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STEROIDAL CONSTITUENTS OF *YUCCA SHIDIGERA* PLANTS AND TISSUE CULTURES

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Key Word Index—*Yucca schidigera*; Agavaceae; steroidal sapogenins; tissue culture; ^{13}C NMR; GC analysis.

Abstract—The glycosides in shoots and rhizomes of *Yucca schidigera* plants contain not only the previously reported sarsasapogenin and markogenin but also smilagenin, samogenin, gitogenin and neogitogenin. Unorganized cell suspension cultures and root organ cultures of *Y. schidigera* contained insignificant concentrations of glycosides and sapogenins. Shoot organ cultures contained the same major sapogenins as the plant shoot but in different concentrations and had a glycoside pattern resembling that of a mature *Y. schidigera* rhizome. None of the five growth regulators studied increased the concentration of the sapogenins in shoot organ cultures.

INTRODUCTION

Extracts containing steroidal saponins of *Yucca schidigera* Roezl. Mohave yucca are used as antiarthritic agents and antistress agents in man [1], as antistress agents in poultry [2] and plants [3], to promote weight gain in cattle [4], and to retard ammonia formation in poultry waste [2]. It is also reported that steroidal glycosides and sapogenins elicit plant growth responses [5].

Steroidal saponins have been reported in a number of *Yucca* species, and the sapogenins found in shoot extracts are principally smilagenin [6], sarsasapogenin [7] and hecogenin [8]. Smilonin is the glycoside of smilagenin and sarsaponin is the glycoside of sarsasapogenin [9]. The glycosides of sarsasapogenin and markogenin are present in *Y. schidigera* leaves and fruiting pods [10, 11].

Numerous efforts have been made to produce sapogenins or their glycosides commercially from plant tissue culture systems. The types and ratios of the steroidal compounds produced from plant tissue culture systems often differ from those present in the plant [12]. Although the plant tissue culture yield of a sapogenin may be as high as 7.8% [13], the system is not yet commercially practical.

We report four sapogenins to be present in *Y. schidigera* plants which were not previously reported. We also report for the first time the sapogenin content of *Y. schidigera* cell, shoot and root cultures and observations regarding the presence of their glycosides.

RESULTS AND DISCUSSION

Plants

Three pairs of C-25 epimers were isolated and identified from the acid-hydrolysed glycoside fraction of *Y. schidigera* mature plant. The three epimeric pairs are sarsasapogenin and smilagenin [(25*S*)-5β-spirostan-3β-ol; (25*R*)-5β-spirostan-3β-ol], markogenin and samogenin [(25*S*)-5β-spirostan-2β,3β-diol; (25*R*)-5β-spirostan-2β,3β-diol] and gitogenin and neogitogenin [(25*R*)-5α-spirostan-2α,3β-diol; (25*S*)-5α-spirostan-2α,3β-diol]. The C-25 epimer has similar chemical and physical properties and is difficult to separate [14–16]. It may be confirmed by IR specific spirostan absorption at 915 and 900 cm^{-1} [6, 7, 10, 15]. However, the ratio of these IR absorption bands does not assure the purity of the isolated epimer.

Thin-layer chromatography (TLC) [14], IR spectroscopy [15], spectrophotometry [16] and gas–liquid chromatography (GC) [17] have previously been used for the quantitative analysis of individual or mixtures of C-25 epimers. We analysed the steroidal constituents of *Y. schidigera* by a rapid (10 min or less) and accurate gas–liquid chromatography (GC) method [coefficient of variance (CV) less than 5%]. Free sapogenins were not identified in the unhydrolysed steroidal fraction, but trace amounts (< 0.04%) of minor unidentified sapogenins were detected. The concentrations of the major sapogenins and epimers were determined in the hydrolysed steroidal fraction of a dried mature plant (shoot 345 g; rhizome 120 g; root 27 g) and a dried young plant (shoot 26 g; rhizome 13 g; root 2 g). The total content of the six sapogenins from the mature plant shoot was much less than that of the rhizome. However, the total sapogenin content of the young plant shoot was similar to that of the rhizome. Interestingly, the total sapogenins in the entire mature plant were approximately those of the young plant (Table 1). It appears that *Y. schidigera* shoots are the active site of sapogenin synthesis and the rhizomes for deposition, as has been reported for *Y. aloefolia* [18]. The sapogenin content reported for 3-year-old *Y. aloefolia* shoots is approximately 1.15% and is principally tigogenin [(25*R*)-5α-spirostan-3β-ol] and three other sapogenins [8, 18, 19]. The sapogenin content reported for *Y. glauca* plant shoots is approximately 1–2% and is principally sarsasapogenin and 12 other sapogenins [6, 7, 12, 19].

