Tumor Inhibitors. XXXV.[1] Myrsine Saponin, the Active Principle of *Myrsine africana* L.[2]

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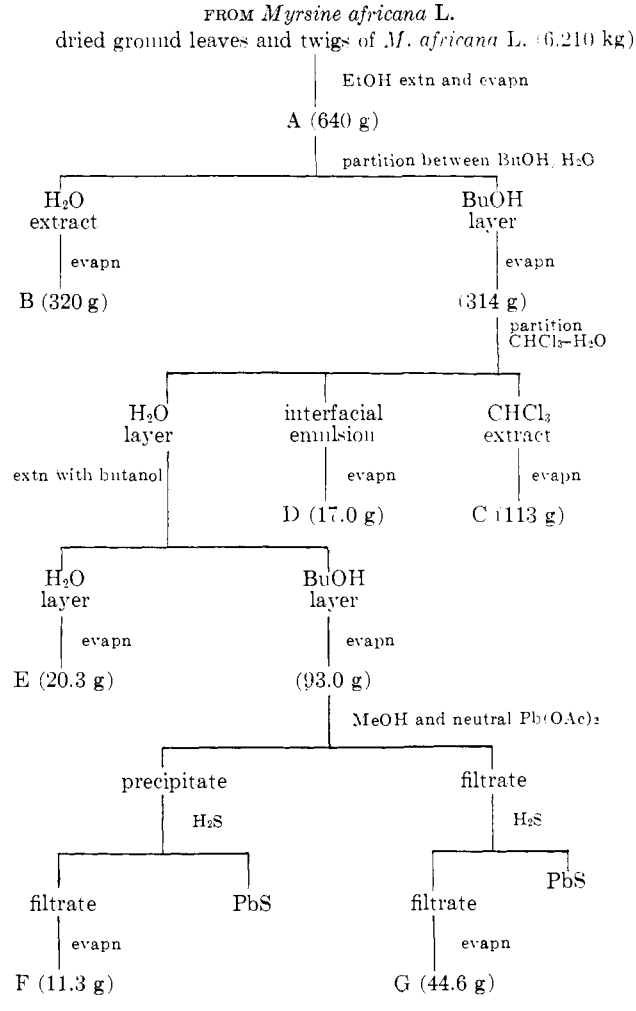
An alcoholic extract of twigs and leaves of *Myrsine africana* L. was found to show significant inhibitory activity against the Walker intramuscular carcinosarcoma 256 in rats. Systematic fractionation of this extract led to the isolation of a new saponin as the active principle. The characterization of the active Myrsine saponin as a glycoside of primulagenin A and the sugars glucose, rhamnose, galactose, and glucuronic acid, in the molar ratio 1:2:1:1, is described.

In the course of a continuing search for tumor inhibitors of plant origin, an alcoholic extract of the twigs and leaves of *Myrsine africana* L.[3] was found to show significant activity against the Walker intramuscular carcinosarcoma 256 in rats.[4] We report herein the systematic fractionation of the active extract of *Myrsine africana* L. and the isolation and partial characterization of the active principle, which we designate as Myrsine saponin. The observations confirm and extend earlier reports of the tumor-inhibitory activity of saponins.[5,6]

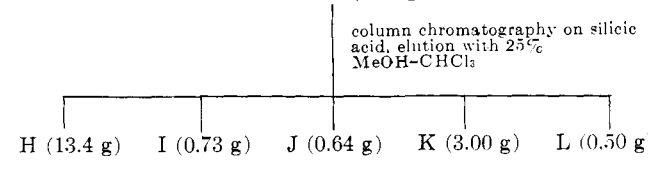
The dried ground plant material was extracted continuously with ethanol to yield an extract (A) which was fractionated according to Scheme I. The bioassay data for the active fractions in a typical experiment are given in Table I. Further fractionation of active fraction G involved adsorption column chromatography on silicic acid, employing 25% methanol in chloroform as eluent (Scheme II). Fraction K in MeOH was purified by adding acetone, which effected the precipitation of the chromatographically homogenous active compound. The precipitate was crystallized from EtOH and clusters of colorless microneedles (M) were obtained. Although the active constituent showed significant and reproducible *in vivo* tumor inhibitory activity, the relatively low ratio between active and toxic doses renders Myrsine saponin less promising as a candidate for clinical trial than saponin P from *Acer negundo*.[6]

An aqueous suspension of the highly polar compound M formed a persistent foam on shaking and showed appreciable hemolytic activity.[6] These properties indicated that the active principle was a saponin, and it was therefore called Myrsine saponin. The characterization was supported by the ir spectrum of the compound, which showed absorption typical of saponin-type compounds. A band in the carbonyl absorption region at 5.78 μ was assigned to CO_2H; this conclusion was verified by treatment of Myrsine saponin with CH_2N_2 to form a less polar methyl ester

SCHEME I
THE FRACTIONATION OF THE TUMOR-INHIBITORY EXTRACT FROM *Myrsine africana* L.



