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POTENTIAL ANTI-TUMOR AGENTS FROM AGAVE PACIFICA  
(TRELEASE) FAMILY AMARYLLIDACEAE

by

Ahmad Ismail Jado

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A Dissertation Submitted to the Faculty of the  
COLLEGE OF PHARMACY

In Partial Fulfillment of the Requirements  
For the Degree of

DOCTOR OF PHILOSOPHY  
WITH A MAJOR IN PHARMACEUTICAL CHEMISTRY

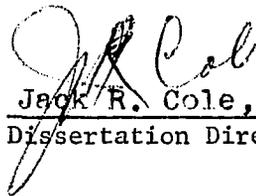
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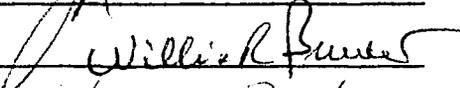
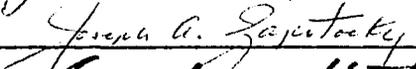
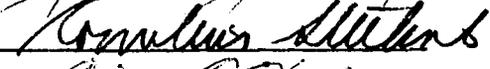
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I hereby recommend that this dissertation prepared under my  
direction by Ahmad Ismail Jado  
entitled Potential Anti-tumor Agents from Agave Pacifica  
(Trelease) Family Amaryllidaceae  
be accepted as fulfilling the dissertation requirement of the  
degree of Doctor of Philosophy

  
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After inspection of the final copy of the dissertation, the  
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A. I. Jado

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## ABSTRACT

In the course of a continuing search for new and more effective anti-tumor substances from the plant kingdom, the methanol extract of the defatted fresh leaves of Agave pacifica, family Amaryllidaceae, was found to possess anti-tumor activity. The activity was obtained in the 5 WM [Walker Carcinoma 256 (intramuscular)] test system of the National Institutes of Health, Cancer Chemotherapy National Service Center.

Preliminary investigation of the methanol extract revealed the presence of a substantial amount of saponins. The saponins were extracted from the plant in a more direct manner using hot water. The concentrated hot water extract was exhaustively extracted with n-butanol and the extract was evaporated to dryness. Preliminary investigation of the n-butanol extract showed it to form a persistent foam upon shaking with water, to hemolyze the red blood cells, and to give a typical saponin absorption upon being subjected to infrared spectroscopy.

The n-butanol extract contained two saponins which were separated, in the acetate forms, by means of dry column chromatography. The individual saponins were

regenerated by the alkaline hydrolysis of their respective acetates.

Acid hydrolysis of the saponins afforded their respective aglycone and sugar moieties.

Based on evidence from thin-layer chromatography, infrared spectroscopy, melting point determinations, and mass spectrometry the aglycones of saponins 1 and 2 were identified as hecogenin and tigogenin, respectively.

The sugar moieties of both saponins were found by paper chromatography to consist of L-rhamnose, D-xylose, and D-glucose. Gas chromatography of trimethylsilyl ether derivatives of the saponins sugar moieties showed sugar moieties of saponins 1 and 2 to consist of L-rhamnose, D-xylose, and D-glucose in the ratio 2:1:4, and 3:1:3, respectively.

Elemental analyses indicated that saponin 1 contained one mole of hecogenin, two moles of L-rhamnose, one mole of D-xylose, and four moles of D-glucose. The molecular weight of saponin 1 was 1503.59 with the corresponding molecular formula of  $C_{68}H_{110}O_{36}$ . Saponin 2 contained one mole of tigogenin, three moles of L-rhamnose, one mole of D-xylose, and three moles of D-glucose. The molecular weight of saponin 2 was 1473.6 with the corresponding molecular formula of  $C_{68}H_{112}O_{34}$ .

## CHAPTER 1

### INTRODUCTION

As a result of the continuing search for plants with potential anti-tumor activity, the fresh ground leaves of Agave pacifica, family Amaryllidaceae were defatted and then extracted with methanol. This extract showed anti-tumor activity against the 5WM (Walker Carcinosarcoma 256 [intramuscular]) test system of the Cancer Chemotherapy National Service Center (1).

A literature search revealed that nothing concerning the nature of the chemical constituents of the plant has been reported to date.

Recently, Bianchi and Cole (2) isolated tumor inhibitory saponins from Agave schottii, family Amaryllidaceae.

The purpose of this investigation is to isolate and identify the material in the extract of Agave pacifica responsible for the anti-tumor activity.

Two preliminary tests were carried out on the methanol extract to determine the possible presence of saponin-like material. The extract formed persistent foam when shaken with water thus indicating the possible presence

of saponins. A second test utilized silica gel G thin-layer chromatography. Two plates were spotted with a 5% alcoholic solution of the extract and were developed using the lower phase of the chromatographic liquid, chloroform : methanol : water, 65:35:10 (solvent system 1) (3). The two chromatograms were treated as described on page 3 under the title "Blood Hemolysis Test". Two major zones in the chromatogram hemolyzed the red blood cells.

#### Occurrence and Description of the Plant

Agave pacifica is found on rocky hillsides and desert flats, lower Sonoran zone and Central Sonora southward to Tepic. However, the plant which was provided for this investigation was collected from Nuevas Minas, Sonora, Mexico in February, 1968.

Species of Agave are known in the United States as Century plants because the plants were believed to flower only when they reach an age of a hundred years. This incorrect belief was probably due to the fact that the cultivated plants rarely bloom. The plants are known in Europe as American Aloes, because of a slight resemblance to old world plants of the genus Aloe of the Liliaceae family.

The plant is acaulescent with very slightly glaucous and zoned yellow-green leaves, 3.5-5 cm wide and 50-75 cm

long. The spine is purplish red-brown in color and 15-25 mm long. It is often abruptly contracted and slender above the decurrent base. The teeth are usually upcurved-triangular, 15-25 mm apart and 3-5 mm long, the intervening cartilaginous margin straight. Flowers are greenish yellow, 50 mm long with openly conical tube half as long as the segments. The capsules are shortly stipitate and beaked, 25 mm broad and 45 mm long (4).

#### Plant Extraction

Since the preliminary examination of the methanol extract indicated the possible presence of saponin material, the isolation of the saponins by a more direct route was undertaken.

Fifty kilograms of the fresh leaves of the plant were ground into a meal-like material and then macerated with hot water for four hours. Filtration of the macerate gave a dark greenish brown filtrate which showed foaming properties. The marc was discarded and the aqueous filtrate was concentrated by air evaporation.

#### Saponin Extraction

The concentrated hot water extract was extracted by means of repeated shake-outs with n-butanol. The butanol phases were combined and washed with water saturated with n-butanol until the reducing sugars were removed from

the extract (5). The butanol extract was then concentrated, first by air evaporation and then dried completely under reduced pressure.

Preliminary Analysis of the  
n-butanol Extract

The following tests were carried out on the n-butanol extract to obtain some indication of the nature of the extract.

1- Solubility Test

The extract was soluble in water, methanol, ethanol, and n-butanol. It was insoluble in acetone, ether, and all other non-polar solvents.

2- Liebermann-Burchard Test

Approximately 10 mg of the extract was dissolved in a few drops of aqueous ethanol. One drop each of acetic anhydride and sulfuric acid was added. The color changed from yellowish to dark blue indicating the probable presence of cholesterol or other pentacyclic-type structures (6, p. 492).

3- Blood Hemolysis Test

Two silica gel G thin-layer plates, were spotted with 10% ethanolic solution of the butanol extract. The

plates were developed using solvent system 1. The plates were then air dried and treated in the following manner:

Chromatogram I was visualized with ceric sulfate charring which revealed the presence of several spots. However, only two spots, which had Rf values of 0.35 and 0.32 were predominant (Fig. 1).

Chromatogram II was placed horizontally on a cold block. A fresh blood-gelatin solution was poured on until the entire plate was covered with a thin film of the blood solution. After 30 minutes, the red blood-gelatin film became transparent in only two major zones while the rest of the film remained opaque and red. Those zones had the same Rf values as the predominant spots in chromatogram I. Since hemolysis of the red blood cells took place at those two zones only, it was assumed that they were probably saponins. The two major spots whose Rf values were .35 and .32 are referred to hereafter as saponins 1 and 2, respectively.

