Isolation of an Antitumor Proteinaceous Substance from *Gutierrezia sarothrae* (*Compositae*)

By AYHAN ULUBELEN*, MARY E. CALDWELL, and JACK R. COLE

Preliminary screening of the aqueous extracts of *Gutierrezia sarothrae* have shown antitumor activity against Sarcoma 180 in mice. The isolation, purification, and partial characterization of a proteinaceous material are reported. The procedures involved the use of solvent extraction, column chromatography, dialysis, and electrophoretic and spectrophotometric techniques.

As a result of a routine screen of southwestern plants for potential antitumor activity, the aqueous extract of *Gutierrezia sarothrae*[1] (Pursh) Britton and Rusby demonstrated activity toward the Sarcoma 180 test system in mice. This screening program was carried out by the Cancer Chemotherapy National Service Center, Bethesda, Md.

The plant is a low growing and glutinous perennial herb or subshrub of the family *Compositae*. It has an inflorescence which is a cymose panicle terminated with numerous, resinous flower heads.

The collection used in this study was obtained in the fall at the base of the Chiricahua Mountains near Portal, Ariz. Through a systematic examination of the parts of the plant it was found that the activity resides in the root portion.

There are no reports in the scientific literature concerning medicinal uses for this plant or the utilization of a proteinaceous substance from higher plants for antitumor purposes. However, some recent publications (1, 2) report proteinaceous antitumor substances from higher fungi.

EXPERIMENTAL

Extraction.—Three kilograms of the dried roots of *G. sarothrae* was extracted with petroleum ether, methanol, and water at room temperature. The extracts in this study were all lyophilized in a Repp Industries model 15 sublimator. A yield of 200 Gm. of crude material was obtained and dissolved in distilled water. The soluble portion was washed with ether and chloroform until the organic phase no longer extracted any material. It was then treated with three times its volume of 95% alcohol. A precipitation occurred.

The precipitate was separated, dissolved in distilled water, and lyophilized. The lyophilized powder was extracted with 83% hot alcohol. This alcohol-extracted solution had a red-brown color at first which, as the extraction proceeded, turned light yellow. The solutions were discarded.

The remaining dark powder was dissolved in cold water and the insoluble portion separated by centrifugation. It was then dissolved in a 0.1 *M* phosphate buffer system of pH 8.04 and dialyzed against distilled water.

After 1 week of dialysis, there appeared a precipitate in the dialysis tube. A precipitate (I) and a solution (II) were separated by centrifuge and lyophilized. The yield of I = 4.1 Gm. and II = 5.06. Both, upon submission for screening, show marked activity against Sarcoma 180 in mice. (Table I.)

A series of column chromatographic experiments were employed in order to separate the active materials. Among the substrates employed were Celite 565, Cellex-D, and Sephadex. The most promising results were obtained by the use of cellulose powder (Cellex-D) and Sephadex. The Celite results were essentially inactive.

Cellulose Powder.—A. A 400-Gm. quantity of Cellex-D (DEAE anion exchange cellulose, Calbiochem, Los Angeles, Calif.) was suspended in a 0.005 *M* phosphate buffer solution of pH 7. After expansion was essentially complete, it was poured into a 40 × 4 cm. column and permitted to settle. It was then washed with the same phosphate buffer solution until the pH of the eluent was maintained at 7.0. The following gradients were then passed through the column: 0.005 *M* phosphate buffer of pH 7.0, 0.02 *M* phosphate buffer of pH 5.7, 0.1 *M* NaCl + 0.05 *M* phosphate solution, and 0.5 *M* NaCl + 0.1 *M* phosphate solution.

One hundred and ten fractions of 25 ml. each were collected in a fraction collector. All demonstrated a positive ninhydrin reaction and absorbed at 275 mμ in the ultraviolet. After dialysis and subsequent lyophilization a total of 89 mg. of material was obtained from the 2 Gm. of crude starting material (mixture of I and II). This material was submitted for test. A T/C of 33% was obtained at a dosage of 25 mg./Kg.

B. A Cellex-D column was used again. Four grams of crude material (mixture I and II) was applied and followed by the following eluents: 0.2 *M* phosphate buffer of pH 6.98, 0.2 *M* phosphate buffer of pH 5.06, 0.1 *M* ammonium sulfate solution, 0.2 *M* ammonium sulfate solution, 0.05 *M* borate

TABLE I.—SARCOMA 180 ACTIVITY IN MICE

	%T/C[a]	Dose, mg./Kg.
I =	18	60
	37	40
II =	37	90
	28	50

[a] The criteria for activity is defined as being a T/C (test/control) value of less than 42 in a satisfactory dose response test (3).

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[1] Identification confirmed by Robert Barr, Research Associate, College of Pharmacy, and Dr. Charles Mason, Curator of the Herbarium, Botany Department, University of Arizona, Tucson. A reference specimen was also deposited.