SCHEME II
THE CHROMATOGRAPHY OF THE ACTIVE FRACTION (G) OF *Myrsine africana* L.



(1) Part XXXIV: S. M. Kupchan, A. Karim, and C. Marcks, *J. Am. Chem. Soc.*, **90**, 5923 (1968).

(2) This investigation was supported by grants from the National Cancer Institute (CA-04500) and the American Cancer Society, (T-275) and a contract (PH 43-64-551) with the Cancer Chemotherapy National Service Center (CCNSC), National Cancer Institute, National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

(3) Leaves and stems were collected in Ethiopia in June 1965. We acknowledge the receipt of the dried plant material from Dr. Robert E. Perdue, Jr., U. S. Department of Agriculture, Beltsville, Md., in accordance with the program developed with the U. S. Department of Agriculture by the CCNSC.

(4) The evaluation of assay results by the CCNSC on a statistical basis in sequential testing is such that a material is considered active if it causes reduction of tumor weight to 42% or less. The procedures were those described in *Cancer Chemotherapy Rept.*, **25**, 1 (1962).

(5) R. F. Nigrelli, *Zoologica, Part 2*, **37**, 89 (1952).

(6) S. M. Kupchan, R. J. Hemingway, J. R. Knox, S. J. Barboutis, D. Werner, and M. A. Barboutis, *J. Pharm. Sci.*, **56**, 603 (1967).

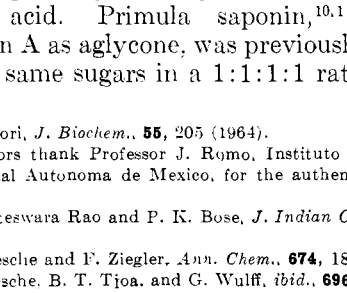
TABLE I

ACTIVITY OF FRACTIONS FROM *M. africana* L. AGAINST WALKER CARCINOSARCOMA 256

Fraction	Dose, mg/kg	Survivors	Animal wt chg dif, g (T − C)	Tumor wt, mg (T/C)	T/C × 100
A	400	0/6			Toxic
	200	1/6	−26	600/4100	Toxic
	100	6/6	−15	2300/4100	56
B–F					Inactive
G	60	0/4			Toxic
	30	4/4	−17	2400/6900	34
	15	4/4	−9	5500/6900	79
H–J					Inactive
K	20	2/4	−17	2200/5300	Toxic
	10	0/4			Toxic
	5	4/4	−19	2300/5300	43
L					Inactive
M	16	0/4	...	...	Toxic
	8	4/4	−18	2500/9000	27
	6	4/4	−14	3100/9000	34
	4	4/4	−7	4700/7500	62

derivative (C=O band at 5.72 μ) and by neutralization with aqueous cesium hydroxide to form a crystalline salt, with CO_2^- absorption at 6.12 μ. The uv spectrum of Myrsine saponin showed no high-wavelength absorption. Analysis of crystalline Myrsine saponin gave results in accordance with the molecular formula $C_{60}H_{98}O_{27}\cdot 4H_2O$. A crystalline permethyl derivative ($C_{76}H_{130}O_{27}$) of myrsine saponin was obtained by methylation by Hakomori's method.[7] This result indicated the probable presence of 16 free OH groups in Myrsine saponin.

Myrsine saponin was subjected to aqueous acid hydrolysis, which yielded $CHCl_3$-soluble and H_2O-soluble fractions. The main component isolated from the organic phase was characterized as primulagenin A (I) by direct comparison with an authentic sample.[8]



I

Acetylation of the aglycone yielded diacetate and triacetate derivatives with physical constants in agreement with those reported for the respective derivatives of primulagenin A. It is noteworthy that primulagenin A has been isolated from the saponins of *Aegiceras majus* Gaertn., which also belongs to the family *Myrsinaceae*.[9] The water-soluble hydrolysate was investigated by paper chromatography and shown to contain the following sugars: glucose, galactose, rhamnose, and glucuronic acid. Primula saponin,[10,11] containing primulagenin A as aglycone, was previously reported to contain the same sugars in a 1:1:1:1 ratio. Myrsine saponin and Primula saponin[12] were therefore compared directly. The comparison indicated differences in melting point, as well as in optical rotation. The two saponins had the same mobility in several solvent systems on silica gel tlc plates. However, in BuOH–AcOH–H_2O (4:1:5), upper phase, Myrsine saponin and Primula saponin appeared as two distinctly different spots at R_f 0.23 and 0.26, respectively. These observations supported the view that Myrsine saponin might contain the same sugars as Primula saponin, but in a different molar ratio.