#### Infrared Analysis

The infrared spectrum of the n-butanol extract was obtained utilizing a potassium bromide pellet. The spectrum (Fig. 2) showed a strong peak at about 2.9 microns, corresponding to a hydroxyl group, a weak peak at 5.9 microns, corresponding to a carbonyl group, a very broad

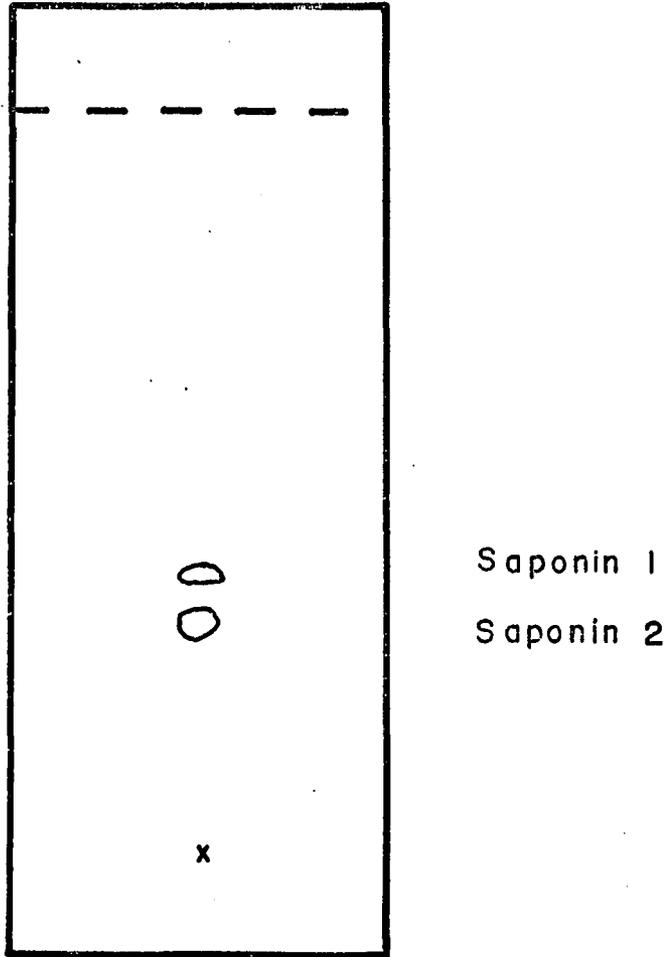


Figure 1. Thin-layer chromatogram of the butanol extract.

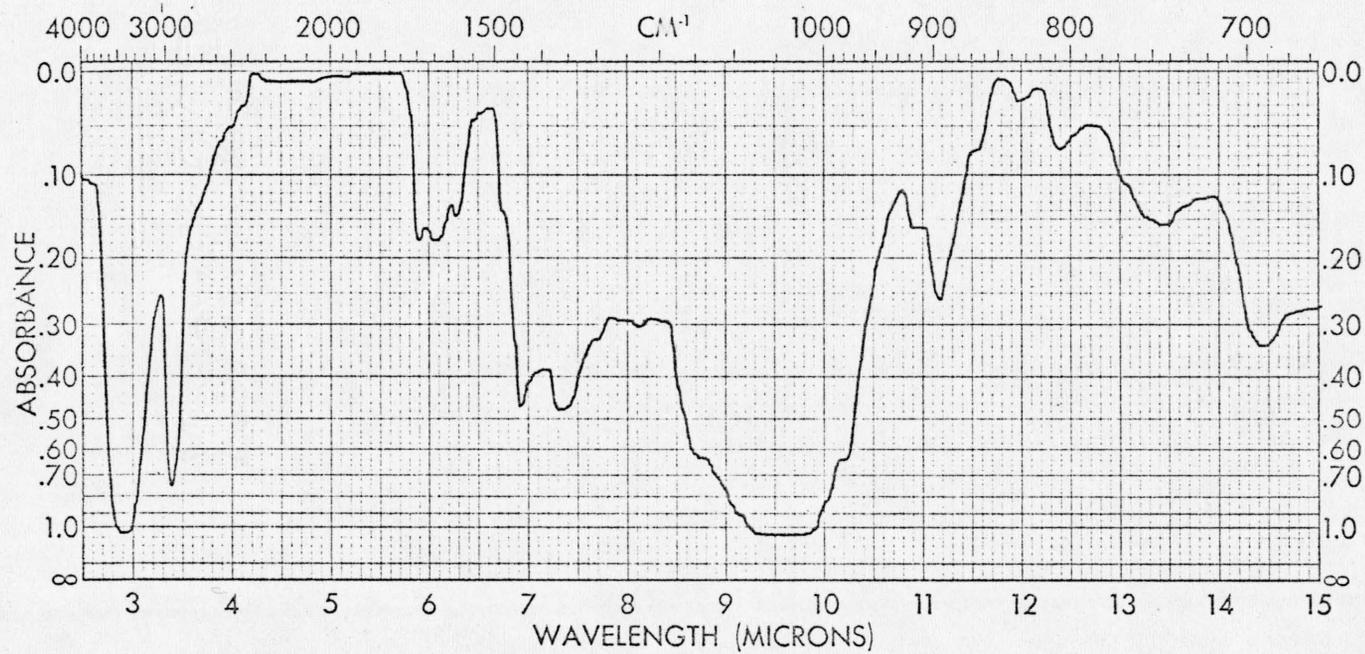


Figure 2. Infrared spectrum of the butanol extract.

peak centered at 9.5 microns, corresponding to an ether group, three small peaks at 10.22, 10.88, and 11.6 microns and a large peak at 11.15 microns.

The spiroketal side chain in steroidal saponins was reported to have a highly specific infrared absorption spectrum, with maxima occurring near 10.18, 10.85, 11.1, 11.55 microns (5). These values correlate with those obtained from the infrared spectrum of n-butanol extract mentioned above.

## CHAPTER 2

### PURIFICATION AND SEPARATION OF THE SAPONIN MIXTURE

It was thought feasible at this point to attempt to purify the butanol extract before actually separating the saponin mixture.

#### Purification of the n-butanol Extract

Since the n-butanol extract was soluble in ethanol and insoluble in acetone an attempt was made to precipitate the saponins from an ethanolic solution of the extract with acetone.

Three hundred-ninety grams of the extract was dissolved in ethanol and filtered. This alcoholic solution was added to an excess amount of acetone with continuous stirring, and a precipitate was formed. This precipitate was washed with acetone and air dried. The yield was 205 g.

#### Thin-layer Chromatography of the Purified Butanol Extract

The precipitate was chromatographed on a thin-layer plate using solvent system 1 with the original butanol extract as a reference. Visualization of the

chromatogram showed the precipitate to contain two slightly contaminated spots (Fig. 3). The two spots had Rf values identical with the Rf values of the two major spots in Fig. 1 (p. 6). The precipitate is referred to hereafter as the saponin mixture.

#### Separation of the Saponin Mixture

Separation of the saponin mixture by thin-layer chromatography using solvent system 1 was attempted. This system failed to resolve large quantities of the mixture although it gave satisfactory resolution with small quantities.

Separation of glycosidic substances is usually carried out by preparing a derivative which is less polar than the glycoside itself and could easily be hydrolyzed back to the original compound. The acetate derivative meets these conditions and it has been successfully used in other investigations (2). The acetates of the saponin mixture were prepared in an attempt to separate the saponin mixture.

#### Preparation of the Saponin Acetates

Seventy-five grams of the dry saponin mixture was acetylated by refluxing it for two hours with 150 ml of dry pyridine and 150 ml of acetic anhydride. The reaction mixture was cooled and then added to ice water with constant

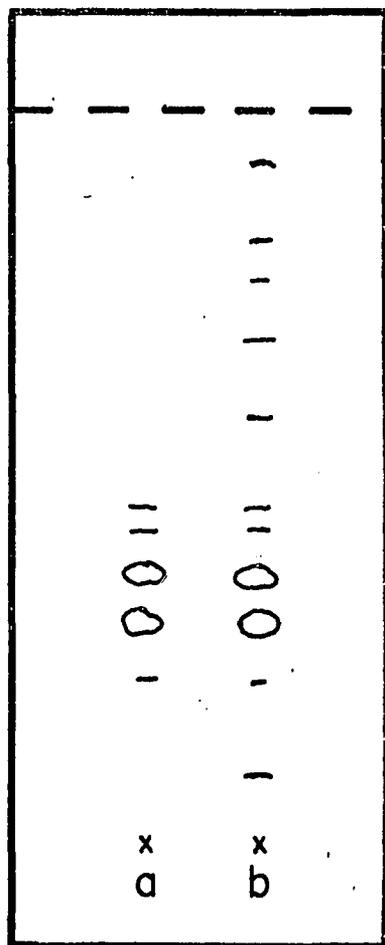


Figure 3. Thin-layer chromatogram of the saponin mixture.

a, saponin mixture; b, butanol extract.

stirring, and a yellowish precipitate formed. The reaction mixture was filtered and the precipitate was washed with 2% hydrochloric acid solution, followed by water, and finally with 5% sodium bicarbonate solution. The precipitate was then placed in a flask and shaken with 10% sodium bicarbonate solution to hydrolyze any unreacted acetic anhydride. The mixture was filtered and the precipitate was washed with water and air dried. This precipitate is referred to hereafter as the saponin acetate mixture. The yield was 80 g.

#### Thin-layer Chromatography of the Saponin Acetate Mixture

A sample of the saponin acetate mixture was spotted on a thin-layer plate and the plate was developed twice using the chromatographic system, ether : petroleum ether (b.p. 30-60°C) : acetone, 9:2:1 (solvent system 2). Visualization of the chromatogram revealed the presence of two major substances. The substance with higher R<sub>f</sub> value is referred to hereafter as saponin acetate 1 and the substance with lower R<sub>f</sub> value is referred to as saponin acetate 2 (Fig. 4).

