Table II. LD_{50}[a] of the Components in Cashew Shell Oil against the Snail *B. glabratus*

constituents	R	LD_{50}, ppm	constituents	R	LD_{50}, ppm
anacardic acid	$C_{15:3}$	0.3	2-methylcardol	$C_{15:3}$	20
	$C_{15:2}$	0.6		$C_{15:2}$	15
	$C_{15:1}$	1.0		$C_{15:1}$	10
cardol	$C_{15:3}$	15	cardanol	$C_{15:3}$	80
	$C_{15:2}$	7		$C_{15:2}$	80
	$C_{15:1}$	7		$C_{15:1}$	>100

[a] LD_{50} values are the lethal doses for 50% mortality.

decreased in inverse proportion to the degree of unsaturation in the side chain. The activity–side-chain relationship concerning the cardanol type compounds was less clear. The activity decreased in the order of cardol > 2 methylcardol ≫ cardanol. It seems that the degree of saturation in the alkyl side chain gives a relatively small change in activity, while the activity is dramatically changed by a change of hydrophilic groups on the ring. Moreover, an additional carboxy group on the ring results in a large increase in activity.

Molluscicides of plant origin are currently receiving considerable attention due to their relatively harmless biodegradative properties (Kools and McCullogh, 1981). Recently, many saponins have been reported as naturally occurring molluscicides (Domon and Hostettmann, 1984; Hostettmann et al., 1982; Sati et al., 1984). Unfortunately, many molluscicidal saponins are also toxic against fish. The toxicity of molluscicides against fish should be considered, since fish are a very important protein source in the countries where schistosomiasis is a problem. However, an application of these nonpolar compounds around the shoreline, the location favored by these snails, would more likely remain in this location than more polar compounds such as saponins. Hence, the physical property of solubility suggests an application of such nonpolar compounds will be less available to fish than an application of saponins.

These compounds were found in the nuts and in the fruit juice of the cashew, which are used for both food and drink. These compounds have also been reported in pistachio nuts *Pistachia vera* (Yalpini and Tyman, 1983) and in several varieties of cereals (Salek and Brudzyński, 1981). Therefore, it would appear that their potential for human oral toxicity either is not serious or has been overlooked.

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Scilliroside and Other Scilla Compounds in Red Squill

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Scilliroside and other bufadienolide glycosides in red squill, *Urginea maritima* (L.) Baker, were identified by isolation, high-performance liquid chromatography and thin-layer chromatography. Scilliroside, the major toxic glycoside, occurs in all plant parts including leaves, flower stalk, scales, and especially the roots and core of this bulbous plant. Other scilla compounds detected include desacetylscilliroside, scillaren A, and the aglycon scillirosidin. A new glucosylscilliroside and a phenolic nonscilla glycoside were also isolated and partially characterized. Scilliroside content of bulbs is highest in late summer after a dormancy period and does not appear to change with age. The scilliroside content of seed-derived varieties differs substantially, indicating a genetic factor affecting toxicant levels in the individual seedling plants. Toxicity of the bulbs is due principally to their content of scilliroside. The 6-acetoxy group of scilliroside contributes substantially to this toxicity.

INTRODUCTION

Red squill is a large onionlike plant whose bulb extracts and dried powders have been used in rodent control since the 13th century (Chitty, 1954; Marsh and Howard, 1975). The bulb and other plant parts contain scilliroside, a high-toxicity bufadienolide glycoside. Scilliroside affects the cardiovascular and central nervous systems, causing convulsions and death. Red squill preparations are emetic to humans (Belt, 1944), dogs and cats (Gold et al., 1950), and pigeons (Crabtree, 1947; Marsh and Verbiscar, 1986). However, rats and mice are unable to vomit, and they die within a few hours after ingesting lethal doses of scilliroside

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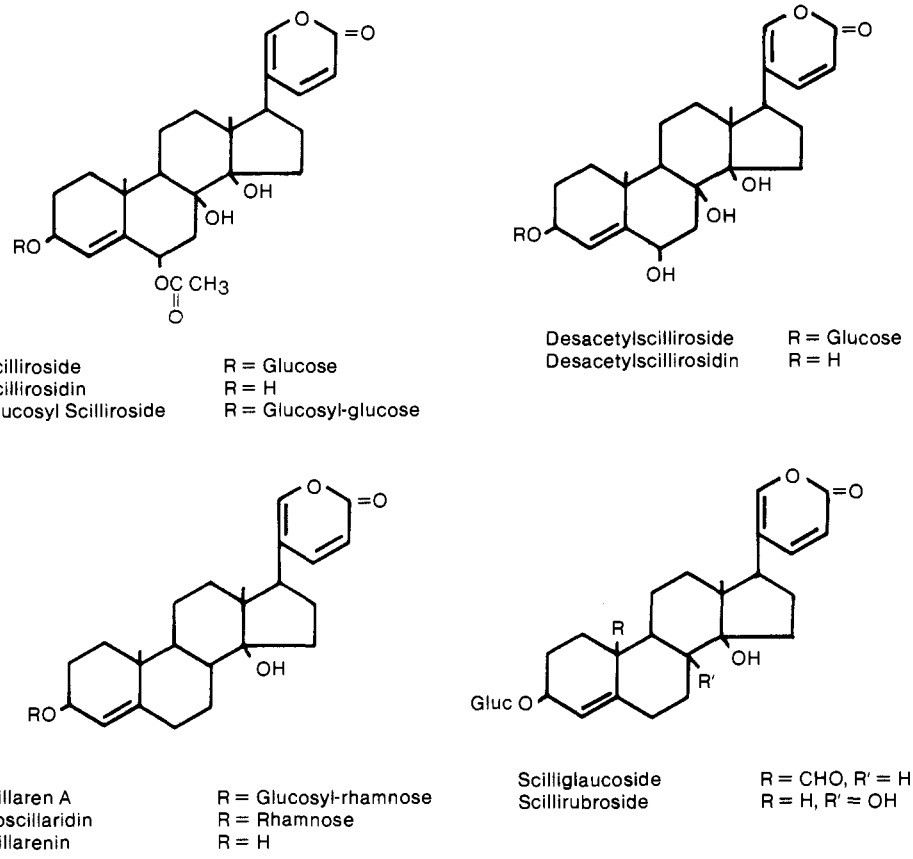