The chemical shifts of sapogenin ^{13}C NMR spectra are stereochemically dependent on the F-ring [20, 21], and the purity and ratio of the C-25 epimer can be established from peak heights such as those for C-22 and C-27. Mature shoots of *Y. schidigera* contained mixtures of sarsasapogenin and smilagenin (2:1) and markogenin and samogenin (3:2). The mature rhizome contained mixtures

Table 1. Sapogenin content of *Y. schidigera* plants*

Sapogenin	Concentration (%) Mature plant Shoot	Rhizome	Root	Young plant Shoot	Rhizome	Root
Sarsaspogenin and smilagenin	0.31	1.54	0.04	1.19	0.79	0.11
Markogenin and samogenin	0.13	0.72	0.02	0.23	0.49	0.07
Gitogenin and neogitogenin	0.02	0.33	0.03	0.04	0.10	0.04
Total	0.46	2.59	0.09	1.46	1.38	0.22

*Mature plants (65 cm tall) were collected in the field and young plants were started from seeds. Both mature and young plants (25 cm tall) were collected after 5 years' growth in a greenhouse environment. The coefficient of variance for triplicate samples was less than 5%.

of sarsasapogenin and smilagenin (2:1), markogenin and samogenin (4:1) and gitogenin and neogitogenin (3:2).

Unorganized tissue cultures

Unorganized *Y. schidigera* cells (4.0 g dry wt, 4 weeks old) established from aseptic seeds 4 years previously did not contain identifiable sapogenins when extracted and examined by our GC procedure. Common sterols such as stigmasterol and sitosterol were reported previously in *Y. schidigera* callus [22] and appeared to be among the compounds resolved by our TLC procedure. It is reported that unorganized callus of *Y. glauca* derived from aseptic germinating seeds contained gitogenin, trace amounts of manogenin [(25*R*)-5α-spirostan-2α,3β-diol-12-one] and a total sapogenin content of 0.45% [23]. The suspension culture contains decreasing amounts of stigmasterol, sitosterol, campesterol and trace amounts of cholesterol [24]. Unorganized callus of *Y. aloefolia* established from plant shoots contained 0.82% dry wt of sapogenins of which 75% was tigogenin and significant amounts were hecogenin [(25*R*)-5α-spirostan-3β-ol-12-one], gitogenin and chlorogenin [(25*R*)-5α-spirostan-3β,6α-diol]. Three-week-old cell cultures derived from *Y. filifera* seeds contained 0.007% sarsasapogenin and 0.004% cholesterol [25]. The shoots of most *Yucca* species contain 1–2% sapogenins and the seeds as much as 12% [6]. We conclude that unorganized cells of *Y. schidigera* produced significantly less and different sapogenins than the plant, as has been reported for *Y. glauca* and *Y. filifera* but not *Y. aloefolia*.

Organized cultures

Previous organized *Yucca* tissue culture studies considered the differentiation of unorganized *Y. gloriosa* callus cultures into plants [26], the influence of light on the morphogenesis of *Y. schidigera* root organ suspension cultures [27], and the *in vitro* propagation of *Yucca* [28, 29]. None of these studies considered the chemistry of the *Yucca* glycosides of sapogenins.

The tissue dry weights of shoot organ cultures analysed from experiments I–VI were 0.56, 2.85, 1.85, 2.26, 2.56 and 1.32 g, respectively, and these tissues contained approximately 85% moisture (Table 2). The dominant sapogenins in *Y. schidigera* plants are sarsasapogenin and smilagenin. Our shoot organ cultures contained approximately equal concentrations of sarsasapogenin and smilagenin and markogenin and samogenin and concentrations of gitogenin and neogitogenin that were higher than in the plant. The shoot culture grown in the absence of a growth regulator grew poorly (experiment I) and had a low total sapogenin content (0.69%).