#### Separation of the Saponin Acetate Mixture by Silica-gel G Dry Columns

Saponin acetates 1 and 2 were successfully separated in pure forms and in large quantities using silica gel G

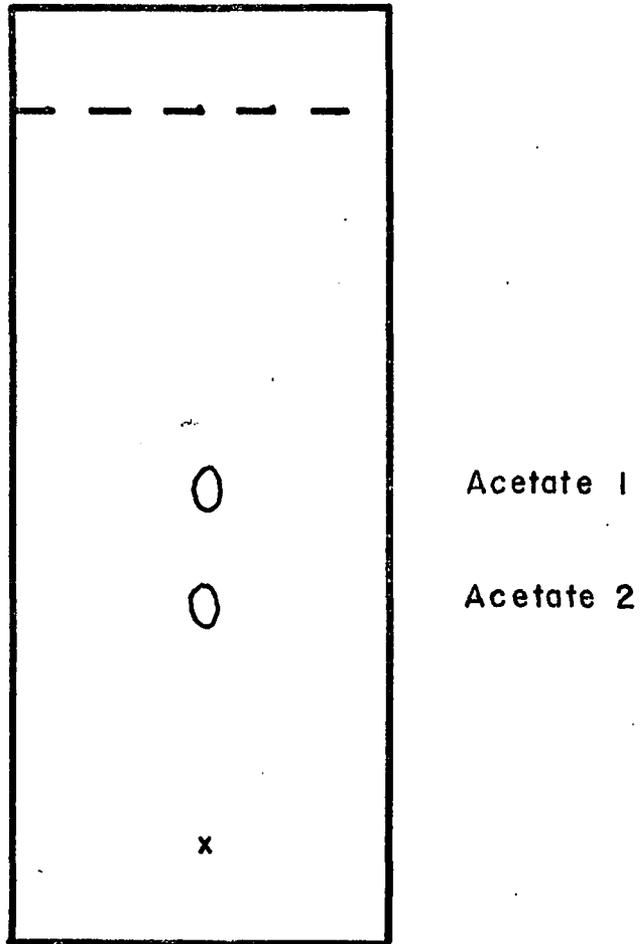


Figure 4. Thin-layer chromatogram of the saponin acetate mixture.

dry columns (2). Three dry columns were utilized to separate 45 g of the saponin acetate mixture using solvent system 2. The yield of saponin acetate 1 and 2 was 2.805 g and 2.264 g, respectively.

Alkaline Hydrolysis of Saponin  
Acetates 1 and 2 (5)

Saponin acetates 1 and 2 were each treated in the following manner: 0.5 g of the saponin acetate was added to 12 ml of 75% methanol and 1.75 ml of methanol saturated with potassium hydroxide. The mixture was refluxed for two hours. The reaction mixture was transferred to an evaporating dish. Fifteen milliliters of water was added to the reaction mixture and the excess methanol was evaporated by passing a stream of air over the reaction mixture. The liquid was neutralized with hydrochloric acid and diluted with water to twice its volume. It was extracted with four 50 ml portions of n-butanol saturated with water. The n-butanol layers were combined, washed three times with 25 ml portions of water saturated with n-butanol, and evaporated to dryness under reduced pressure. Saponin acetates 1 and 2 yielded respectively 0.265 g and 0.253 g of crude saponins.

### Purification of the Crude Saponins

Each crude saponin was purified in the following manner: the substance was dissolved in 5 ml of ethanol and the solution was added to 100 ml of acetone with stirring, and an amorphous precipitate was formed. The precipitate was removed by centrifugation and was then crystallized from hot methanol to give a white crystalline saponin.

### Thin-layer Chromatography of Saponins 1 and 2

Both crystalline saponins were chromatographed on a thin-layer plate using solvent system 1, with the original saponin mixture being used as a reference. Visualization of the plate revealed that saponin acetate 1 yielded saponin 2 and saponin acetate 2 yielded saponin 1.

## CHAPTER 3

### CHARACTERIZATION OF THE SAPONINS

Proposed structures for the two saponins isolated from the plant extract have been made. These proposals were based upon the information obtained from the identification of the aglycone and the sugar moieties of the saponins and the respective carbon-hydrogen analyses. Each saponin was hydrolyzed to its two moieties, the aglycone and the sugar, which were then isolated and identified using various methods. These methods will be discussed below.

#### Acid Hydrolysis of the Saponins

Acid hydrolysis of the saponins has been reported to cause decomposition of the sugars. Therefore, the hydrolysis of each saponin was carried out under mild conditions in the following manner: 100 mg of the saponin was dissolved in a mixture of 10 ml of dioxane and 20 ml of 2N sulfuric acid solution. The mixture was refluxed on a steam bath for five hours (7). Forty milliliters of water was added to the reaction mixture, which was then allowed to cool to room temperature. The excess dioxane was removed in an air stream. The mixture was then extracted four

times with 100 ml portions of ether and the ether layers were then combined.

The aqueous layer was placed in a beaker and anhydrous barium carbonate was added slowly with stirring until the solution became neutral and carbon dioxide evolution had ceased (8). The resulting slurry was filtered through a fine sintered-glass funnel and the precipitate was washed twice with 20 ml portions of water. The filtrate and the washings were combined, placed in an evaporating dish, and air evaporated, resulting in a yellowish white residue. The residue was dissolved in 10 ml of water, filtered, and concentrated to a syrup under reduced pressure at 25°C. The two sugar moieties thus obtained from the acid hydrolysis of saponins 1 and 2 are referred to hereafter as sugar moieties 1 and 2, respectively. They were labeled as such and set aside for further investigation.

The ether solution was washed twice with 50 ml portions of water, once with 50 ml of 10% sodium bicarbonate solution, and again twice with 50 ml portions of water until the aqueous washings were neutral. The ether solution was dried with anhydrous magnesium sulfate, filtered, and evaporated to dryness under reduced pressure to yield a brown colored residue. The yield of the crude sapogenins obtained from saponins 1 and 2 was 27.0 and 26.8 mg, respectively.

### Purification of the Crude Sapogenins

Each crude sapogenin was purified in the following manner: The sapogenin was dissolved in 5 ml of chloroform and introduced into an alumina (III) column. The column was eluted with chloroform, and the colorless eluate was collected and evaporated to dryness under reduced pressure. The residue was recrystallized from acetone to yield a white crystalline substance. The sapogenins of saponins 1 and 2 are referred to hereafter as sapogenins 1 and 2, respectively.

### Thin-layer Chromatography of the Sapogenins

Sapogenins 1 and 2 were spotted on a thin-layer plate, along with authentic samples of the genins: chlorogenin, hecogenin, yuccagenin, tigogenin, and gitogenin (obtained from Syntex Laboratories, Palo Alto, California), which were used as references. The plate was developed using the solvent system, chloroform : methanol : water, 188:12:1 (solvent system 3) (9). Visualization of the chromatogram revealed that sapogenin 1 and hecogenin had identical R<sub>f</sub> values, 0.77; and that sapogenin 2 and tigogenin had identical R<sub>f</sub> values, 0.92 (Fig. 5).

Another thin-layer plate was spotted with the following samples; sapogenin 1, a 1:1 mixture of sapogenin 1

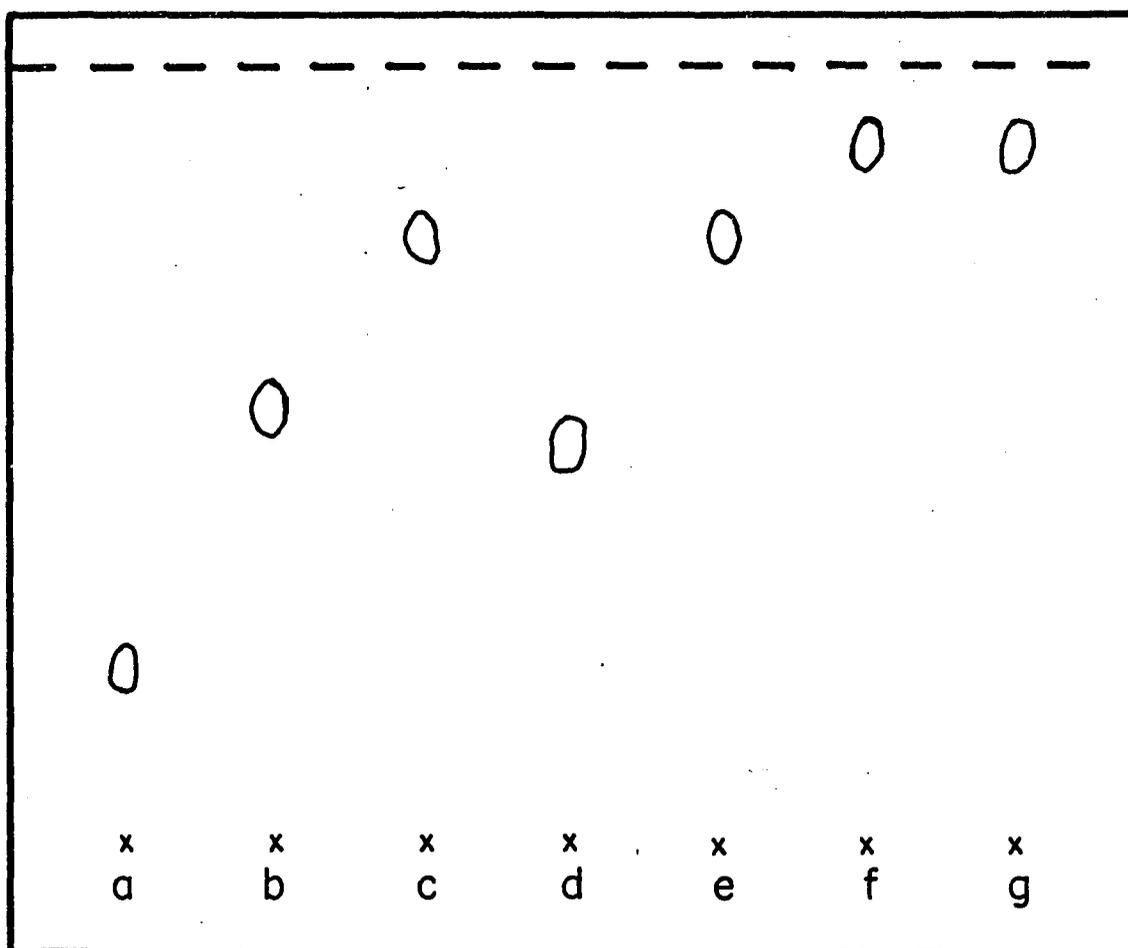


Figure 5. Thin-layer chromatogram of the sapogenins.