Figure 1. Scilla compounds in red squill.

or red squill formulated products (Rothlin and Schalch, 1952; Dybing et al., 1952). Scilliroside is the major bufadienolide in red squill (Stoll and Renz, 1942) but varieties contain lesser amounts of other scilla glycosides including scillaren A, scilliglaucoside, scillirubroside, and scillarenin β-D-glucoside (Stoll et al., 1943; von Wartburg and Renz, 1959; von Wartburg, 1966) (Figure 1).

According to an informal Department of Commerce report, 1.36×10^6 lbs of red squill powder were imported into the United States in 1944, but this declined substantially and has ceased in recent years. This was partly due to the introduction of warfarin and other anticoagulant type rodenticides. Also domestic use of red squill has been irregular due to the variable toxicity of imported lots of wild bulb powder, and only an expensive rat bioassay was available. It is now well-known that rodents develop a resistance to warfarin and other anticoagulant rodenticides over a period of years. The different nature of the toxicity of red squill makes it an attractive corodenticide for complete eradication. It is a potential new economic crop for the Southwest United States where climatic conditions are similar to the natural habitat of this species in lands around the Mediterranean Sea.

A domestic supply of selected high-toxicity clones of red squill has been developed over the last 30 years at the Gentry Experimental Farm in California. High variability in toxicity among individual red squill bulbs propagated from seed indicates a genetic basis for toxicant content. An initial objective was to develop an accurate assay of these different clones using high-performance liquid chromatography. Such an assay could then be used to guide selection, propagation and breeding studies, supplanting the rat bioassay. The assay of red and white squill bufadienolides has previously been the subject of several investigations. An attempt was made to relate rat bioassays with concentration of scilla glycosides in extracts as determined by absorption at 297 nm (Kaplan et al., 1950). A paper chromatographic method using photometric quantitation was reported for red and white squill glycosides (Wichtl and Fuchs, 1962). Thin-layer chromatographic systems have been reported by several groups (Gorlich, 1960, 1965; Elkiey et al., 1965, 1967; Kraus et al. 1969; Steinegger and Van der Walt, 1961). TLC and reversed-phase HPLC assays for red squill extracts, but not plant materials, have been reported (Binkert, 1979). Our TLC and HPLC methods in this study followed assay methods we developed for simmondsin and other jojoba glycosides (Verbiscar and Banigan, 1978; Verbiscar et al., 1980).

A number of toxicological studies report the effect of red squill powders and scilliroside in rats and other animals (Winton, 1927; Lubitz and Fellers, 1941; Stoll and Renz, 1942; Gold et al., 1947; Rothlin and Schalch, 1952; Dybing et al., 1952). These studies demonstrate that the nature of the toxicity of pure scilliroside is identical with the toxicity of red squill powders and extracts. The scilliroside content of red squill was expected to be the major factor for the identification of high-toxicity clones. The isolation and identification of other scilla glycosides and aglycons that occur in red squill and may contribute to its toxicity was another objective of this study.

EXPERIMENTAL SECTION

Materials. Plant material from annotated clonal lines of red squill was supplied by Howard Scott Gentry from the Gentry Experimental Farm, Murrieta, CA. These bulbs had been propagated vegetatively from wild bulbs selected for high toxicity from 1946 to about 1960 by the Agricultural Research Service, La Jolla, CA, and the U.S. Fish and Wildlife Service, Denver Wildlife Research Center, Denver, CO. Bulbs of clone #333 were propagated from seed from a single plant. The crop Protection De-

partment, Sandoz, Inc., San Diego, CA, supplied a sample of a technical red squill extract powder containing 28% scilliroside. High-purity samples of scilliroside, scillaren A, proscillaridin, and scilliglaucoside were provided through the courtesy of A. von Wartburg, Sandoz Ltd., Basle, Switzerland. Additional scilla glycosides and other products were isolated from bulbs of clone #871 described here. Naringinase was obtained from Sigma Chemical Co., St. Louis, MO.