Myrsine saponin and Primula saponin were subjected separately to aqueous acid hydrolysis under identical conditions. The water-soluble fractions were converted into trimethylsilyl derivatives[13] and examined by glpc. Complex chromatograms were obtained, due to the formation of equilibrium mixtures from each monosaccharide during the acid hydrolysis. This resulted in the formation of two to four peaks per parent sugar on the chromatogram. Equilibrium mixtures of each of the four monosaccharide moieties (*viz.*, glucose, rhamnose, galactose, and glucuronic acid) were prepared by treatment of the individual sugars under the same hydrolytic conditions. The trimethylsilyl derivatives were subsequently prepared and investigated by glpc. This led to the assignment of appropriate peaks in the chromatogram for the respective sugars. This study indicated that, in the case of Myrsine saponin, the sugars occurred in a molar ratio of glucose:rhamnose:galactose:glucuronic acid as 1:2:1:1, compared to the 1:1:1:1 ratio in Primula saponin. The latter conclusion was confirmed by the similarity in glpc patterns of a standard solution of the trimethylsilyl derivatives of an equilibrium mixture of the sugars to those of the derivatives of the sugars obtained by hydrolysis of the Myrsine saponin.

Experimental Section[14]

Extraction and Preliminary Fractionation.—The ground twigs and leaves of *M. africana* L. (6.20 kg) were extracted continuously with EtOH and the extract was concentrated under reduced pressure to yield a dark gum (A, 640 g). The gum A was partitioned between H_2O and BuOH (600 ml of each). The resulting H_2O layer was washed (BuOH, 1.5 l.) and the BuOH layer was washed with H_2O (600 ml); the emulsions which formed at the interfaces were separated by centrifugation. The aqueous fraction was evaporated under reduced pressure to a gum (B,

(7) S. Hakomori, *J. Biochem.*, **55**, 205 (1964).

(8) The authors thank Professor J. Romo, Instituto de Quimica, Universidad Nacional Autonoma de Mexico, for the authentic sample of primulagenin A.

(9) K. Venkateswara Rao and P. K. Bose, *J. Indian Chem. Soc.*, **36**, 358 (1959).

(10) R. Tschesche and F. Ziegler, *Ann. Chem.*, **674**, 185 (1964).

(11) R. Tschesche, B. T. Tjoa, and G. Wulff, *ibid.*, **696**, 160 (1966).

(12) The authors thank Professor R. Tschesche, Organisch-Chemisches Institut der Universität, Bonn, West Germany, for an authentic sample of Primula saponin.

(13) C. C. Sweeley, R. Bentley, M. Makita, and W. W. Wells, *J. Am. Chem. Soc.*, **85**, 2497 (1963).

(14) Melting points were determined on a Hoover Uni-Melt melting point apparatus and are corrected. Ir spectra were determined on a Beckman IR-5A infrared spectrophotometer. Tlc was carried out on silica gel G (E. Merck) plates, and the chromatograms were sprayed with a $Ce(SO_4)_2$–H_2SO_4 solution followed by heating until brown spots appeared. Paper chromatographic separations of the sugar components were carried out on Whatman No. 1 filter paper by the descending method in glass chambers lined with filter paper. The sugars were visualized by dipping the papers in HIO_4 solution followed, after 4 min, by benzidine solution, to give white spots on a blue background.[15] Glpc was carried out on an F & M Laboratory Chromatograph Model 700, with H_2 flame ionization detector, employing a coiled stainless steel column, 2 m × 6 mm o.d. packed with 10% SE-30 silicone rubber on Chromosorb (60–80 mesh). The instrument was programmed for a linear temperature increase of 3°/min, initial temperature 160°, and holding isothermal conditions when 240° was reached. Inlet pressure of carrier gas (N_2) was 2.76 × 10^5 dynes/cm², with a flow rate of 60 ml/min. These conditions were employed throughout the glpc analysis of all the sugar derivatives.

(15) H. T. Gordon, W. Thornburg, and L. N. Werum, *Anal. Chem.*, **28**, 849 (1956).