a, chlorogenin; b, yuccagenin; c, sapogenin 1; d, gito-  
genin; e, hecogenin; f, sapogenin 2; g, tigogenin.

and hecogenin; sapogenin 2, a 1:1 mixture of sapogenin 2 and tigogenin, and tigogenin. The plate was developed using solvent system 3. Visualization of the plate revealed that sapogenin 1, sapogenin 1-hecogenin mixture, and hecogenin each gave a single spot with identical Rf value of 0.77. Sapogenin 2, sapogenin 2-tigogenin mixture, and tigogenin each gave a single spot with identical Rf value of 0.92.

#### Infrared Spectrum of Sapogenin 1

The infrared spectrum of sapogenin 1 was obtained utilizing a potassium bromide pellet. The spectrum showed all the four characteristic peaks of the spiroketal side chain at 10.22, 10.9, 11.15 and 11.6 microns. In addition, the spectrum contained a medium peak at 2.85 microns, corresponding to a hydroxyl group, and a strong peak at 5.88 microns, corresponding to a carbonyl group. There were also several peaks between 6.9 and 10 microns (Fig. 6).

#### Infrared Spectrum of Sapogenin 2

The infrared spectrum of sapogenin 2 was obtained utilizing a potassium bromide pellet. The spectrum (Fig. 7) showed all the four characteristic spiroketal side chain peaks at 10.25, 10.9, 11.15 and 11.6 microns. The spectrum had a medium peak at 3.0 microns, corresponding to a

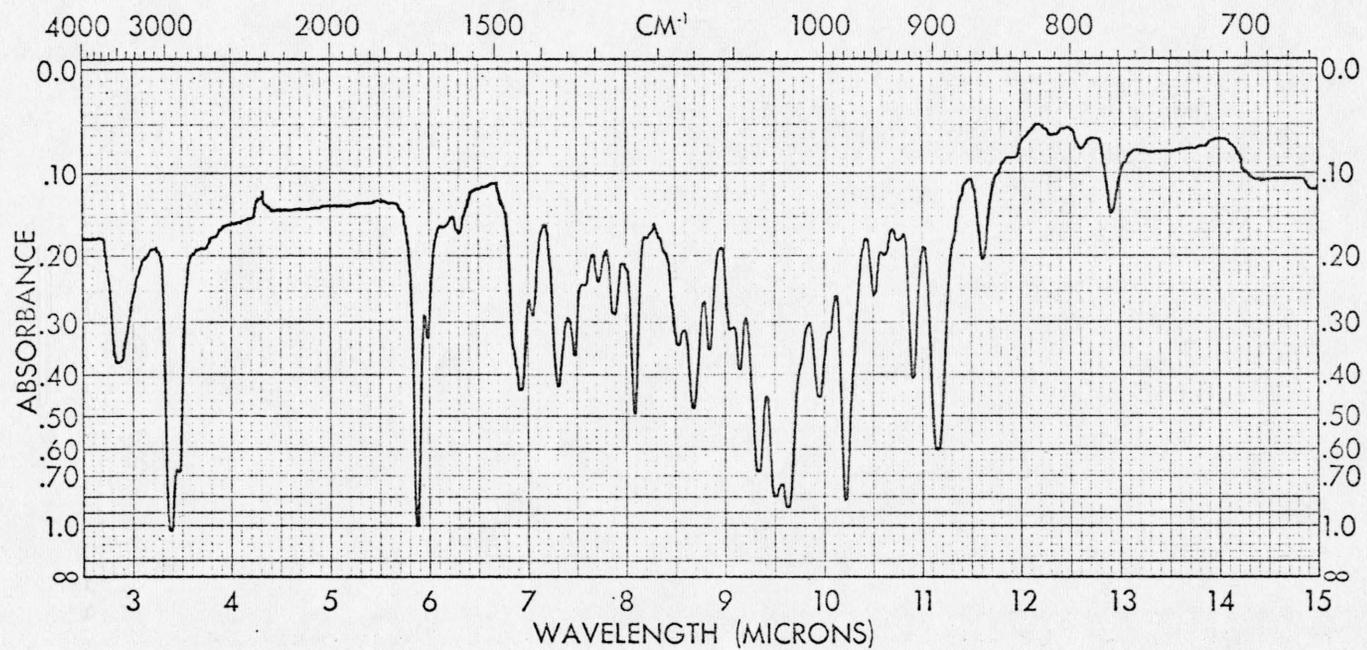


Figure 6. Infrared spectrum of sapogenin 1.

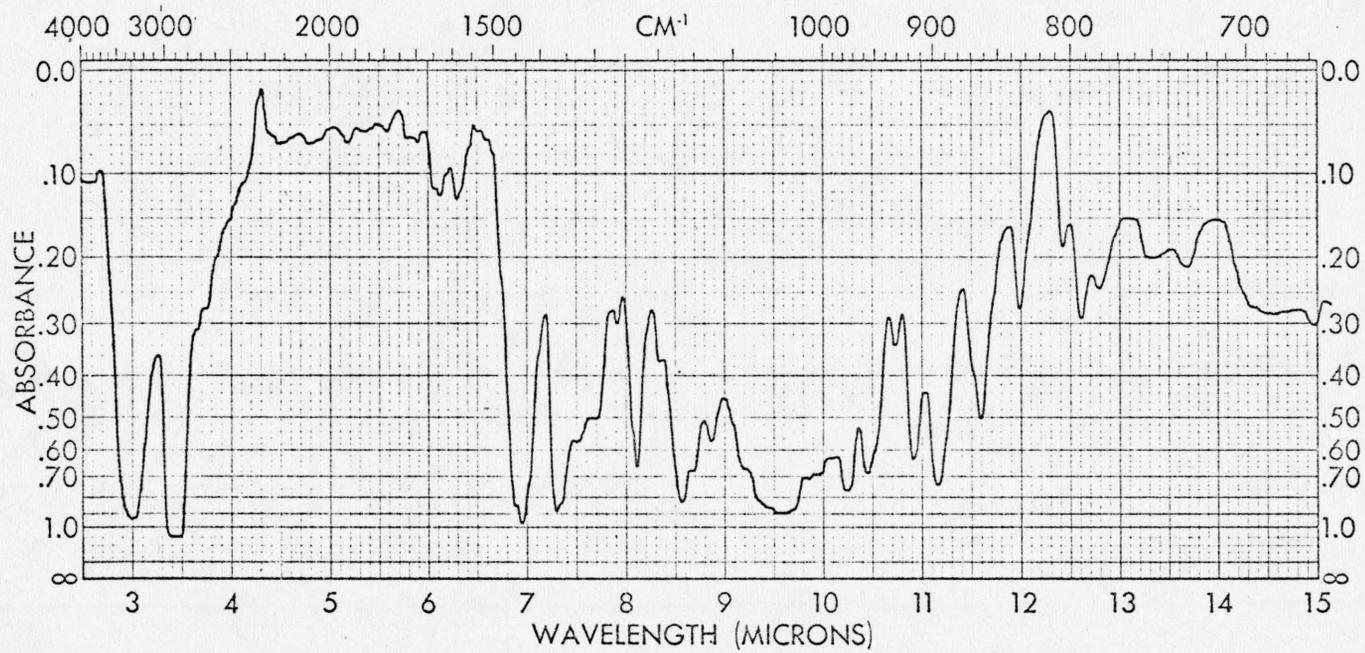


Figure 7. Infrared spectrum of sapogenin 2.

hydroxyl group, and several peaks between 6.1 and 10 microns. It lacked any absorption in the ketone absorption region.

Infrared spectra of authentic samples of hecogenin and tigogenin were obtained utilizing potassium bromide pellets. The two spectra were compared to those of the sapogenins. The spectrum of hecogenin was identical with that of sapogenin 1 and the spectrum of tigogenin was identical with that of sapogenin 2.

In addition to the distinctive spiroketal side chain peaks in the infrared spectrum of saponins and sapogenins, information is also revealed concerning the stereochemical form of the saponin and sapogenin side chain (6).