Thin-Layer Chromatography. TLC was carried out on glass plates coated with 250 μm of Merck silica gel G, heated for 15 min at 110 °C. Developer solvents: (A) acetonitrile–water (9:1); (B) acetonitrile–2-propanol–water (94:4:2); (C) acetonitrile–water (97:3); (D) ethyl acetate–ethanol (7:5); (E) ethyl acetate–methanol–water–acetic acid (4:1:1:1). Several detection agents were used. A 10% sulfuric acid spray with gentle heating caused scilliroside and other 6-acetoxy or 6-hydroxy scilla glycosides to fluoresce green under long-wavelength UV light (UVL). Under the same conditions the other scilla glycosides fluoresce dull orange. As an example, using solvent B and UVL, the following compounds fluoresce green with R_f values noted: scillirosidin, 0.79; desacetylscillirosidin, 0.62; scilliroside, 0.33; desacetylscilliroside, 0.19. The following compounds fluoresce orange: proscillaridin, 0.55; scilliglaucoside, 0.25; scillaren A, 0.11. Carbohydrates and glycosides fluoresce blue or green-blue under UVL after heating a *p*-anisaldehyde in sulfuric acid sprayed plate. Antimony chloride spray reagent is most sensitive causing carbohydrates and glycosides to fluoresce blue under UVL. Reducing sugars and other aldehydes or ketones were detected with a 1% *p*-anisidine + phthalic acid spray reagent and heat. Phenolics were detected by spraying with alcoholic ferric chloride followed by heat to develop a yellow to brown spot.

High-Performance Liquid Chromatography. HPLC was carried out on an Altex Model 110 pump with a variable-wavelength Altex-Hitachi UV–vis detector set at 295 nm, the λ_{max} of scilliroside (Stoll and Renz, 1942). Two columns, 3.2 × 250 mm of Lichrosorb Si 60 (5 μm) were protected by a precolumn, 3.2 × 40 mm of Porasil A, 37–75 μm. In this system using solvent C for elution at a flow rate of 1.0 mL/min, the retention times of red squill glycosides are as follows: proscillaridin, 6.7 min; phenolic glucoside, 10.1 min; scilliroside, 12 min; scilliglaucoside, 12.6 min; desacetylscilliroside, 16.5 min; scillaren A, 17.6 min. With solvent F, acetonitrile–water (96:4), for elution, retention times of aglycons are as follows: scillirosidin, 5.0 min; desacetylscillirosidin, 6.7 min. Peak areas were quantitated with a Shimadzu C-EIB integrating data processor.

Scilliroside Assay. Plant materials were dried in a forced-air oven at 65–70 °C. Sixteen hours of drying was required for bulbs that are 80% water. The bulbs were first sliced into thin pieces, or a representative wedge section was taken vertically through the scales, core, and pad of the bulb. Roots, leaves, pad scales, flower stalk, core, and skins were all dried to a moisture level of 5–7% or less. The dry plant material was then ground to a powder through a hammer mill with a 0.05-in. slotted screen or a 0.04-in. round-hole screen. Powdered samples were stored in dark bottles to minimize scilliroside decomposition. For assay, 5 g of dried and ground plant part was weighed into a 50-mL plastic test tube. The powder was extracted with 20 mL of acetone–methanol (9:1) on a Tekmar homogenizer at high speed for 5 min. Ethanol or acetone–methanol (1:1) was also used successfully at various times. The extraction mixture was centrifuged, and the solvent was decanted. Reextraction was carried out three more times. The combined extracts were then treated with 5 mL of 5% aqueous lead acetate, stirred, kept for at least 1 h to precipitate the yellow phenolic lead salts, and then filtered, washing the precipitate with solvent. Lead acetate treatment effectively removes phenolics from red squill extracts but does not affect scilliroside content. The filtrate was concentrated to about 3 mL under vacuum on a rotary evaporator. The concentrate was transferred to a 10-mL volumetric flask, brought to volume with methanol, and then filtered again into a bottle for storage under refrigeration. The resulting solution was evaluated for scilla glycosides and aglycons by TLC and assayed for scilliroside by HPLC.

Extraction and Workup of Glycosides and Aglycons. Dry powder (650 g) from clone #871 bulbs was batch extracted twice with stirring with a total of 4 L of acetone–methanol (1:1). The solvent was removed under vacuum, leaving 52 g of plant extract. This material was dissolved in 100 mL of methanol, and 300 mL of acetone was added. A red precipitate that formed on refrigeration was discarded. The filtrate was further diluted with acetone and treated with 200 mL of 5% aqueous lead acetate to precipitate phenolics as yellow salts. The filtrate was further freed of lead salts by concentrating and adding acetone several times. Final concentration yielded a syrup that was triturated with hexane–acetone (97:3) to remove lipid solubles. The residue was then extracted with acetone, filtered, and dried to yield 1.67 g of crude product. This crude extract was dissolved in methanol and mixed with 10 g of Merck silica gel 60 (230–400 mesh). After drying, the mixture was placed on a column with the same packing. The column was eluted with chloroform followed by chloroform containing increasing amounts of methanol from 3 to 20% to pure methanol. TLC and HPLC were used to monitor the 24 × 100 mL fractions collected. Fractions 8–11 were enriched with aglycons, fraction 15 was principally scilliroside, and fractions 16–24 contained diglycosides and other more polar compounds. Fraction 15 was concentrated to dryness and crystallized from ethyl acetate and hexane to yield 414 mg (0.063%) of amorphorus, colorless scilliroside from 650 g of the red squill dry powder. The dried aglycon fraction weighed 375 mg and the more polar fraction 700 mg.