It is difficult to be certain if growth regulators directly affect the production of saponin glycosides and sapogenins or their production indirectly through morphogenisis. The production of the sapogenin diosgenin is promoted more by nutrients and stress elicitors [30] and the growth regulator 2,4-dichlorophenoxyacetic acid (2,4-D) [31] than by either root [32] or leaf morphology [33]. Benzyladenine, when used in appropriate concentrations, promotes bud and leaf formation. Benzyladenine depressed *Agave sisalana* cell growth and sapogenin production [34] and diosgenin production in *Dioscorea deltoidea* cell cultures [31]. *Yucca schidigera* shoot organ cultures treated with growth regulators consistently contained the same six sapogenins identified in the plant (Tables 1 and 2). The total sapogenin content of *Y. schidigera* shoot organs treated with benzyladenine or 2,4-D was similar (> 1.04%) to that of young plant shoots (1.46%). However, untreated shoots, chlorocholine chloride or giberellic acid treated shoots contained a lower sapogenin concentration (< 0.85%) than young plant shoots (1.46%). The root promotors indoleacetic acid (IAA) and indolebutyric acid increased steroid production in *Agave* [34] and *Dioscorea* [31] cultures, respectively. Root organ cultures of *Y. schidigera* can be grown in the light and become green, or in the dark and remain cream coloured [27]. When IAA was present in the dark-grown culture medium, more elongated roots formed than with naphthalene acetic acid (NAA) (Table 3). The amount of tissue analysed from either IAA or NAA cultures was approximately 8.0 g dry wt and the medium pH varied from 4.4 to 6.5. Neither IAA root organ cultures of NAA root cultures grown in the light or dark contained measurable amounts of sapogenins (Fig. 1). These data support the concept that the primary site of *Y. schidigera* sapogenin synthesis is in the shoot.

Sterols and steroidal glycosides

The ether fraction of *Y. schidigera* plants and tissue cultures did not contain identifiable sapogenins when examined by TLC. However, the TLC plates of the fraction did contain yellow fatty acid zones and a purple-coloured free sterol zone ($R_f > 0.5$). The root and un-

Table 2. Growth regulator effects on the sapogenin content in *Y. schidigera* shoot organ cultures

	Concentration (%) Growth regulator treatments*					
Sapogenin	I	II	III	IV	V	VI
Sarsasapogenin and smilagenin	0.26	0.47	0.43	0.45	0.35	0.33
Markogenin and samogenin	0.27	0.44	0.48	0.42	0.35	0.34
Gitogenin and neogitogenin	0.16	0.16	0.15	0.17	0.16	0.18
Total	0.69	1.07	1.06	1.04	0.86	0.85

*Growth regulator treatments: I, no growth regulator; II, low benzyladenine (control); III, high benzyldenine; IV, 2,4-D; V, chlorocholine chloride; VI, gibberellic acid. I, II, 5 weeks; III–VI, 3 weeks. The coefficient of variance for triplicate analyses was less than 5%. Treatment details are given in the Experimental.

Table 3. Growth index of *Y. schidigera* root organ cultures

Growth regulators (ppm)	Age (weeks)	Growth index* (GI)
NAA	3	1.7 ± 0.3
(3 ppm)	6	2.3 ± 0.3
	9	2.1 ± 0.6
IAA	4	2.5 ± 0.1
(2 ppm)	4†	3.3 ± 0.2
	6	2.3 ± 0.6
	6†	2.9 ± 1.4

*GI: Initial inoculum for each flask was *ca* 50 g wet wt. All conditions were determined in either duplicate or triplicate.

†The last 2 weeks of tissue growth were without light.

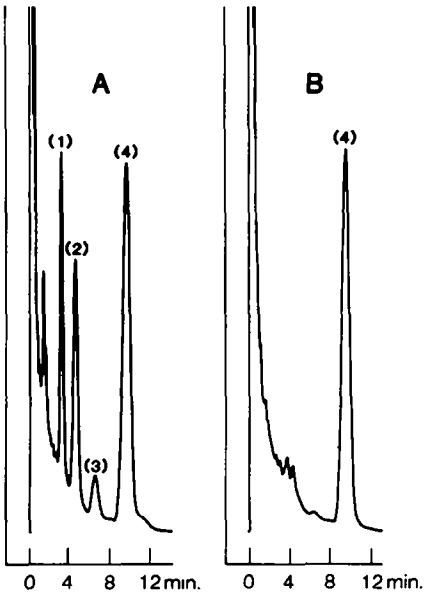

Fig. 1. Gas–liquid chromatogram of *Y. schidigera* tissue cultures. Conditions for analysis are given in the Experimental. A, Shoot culture; B, root organ culture. (1) Sarsasapogenin and smilagenin-TMSi; (2) markogenin and samogenin-TMSi; (3) gitogenin and neogitogenin-TMSi; (4) cholesteryl *n*-caproate (internal standard).

organized cell cultures also contained three or four significant purple-coloured sterol glycosides at a lower R_f (< 0.5).

