5-hydroxyindole, 1953-54-4; 5-methylindole-3-acetic acid, 1912-47-6; indole-3-butyric acid, 133-32-4; tryptophol, 526-55-6; 1,3-dimethylindole, 875-30-9; indole-2-carboxylic acid, 1477-50-5; *N,N*-dimethyltryptamine, 61-50-7; *N*-hydroxy-*N*-methyl-1*H*-indole-3-ethanamine, 57383-99-0; 2-hydroxy-*N*-methyltryptamine, 106987-89-7; gramine, 87-52-5.

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Modification of Red Squill by *Aspergillus niger*

Anthony J. Verbiscar,* Thomas F. Banigan, and Robert A. Schatz

Five strains of *Aspergillus niger* produced a β-glucosidase that cleaved scilliroside to its aglycon scillirosidin. After incubation for 6 days in submerged cultures, yields of scillirosidin for each strain ranged from 23 to 100%. Red squill bulb extracts were treated with *A. niger* and naringinase, a mixed-function enzyme derived from *A. niger*. Aglycons were isolated and showed a toxicity to rats that parallels their scillirosidin content. The scillirosidin aglycons are more toxic to female rats than to males.

Red squill (*Urginea maritima, lilliaceae*) has been used as a rodenticide since the Middle Ages (Chitty, 1954). The bulbs and roots contain scilliroside and other toxic scilla glycosides and are generally formulated into rodent baits as dried powders. The toxicity of the dried bulb powders varies substantially due to genetic variants in the wild seed propagated plants, differences in harvest time, and decomposition on storage (Verbiscar et al., 1986; Gentry et al., 1987). Bioavailability may also affect toxicity. The dry powders are composed of hard mucilagenous granules from which the scilla glycosides may not be readily extracted into rodent stomach fluids, and hence not absorbed. Solvent extracts of red squill have been used in rat baits in an attempt to solve these problems. However, the extracts of scilla glycosides are extremely bitter, and rodents quickly learn to avoid such baits. The extracted glycosides are more readily available to taste receptors in the rodent palate than are the glycosides in dry powders. The utility of red squill preparations for rodent control is related to acceptability as well as toxicity.

A goal of this study was to convert the scilla glycosides in red squill to their aglycons, thereby increasing palatability. Scilliroside, the major toxic glycoside in red squill, is bitter, whereas its aglycon scillirosidin is tasteless. Scillirosidin is equally or more toxic than the parent glucoside (Rothlin and Schalch, 1952; Stoll and Renz, 1950). Chemical methods designed to cleave the glucose from scilliroside are too drastic, causing hydrolysis of the lactone and acetyl grops (von Wartburg and Renz, 1959), resulting in decreased toxicity (Verbiscar et al., 1986). Accordingly, enzymatic methods were chosen for this conversion.

Free scillirosidin has been detected in some bulb clones, but red squill does not contain the enzyme necessary to cleave glucose from scilliroside. Commercial β-glucosidase from bitter almonds does not cleave this glucoside (Stoll and Renz, 1942; Verbiscar et al., 1986). The enzymatic conversion of scilliroside to scillirosidin was successful on extracts of the seeds from *Coronilla glauca* and *Medicago sativo* (Stoll and Renz, 1950). Scilliroside has also been cleaved by enzymes in fungi including *Penicillium sp.*, *Aspergillus sp.*, and others (Stoll et al., 1951a,b). Naringinase, a mixed-function enzyme from *Aspergillus niger*, cleaves first rhamnose and then glucose from naringen (Horowitz, 1981). We found that naringinase cleaved the glucose from scilliroside, desacetylscilliroside, and scillaren A, providing scillirosidin, desacetylscillirosidin, and proscillaridin, respectively (Verbiscar et al., 1986). With this basis, five strains of *Aspergillus niger* were screened for their ability to produce a β-glucosidase that would cleave scilliroside to its aglycon (Figure 1).

Anver Bioscience Design, Inc., Sierra Madre, California 91024 (A.J.V., T.F.B.), and College of Pharmacy and Allied Health Professions, Northeastern University, Boston, Massachusetts 02115 (R.A.S.).