A. Aglycons. The aglycon-enriched fraction was rechromatographed on silica gel with chloroform–methanol (98:2) eluant. TLC was used to monitor the fractions with chloroform–methanol (95:5) for development and 10% sulfuric acid spray plus mild heat to detect UVL fluorescing compounds. One fraction contained two aglycons that fluoresce. Two corresponding peaks were observed in an HPLC scan eluting with acetonitrile–water (95:5:0.5). The green fluorescing spot with a retention time of 6.8 min is scillirosidin, identified by its infrared spectrum and by comparison with an authentic reference sample prepared here. A second green fluorescing spot at R_f 0.78 has a retention time of 5.4 min, is not scillarenin, but was not further identified.

B. Polar Glycosides. The more polar diglycoside fractions 16–24 from the original column were rechromatographed on a silica column with chloroform containing increased amounts of methanol as the eluant. Fourteen 100-mL fractions were analyzed by TLC using solvent B and HPLC using solvent F. One fraction was found to contain a phenolic glucoside (R_f 0.50; RT 10.5 min); scilliroside (R_f 0.43; RT 12.4 min); desacetylscilliroside (R_f 0.37; RT 18 min); and two diglycosides (R_f 0.22; RT 30 min/R_f 0.16; RT 36 min). Similar fractions were combined and

separated further by preparative TLC on 500-μm layers of silica gel G using solvent C developer. One diglycoside was identified as scillaren A by comparison with a reference standard. Characteristics of the phenolic glycoside and the second diglycoside are in the following sections.

C. Phenolic Glycoside. Preparative TLC of a chromatographed fraction of clone #871 yielded 25 mg of a compound that proved to be a phenolic glycoside. It ran faster than scilliroside on TLC and HPLC and is probably residual phenolic not removed by the lead acetate treatment. Phenolic character was demonstrated by a positive ferric chloride test in solution and on a TLC plate. It showed no fluorescence under UVL as do the scilla compounds. Hydrolysis in 1 N hydrochloric acid at 100 °C for 1 h yielded glucose and an aglycon. Both hydrolysis compounds, but not the starting phenolic glucoside, were detected with *p*-anisidine reagent on TLC, which is positive for aldehyde and ketone carbonyl groups. An infrared spectrum showed very strong OH, weak CH, no lactone or ester, strong 1635 cm^{-1}, aromatic at 1610 and 1510 cm^{-1}, C–O at 1070 cm^{-1}, and strong absorption at 830 cm^{-1}.

D. Glucosyl Scilliroside. Preparative TLC of the clone #871 glycoside fractions yielded 123 mg of a fraction rich in a product that was apparently a diglucoside. Recrystallization from absolute ethanol with cooling in a freezer provided a white hygroscopic precipitate. An infrared spectrum showed strong –OH and lactone. TLC using solvent A developer and sulfuric acid spray plus heat revealed this compound as a bright green fluorescent spot at R_f 0.36, compared to bright green fluorescent desacetylscilliroside at R_f 0.54 and dull orange scillaren A at R_f 0.52. The green fluorescence is indicative of a 6-hydroxyl or 6-acetoxy group in the aglycon moiety. Naringinase treatment for 24 h completely hydrolyzed off the first glucose, giving scilliroside, scillirosidin, and glucose as shown by TLC. The second glucose was removed more slowly, but after 15 days the hydrolysis to scillirosidin was nearly complete. The presence of scilliroside and scillirosidin in the enzymatic hydrolysate was confirmed by HPLC. A quantity of glucose was found in the final hydrolysate using TLC.

Scillirosidin. A solution of 400 mg of 82% pure scilliroside isolated from a technical sample containing 28% scilliroside was dissolved in 80 mL of distilled water and treated with 400 mg of naringinase at 38–39 °C in an incubator with magnetic stirring of the slurry. After 4 days, a TLC monitor indicated the reaction was complete and the mixture was filtered. The filtrate was extracted with 6 × 15 mL of chloroform; the extracts were dried over anhydrous sodium sulfate and then concentrated to dryness under vacuum. The residue was triturated with hexane to yield 103 mg of light yellow crystalline scillirosidin, homogeneous on TLC and HPLC.

Desacetylscilliroside. A solution of 115 mg of 82% pure scilliroside in 15 mL of methanol was treated with six drops of concentrated ammonium hydroxide. The solution was stirred at 30 °C for 124 h until an HPLC monitor indicated that deacetylation was essentially complete. The solution was concentrated to dryness under reduced pressure, and the residue was dissolved in warm acetone. The solution was washed through a silica gel 60 column with an acetone eluant. Fraction 1 containing most of the desacetylscilliroside was concentrated to a small volume and further purified on preparative TLC. The yield was 65 mg of hygroscopic product, approximately 90% pure by HPLC.