The *n*-butanol extracts of *Y. schidigera* contained ten or more steroidal glycoside zones when examined by TLC. The mature plant shoot and rhizome extracts contained three or four major dark-brown steroidal glycoside zones at $R_f > 0.5$ or < 0.5, respectively. The TLC glycoside pattern of the shoot tissue fraction resembled that of the plant rhizome rather than the plant shoot. Young plant shoot and rhizome contained approximately ten glycoside zones of equal intensity. Root and unorganized cell cultures did not contain any major dark-brown steroidal glycoside zones. Although a glycoside precursor to dioscin has been identified in dividing cells of *Dioscorea* [35], it is suggested that the major site for steroidal glycoside synthesis resides in *Y. schidigera* shoot cultures.

EXPERIMENTAL

Plants. Mature *Y. schidigera* plants (approximately 65 cm tall) and seeds were collected in the vicinity of Essex, CA , in April 1979. Additional plants were started from the collected seeds in September 1979 and were approximately 25 cm tall when harvested. Both mature and seedling plants were grown in the greenhouse of the College of Pharmacy, University of Minnesota, until September 1984, when both were collected for chemical analysis.

Tissue cultures. All *Y. schidigera* tissue cultures studied were initiated from aseptically germinated seeds in November 1979 on Murashige and Skoog's Revised Tobacco medium (RT) [36] containing 1.0% agar and 2,4-dichlorophenoxyacetic acid (2,4-D; 1 ppm) for callus and benzyladenine (BA; 0.3 ppm) for shoot induction. Callus cultures were transferred to RT medium containing naphthalene acetic acid (NAA; 3 ppm) to initiate root cultures [27]. All cultures were transferred to fresh medium at approximately 3–4 week intervals.

Shoot organ cultures were studied for growth regulator effects on steroid production. The shoot cultures were grown on 300 ml RT medium containing 1.0% agar in 64-oz jars, 20 cm tall, at 25°C under a 16-hr day-light cycle (500 f.c. Fluorescent Plant-Grow Bulbs, 40 W, cool-light, Sears Roebuck). Each jar contained two *Yucca* shoots approximately 10 cm in height grown on RT medium without growth regulators (expt I—RTo), RT control medium with 0.3 ppm BA (expt II—RT BA 0.3). The filter-sterilized growth regulator supplement (2 ml) was applied to the base of 2-week-old shoots growing on control medium [expt III—2 ppm BA; IV—0.1 ppm 2,4-D; V—300 ppm chlorocholine chloride (CCC); and VI—200 ppm gibberellic acid (GA)]. All shoot cultures were collected for chemical analysis after 5 weeks of growth.

Root organ cultures were studied for the effects of light and growth regulators on steroid production. Root organ cultures were grown in 2 l. Erlenmeyer flasks containing 500 ml of liquid RT medium with either 2 ppm IAA or 3 ppm NAA on a gyratory shaker (80 rpm). Root cultures were grown at the same temp. and light conditions as the shoot cultures except for some root cultures (2 ppm IAA) grown in the dark. Root cultures were collected for chemical analysis at the weeks of growth stated in Table 3.

Extraction, separation and characterization of sapogenins from Yucca *plant.* Dry rhizome powder (100 g) of a mature plant (Table 1) was macerated and extracted with MeOH (3 × 1 l.) for 1 hr at 65°. The MeOH was evaporated and the extract was dissolved in 200 ml H_2O and washed with 200 ml Et_2O. The H_2O layer was treated with *n*-BuOH (2 × 200 ml). The *n*-BuOH extract was hydrolysed with 500 ml 2 M HCl–MeOH (1:1) for 3 hr at 90° and MeOH was removed *in vacuo*. The aq. soln was extracted with $CHCl_3$ (2 × 200 ml). This $CHCl_3$ extract was chromatographed on silica gel 60 (Merck) and eluted with C_6H_6 and C_6H_6–Me_2CO (10:1) and gave sarsasapogenin and smilagenin (940 mg), markogenin and samogenin (380 mg) and gitogenin and neogitogenin (30 mg) (TLC, IR, CIMS, ^{13}C NMR). Dry shoot powder (320 g) of a mature plant (Table 1) was treated in the same way as the rhizome and gave sarsasapogenin and smilagenin (500 mg) and markogenin and samogenin (200 mg) (TLC, IR, CIMS, ^{13}C NMR). ^{13}C NMR spectral data of sarsasapogenin and smilagenin and gitogenin and neogitogenin are given in refs [20, 21]; ^{13}C NMR of markogenin and samogenin ( ), $CDCl_3$–CD_3OD (10:1), 25°, TMS; 38.1 (C-1), 69.8 (C-2), 67.5 (C-3), 32.5 (C-4), 36.8 (C-5), 25.8 (C-6), 26.5 (C-7), 35.5 (C-8), 41.4 (C-9), 35.5 (C-10), 21.1 (C-11), 40.3 (C-12), 40.7 (C-13), 56.4 (C-14), 31.7 (C-15), 81.0 (C-16), 62.2 (62.4) (C-17), 16.5 (C-18), 23.8 (C-19), 42.2 (41.7) (C-20), 14.3 (14.4) (C-21), 109.8 (109.4) (C-22), 25.8 (31.4) (C-23), 26.0 (28.8) (C-24), 27.1 (30.3) (C-25), 65.2 (66.9) (C-26), 16.0 (17.1) (C-27).