320 g). The active BuOH fraction was evaporated under reduced pressure to a gum (314 g), which was partitioned between $CHCl_3$ and H_2O (1.5 l. of each). The resultant emulsion was separated by centrifugation and the $CHCl_3$ and emulsion layers were concentrated under reduced pressure to yield fractions C (113 g) and D (17.0 g), respectively. The active material was extracted from the H_2O layer with BuOH (1.5 l.). The aqueous layer was subsequently evaporated under reduced pressure to yield E (20.3 g). The BuOH layers were washed (H_2O, 600 ml) and evaporated to dryness under reduced pressure (93.0 g). The latter material was taken up in MeOH and treated with a solution of neutral $Pb(OAc)_2$. The precipitate was removed by centrifugation and washed twice with MeOH. The combined supernatant MeOH solution was freed from excess lead by treatment with H_2S. The PbS was removed by filtration, and the filtrate was evaporated under reduced pressure to yield G (44.6 g). The originally formed precipitate was suspended in MeOH and H_2S bubbled through the suspension. The PbS was filtered off after complete precipitation and the filtrate was evaporated to yield F (11.3 g).

Isolation of Myrsine Saponin.—Fraction G was further fractionated by adsorption chromatography on a silicic acid column (1.0 kg). Fraction G (21.3 g) in 25% MeOH in $CHCl_3$ (45 ml) was applied to the column. The column was eluted with 25% MeOH in $CHCl_3$. All fractions were investigated by means of tlc on silica gel plates in BuOH–HOAc–H_2O (4:1:5), upper phase. The active material was combined as indicated by bioassay to yield fraction K (3.00 g). Fraction K in MeOH was treated with Me_2CO to yield a precipitate (1.63 g). The precipitated material was crystallized from EtOH to yield M (700 mg), as clusters of microneedles, mp 259–260°. Treatment of the MeOH mother liquor with more Me_2CO led to the precipitation of additional homogeneous saponin (220 mg) identical with M. M showed $[\alpha]^{30}D$ −35° (*c* 0.94, MeOH); λ_{max}^{KBr} 2.93, 3.40, 5.78, 6.16, 7.20, 7.35, 9.25, and 9.50 μ. *Anal.* ($C_{60}H_{98}O_{27}\cdot 4H_2O$) C, H; neut equiv: calcd for $C_{60}H_{98}O_{27}$, 1250; found, 1196.

Permethylation of Myrsine Saponin.—A mixture of NaH (110 mg, 50% oil dispersion) and dry DMSO (3.0 ml) was stirred under dry N_2 gas for 20 min at room temperature. Myrsine saponin (100 mg) in DMSO (3.0 ml) was added to the reaction mixture and stirring was continued for a further 20 min. Excess MeI (1.0 ml) was added and the mixture was stirred for a further 25 min. Excess HOAc was added and the mixture was partitioned between $CHCl_3$ and H_2O. The organic phase was concentrated and the residue was partitioned between 95% MeOH and petroleum ether (bp 60–68°). The aqueous MeOH layer was concentrated under reduced pressure and the residue (125 mg) was added to a column of silica gel (15 g). Elution with $CHCl_3$–MeOH–Me_2CO (100:3:2) gave the permethyl derivative of Myrsine saponin (65 mg), mp 243–245° (from MeOH), λ_{max}^{KBr} 5.70 μ. *Anal.* ($C_{76}H_{130}O_{27}$) C, H.

Treatment of Myrsine Saponin with Diazomethane.—Myrsine saponin (10 mg) in MeOH (20 ml) was treated with excess ethereal CH_2N_2 for 15 min. A drop of HCO_2H was added and the solvent was removed under reduced pressure to yield Myrsine saponin methyl ester (10 mg), λ_{max}^{KBr} 5.72 μ.

Treatment of Myrsine Saponin with Aqueous Cesium Hydroxide.—Myrsine saponin (50 mg) was added to 50% aqueous MeOH containing a drop of phenolphthalein. Dilute aqueous CsOH was added carefully to the reaction mixture until a faint pink color persisted, and a small drop of diluted HOAc was then added. The solvent was removed under reduced pressure at room temperature and the residue was crystallized from a mixture of MeOH and EtOH to yield long thin colorless crystals, mp 274–276°, λ_{max}^{KBr} 6.12 μ.