EXPERIMENTAL SECTION

Materials. Five strains of *A. niger* (NRRL 3, 330, 337, 372, 6411) were obtained from the Culture Collection, Northern Regional Research Laboratory, U.S. Department of Agriculture, Peoria, IL, courtesy of C. W. Hesseltine. Naringinase derived from *A. niger* was obtained from Sigma Chemical Co. Red squill bulbs were provided courtesy of H. S. Gentry, Gentry Experimental Farm, Murrieta, CA. Malt extract broth was obtained from Becton Dickenson and peptone from Difco.

Culture Conditions. The *A. niger* strains were maintained on slants of Blakeslee's formula consisting of 5 g of malt extract broth, 5 g of dextrose, 5 g of agar, and 250 mg of peptone brought to 250 mL with distilled water. Submerged cultures were carried out in Czapek Dox broth (Difco Manual, 1953) containing 30 g of sucrose, 3 g of sodium nitrate, 1 g of dipotassium phosphate, 0.5 g of magnesium sulfate, 0.5 g of potassium chloride, and 0.01 g of ferrous sulfate made up to 1 L with distilled water. Media were autoclaved for 15 min at 121 °C. Milk filter disk closures were used for the *Aspergilli*, which were incubated at 30 °C for propagation and growth. A gyratory shaker at 150–200 rpm was used to increase aeration, growth rate, and scilliroside hydrolysis rate. The propagation slants were stored refrigerated at 8 °C.

Analytical Methods. Thin-layer chromatography (TLC) was run on Merck silica gel G using various de-

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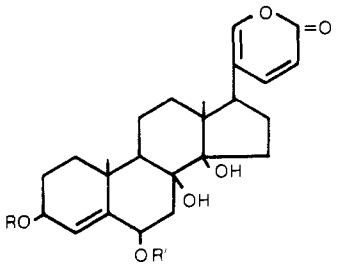

Scilliroside	R = Glucose, R' = Acetyl
Scillirosidin	R = H, R' = Acetyl
Desacetylscilliroside	R = Glucose, R' = H
Desacetylscillirosidin	R = R' = H

Figure 1. Scilla glycosides and aglycons in red squill.

velopment solvents and detection methods. Unless otherwise noted, high-performance liquid chromatography (HPLC) was carried out on two Lichrosorb Si 60 5-μm columns, 3.2 × 250 mm each, protected by a Porasil A column, 3.2 × 40 mm, using acetonitrile–water–2-methoxyethanol (98:1:1) eluant at 1.0 mL/min, with the UV–vis detector set at 300 nm. In this system retention times are as follows: scillirosidin, 5.6 min; desacetylscillirosidin, 7.5 min; scilliroside, 17.6 min. Assays of microorganism-treated preparations were carried out with pure scilliroside and scillirosidin as reference standards (Verbiscar et al., 1986). Desacetylscillirosidin was estimated on the basis of the scillirosidin reference peak. Peak areas were quantitated with a Shimadzu C-E1B integrating data processor.

***A. niger* Treatment of Naringin.** Culture flasks, 125 mL, containing 15 mL of sterile Czapek Dox broth were inoculated with the five *A. niger* strains and incubated at 30 °C on a gyratory shaker. Within 7 days mycelia growth was heavy and surmounted by black spores. After 8 days 300 mg of naringin was added to each flask, and shaking was continued for 4 days more. The total-growth masses and medium were then diluted with methanol and filtered. No residual naringen was detected on TLC using acetonitrile–water (9:1) developer and long-wavelength UVL detection where reference standard naringin fluoresces at R_f 0.60. With cyclohexane–ethyl acetate (9:2) developer and alcoholic ferric chloride spray detection, naringinen appeared at R_f 0.20 as a yellow spot on warming. On HPLC using ethyl acetate for elution naringenin had a retention time of 3.6 min. By these methods no uncleaved naringen was detected in any of the five samples, whereas the aglycon naringenin was clearly present in significant quantities.

