Antitumor Agents from *Agave schottii* (Amaryllidaceae)

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Abstract □ Extracts of *Agave schottii* Engelm. (Amaryllidaceae) were shown to be effective inhibitors of the Walker carcinoma 256 (intramuscular) tumor system of the CCNSC. The active material was shown to be saponin. By means of a Silica Gel G dry-column technique, an effective separation of the acetates of the saponins present was accomplished. Gitogenin was identified as the genin of all saponins present. While the sugar associated with the saponin showing the highest degree of activity was galactose, the sugars associated with the three other saponins were identified as a mixture of galactose and glucose.

Keyphrases □ Saponins, extraction—*Agave schottii* □ Antitumor screening—*Agave schottii* saponins □ Dry Column chromatography—separation □ TLC—identity

As a result of the continuing search for Southwestern plants having tumor inhibitory constituents, the authors found that the *n*-butanol extraction of a water extract of the inflorescence of *Agave schottii* Engelm. (Amaryllidaceae) was active against the Walker carcinoma 256 (intramuscular) tumor system (5WM) of the Cancer Chemotherapy National Service Center (CCNSC). Activity was detected in Sprague rats at a level of 7% T/C (test/control) at 75 mg./kg., and 28% T/C at 37.5 mg./kg. Activity in this system is defined as a percent T/C value of less than 60 in a satisfactory dose response test (1). The plant[1] was collected in Pima County, Arizona, during the month of June 1968.

A preliminary examination of the *n*-butanol extract revealed that saponins were responsible for the tumor-inhibitory effects. Separation and subsequent testing of the saponin-containing fraction showed that one of the saponins was substantially more active than the others. The resolution of six detectable saponins was effected by utilizing a modified dry-column chromatographic separation (2–5) of the acetylated saponin mixture from which free reducing sugars had been removed (6). This modification resulted in purer materials and was especially useful for preparing large quantities. Better resolution was achieved using this technique since the solvents passed through the columns in a manner which resulted in a distribution of the components along the total length of the columns.

This procedure involved the sectioning of the dry-column in order to determine the position of each of the components in the acetylated saponin mixture. The *Experimental* portion describes the detail. Exploratory samples taken from the columns were run on TLC in order to determine where each of the components was located. Therefore, it was possible to cut the column in such a way that each component could be separated.

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

Of the six saponins separated, No. 4 was the most active. Since Nos. 5 and 6 were inactive, no further work was performed on these materials. Saponin No. 4 when tested against the 5WM tumor system showed activity of 17% T/C at 65 mg./kg., and 22% T/C at 33 mg./kg.

EXPERIMENTAL

Extraction—Twenty-five kilograms of the ground fresh inflorescence of *Agave schottii* was macerated with approximately 100 l. of distilled water for 24 hr. The aqueous extract was filtered and evaporated to approximately 10% its volume. The marc was discarded. The aqueous extract was then exhaustively extracted by means of repeated shake-outs with *n*-butanol, until a butanol extract could be evaporated to dryness without leaving an appreciable residue. The butanol extract was washed with water saturated with butanol until the Benedict's reagent test for reducing sugars was negative (6). The butanol extract was evaporated *in vacuo* until completely dry. One hundred sixty-five grams of essentially pure sugar-free saponins was obtained. The yield was 0.66% from the fresh plant.

TLC analyses of portions of the saponin-containing extract were performed by allowing the lower phase of a chloroform–methanol–water (65:25:10) mixture (7) to ascend twice up plates of Silica Gel G (Merck). Visualization with cerium sulfate solution showed the presence of six compounds. TLC performed as above and visualized with defibrinated blood indicated that all six compounds were saponins (8).

Acetylation of the Saponin Mixture—Sixty grams of the saponin mixture was acetylated by refluxing for 2 hr. with a mixture of 400 ml. of pyridine–acetic anhydride (1:1). The reaction mixture treated in the conventional manner yielded 64 g. of a saponin acetate mixture (6). This material run on TLC, Silica Gel G, using ether as the solvent system, indicated six compounds.