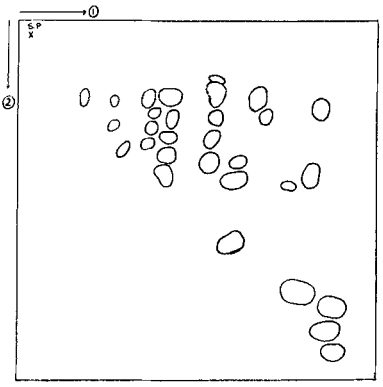

Fig. 1.—Paper chromatography of hydrolyzed compound III. Key: SP, = starting point; 1, phenol–water (3:1); 2, butanol–formic acid–water (7:1:3).

buffer of pH 9, and 0.05 *M* carbonate + borate buffer of pH 10.

Six fractions which showed positive ninhydrin tests were obtained from the chromatographic analysis indicated above and submitted for test. One fraction exhibited a T/C of 22% at 50 mg./Kg.

Sephadex Treatment.—Ten grams of anion exchanger DEAE A-50 Sephadex (medium) (Pharmacia Uppsala, Sweden) was treated by the addition of water, 0.5 *M* hydrochloric acid, and 0.5 *M* sodium hydroxide. It was then washed with a 0.02 *M* phosphate buffer of pH 6.6 and placed in a 20 × 3 cm. column. One gram of crude product (I) was dissolved in the phosphate buffer indicated above and placed at the top of the column. It was eluted with this same buffer and ninhydrin positive fractions were collected which were subsequently dialyzed and lyophilized. A 321-mg. quantity of compound was obtained; 200 mg. of this material was chromatographed on a cation exchange column [CM Sephadex C-50 (medium), Pharmacia Uppsala, Sweden], 20 × 3 cm. which was prewashed with 0.005 *M* acetate buffer pH 5.6. The brown material (156 mg.) was collected. It was found to be positive to ninhydrin and then was dialyzed, lyophilized, and submitted to test. The colorless column was then washed with 0.1 *M* acetate buffer of pH 5.6. The solution obtained was ninhydrin positive and was also dialyzed, lyophilized, and submitted for test. This material (III) was in a highly purified state (40 mg.). A result of 33% at 10 mg./Kg. was obtained.

The same treatment as indicated above was applied to 1 Gm. of the crude product II. The yield was 407 mg. from the anion exchange column. A 300-mg. quantity was applied to the cation exchange column. The brown material obtained was 134 mg. The purified material (IV) yield was 14.2 mg.

Physical and Chemical Characteristics.—The purified compounds obtained above (III and IV) migrated toward the cathode pole as single compounds in a paper electrophoretic experiment. A barbital buffer of pH 8.6, ionic strength 0.05, was used in a Spinco model Beckman instrument. The time was 2 hr. and the current was 2.5 ma. The mobility was measured as 5.9×10^{-7} cm.²/sec. v. for III and 3.6×10^{-6} cm.²/sec. v. for IV.

Also a phosphate buffer of pH 7.7, ionic strength 0.1, was used for 4 hr. The mobilities were: III, 1.2×10^{-7} cm.²/sec. v.; IV, 6.6×10^{-8} cm.²/sec. v.

Compound III was hydrolyzed in 6 *N* HCl for 24 hr. in an evacuated and sealed glass tube. The presence of amino acids was detected by two dimensional paper chromatography using secondary butanol–formic acid–water (7:1:3) for the first dimension and phenol–water (3:1) (4) for the second dimension.

The spots were developed by spraying with ninhydrin reagent. Thirty spots were detected (Fig. 1). Some of these could possibly be attributed to polypeptides and other amino compounds. Hydrolysis of compounds III and IV with 5 *N* HCl and then paper chromatographic analysis using butanol–acetic acid–water (4:1:5) as the solvent system and subsequent spraying with phytalic acid–aniline reagent showed three sugar spots. When compared to known samples, the presence of glucose, glucuronic acid, and possibly acetylglucose amine were noted.

Nitrogen determinations using the micro-Kjeldahl method showed 6.4% N for III and 6.2% N for IV which corresponds to 40% proteinaceous substance in the first and 38.5% in the second compound.

Ultraviolet curves in the case of III show two maximum absorption peaks at 258 and at 235 mμ and a minimum peak at 248 mμ. Compound IV shows maximum at 260 and 205 mμ and a minimum at 250 mμ. The infrared spectrum of compound III run immediately after lyophilization (Fig. 2), gave absorption bands at 3460 cm.$^{-1}$ which was assigned to N—H stretch and a band at 1650 cm.$^{-1}$ called amide I band which was assigned to C=O stretch and a band at 1460 cm.$^{-1}$ which was ascribed the amide II band and was assigned the amide deformation mode.