***A. niger* Treatment of Scilliroside.** Cultures of the five strains of *A. niger* were prepared as described in the prior experiment. After 13 days of growth, 40 mg of technical scilliroside (28%, Sandoz) was added to each flask with an additional 5 mL of distilled water. Shaking was continued for 6 days longer at 30 °C. The growth masses and medium were mulled with methanol and filtered, and the mycelia spore pads were washed with more methanol. The combined filtrates were evaporated to a low volume under vacuum, extracted into chloroform, and brought to 25 mL. TLC with acetonitrile–water (9:1) developer showed complete conversion of scilliroside (R_f 0.65) to scillirosidin (R_f 0.86) plus other compounds. HPLC was used to quantitate scillirosidin (Table I). The pHs of these growth media decrease due to the production of citric acid (Moyer, 1953).

***A. niger* Treatment of Red Squill Extract.** A 700-g quantity of a red squill clone 841 bulb was sliced and homogenized in a Waring Blendor with 700 mL of distilled water. The homogenate was filtered, washing the cake with 100 mL of water to provide 900 mL of clear red extract. The extract was sterilized in an autoclave and brought to a volume of 1000 mL with distilled water. Assay by HPLC indicated a scilliroside content of 320 mg.

Three selected strains of *A. niger* (NRRL 3, 330, 372) were grown in 10 mL of Czapek Dox broth in 125-mL culture flasks for 4 days at 30 °C on a gyratory shaker at 150 rpm. To each inoculum was added 40 mL of the above sterile extract, and the treatment was continued for 6 days. The samples were filtered; the mycelia pad was washed with water and dried. The filtrate was extracted with methylene chloride that was dried over anhydrous sodium sulfate. The solvent was evaporated, and the oil that remained was reconstituted to 10 mL with acetone and assayed for scillirosidin by HPLC (Table II).

Aglycon Preparation with Naringinase. Two bulbs of clone 841 were cleaned and weighed with roots, yielding 3650 grams of plant material. This was reduced to a mash in a centrifugal Acme juicerator. Further maceration and extraction was carried out in a Waring Blendor with added water. The slurry was centrifuged, and the combined aqueous extracts, 7.5 L, were autoclaved in a 5-gal bottle fitted with a mechanical stirrer. The filter cake was dried and assayed by HPLC, which showed only 30 mg of scilliroside present. An HPLC assay of the extract showed it contained 2.02 g of scilliroside (98% recovery).

The sterilized extract was treated with 2.0 g of naringinase and the resultant mixture stirred slowly at ambient temperature. The treatment was continued for a total of 6 weeks, monitoring the slow, progressive decrease in scilliroside by TLC and HPLC. The mixture was then extracted with methylene chloride. After the mixture was dried over sodium sulfate, evaporation of the solvent under vacuum resulted in an oil. The oily product was triturated with hexane to yield 1.56 g of a brown crude aglycon powder (0-102-1). TLC of the powder using acetonitrile–water (98:2) developer, with a 10% sulfuric acid spray plus light heat detection, showed four green fluorescent scilla compounds under UVL corresponding to scillirosidin (R_f 0.80), desacetylscillirosidin (R_f 0.58), scilliroside (R_f 0.20), and an unknown (R_f 0.35). HPLC assay identified the aglycon extract as a mixture of about 300 mg/g of scillirosidin, 430 mg/g of desacetylscillirosidin, and 70 mg/g of scilliroside (Figure 2).

Aglycon Preparation with *A. niger*. Three bulbs of clone 841 were cleaned and weighed with roots, yielding 3530 g. They were sliced and macerated with the Acme juicerator and Waring Blendor as described previously to yield 6.8 L of a clear red solution. The solution was autoclaved in a 5-gal bottle fitted with a mechanical stirrer and tube with a fritted dispenser for the addition of filtered air.