The spiroketal side chain can exist in two different stereochemical forms (10), neo(methyl axial) and iso(methyl equatorial) (Fig. 8). The relative intensities of the peaks at 11.1 and 10.85 microns distinguish between the two stereochemical forms (11). If the peak at 11.1 microns is of greater intensity than the peak at 10.85 microns, the spiroketal side chain is in the iso form. On the other hand, if the peak at 10.85 microns is of greater intensity than the peak at 11.1 microns, the spiroketal side chain is in the neo form. Examination of the infrared spectra of sapogenins 1 and 2 revealed that they exist in the iso form.

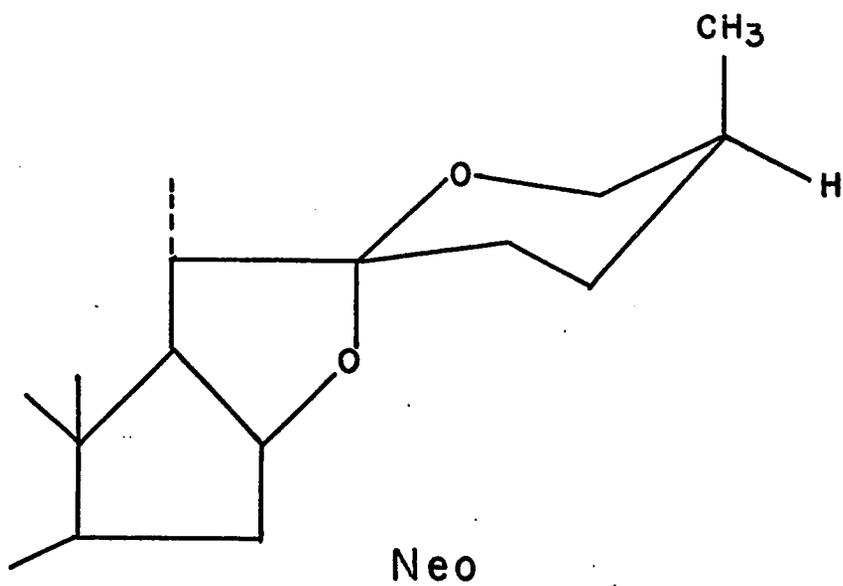
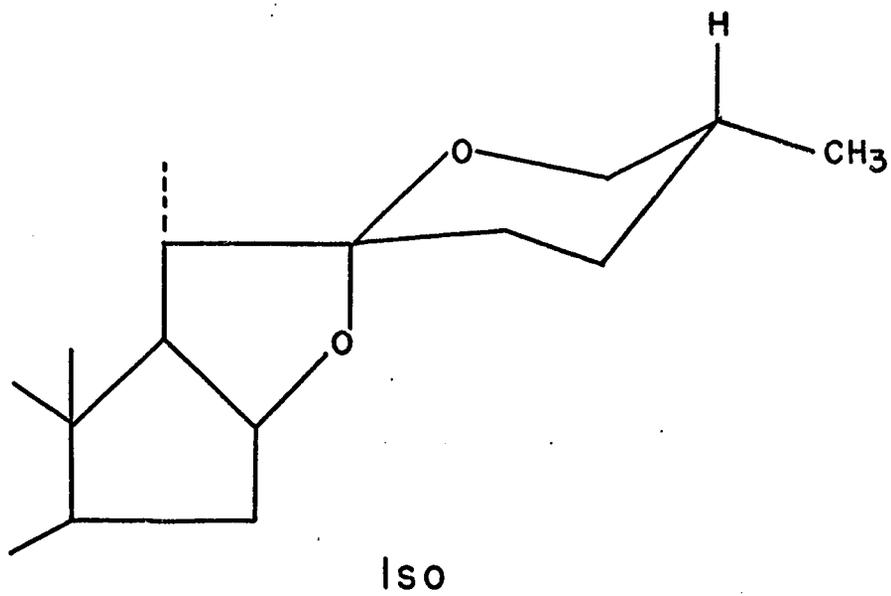


Figure 8. Iso and neo forms of the spiroketal side chain.

### Melting Point Determinations

The melting points of sapogenins 1 and 2 were determined. Sapogenin 1 melted at 250-251°C. Approximately 1:1 mixture of hecogenin and sapogenin 1 was prepared and the melting point of the mixture was determined. No depression in the melting point (of the mixture) was observed. Sapogenin 2 melted at 203-203.5°C. A mixture of sapogenin 2 and tigogenin showed no depression in the melting point.

### Mass Spectrometry of the Sapogenins

The mass spectra of the two sapogenins were obtained using the Hitachi Perkin-Elmer, RMU-6E Mass Spectrometer. The spectra of sapogenins 1 and 2 showed parent peaks at 430 m/e and 416 m/e, respectively. The calculated molecular weights of hecogenin and tigogenin are 430.61 and 416.62, respectively.

### Identification of Sapogenins 1 and 2

On the basis of thin-layer chromatography, infrared spectroscopy, melting points, and mass spectrometry, sapogenin 1 is hecogenin and sapogenin 2 is tigogenin.

### Paper Chromatography of the Saponin Sugar Moieties

The two saponin sugar moieties were subjected to paper chromatography using the chromatographic solvent

system, n-butanol ; pyridine : water, 6:4:3 (solvent system 4). Authentic samples of D-glucose, D-galactose, D-mannose, L-rhamnose and D-xylose (obtained from Aldrich Chemical Company, Milwaukee, Wisconsin) were used as references.

The chromatographic tank was previously saturated in the following manner: a 200 ml beaker half full with solvent system 4 was placed in the middle of the stainless steel stand-up support rack. The tank was then closed with the crystal lid and left as such for 48 hours.

A Whatman number 1 filter paper 57 cm long and 22.5 cm wide was spotted with 10% aqueous solutions of the saponin sugar moieties 1 and 2 along with the five reference sugar samples. The spots were 2.5 cm apart and 10 cm distant from the top of the filter paper. The paper was mounted on one of the troughs and held in place by means of the glass rod anchor as usual for descending chromatography. Fifty milliliters of solvent system 4 was poured in the trough and the tank was then closed. The chromatogram was run for 14 hours after which it was removed and air dried. Visualization of the chromatogram was accomplished by spraying it with aniline phthalate spraying reagent (12). The chromatogram was then placed in the oven for 10 minutes at 110°C until the spots appeared. Sugar moieties 1 and 2

each showed three spots with Rf values identical to those of D-glucose, D-xylose and L-rhamnose (Fig. 9).

#### Gas Chromatography of the Saponin Sugar Moieties

The analysis of sugar components in steroidal glycosides and polysaccharides has been carried out by paper chromatographic separation followed by colorimetric methods of identification. Recently, Sweeley et al. (13) developed a new method for a more rapid and more accurate analysis of the sugars. The method utilizes trimethylsilyl (TMS) ether derivatives of the sugars for analysis by gas-liquid chromatography (glc). The TMS ether derivative was prepared by dissolving the dry sugar sample in anhydrous pyridine followed by the addition of hexamethyldisilazane and trimethylchlorosilane. However, for the reaction to be quantitative the sugar sample must be completely dry. Anhydrous conditions are usually recommended as the silylated derivatives are sensitive to water in varying degrees. A 10- to 50-fold equivalent ratio of silylating agent to functional group being silylated is ordinarily taken. This will consume small amounts of water and help preserve the silyl derivative if kept in the reaction charge. However, in the presence of a moderate amount of water, an excess of the reagent will be hydrolyzed by the water and the yield of the silylated sugar will be poor (14, p. 10).

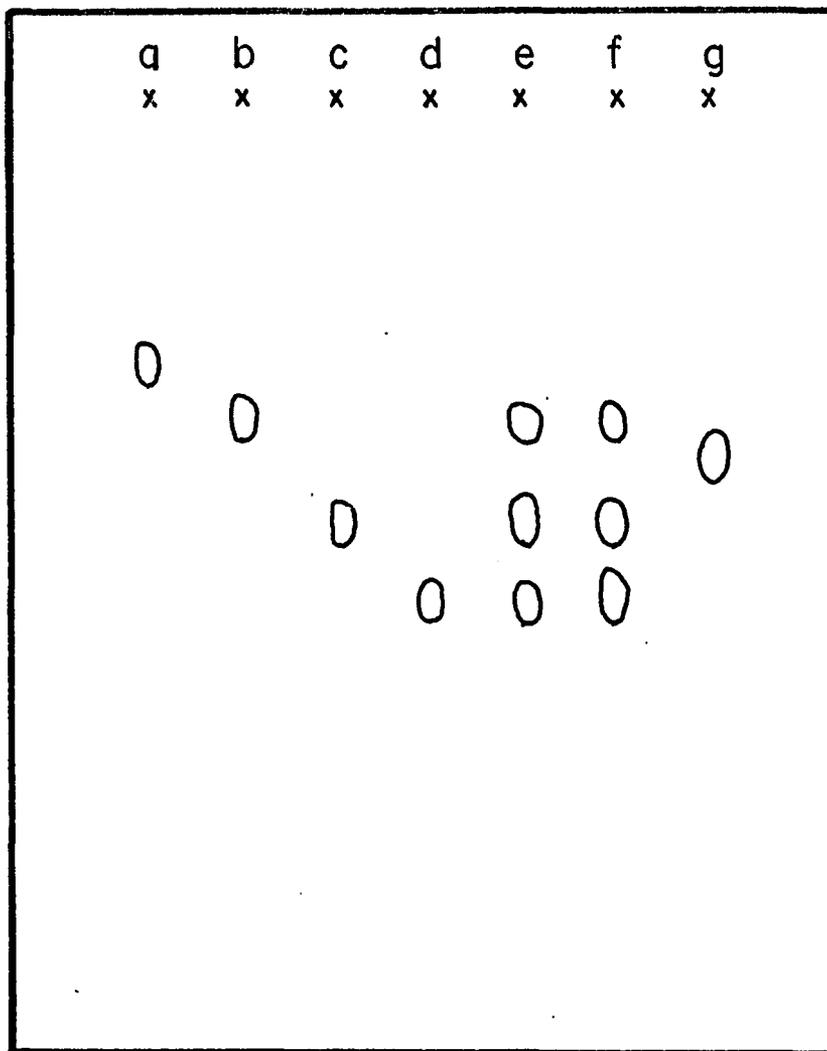


Figure 9. Paper chromatography of the two saponin sugar moieties.