A 2.5-g. sample of saponin acetate was subjected to a second acetylation using the above conditions. The reaction product chromatographed on TLC indicated that no further acetylation occurred.

Separation of the Saponin Acetates by Silica Gel G Dry Columns—Four columns were prepared in the following manner. One end of cellophane dialysis tubing (No. 4465-A2, Arthur H. Thomas Co., Philadelphia, Pa.) 1 m. long and 9 cm. in diameter was attached to a constricted glass joint and tied with a string and held by a clamp. The other end was moistened around the lip to a depth of approximately 2.5 cm. A Büchner funnel fitted with filter paper of approximately the same diameter as the tubing was introduced into the lower part of the tubing and was tied with a string. A sintered-glass funnel could also be used for this purpose. In order to prevent the column from collapsing when packed, the funnel was supported by a ring. Compressed air was applied gently on the upper part of the constricted glass joint in order to open the flat tubing into a cylindrical shape. Approximately 1.8 kg. of Silica Gel G, previously activated overnight in an oven at 120°, was poured into the column in three portions. The first portion (900 g.) was poured into the column while patting and vibrating it with the hand in order to get the Silica Gel G as homogeneous as possible. Compressed air gently applied occasionally aids in packing. A second portion (600 g.) was poured into the column following the procedure described above. The remainder of the Silica Gel G was then added. The height of the Silica Gel G was 65 cm.

After packing the column, 60 g. of saponin acetate mixture was dissolved in 400–500 ml. of chloroform and adsorbed on 200 g. of Silica Gel G. The mixture was allowed to dry completely in the air with occasional stirring. The dried material was divided into four equal parts, one for each column. The material was then poured slowly and carefully into each column so as not to upset the upper

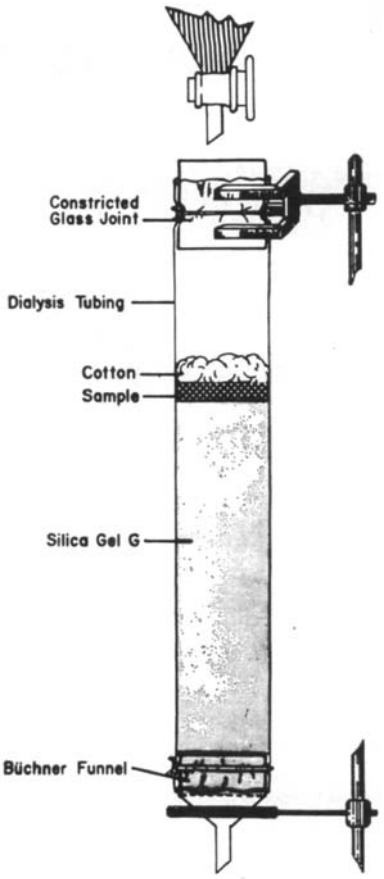

Figure 1—*Graphic representation of dry chromatographic column.*

surface of the Silica Gel G. A large piece of cotton was then placed upon this mixture so that the solvent dripping into the column would not disturb it or the Silica Gel G. Figure 1 is a graphic representation of the dry-column.

The chromatographic solvent system employed was ether–petroleum ether (b.p. 30–60°)–acetone (9:2:1). A 2-l. funnel was placed over the column, filled with the liquid mixture, and allowed to drip at a rate preventing overflow on the upper part of the column. The dripping was allowed to continue overnight. When approximately 3 l. of the liquid had entered the column, the liquid began to drip from the funnel at the bottom end of the column and was collected.

Since the distribution of the substance in the columns is the same as on the Silica Gel G-ether plates, the collected liquid was run on TLC periodically using the original mixture of saponin acetates as reference. The column was allowed to run in this manner until the eluate from the column when chromatographed on TLC showed all of the less polar material which precedes the saponin acetate No. 4. At this point about 10 l. of the chromatographic liquid had been added to the column.