After a period of 2 hr. the infrared spectrum was run again on the same sample, and a shift in the three peaks indicated above was observed (Fig. 2). The first peak shifted from 3460 to 3300 cm.$^{-1}$. This change can be accounted for on the basis of hydrogen bonding which is relatively conformation independent (5).

SUMMARY AND DISCUSSION

G. sarothrae has been the subject of a preliminary phytochemical investigation to determine the chemical agent responsible for its activity against Sarcoma 180 in mice. Based on the isolation procedure, qualitative tests, and hydrolysis products of the active material to a number of identifiable amino acids, it would appear that the agent is a proteinaceous substance. Utilizing column chromatographic analysis, it was shown that this material still had some carbohydrate character, and subsequent refinement

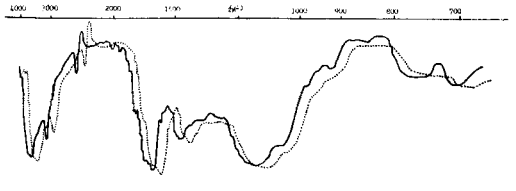

Fig. 2.—Key: ——, infrared spectrum of compound III before shift; - - -, infrared spectrum of compound III after shift.

of the material by means of paper electrophoresis to a single component would lead to the speculation that the material was of the glycoprotein type.

Further characterization of the precise nature of the active agent will be presented after screening is completed.

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Monoamine Oxidase Inhibitors. Synthesis of a Series of Isopropylidine and Isopropyl Derivatives of Some Aryl and Arylalkyl Acid Hydrazides

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Six isopropylidene hydrazides and six isopropyl hydrazides were prepared. This investigation was undertaken since recent derivatives of hydrazines and hydrazides have shown considerable activity as monoamine oxidase inhibitors. The hydrazides were synthesized from the aryl and arylalkylesters and hydrazine. From these hydrazides the isopropylidene derivatives were prepared by refluxing with acetone. Reduction of the isopropylidene hydrazides, using hydrogen and 5 per cent platinum-on-charcoal catalyst, gave the isopropyl hydrazides. The hydrazides prepared were of benzoic acid, phenylacetic acid, α-naphthoic acid, β-naphthoic acid, α-naphthylacetic acid, and β-naphthylacetic acid.

INTEREST in acyl hydrazine derivatives started in 1952 when Fox and Gibas (1) observed the tuberculostatic activity of isonicotinic acid hydrazides (isoniazid). Many derivatives of this compound were prepared, and one of these derivatives was 1-isonicotinyl-2-isopropyl hydrazide (iproniazid) (2) which was tested clinically as a tuberculostatic agent. During the investigation, it was noted (3) that iproniazid had a side effect of central nervous system stimulation. This effect appeared at the normal dosage levels used in the treatment of tuberculosis.

Further investigation by Zeller (4) in 1952 proved iproniazid to be an inhibitor of monoamine oxidase. The discovery of the clinical activity of iproniazid led to the modification of its structure (5) in an attempt to obtain a compound that would be more rapid in onset and of a lower toxicity.

The compounds chosen for synthesis in this study have the isopropyl hydrazine moiety in common with the monoamine oxidase inhibitor, iproniazid. The isonicotinyl portion has been replaced with aryl and arylalkyl groups.

The general synthesis of these compounds is shown in Scheme I.

The physical properties of the products obtained by this procedure are recorded in Tables I and II.

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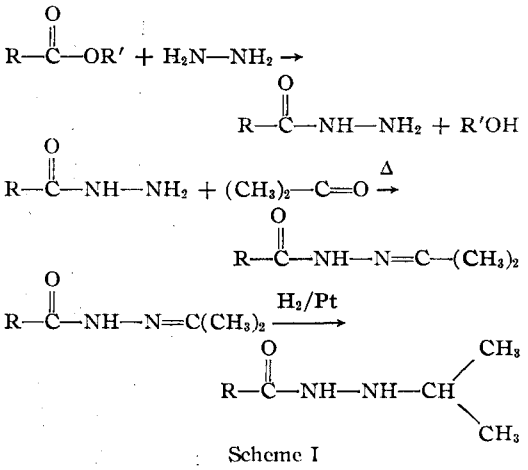
EXPERIMENTAL

The preparation of most of the compounds prepared in this investigation were obtained through a four-step synthesis. This involved the preparation of methyl or ethyl esters of the various aryl and arylalkyl carboxylic acids. The procedure used was described by Vieth (6) by reacting the corresponding acyl chloride with absolute methyl alcohol with slight warming. The next step involved the preparation of the hydrazides, and for this the method employed by Fox and Gibas (1) was used. The esters of the aryl and arylalkyl carboxylic acids were refluxed in alcoholic solution with an excess of 95% hydrazine.

The isopropylidene derivatives were then pre-



Scheme I