB active compounds (Scheme I). Testing of the fractions derived from this procedure gave the results shown in Table I. Even though the KB activity of these fractions is less than that observed for the crude methanol extract, it is clearly concentrated in Fractions B (chloroform extract) and J (water extract).

Further fractionation by basic extraction of chloroform Fraction B gave a precipitate (Fraction E), which was recrystallized from methanol–acetone to yield a compound that did not melt even when heated to 315°. The IR spectrum of this compound showed bands characteristic of a carboxylic acid salt. Acidification and recrystallization of the salt gave a compound identified as betulinic acid by reduction to betulin with lithium aluminum hydride, followed by a direct comparison of IR, NMR, and mass spectra with those of an authentic sample of betulin. Betulinic acid was then shown to be the major constituent of the beige solid remaining after the initial methanol extraction. The presence of KB activity in this fraction suggested that betulinic acid might be the active component.

Samples of betulinic acid, its methyl ester, the acid acetate, its keto derivative (betulonic acid), and the keto ester (methyl betulonate) were prepared (8) and sent to the National Cancer Institute for testing (Table II). These data clearly indicated that betulinic acid was not the active constituent of the plant, even though the active chloroform extract is over 80% betulinic acid by weight.

Chromatography of the chloroform Fraction B after removal of betulinic acid by fractional crystallization resulted in the isolation of the known tumor inhibitor betulin (7). The mass spectrum of betulin showed an M^+ peak at 442, and the NMR spectrum indicated the appropriate signal for vinyl protons. The IR spectra of the compound and its diacetate were superimposable with authentic samples (8).

EXPERIMENTAL

Isolation of Betulinic Acid—The white needle crystals (Fraction D) were recrystallized four times from benzene–methanol (1:4) to yield a white crystalline material, mp 287–290°, which was identical to the acid from the beige solid Fraction F. Fraction F was recrystallized from hot methanol–acetone to yield a white crystalline material which did not melt when heated to 315°. The partially dissolved salt in 10% HCl was continuously extracted with chloroform. The chloroform layer was dried over anhydrous magnesium sulfate and concentrated *in vacuo* to yield an off-white solid which could be recrystallized from ether, mp 290–292° [lit. (9) for betulinic acid mp 316–318°]. The IR spectra of both acids from Fractions D and F were identical with that of a sample of betulinic acid[6] (~90%), mp 295–298°.

Isolation of Betulin—Fraction E (80 g) was dissolved in 300 ml benzene–chloroform (8:1). The soluble part (about 30 g) was chromatographed on a neutral alumina column (1.3 kg, activity I, 7 × 27 cm) prepared in hexane. The column was eluted first with hexane and then with solvents of increasing polarity. Each fraction (2 liters) was examined by TLC (silica gel GF) using acetone–benzene–chloroform (1:2:17) as the solvent system and potassium dichromate–sulfuric acid as the developing agent. Fractions 1–13 of the chloroform–benzene (3:4) elution were combined and purified twice by preparative TLC [0.75 mm, silica gel GF, acetone–benzene–chloroform (1:2:17)], yielding betulin as a white crystalline solid (35 mg), mp 214–217°; IR (KBr pellet): 3300, 1630, and 875 cm^{-1}; NMR ($CDCl_3$): δ 0.76 (3H, s), 0.84 (3H, s), 0.99 (3H, s), 1.02 (3H, s), 1.05 (3H, s), 1.71 (3H, s), 3.58 (2H, d of d, J = 11 Hz), and 4.67 (2H, d, J = 5 Hz); mass spectroscopy: parent ion at M^+ = 442; calculated for $C_{30}H_{50}O_2$, M^+ = 442. The precise mass deter-

[6] Obtained from Aldrich Chemical Co.

mination using polyfluorokerosine as the standard gave M^+ = 442.3745 (calc. M^+ = 442.3811).

The difference in melting points [lit. (10) betulin mp 252–253°] was due to traces of betulinic acid. The IR spectra of the isolated betulin (KBr pellet) and that of an authentic sample of betulin plus traces of betulinic acid were in complete agreement.

Preparation of Betulin Diacetate—Betulin (12 mg), pyridine (0.5 ml), and acetic anhydride (0.5 ml) were refluxed for 1 hr. After cooling, the reaction mixture was poured over crushed ice. The precipitated compound was collected and washed thoroughly with water until free of acetic acid and pyridine. After drying under vacuum, the compound was recrystallized from absolute ethanol. Approximately 10 mg of solid needles of betulin diacetate was obtained, mp 218–221° [lit. (10) mp 223–224°]. The IR spectra of the prepared betulin diacetate and that of an authentic sample were in complete agreement.

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Anti-Inflammatory Activity of *para*-Substituted *N*-Benzenesulfonyl Derivatives of Anthranilic Acid

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Abstract □ Several *para*-substituted *N*-benzenesulfonyl derivatives of anthranilic acid were prepared and evaluated for anti-inflammatory activity using erythrocyte membrane stabilization and carrageenin-induced rat paw edema assays. *N*-*p*-Bromobenzenesulfonylanthranilic acid possessed greater activity than phenylbutazone in both assays. Quantitative structure–activity correlations employing the Hansch method were unsuccessful in the erythrocyte membrane stabilization assay. However, correlations were obtained in the rat paw edema assay, and it appears that the activity of these derivatives depends solely on the lipophilic character of the substituents on the benzenesulfonyl ring.

Keyphrases □ Anthranilic acid, *para*-substituted *N*-benzenesulfonyl derivatives—synthesized and screened for anti-inflammatory activity □ Anti-inflammatory activity—*para*-substituted *N*-benzenesulfonyl derivatives of anthranilic acid, synthesis and screening □ Structure–activity relationships—*para*-substituted *N*-benzenesulfonyl derivatives of anthranilic acid

Considerable effort continues to be expended in the search for effective nonsteroidal anti-inflammatory agents. Various chemical classes have been demonstrated to possess therapeutically useful properties—*viz.*, salicylates, carboxylic acid amides, arylalkanoic acids, pyrazolidinediones, and anthranilic acids. Recently, significant anti-inflammatory activity was reported for members of a series of *para*-substituted *N*-benzenesulfonyl derivatives of amino acids, most notably phenylalanine (1). While phenylalanine itself possesses anti-inflammatory activity (2), the *N*-benzenesulfonyl moiety was found to enhance this activity significantly. In view of the pronounced anti-inflammatory activity of the anthranilic acid derivatives mefenamic acid[1] and flufenamic acid[2], earlier studies were extended to the synthesis of various *N*-*para*-substituted benzenesulfonyl derivatives of anthranilic acid (I–VI) and the examination of the influence of the *para*-substituent through quantitative structure–activity correlations.

EXPERIMENTAL[3]

General Synthetic Procedure—A solution of the appropriate benzenesulfonyl chloride (1.2 moles) in dioxane was added to a solution of anthranilic acid (1.0 mole dissolved in sufficient 2 *N* NaOH) at such a rate as to maintain the pH of the mixture between 9.5 and 10.5. Additional 2 *N* NaOH was added when the

[1] Ponstel, Parke, Davis and Co.

[2] Arlef, Parke, Davis and Co.

[3] Melting points were determined on a Thomas-Hoover Unimelt apparatus and are uncorrected. Elemental analyses were performed by Chemalytics, Inc., Tempe, Ariz. The IR spectra (KBr, Perkin-Elmer model 256) and NMR spectra (dimethyl sulfoxide-d_6, Jeolco model C-60-HL) were consistent with the proposed structures.