a, D-galactose; b, D-glucose; c, D-xylose; d, L-rhamnose; e, sugar moiety 1; f, sugar moiety 2; g, D-mannose.

The compound N-trimethylsilylimidazole (TSIM) was found to be extremely effective in the silylation of sugar syrups in the presence of moderate amounts of water (14, p. 22). This method was therefore adopted for the preparation of the TMS ether derivatives of the saponin sugar moieties using the prepared reagent Tri-Sil'Z' (obtained from Pierce Chemical Company, Rockford, Illinois). It is formulated from TSIM in dry pyridine and is claimed by the manufacturer to silylate sugars very rapidly and smoothly with less anomerization than has been observed with any other method.

One-half microliter of the TMS ether derivative of saponin sugar moiety 1 was drawn into a 10 microliter Hamilton syringe (Hamilton Company, Whittier, California) and injected directly into the gas chromatograph. The resultant chromatogram showed three single peaks with retention times of 8.6, 12.7 and 35.4 minutes. The ratio of the peak areas was 2:1:4, respectively. The peak areas are proportional to the amount of material present. Each peak area was measured by multiplying the peak height by the peak width at half height.

A similar amount of the TMS ether derivative of saponin sugar moiety 2 was treated as described above. An identical chromatogram was obtained except for the ratio of the peak areas which was 3:1:3, respectively.

TMS ether derivatives of L-rhamnose, D-xylose and D-glucose were subjected separately to glc according to the same procedure described above. A single peak was obtained from each sugar derivative. The retention times were 8.6, 12.7 and 35.4 minutes, respectively. Therefore saponin sugar moieties 1 and 2 consist of L-rhamnose, D-xylose and D-glucose in the ratio 2:1:4 and 3:1:3, respectively.

#### Structure Proposals for the Saponins

Saponin 1 was shown to contain hecogenin as its aglycone. Sugar moiety 1 consisted of L-rhamnose, D-xylose, and D-glucose in the mole ratio 2:1:4, respectively.

Assuming this ratio to be the actual number of the sugar units in the saponin, the molecular formula of the saponin would be  $C_{68}H_{110}O_{36}$ . This formula corresponds to a molecular weight of 1503.59. Analytically calculated for  $C_{68}H_{110}O_{36}$  : C, 54.32; H, 7.37; found C, 54.23; H, 7.91.

Therefore, the sugar moiety of saponin 1 consists of seven units of the three sugars in the same molecular ratio shown above.

Based on the above, saponin 1,  $C_{68}H_{110}O_{36}$ , contains as its sugar moiety two units of L-rhamnose, one unit of D-xylose and four units of D-glucose. Its aglycone is hecogenin and the sugar units are linked to the three position (Fig. 10).

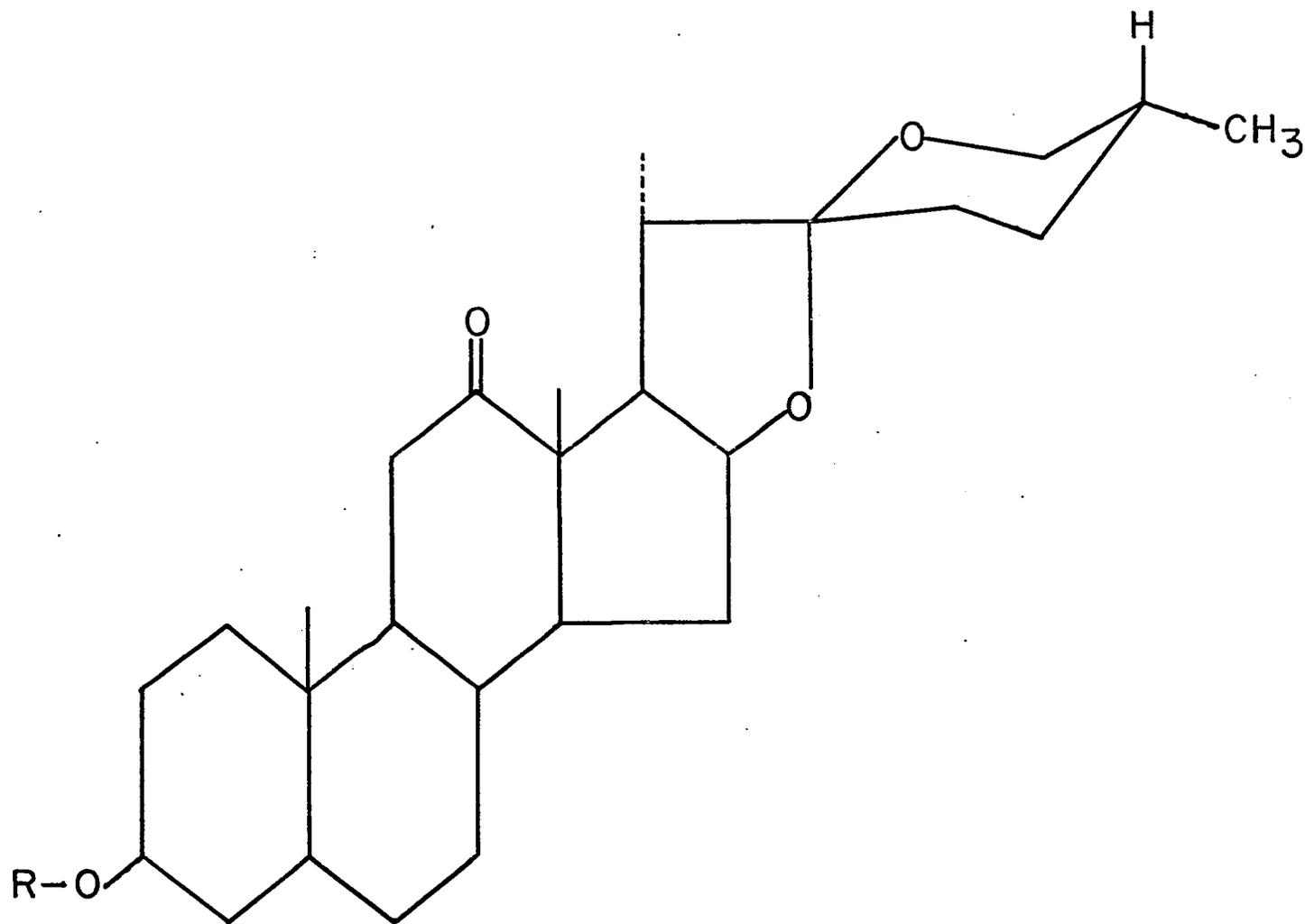


Figure 10. Proposed structure of saponin 1.

R = one unit of D-xylose, two units of L-rhamnose and four units of D-glucose.

Saponin 2 was shown to contain tigogenin as its aglycone. The sugar moiety 2 consisted of L-rhamnose, D-xylose, and D-glucose in the molecular ratio 3:1:3, respectively.

Assuming this ratio to be the actual number of the sugar units in the saponin, the molecular formula of the saponin would be  $C_{68}H_{112}O_{34}$ . This formula corresponds to a molecular weight of 1473.65. Analytically, calculated for  $C_{68}H_{112}O_{34}$  : C, 55.42; H, 7.66; found C, 55.31; H, 7.44.

Therefore, the sugar moiety of saponin 2 consists of seven units of the three sugars in the same molecular ratio as described above.

Based on the above, saponin 2, molecular formula  $C_{68}H_{112}O_{34}$ , contains as its sugar moiety three units of L-rhamnose, one unit of D-xylose and three units of D-glucose. Its aglycone is tigogenin and the sugar units are linked to the three position (Fig. 11).