Table I. *A. niger* Treatment of Technical Scilliroside

NRRL strain	aqueous–MeOH extract		
	appearance	pH	scillirosidin, %
3	yellow	4.5	100
330	yellow	5.2	88
337	dark	4.0	37
372	dark	6.8	83
6411	dark	4.7	75

Table II. *A. niger* Treatment of Red Squill Bulb Extract

NRRL strain	dry mycelia pad		scillirosidin yield	
	grams	appearance	mg	% theory
3	1.45	tan	14	100
330	1.40	tan	12	94
372	1.10	dark	3	23

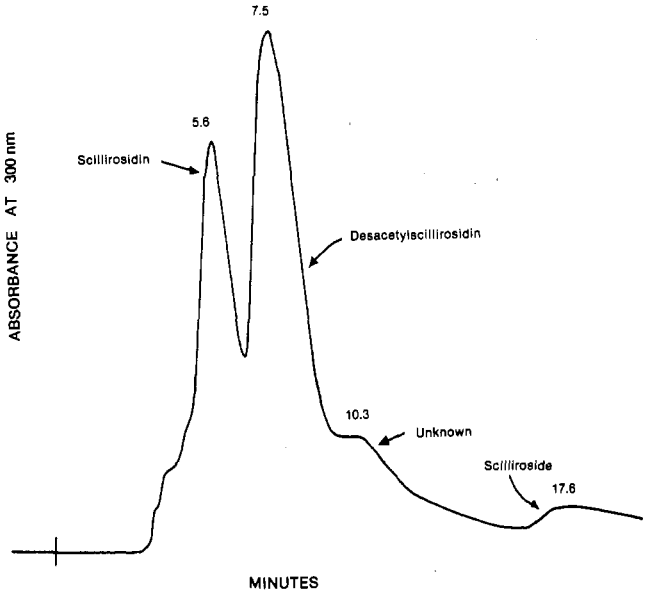

Figure 2. HPLC of aglycon mixture (0-102-1) from treatment of red squill extract with naringinase.

A. niger NRRL 3 was grown in three culture flasks each containing 250 mL of Czapek Dox broth. This inoculum was added to the sterilized bulb extract, and the culture was stirred slowly at ambient temperature. A diaphram pump was used to supply moderate aeration to the fritted dispenser through a micropore filter. Mycelial growth was abundant in 2 days. After 7 days TLC and HPLC monitors showed conversion of scilliroside to scillirosidin was 30–40%, and after 11 days it was 95%. The aglycons were recovered by multiple methylene chloride extractions of the aqueous solution and mycelia filter cake. The extracts were dried over sodium sulfate (anhydrous) and evaporated under vacuum to an oil. Trituration with hexane yielded 2.07 g of a yellow crystalline powder (0-102-3). HPLC assay showed this contained about 600 mg/g of scillirosidin and 320 mg/g of desacetylscillirosidin (Figure 3). TLC showed the presence of a third green fluorescing unknown compound at R_f 0.35 as in the naringinase run. No scilliroside or desacetylscilliroside was detected.

RESULTS AND DISCUSSION

Naringinase is a commercial enzyme obtained from the fungus *A. niger*. Our previous report described the use of this enzyme to cleave glucose and rhamnose from scilla glycosides (Verbiscar et al., 1986). Our choice of *A. niger* for the current study was dictated by our success with naringinase and the ready availability of this relatively nontoxic Aspergilli (Raper and Fennel, 1965).

Naringin, 7-rhamnosylglucosylnaringenin, obtained from grapefruit rind, is a substrate for naringinase. This enzyme, or mixture of enzymes (Pazur et al., 1971) cleaves rhamnose then glucose from naringin. These enzymatic actions were used as a model to test for naringinase activity in five strains of *A. niger*, for potential application to the scilla glycosides. All five strains (NRRL 3, 330, 337, 372, 6411) grew well in a synthetic medium and completely cleaved both carbohydrates from naringin within 4 days, providing the aglycon naringenin. The intermediate glucosylnaringenin was not found on TLC monitors of this reaction, indicating that all five strains of *A. niger* produced quantities of the mixed-function enzyme naringinase.