Table I—Saponin Acetates from Exploratory Samples of the Dry Column

Sample(s)	Saponin Acetate(s) Present	Section
1	No. 4 contaminated by less polar material	I
2, 3, 4, 5	No. 4	II
6	No. 3 and No. 4	III
7, 8	No. 3	IV
9	No. 3 and No. 2	V
10, 11	No. 2	VI
12	No. 2 and No. 1	VII
13	No. 1 and No. 2	VIII
14	No. 1	IX
15, 16	Discarded	

The columns were allowed to stand from 48–60 hr. Sixteen exploratory samples at a distance of about 2.5 cm. from each other starting at the bottom were taken from the column in the following manner: A small window was cut in the column with the top and sides open and the bottom uncut. The flap was pulled down and a small amount of the wet Silica Gel G was taken from the opening with a spatula and placed in a 50-ml. conical flask. Acetone was added to the flask. The flap was then pulled up and covered with cellophane tape in order to prevent further evaporation of the liquid. After 2.5 hr. the contents of the flasks were filtered and the acetone was evaporated. The residues of each flask were dissolved in a few drops of chloroform and chromatographed on a 20 × 20-cm. TLC plate with the saponin acetate mixture being used as reference and ether as the solvent system. Figure 2 represents the chromatographic analysis of the 16 exploratory samples. These have been represented by progressive Arabic numbers starting from the bottom and going up to the top part of the column.

According to the information from the TLC and the location of the spots of the saponin acetates 1, 2, 3, and 4, the column was cut into sections (Table I). The elution was made by adding acetone and stirring with a magnetic stirrer overnight. The following day the material of each section was filtered and the acetone solution was evaporated. The residue of each section was then run on TLC (Silica Gel G–ether, with the acetate mixture as a reference). A very satisfactory resolution was obtained. All four columns gave approximately the same yield of saponin acetate No. 4 (1.5 g.). From the four columns, a total of 5.388 g. of saponin acetate No. 4 was obtained. Also obtained were 4.985 g. of saponin acetate No.

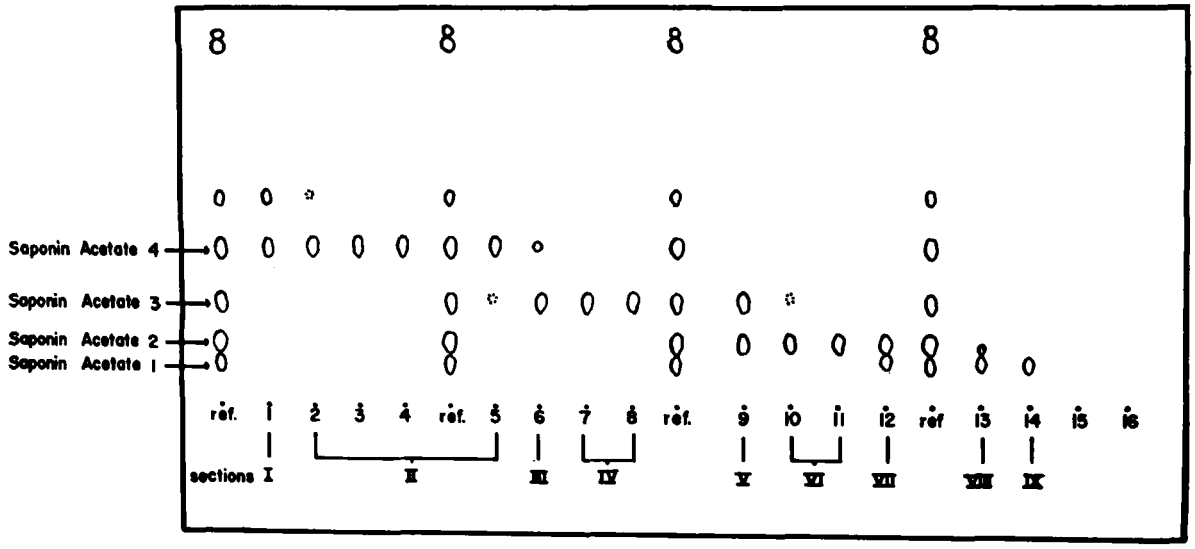

Figure 2—*Relative positions of saponin acetates on TLC plates. Roman numerals indicate which samples were combined.*

3, 3.598 g. of saponin acetate No. 2, and 1.785 g. of saponin acetate No. 1.