Desacetylscillirosidin. A solution of 150 mg of 82% pure scilliroside in 15 mL of methanol was treated with six drops of concentrated ammonium hydroxide. After 184 h at 30 °C on a magnetic stirrer the deacetylation was 98% complete. The solvent was removed under vacuum, and the residue was dissolved in 50 mL of distilled water. Naringinase (400 mg) was added, and the slurry was stirred at 30 °C with TLC to monitor the hydrolysis of the glucoside. After 136 h, the reaction was complete and the solution was at pH 4.5. The filtered solution was extracted with 6 × 10 mL of chloroform; the extract dried over anhydrous sodium sulfate and then evaporated to dryness. The residue was triturated with hexane to yield 49 mg of crystalline desacetylscillirosidin.

3-*O*-Acetylscillirosidin. Following the procedure of von Wartburg and Renz (1959), 80 mg of scillirosidin was dissolved in 1 mL of acetic anhydride and 4 mL of pyridine and the resultant mixture stirred for 24 h at room temperature. The reaction mixture was evaporated to near dryness under vacuum, and the residue was dissolved in methanol. As the solvent was allowed to evaporate, 60 mg of fine white crystals separated. This 3-*O*-acetylscillirosidin on TLC using ethyl acetate–2-methoxyethanol developer had R_f 0.90 compared to scillirosidin at R_f 0.71. This 3,6-diacetoxy compound was homogeneous and strongly fluorescent under UVL after the sulfuric acid spray with light heat.

Scillarenin. A solution of 5 mg of scillaren A in 1.0 mL of distilled water was treated with 5 mg of naringinase. The slurry was incubated at 37 °C on a gyratory shaker at 150 rpm for 14 days. On day 6, 0.5 mL of acetate buffer (pH 4.0) was added to increase the hydrolysis rate. Periodic TLC monitors of the reaction showed considerable quantities of the rhamnoside intermediate proscillaridin, which was eventually itself further hydrolyzed to scillarenin. A final TLC using solvent A showed a major spot for scillarenin at R_f 0.89, a minor spot for proscillaridin at R_f 0.71, and no scillaren A at R_f 0.41. A minor contaminant at R_f 0.82 could not be identified. The scillarenin was extracted into chloroform for use as a reference standard.

Rat Testing. These tests were carried out at the Northeastern University, Toxicology Program, utilizing male or female Charles River CD rats (150–250 g). The animals were allowed at least a 2-day acclimation period prior to dosing. Rats were housed by sex, earmarked, and placed three per cage. Subjects were fasted from food and water 10–14 h before dosing. The red squill powders were suspended in 0.25% agar. Suspensions of powders and compounds were prepared to yield five different concentrations in 20-mL volumes. During constitution the suspension was homogenized for 15 s with a Brinkman Polytron set at 50% maximum speed to ensure uniform preparations and optimal bioavailability. Gavage volumes of 10 mL/kg were administered p.o. for a 48-h LD_{50} observation period. Animals surviving after 2 days were monitored for another 12 days. Rats not dying within 2 days generally survived although deaths were occasionally noted as late as 10 days. In some cases LD_{100} values were not much higher than LD_{50} values. Statistical analysis was carried out by the method of Litchfield and Wilcoxon (1949) (Table III, ref *a* and *b*).

RESULTS AND DISCUSSION

Scilliroside is the major rodent toxicant in red squill. Scilliroside occurs in cell plant parts including leaves, flowering stalk, red skins, scales, core, pad, and root. Leaves, skins, and flowering stalks have low scilliroside levels when they are fresh and lower levels when dry. In

Table I. Scilliroside Content of Selected Red Squill Clones and Plant Parts

clone-bulb	plant part	plant age, yr	scilliroside, % in dry powders
297	wedge[a]	7	0.13
324	wedge[a]	6	0.03
638-A	wedge[a]	5	0.16
638-C	wedge, 7 bulbs	6	0.15
843-B	wedge, 5 bulbs	7	0.07
871-B	whole bulb	3	0.15
333-22[f]	wedge	22	0.35
333-28	wedge	22	0.26
333-38[f]	wedge	22	0.02
868-A	wedge	unknown	0.53
466-B	scales	7	0.04
466-B	flowering stalk[b]	7	0.04
466-B	red skin	7	0.05
466-B	core[c]	7	0.17
466-B	roots	7	0.22
466-D	leaves, fresh	8	0.03
466-E	flower stalk, dry[d]	unknown	<0.01
466-G	planting wedge[e]	unknown	1.25
466-H	whole bulb	1	0.08
466-I	wedge	2	0.09
466-J	wedge, 5 bulbs	4	0.08
466-K	wedge, 5 bulbs	5	0.09
466-L[g]	wedge, 5 bulbs	5	0.09
466-M[h]	wedge, 5 bulbs	5	0.09
466-N	wedge, 5 bulbs	7	0.12
466[i]	wedge, 5 bulbs	6	0.30
841[i]	wedge, 5 bulbs	6	0.27
843[i]	wedge, 5 bulbs	6	0.21
868[i]	wedge, 5 bulbs	6	0.50