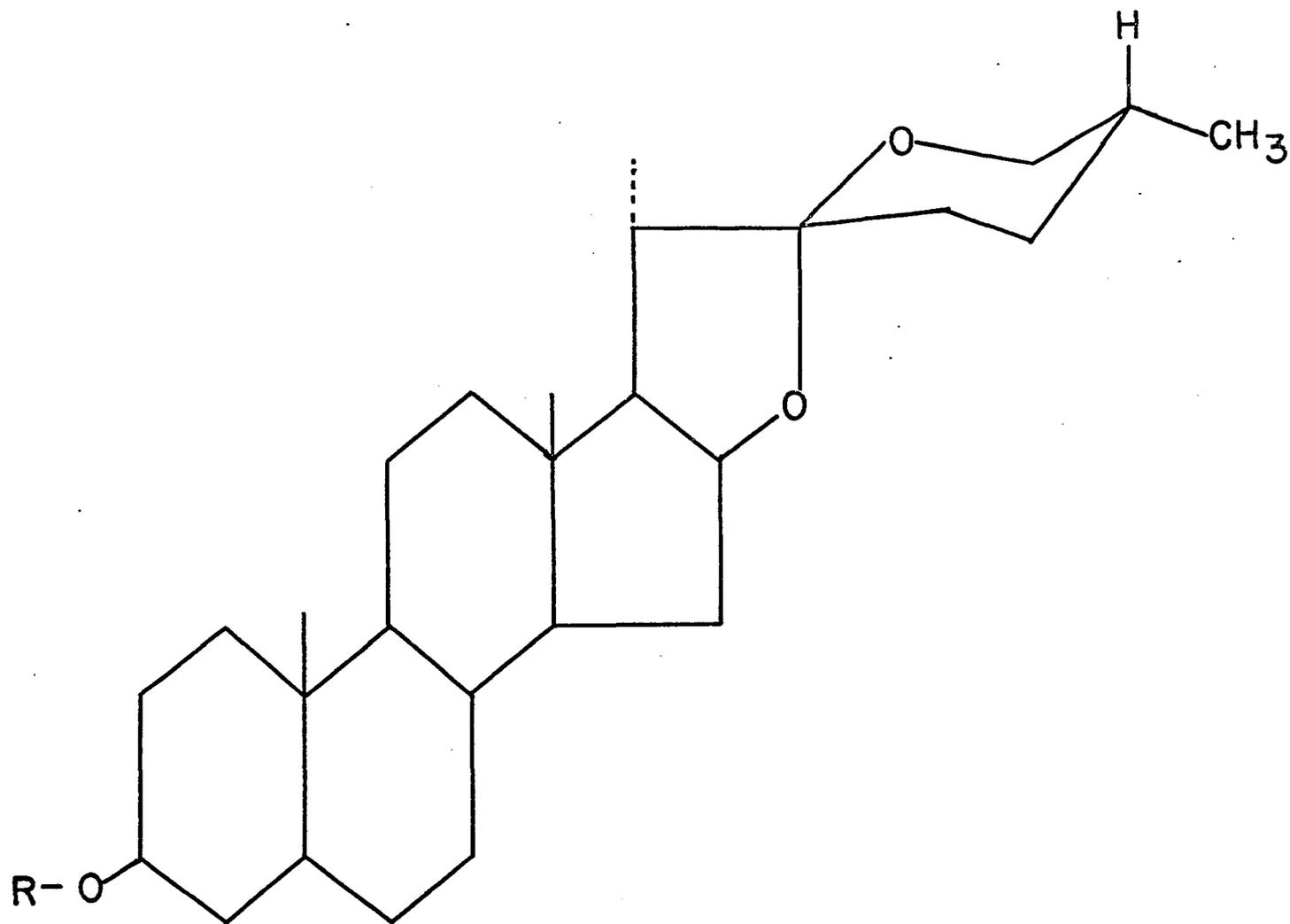


Figure 11. Proposed structure of saponin 2.

R = one unit of D-xylose, three units of L-rhamnose and three units of D-glucose.

## CHAPTER 4

### EXPERIMENTAL

This chapter is concerned with the detailed description of the different methods and techniques used throughout this investigation.

Identification of Agave pacifica, family Amaryllidaceae, was confirmed by Dr. Charles Mason, Director of the Herbarium, University of Arizona, Tucson, Arizona. The plant which was provided for this investigation was collected in February, 1968. A reference specimen was also deposited in the University of Arizona Herbarium.

#### Plant Grinding

Fifty kilograms of the fresh leaves of the plant were ground into a meal-like material in a Wiley mill manufactured by Arthur H. Thomas Company, Philadelphia, Pennsylvania. The mill was equipped with a 4 mm size screen.

#### Preparation of Plant Extracts

##### Methanol Extract of the Defatted Plant

Two hundred grams of the plant's ground leaves were defatted by extraction with 1.5 liters of petroleum ether.

The plant was air dried and was then extracted with 1.5 liters of anhydrous methanol. The methanol extract was evaporated to dryness under reduced pressure. The extract thus obtained is referred to as the methanol extract.

#### Hot Water Extract

Forty-five kilograms of the ground leaves of the plant were macerated with approximately 20 liters of hot water for four hours with occasional stirring. The macerate was filtered and the filtrate was concentrated to about 10 liters by air evaporation.

#### Anti-tumor Activity

The methanol extract was active against the Walker carcinosarcoma 256 (intramuscular) tumor system of the Cancer Chemotherapy National Service Center, National Cancer Institute, Bethesda, Maryland. Activity was detected in non-inbred albino rats at a level of 24% T/C (test/control) at 100 mg/kg, and 33% T/C at 67 mg/kg. Activity in this system is defined as a percent T/C value of less than 60% in a satisfactory dose response test.

#### Saponin Extraction

The concentrated aqueous extract was divided equally among ten 2-liter separatory funnels. Each portion was extracted twice with 500 ml portions of n-butanol, and the emulsion which formed was broken with a saturated

solution of sodium chloride. The butanol phase of each funnel was washed with water saturated with n-butanol, and the aqueous phase was tested for the presence of reducing sugars. Approximately 3 ml of the aqueous phase was placed in a test tube and one drop of Benedict test solution was added. The test tube was heated in a steam bath for five minutes. An orange precipitate was formed, indicating a positive test. The washing of the butanol phase was repeated until the aqueous phase gave a negative Benedict test, indicating the removal of all reducing sugars from the butanol phase.

#### Preparation of Blood Gelatin Solution

Fifty milliliters of a 0.9% sodium chloride solution was added to 2.25 g of gelatin powder, and after standing for 30 minutes at room temperature, the mixture was heated, with stirring, to about 80°C in a water bath. The gelatin solution was cooled to 45°C and, while stirring, 6 ml of defibrinated cow's blood was added. This blood gelatin suspension was immediately poured on the horizontally placed chromatogram until a thin film of the suspension covered the entire chromatogram. To prevent spilling of the blood gelatin suspension, an adhesive tape, about 1 cm wide, was placed around the edge of the thin-layer plate to form a kind of trough.

### Defibrinated Cow's Blood

Approximately 500 ml of blood from a freshly slaughtered cow was collected in a 1 liter wide-necked flask, and stirred thoroughly with a wooden stick until the fibrin agglutinated. This was filtered through several sheets of cheesecloth to separate the gelatinous residue. The filtrate contained the defibrinated blood.

### Thin-layer Chromatography

All the silica gel G, which was used for thin-layer chromatography was made by Merck and distributed by Brinkman Instruments, Inc., Westbury, New York. The plates were 20 x 20 cm and the thickness of the silica gel G layer was 0.3 mm. An automatic plate maker was employed for the preparation of the plates, which were activated by placing them in an oven at 110°C for at least one hour prior to their use. The developed plates were revealed by spraying them with ceric sulfate solution then placing them in an oven at 110°C until the spots appeared. The ceric sulfate solution was prepared by mixing 20 g of ceric sulfate, 56 ml of sulfuric acid, and 1 liter of water.

### Infrared Spectroscopy

All infrared spectra were obtained using a Model 137B Infracord Spectrophotometer manufactured by

Perkin-Elmer Corporation, Norwalk, Connecticut. All infrared samples were prepared using potassium bromide pellets.

#### Melting Point Determinations

A Kofler block was used to determine melting points. All melting points were uncorrected.

#### Purification of the n-butanol Extract

Three hundred ninety grams of the extract was dissolved in 150 ml of 95% ethanol. The solution was slowly added to 2 liters of acetone in a beaker with constant stirring and a greenish yellow precipitate was formed. The mixture was left overnight to effect complete precipitation. The mixture was then filtered and the precipitate was washed twice with 200 ml portions of acetone and air dried for 24 hours. The yield was 206 g.

#### Preparation of Saponin Acetate Mixture

Pyridine was dried by refluxing it for two hours with barium oxide. The freshly distilled pyridine was used for the preparation of the saponin acetates.

Seventy-five grams of the saponin mixture was dissolved in 150 ml of anhydrous pyridine, then 150 ml of acetic anhydride was added and the mixture was refluxed for two hours. The reaction mixture was cooled and added

slowly to 750 ml of ice water. An oil was formed at first, but upon stirring the mixture continuously for approximately one hour, a yellow precipitate was obtained. This precipitate was filtered and washed once with 200 ml of water and three times with 150 ml portions of 2% hydrochloric acid solution until the pyridine was completely removed. The precipitate was then washed with water until the washings were almost neutral. The precipitate was transferred to a flask and mixed with 100 ml of 10% sodium bicarbonate solution with occasional shaking for one hour to hydrolyze any unreacted acetic anhydride. The mixture was filtered and the precipitate was washed three times with 150 ml portions of water until the washings were neutral. The yield of air dried precipitate was 80 g.