Analysis of steroidal constituents of Yucca *plants and tissue cultures.* Mature and young *Yucca* plant materials (Table 1), shoot organ cultures (Table 2) and root organ cultures (Table 3) were analysed by TLC or GC.

Samples for TLC analysis were prepared as follows: a 40 mesh powder (0.2–0.5 g) was macerated and extracted with 50 ml MeOH for 15 min at 65°. The MeOH extract was dissolved in 20 ml H_2O and the aq. soln extracted with 50 ml Et_2O. The conc Et_2O extract was compared with reference sterol and sapogenin compounds using a Merck Kieselgel TLC plate and solvent systems I: C_6H_6, II: C_6H_6–Me_2CO (5:1); III: $CHCl_3$ and IV: $CHCl_3$–MeOH (20:1) and 10% H_2SO_4 and heat for detection. The residual aq. soln was then extracted with 20 ml *n*-BuOH and the conc *n*-BuOH fraction was compared using Merck Kieselgel plate and solvent systems V: $CHCl_3$–MeOH–H_2O (7:3:1, lower layer) and VI: $CHCl_3$–MeOH–H_2O (7:4:1) and 10% H_2SO_4 and heat for detection of steroidal glycosides.

Samples for GC detection of sapogenins were prepared as follows: (A) a 40 mesh powder (100–500 mg) was refluxed with MeOH (3 × 40 ml) for 15 min at 65°. The MeOH was evaporated and the residue was dissolved in 10 ml H_2O and extracted with Et_2O (2 × 40 ml). The Et_2O layer was washed with 10 ml H_2O, dried with Na_2SO_4 (1 g), filtered and 1.5 mg cholesteryl *n*-caproate was added. The Et_2O fraction was evaporated and treated with 0.5 ml TMS-HT for 20 min at 80° and centrifuged. This supernatant was used for the analysis of unhydrolysed sapogenins. (B) A 40 mesh powder (50–200 mg) was hydrolysed with 10 ml 2 M HCl–EtOH (1:1) for 3 hr at 90° and extracted with Et_2O (2 × 40 ml). The Et_2O layer was treated the same as in preparation (A). The supernatant was used for analysis of both the unhydrolysed and hydrolysed sapogenins (total sapogenins).

The GC conditions used were: FID detector, injection temp. 270°, column temp. 250°, N_2 at 40 ml/min, glass column 1 m × 3 mm (i.d.) packed with 1% OV-1 (Chromosorb Q). The standard calibration curve was prepared as follows: Peak height ratio (Y) of TMSi of sarsasapogenin and smilagenin (0.24, 0.48, 0.72, 0.96 mg), R_t = 3.5 min, that of markogenin and samogenin (0.24, 0.48, 0.72, 0.96, 1.20 mg), R_t = 4.9 min, and that of gitogenin and neogitogenin (0.04, 0.08, 0.12, 0.16 mg) (range × 8), R_t = 7.0 min, (reference cholesteryl *n*-caproate, 1.5 mg, R_t = 10.4 min), were compared with their weight ratio (X): sarsagenin and smilagenin, $Y = 2.455X + 0.016$ ($r = 1.000$, $n = 4$); markogenin and samogenin, $Y = 1.880X - 0.014$ ($r = 1.000$, $n = 5$); gitogenin and neogitogenin $Y = 7.850X - 0.013$ ($r = 0.999$, $n = 4$).

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