[a] Longitudinal wedge section of scales, core, pad, and skin. [b] Fresh stalk while flowering. [c] Center core from pad to top of bulb. [d] Dried-up flower stalk. [e] Residue of a planting wedge attached to a growing bulb. [f] High- and low-scilliroside selections from 42 seedling-grown bulbs. [g] Irrigated. [h] Irrigated and fertilized. [i] Sampled during flowering period.

comparison, scilliroside content of the core, pad, and roots is consistently high. A selection of scilliroside assays in red squill bulb and plant parts is summarized in Table I. The scilliroside content of bulbs assayed ranged from 0.01% to 0.53%.

Plant samples were prepared for analysis by drying and then grinding to a powder with a hammer mill. Hard musciligenous macrogranules resulted from bulb samples, which required multiple extractions of the scilla glycosides using a Tekmar Tissumizer run at high speed. Three or more 5-min extractions in the homogenizer further comminuted the granules and was more efficient than batch or Soxhlet extraction. The bulbs contain a considerable quantity of phenolic material. This was removed by treatment of the extracts with aqueous lead acetate that precipitates the phenolics as yellow lead salts.

A simplified workup of assay sample was investigated with fresh bulb wedge sections macerated and extracted with acetone in a Waring blendor. The bulbs contain 80% or more of water, which serves as a cosolvent. Scilliroside content by HPLC checked well with the standard dry-powder assay for one clone sample but was in error for another due to excessive phenolic interference. Semiquantitative TLC gave accurate comparative values for both clones. 2-Propanol extraction of the undried bulb slices also led to more phenolic compounds being extracted. Larger amounts of lead acetate were needed to purify these samples adequately for successful quantitation in our HPLC system (Figure 2).

After examination of several sampling techniques including whole-bulb assays, we found wedge sections to be both representative of the bulb and useful to help conserve

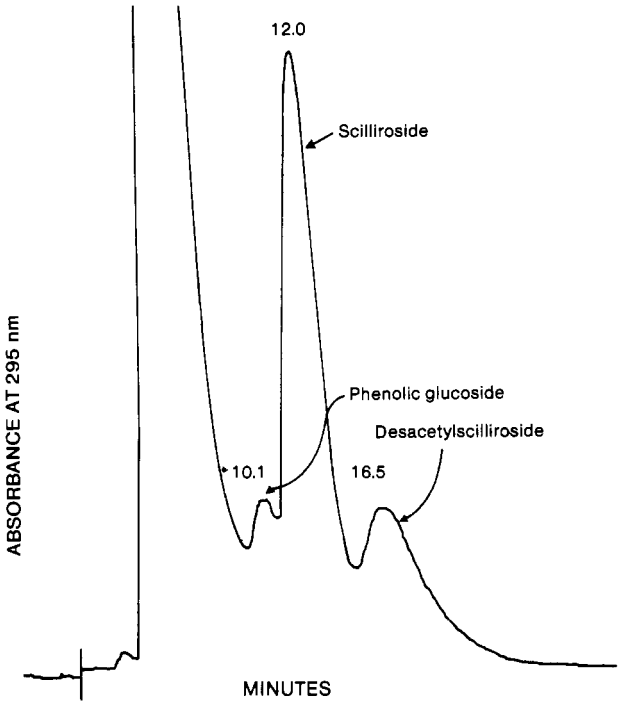

Figure 2. HPLC of extract of clone #333–28 using acetonitrile–water (97:3) at a flow rate of 1.0 mL/min.

tissue for clonal propagation. The wedge section is a vertical slice of the bulb including skin, scales, pad, and core. This sampling resulted in some variations because root or pad material and core contain the highest scilliroside content and more or less of these parts in the wedge can affect the accuracy of the assay.

The scilliroside content of 42 bulbs of the same age (22 years) grown from seedlings from a single clone #333 bulb varied from 0.01% to 0.35% with an average of 0.10%. Each seed from this same plant produced a variety with a scilliroside content different from the parent bulb. This large variation in seed-propagated varieties may partially explain some of the great differences in toxicity for imported red squill dry powders as measured by rat bioassay. Scilliroside content has genetic controls. Scilliroside percent is not related to the size of the bulb although larger bulbs have more total scilliroside. Bulbs chosen for propagation should be considered for the total scilliroside content of the bulb on the basis of size and percent. We observed that generally the red color of the bulb was not related to scilliroside percent.

A review of the assays of clone #466 samples of bulbs of various ages from 1 to 7 years indicated that age has little bearing on percent scilliroside. Also shown was the apparent absence of effect of irrigation and fertilization on scilliroside percent. However, these cultural practices improved growth rate of the leaves and total weight of the bulbs for a higher yield per acre.