#### Thin-layer Chromatography of the Saponin Acetate Mixture

The thin-layer plates were spotted with a 5% saponin acetate solution in chloroform. All thin-layer plates spotted with the saponin acetate mixture were developed twice using solvent system 2 in the following manner: The plate was developed by placing it in the chromatographic chamber and allowing solvent system 2 to ascend up to approximately 2 cm from the top of the plate. It was then removed from the chamber and left in the air to dry. This process was repeated.

Separation of the Saponin Acetate Mixture  
Using Silica Gel G Dry Columns

The silica gel G utilized for the dry column was the same as the material used in the preparation of thin-layer plates. Bianchi and Cole (2) reported on the use of this type of column. In place of glass columns they used cellophane dialysis tubing No. 4465-A2 manufactured by Arthur H. Thomas Company, Philadelphia, Pennsylvania.

Three columns were utilized. Each one was prepared in the following manner: One end of the cellophane dialysis tubing, 1 m long and 9 cm in diameter, was attached to a constricted glass joint and tied with a string held by a clamp. A nylon reinforced tape was also used to further bind the tubing to the glass joint. The other end was moistened around the lip to a depth of approximately 3 cm. A Buchner funnel, with a piece of filter paper inserted in the usual manner for filtration, was attached to the bottom of the dialysis tubing in the same manner the tubing was attached to the glass joint at the top of the column. In order to prevent the column from collapsing when packed, the funnel was supported by a ring. A rubber stopper connected to a source of compressed air was attached to the glass joint at the top of the columns and air was gently forced into the column in order to open the flat tubing into a cylindrical shape. Approximately 1.9 kg of

silica gel G, previously activated overnight in the oven at 110°C was added to the column in three equal portions. The column was patted and vibrated manually with the hand in order to obtain a homogeneous packing. Compressed air was applied occasionally to aid in packing.

Fifteen grams of the saponin acetate mixture was dissolved in 100 ml of chloroform and adsorbed on 40 g of silica gel G. The mixture was occasionally stirred until it became completely dry. The dried material was sprinkled carefully on the top of the packed column so as not to upset the upper surface of the silica gel G.

A pledget of cotton was placed onto the top of the column so that the chromatographic liquid, solvent system 2, dripping into the column would not disturb the silica gel-acetate mixture. A one liter separatory funnel was filled with solvent system 2 and allowed to drop slowly onto the cotton so as not to allow a solvent head to build up. The dripping was continued until approximately 3 liters of the chromatographic liquid had entered the column. At that time the liquid began to drop from the funnel at the bottom end of the column and was collected.

Since the distribution of the saponin acetate mixture in the column is the same as on thin-layer plates, the collected liquid was periodically chromatographed using the original saponin acetate mixture as a reference.

The chromatographic liquid was allowed to drop until the eluate from the column when chromatographed on a thin-layer plate showed all of the less polar material which precedes saponin acetate 1. At this point approximately 9 liters of the chromatographic liquid had been added to the column. The three columns were then allowed to stand for 48 hours.

#### Exploration of the Dry Columns

Each dry column was explored in the following manner: Fifteen exploration samples at a distance of one inch apart, starting at the bottom of the column were taken. A small window was cut in the column with the top and the sides opened and the bottom uncut. The flap was pulled down and a small sample of the wet silica gel was removed from the opening with a spatula and placed in a 50 ml Erlenmeyer flask. Ten millileters of acetone was added to the flask. The flap was quickly pulled up and covered with cellophane tape in order to prevent further evaporation of the chromatographic liquid. The 15 flasks containing the exploratory samples in acetone were shaken occasionally, the contents of each flask was filtered after four hours, and the acetone was evaporated to dryness.

Thin-layer Chromatography of the  
Exploratory Samples

The exploratory samples from each column were chromatographed in the following manner: The residue of each flask was dissolved in a few drops of chloroform and chromatographed, along with the saponin acetate mixture which was used as a reference, on a thin-layer plate using solvent system 2. Visualization of the chromatogram revealed that samples 1-6 and samples 13-15 were void of any material, samples 7 and 8 contained saponin acetate 1, samples 9 and 10 contained saponin acetate 1 and 2, and samples 11 and 12 contained saponin acetate 2.

Cutting of the Dry Columns

Each column was sealed at the upper end and placed on its side. On the basis of the information from the thin-layer chromatography of the exploratory samples, each column was cut, using a sharp knife, in such a manner as to combine sections 7 and 8, 9 and 10, and 11 and 12. Each combined section was placed in a 1 liter Erlenmeyer flask containing 600 ml of acetone. The acetone-silica gel mixture was stirred with a magnetic stirrer overnight. The following day the material of each section was filtered and the acetone solution was evaporated under reduced pressure. The residues of the same sections of the three columns were combined.

### Alumina III Columns

An alumina III column was used to purify the crude sapogenins obtained from the acid hydrolysis of their respective saponins. Alumina III was prepared from alumina (obtained from Fisher Scientific Company, Fair Lawn, New Jersey) by adding 6% water, by weight, to the alumina. The mixture was then shaken overnight on a shaker. Water was added to partially de-activate the alumina which otherwise would have trapped almost any material adsorbed on it.

A glass column 2 cm in diameter and 50 cm high was employed. A pledget of cotton was introduced into the column and held in its bottom with a glass rod. The column was filled in half its height with chloroform, the stopcock was opened, and the chloroform was allowed to drop from the column while alumina III was added to the column to a height of 6 cm. The glass rod was then slowly removed from the column and the chloroform was allowed to continue dropping until its level was approximately 1 cm above the alumina III. The stopcock was closed and the crude sapogenin dissolved in 5 ml of chloroform was added to the column. The stopcock was opened again to allow the solvent to pass through the column until there was no chloroform visible above the top of the alumina. Chloroform was then added

cautiously so as not to disturb the column, until the column was filled. The column was then eluted with chloroform.

#### Paper Chromatography

The apparatus which was employed for paper chromatography was a glass jar, 30 x 30 cm square and 60 cm high with crystal glass lid, manufactured by Scientific Glass Apparatus Company, Inc., Bloomfield, New Jersey. The jar contained a stainless steel stand-up support rack to hold four trough assemblies. Each assembly consisted of one glass solvent trough, one stainless steel trough holder, two glass antisiphon rods and one glass anchor rod.

#### Preparation of TMS Ethers of the Saponin Sugar Moieties

The TMS ether derivative of each sugar moiety was prepared in the following manner: A 10 mg sample of the sugar moiety was placed in a 10 ml Erlenmeyer flask. One milliliter of Tri-Sil 'Z' reagent was added to the flask which was then stoppered and shaken well. The flask was warmed in a water bath at 60-70°C for five minutes until all of the sample dissolved. The flask was set aside at room temperature for five minutes and the product was then submitted to glc. The TMS ether derivatives of D-glucose, D-xylose, and L-rhamnose were prepared in the same manner as described above.

### Gas Chromatographic Analysis

The gas chromatographic analyses were carried out using a model 880 gas chromatograph manufactured by Perkin-Elmer Corporation, Norwalk, Connecticut. The detector was of the flame ionization type. Two coiled pyrex glass columns were employed as the sensing and the reference columns. They were packed with a 2.5% Silicone Rubber Gum SE-30 adsorbed on chromosorb G (80-100 mesh), acid-washed and dimethyldichlorosilane treated. The packing material was obtained from Perkin-Elmer Corporation. Nitrogen was employed as the carrier gas. The nitrogen flow for both columns was held constant throughout all determinations at the rate of 75 ml per minute. The gas chromatograph was programmed for a linear temperature increase of 10°C per minute with an initial temperature of 50°C.

### Carbon-hydrogen Analysis

Fifteen milligram samples of saponins 1 and 2 were dried under reduced pressure at 50°C for 48 hours and sent to Huffman Laboratories, Inc., Wheatridge, Colorado, for carbon-hydrogen analysis.

Preparation of Aniline Phthalate

Freshly distilled aniline (0.93 g) was added to 1.67 g of phthalic acid dissolved in 100 ml of n-butanol saturated with water.

## CHAPTER 5

### SUMMARY AND CONCLUSIONS

Two saponins were isolated from the hot water extract of the fresh leaves of Agave pacifica. Separation of the saponins was accomplished by a modified silica gel G dry column technique. Saponin 1 was shown to possess hecogenin as its aglycone and, as its sugar moiety, seven carbohydrate units consisting of L-rhamnose, D-xylose, and D-glucose in the ratio 2:1:4, respectively. Saponin 2 was shown to possess tigogenin as its aglycone and as its sugar moiety, seven carbohydrate units consisting of L-rhamnose, D-xylose, and D-glucose in the ratio 3:1:3, respectively.

In order to determine whether or not these saponins have tumor inhibitory properties, it will be necessary to isolate them in substantially larger quantities. It is very hopeful that these compounds will possess anti-tumor activity since the original methanol extract, which contained a substantial amount of the saponins, showed anti-tumor activity. It is also encouraging to note that related saponins isolated from other Agave species have shown tumor inhibitory properties.

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