The five strains of *A. niger* cleaved the glucose from scilliroside at varying rates (Table I). After 6 days yields of scilliroside ranged from 37 to 100%. NRRL-337 grew slowly as evidenced by a lower quantity of mycelia and

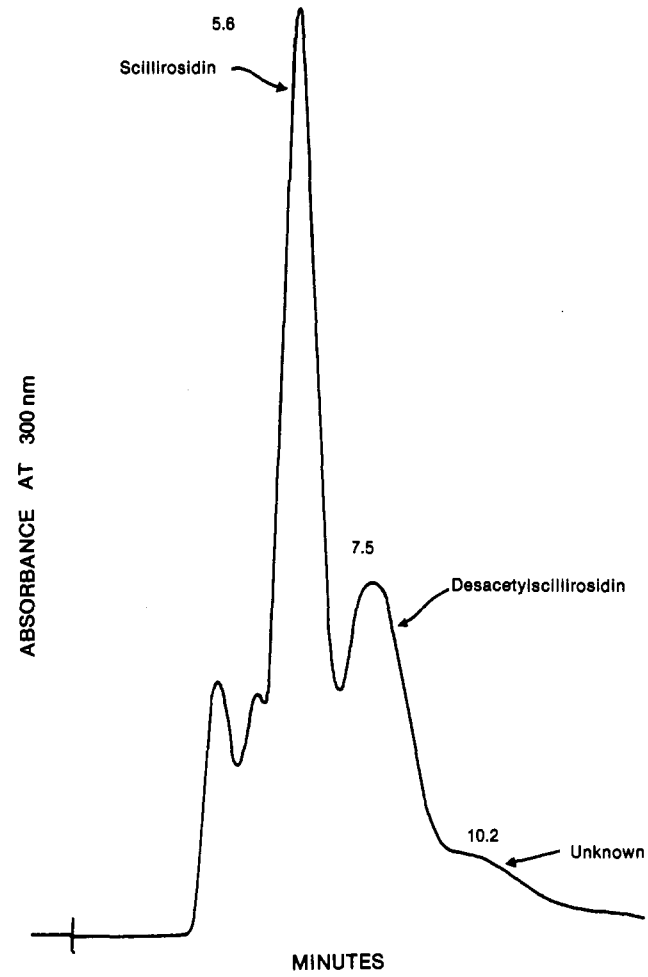

Figure 3. HPLC of aglycon mixture (0-102-3) from treatment of red squill extract with *A. niger*.

produced the lowest yield of scillirosidin. The rate of reaction of the strains with scilliroside is related to the amount of naringinase produced and the rate of growth of the fungus in the synthetic medium.

The three highest producing strains of *A. niger* (NRRL 3, 330, 372) were next grown in a sterile extract of red squill bulb clone 841 for 6 days at 30 °C on a rotary shaker. *A. niger* NRRL 3 showed a 100% conversion of scilliroside to scillirosidin, whereas NRRL 372 gave only a 23% yield when assayed by HPLC (Table II). Again, the higher weight of the mycelia correlated with higher enzyme activity in the *A. niger* strain.

Preparation of test quantities of mixed aglycons were carried out on aqueous extracts of freshly harvested bulbs of clone 841. The whole bulb with roots was macerated in water to extract the scilliroside and other scilla glycosides from the large transparent cells. The extracts were sterilized and then treated with commercial naringinase and *A. niger* NRRL 3 at room temperature. Aeration was required for the growth of *A. niger*. Following HPLC and TLC monitors, the mixed aglycons were recovered by solvent extraction and isolated as crystalline powders.

In the case of naringinase, 3650 g of plant material treated with 2 g of naringinase for 6 weeks yielded 1.56 g of crude mixed aglycons consisting in a 0.47 g of scillirosidin, 0.67 g of desacetylscillirosidin, and 0.11 g of scilliroside (Figure 2). TLC showed the presence of two other minor components including a green and an orange fluorescing spot, indicative of scilla compounds.