Fresh leaves contain scilliroside, but dry leaves are devoid of this compound. This is consistent with the observation (Gentry et al., 1987) that leaf cell sap moves down into the bulb just prior to the summer dormancy period, causing the bulbs to swell. This is also consistent with our results on clone #466 that show slightly higher scilliroside content in bulbs harvested in June compared to bulbs harvested in February at the height of the growth periods. Dry stalks occurring after the flowering period in September are also devoid of scilliroside, although fresh stalks contain a small amount. In contrast to dry leaf and stalk, a dry shriveled planting wedge from which a new bulb had grown showed a 1.25% level of scilliroside. Apparently the

Table II. Storage of Red Squill Powders

clone-bulb	1st assay, % scilliroside	2nd assay,[c] %		3rd assay,[d] %	
		scilliroside	moisture	scilliroside	moisture
466-A	0.09[a]	0.05	7.0	0.06	9.4
871-A	0.10[a]	0.10	7.8	0.06	8.9
1372	0.10[a]	0.09	5.7	0.10	10.2
333-4	0.16[b]	0.07	7.7	0.06	9.5
333-10	0.17[b]	0.05	7.3	0.05	9.3

[a] Harvested June 1980. [b] Harvested July 1981. [c] Assayed Dec 9, 1982, after storage in closed bottles. [d] Assayed March 31, 1983, affter 112 days in open beakers.

new bulb growing from the pad on the planting wedge absorbed nutrients but not scilliroside from the old wedge. Clonal propagation by planting wedge sections containing pad has been a successful method for maintaining high-toxicity varieties (Gentry et al., 1987). Bulbs assayed during their flowering period in September contain the highest levels of scilliroside (Table I).

Red squill powders stored in bottles or in open beakers generally showed lower scilliroside levels with time (Table II). Decomposition of scilliroside can occur on storage and when exposed to the atmosphere. Samples of red squill powders allowed to stand in open beakers on a bench top for 112 days under ambient conditions showed minor weight variations of no more than 5%. These weight changes were related mainly to changes in humidity from day to day. There was minimal caking but some further degradation of scilliroside.

Microscopic examination of bulb tissue before and after drying and communition in hammer mill was informative. Fresh red squill cells are transparent, full of water, with easily ruptured cell walls. The particles in dry powders from hammer mills are not homogeneous, with large variations in size and shape. The small hard granules of red squill powders may protect occluded scilliroside against degradation via lactone or acetyl group hydrolysis by moisture in the air or by oxidation of double bonds. The variability in size of dry-powder granules can affect stability of the powders and bioavailability of scilliroside in rodent stomachs. The latter could lead to variability in rat bioassays for toxicity.

Column chromatography of the aglycon fraction led to the identification of two scilla aglycons in the #871 extracts. One aglycon was definitely identified as scillirosidin, as determined by HPLC and green fluorescence on TLC. A second compound ran slightly faster than scillirosidin on TLC and HPLC and corresponded to scillarenin, but fluoresced green. Scillarenin fluoresces orange. These minor compounds may be precursors to scilliroside and other scilla glycosides.

The polar fraction from a clone #871 extract was also rechromatographed on a column and on preparative TLC plates, leading to the identification of several glycosides. A phenolic glucoside was isolated and partially characterized. On TLC and HPLC it ran faster than scilliroside, corresponding to the interferring phenolic removed by lead acetate. The compound occurs in quantity in red squill bulbs and may be a glucosyl flavonoid.

A new scilla diglucoside was isolated from the polar extracts of #871. The product was obtained as highly hygroscopic white crystals. An infrared spectrum showed the presence of a lactone. The glucoside fluoresced green under UVL on TLC plates, indicating the probable presence of a 6-hydroxy or 6-acetoxy group. Naringinase cleaved both glucoses, giving the intermediate scilliroside, scillirosidin, and glucose on TLC and HPLC. HPLC also indicated that this is a diglucoside, comparable in retention time to scillaren A. This glucosylscilliroside is a hitherto unknown compound. No further work was done on the position of attachment of the second glucose unit to scilliroside. A best estimate is that it is attached to the first glucose, following on the structural attachment of glucose and rhamnose in scillaren A.

Desacetylscilliroside was identified by comparison with authentic reference sample prepared by treatment of scilliroside with ammonium hydroxide. It probably forms as an artifact in the workup of red squill extracts because the acetoxy group is relatively labile to hydrolysis. No procillaridin or scilliglaucoside (Stoll and Kreis, 1951) was found in clone #871 extract. Scillirubroside (von Wartburg, 1966), another red squill scilla glycoside, was not checked because a sample was not available.

Free glucose but no other reducing carbohydrates were found in the aqueous acetone extracts from bulbs of clones #324 and #868. Glucose was confirmed by TLC in two solvent systems, D (R_f 0.53) and E (R_f 0.55).

The aglycons scillirosidin, desacetylscillirosidin, and scillarenin were needed as reference standards for TLC and HPLC assays of red squill extracts and preparations. The enzymes necessary to split glucose from scilliroside are not present in red squill. Acid treatment causes ester and lactone hydrolysis. Commercially available β-glucosidase from bitter almonds does not affect scilliroside. Scillirosidin has been prepared by treatment of scilliroside with aqueous extracts of seeds from *Coronilla glauca* L. and *Medicago sativa* L. (Stoll and Renz, 1950; von Wartburg and Renz, 1959). Enzymes from various fungi were shown to split the glucose from scilliroside (Stoll et al., 1951).