Acid Hydrolysis of Myrsine Saponin.—A solution of Myrsine saponin (1.064 g) in MeOH (50 ml) was treated with 1 *N* HCl (100 ml) and heated under reflux for 4 hr. The MeOH was removed under reduced pressure and the aqueous suspension was extracted with $CHCl_3$. The aqueous phase was retained to study the sugar components. The organic phase was concentrated under reduced pressure and the residue (400 mg) was separated by chromatography on silica gel (25 g). The major component was eluted with $CHCl_3$. Crystallization of the latter from MeOH–$CHCl_3$ gave colorless crystals (176 mg): mp 242–244°; $[\alpha]^{26}D$ +28° (*c* 0.75, absolute EtOH); λ_{max}^{KBr} 2.93, 3.41, 6.16, 9.30, and 9.62 μ. *Anal.* ($C_{30}H_{50}O_3$) C, H. An authentic sample of primulagenin A (I)[8] showed mp 243–245°. The melting point of our material was not depressed by admixture with the authentic primulagenin A, and the ir spectra were superimposable. The two samples showed identical R_f on tlc (3% MeOH in $CHCl_3$).

Acetylation of Myrsine Sapogenin.—The sapogenin (50 mg) in pyridine (3 ml) was treated with Ac_2O (1.0 ml) and left at room temperature for 48 hr. The reaction mixture was worked up in the usual way and yielded a mixture (62 mg) of two compounds (tlc). The mixture was separated by chromatography on Woelm neutral Al_2O_3 (3 g, activity grade II) with PhH as eluent. The less polar compound was crystallized from MeOH (mp 154–156°). The reported melting point for primulagenin triacetate is 152–153°.[16] The more polar compound was crystallized from petroleum ether and characterized as the diacetate of primulagenin A (mp 190–193°), lit.[16] mp 187–188°.

Paper Chromatographic Identification of the Sugar Components.—The aqueous phase obtained from the hydrolysis of Myrsine saponin was compared with authentic sugar samples on Whatman No. 1 filter paper. The following solvent systems were employed: solvent A,[17] BuOH–HOAc–H_2O (4:1:5), upper phase, the chamber saturated with the lower phase; solvent B,[18] AcOEt–Pyr–H_2O (2:1:2), upper phase, the chamber saturated with both phases; solvent C,[19] AcOEt–HOAc–HCOOH–H_2O (18:3:1:4), chamber saturated with the solvent; solvent D,[20] Me_2CO–EtOH–*i*-PrOH–borate pH 10 buffer (3:1:1:2), chamber saturated with the solvent.

The following sugars were found present and identified in the solvent system mentioned: glucose and galactose (B), rhamnose (A–C), and glucuronic acid (D).

Glpc Study of the Sugar Components of Myrsine Saponin. Trimethylsilylation of the Sugars of Myrsine Saponin and Primula Saponin.—The saponin (20 mg) in MeOH (5 ml) was treated with 1 *N* HCl (10 ml) and heated under reflux for 4 hr. The MeOH was removed at room temperature under reduced pressure and the aqueous residue was extracted with $CHCl_3$ (four 20-ml portions). The aqueous residue was concentrated to complete dryness *in vacuo* at room temperature. The residue (*ca.* 12 mg) was taken up in anhydrous pyridine (1.0 ml) and treated with hexamethyldisilazane (0.2 ml) and trimethylchlorosilane (0.1 ml). The mixture was shaken vigorously for about 30 sec and then left for 5 min or longer at room temperature prior to chromatography. A suitable aliquot was used for injection into the gas chromatograph.

Preparation of Equilibrium Mixtures of the Individual Sugars Present in Myrsine Saponin.—The sugar, *viz.*, glucose, rhamnose, galactose, and glucuronic acid (10 mg), in MeOH–1 *N* HCl (1:2) (10 ml) was heated under reflux for 4 hr. The solvent was removed *in vacuo* and the dry residue was trimethylsilylated as described. An aliquot of each sugar was injected into the gas chromatograph.

Preparation of a Standard Solution of Equilibrated Trimethylsilylated Sugars Analogous to That Obtained by Hydrolysis of Myrsine Saponin.—A solution of rhamnose (10 mg), glucose (5 mg), galactose (5 mg), and glucuronic acid (5 mg) in MeOH–1 *N* HCl (1:2) (50 ml) was heated under reflux for 4 hr. The solvent was removed *in vacuo* and the dried residue was trimethylsilylated as described. An aliquot was injected into the gas chromatograph and produced a chromatogram identical in detail with that obtained from the aqueous hydrolysate of Myrsine saponin.

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(20) H. Mukerjee and J. Sri Ram, *ibid.*, **14**, 551 (1964).