The exhausted Silica Gel G treated for 8–10 hr. in a muffle furnace at 800° was used for the preparation of TLC plates which gave the same results as the original Silica Gel G.

Alkaline Hydrolysis of Saponin Acetate No. 4 (6)—To 1.533 g. of saponin acetate No. 4, 35 ml. of 75% methanol and 5.25 ml. of a saturated solution of potassium hydroxide in methanol were added and refluxed for 2 hr. To the reaction mixture 50 ml. of water was added and the excess of methanol removed in an evaporating dish by evaporation. The liquid was neutralized with HCl, diluted with water (1:2) and extracted three times with 50 ml. of *n*-butanol saturated with water. The *n*-butanol extract was washed twice with 5 ml. of water saturated with *n*-butanol. The liquid was evaporated *in vacuo*. 0.887 g. of saponin No. 4 was obtained. The purity was observed on TLC, Silica Gel G, using a solvent system of chloroform–methanol–water (65:25:10), lower phase, with the natural saponin mixture being used as a reference. The plate was run three times. Saponin No. 4 showed slight contamination of saponins 2 and 3.

Acid Hydrolysis of Saponin No. 4—(*A*) *Identification of the Genin*—To 58 mg. of saponin No. 4, 1.5 ml. of a mixture of 20 ml. of concentrated HCl in 80 ml. of ethanol was added and refluxed for 3 hr. After cooling, 1.5 ml. of water was added and the excess ethanol was removed in an air stream. It was then extracted with 5 ml. of ether three times. The aqueous liquid was tested on paper chromatography for sugars as described below.

The combined ether layers were washed with water and a diluted solution of $NaHCO_3$. It was dried on $MgSO_4$, filtered, and evaporated. The brown-colored residue was dissolved in chloroform and was passed through a short Al_2O_3 (III) column. The colorless eluate after evaporation yielded a crystalline material which was recrystallized from methanol–ether, m.p. 268–270°. On the basis of a mixed melting point, IR, and TLC, Silica Gel G, chloroform–methanol–water (188:12:1), the data indicated the presence of gitogenin. Comparison of the above data using an authentic sample[2] confirmed the presence of this compound.

(*B*) *Identification of the Sugars*—The aqueous acid solution after being extracted with ether was examined for the presence of sugars by means of paper chromatography using Whatman No. 1 filter paper and *n*-butanol–pyridine–water (6:4:3) as the solvent system. The chromatogram was run for 48 hr. together with authentic samples of several sugars. When developed with aniline phthalate, the sugar was identified as galactose.

Alkaline and Acid Hydrolysis of the Saponin Acetates No. 1, No. 2, and No. 3—The alkaline hydrolysis of these other three saponin acetates was carried out in the same manner as described above and gave the following results:

Saponin acetate No. 1 yielded saponin No. 1
Saponin acetate No. 2 yielded saponin No. 3
Saponin acetate No. 3 yielded saponin No. 2

The acid hydrolysis of each was also carried out in the same manner and yielded gitogenin, galactose, and glucose which were identified as described above.

[2] Authentic sample of gitogenin obtained from Syntex Laboratories, Palo Alto, Calif.

SUMMARY

Agave schottii has been the subject of a phytochemical investigation in order to determine the chemical constituents responsible for its antitumor activity. It has been shown that saponins were, in fact, responsible for this tumor inhibitory property. Separation of the saponins by means of column chromatography employing a modified Silica Gel G dry-column technique has been described. The most active saponin of the series as well as three other saponins have been analyzed in order to determine the genin and related sugars. In each case the genin was gitogenin. The sugar was galactose in the most active saponin and galactose and glucose were in the other three. Further testing in other tumor systems is now under way.

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