Naringinase has been used to sequentially split the rhamnose and then glucose from naringen, a grapefruit flavonoid (Horowitz, 1981). This enzyme preparation when incubated with scilliroside split off glucose within 4 days at 38 °C, providing high-purity scillirosidin. Desacetylscillirosidin was also readily prepared from the glucoside with naringinase in distilled water at 30 °C for about 6 days. The conversion of scillaren A to the aglycon scillarenin required 14 days at pH 4 and 37 °C with shaking. The glucose was readily split from scillaren A, providing the rhamnoside intermediate proscillaridin, but the removal of the rhamnose was slower. Naringinase also removed the first glucose from our new glucosylscilliroside faster than it did the second glucose unit.

Rothlin and Schalch (1952) studied the toxicity of scilliroside and scillirosidin in 12 different laboratory animals and wild rodents. When it was administered orally to white, mature female Glaxo rats, the LD_{50} values were as follows: scilliroside, 0.43 mg/kg; scillirosidin, 0.35 mg/kg; acetylscillirosidin, 1.35 mg/kg. For white male Glaxo rats the LD_{50} value for scilliroside was 1.35 mg/kg, for a male/female toxicity ratio of 3.1. Higher lethality to females compared to male rats was previously reported (Winton, 1927; Stoll and Renz, 1942; Dybing et al., 1952). The variations in toxicity of scilliroside to the 11 other species ranged from 0.17 mg/kg in a gray house mouse, 1.28 mg/kg for male guinea pigs, 6 mg/kg for cats, 7.7 mg/kg for rabbits, and 37 mg/kg for field mice. Sex differences in response were only significant in a few species in addition to the Glaxo rats. Lubitz and Fellers (1941) also noted large differences in toxicity of red squill powder among chickens, rabbits, and guinea pigs, with rabbits most susceptible to the lethal action.

The toxicity studies summarized in Table III were carried out with Charles River CD rats. The male/female toxicity ratio varies from 2.7 to 5.1 for these Charles River rats, which compares with 3.1 for the Glaxo rats. At doses up to 5 mg/kg, 6-desacetylscillirosidin and proscillaridin

Table III. Toxicity of Red Squill Compounds and Powders to Charles River CD Rats, 2-Day Criteria[a]

preparation	LD_{50},[b] mg/kg	scilla compd,[c] %	ratio male/female
scilliroside, prep		82	3.8
male	5.3 (4.4–6.4)		
female	1.4 (0.99–1.85)		
scilliroside		100	
female	1.7 (0.96–2.99)		
scillirosidin		100	
male	4.2 (3.75–4.70)		
desacetylscillirosidin		100	
female	>5.0[d]		
proscillaridin		100	
male	>5.0[d]		
bulb #1372 powder[e]		0.10	3.5
male	435 (363–524)		
female	125		
bulb #871 powder[e]		0.10	2.7
male	446 (317–629)		
female	165 (109–250)		
bulb #466 powder[e]		0.09	5.1
male	960 (716–1286)		
female	190 (155–234)		

[a] Statistical analysis where $N = 5$ rats/dose (Litchfield and Wilcoxon, 1949). [b] Numbers in parentheses represent 5–95% confidence limits. [c] Scilliroside unless otherwise noted. [d] No lethality up to 5.0 mg/kg. [e] Whole bulbs dried and powdered.

show no toxicity to female and male rats, respectively. Neither of these scilla compounds contains a 6-acetoxy group as does scilliroside, indicating that this functional group contributes substantially to toxicity. Following oral administration, the toxicity of scillirosidin is similar to that of scilliroside, confirming a previous report (Rothlin and Schalch, 1952).

The toxicity of scilliroside administered i.v. to cats is 0.20×10^{-3} mol/kg, whereas scillirosidin is 0.12×10^{-3} mol/kg (Stoll and Renz, 1950). This is a clear indication that the aglycon rather than the glucoside is the active toxic metabolite. Although scilliroside itself may have cardiovascular activity per se, it is unlikely that it will pass the blood–brain barrier to cause convulsions, paralysis, and death. However, animal organs have been shown to split scilliroside to scillirosidin if scilliroside is absorbed intact from the gastrointestinal tract following an oral dose (Stoll and Renz, 1951). It seems more likely that microorganisms in the GI tract produce β-glucosidases that can split the glucose from scilliroside, releasing scillirosidin, which will be more readily absorbed. Differences in gut bacteria in different species and in individual animals may be part of the explanation for the wide differences in toxicity for scilliroside and red squill powders. Cats for example show very large differences in their time response to scilliroside as an emetic (Gold et al., 1950). Additional toxicological studies should resolve this question.

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Registry No. Scilliroside, 507-60-8; desacetylscilliroside, 80496-58-8; scillaren A, 124-99-2; scillirosidin, 507-59-5; scillarenin, 465-22-5; desacetylscillirosidin, 7004-95-7; proscillaridin, 466-06-8.

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