For the fungus treatment, 3530 g of fresh bulb and root of clone 841 was extracted and inoculated with *A. niger* NRRL 3. After 11 days this system produced 2.07 g of

Table III. Toxicity of Red Squill Aglycons to Charles River CD Rats (2-Day Criteria)

preparation	LD_{50},[a] mg/kg	% scilla compd[b]	ratio male to female
scillirosidin			
male	4.2 (3.7–4.7)		
desacetylscillirosidin			
female	>5.0		
aglycons 0-112-1[c]			
male	13.0 (9.8–17.3)	4.8	
female	8.1 (4.4–15)	3.0	1.6
aglycons 0-102-3[d]			
male	8.0 (4.4–14.1)	4.8	
female	2.8 (1.8–4.4)	1.7	2.8

[a] Numbers in parentheses represent confidence limits; $N = 5$ rats/dose level. [b] Total scillirosidin plus scilliroside. [c] Naringinase derived: scilliroside, 7%; scillirosidin, 30%; desacetylscillirosidin, 43%. [d] *A. niger* derived: scillirosidin, 60%; desacetylscillirosidin, 32%.

crystalline product containing 1.24 g of scillirosidin, 0.66 g of desacetylscillirosidin, and no unreacted scilliroside as determined by HPLC (Figure 3). Unidentified green and orange fluorescing spots were visible on TLC. It is clear that *A. niger* NRRL 3 produces large amounts of naringinase. Shorter reaction times decrease the amount of desacetylscillirosidin in the cultures.

The toxicity of the aglycon preparation was studied in Charles River CD rats following procedures previously reported (Verbiscar et al., 1986). For these tests, the preparations were administered to male and female rats orally at five dose levels ($N = 5$ rats per dose level). Behavioral effects and lethality were monitored for 2 days. For scillirosidin the dose levels for males ranged from 3.2 to 5.0 mg/kg. The LD_{50} was 4.2 mg/kg with a confidence level of 95% (Litchfield and Wilcoxon, 1949) (Table III). The aglycon preparations from naringinase (0-102-1) and *A. niger* (0-102-3) were administered at five dose levels from 2.5 to 20 mg/kg to both males and females. Toxicity is due to scillirosidin plus residual scilliroside. In accord with prior reports (Rothlin and Schalch, 1952; Verbiscar et al., 1986), the toxicity of scilliroside is higher in females than males. This is also the case for scillirosidin where male to female toxicity ratios are 1.6 and 2.8 for the two aglycon preparations (Table III). On the basis of higher toxicity to females than males, the absence of toxicity of desacetylscillirosidin at 5.0 mg/kg to females is notable. *A. niger* is an effective fungus for the production of naringinase to cleave scilliroside to scillirosidin, but reaction time should be minimized to limit hydrolysis of the 6-acetyl group, which contributes substantially to toxicity of scillirosidin.

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Registry No. β-Glucosidase, 9001-22-3; scilliroside, 507-60-8; scillirosidin, 507-59-5; maringinase, 9068-31-9; desacetylscillirosidin, 7004-95-7.

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Novel 1,3,4-Oxadiazol-2(3*H*)-ones as Potential Pest Control Agents

Jamin Huang* and Dean F. Bushey

The biological profile of a series of novel 5-methoxy-1,3,4-oxadiazol-2(3*H*)-ones with aliphatic moieties on nitrogen was determined. Relative to the known insecticidal aromatic analogue, these molecules are considerably less active. On the basis of chemical model studies with both aliphatic and aromatic substituted oxadiazolones, a reactive site is postulated that may be operational at the enzymatic level.

In recent years a number of 3-aryl-5-alkoxy-1,3,4-oxadiazol-2(3*H*)-ones have been claimed as anthelmintics (Boesch, 1978) and insecticides (Boesch, 1979; Tieman, 1981). Compound **1A**, a member of this class, has been widely tested under the code number RP 32,861 and is claimed to be particularly effective in controlling piercing and sucking insects such as aphids and rice hoppers (Ambrosi et al., 1979, 1980). This compound was reported to be an antiesterase agent (Ambrosi et al., 1980). Its enzymatic inhibitory activity on housefly head acetylcholinesterase was found to be $I_{50} = 8 \times 10^{-7}$ M. The oxadiazolone **2** (Tieman, 1981), which is a better inhibitor of

Union Carbide Agricultural Products Company, Inc., Research Triangle Park, North Carolina 27709.

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