Oediger, H.; Moller, F.; Eiter, K. *Synthesis* **1972**, 591.
Philpot, R., National Institutes of Environmental Health Sciences, Research Triangle Park, NC, personal communication, 1983.
Pillot, J.-P.; Bennetau, B.; Dunogues, J.; Calas, R. *Tetrahedron Lett.* **1980**, *21*, 4717.
Price, C. C.; Judge, J. M. "Organic Syntheses"; Wiley: New York, 1973; Collect. Vol. V, p 255.
Ringold, H. J.; Malhotra, S. K. *Tetrahedron Lett.* **1962**, 669.
Sachs, C. E.; Fuchs, P. L. *Synthesis* **1976**, 456.
Sharpless, K. B.; Lauer, R. F.; Teranishi, A. Y. *J. Am. Chem. Soc.* **1973**, *95*, 6137.
Srogl, J.; Janda, M.; Stibor, I. *Collect. Czech. Chem. Commun.* **1970**, *35*, 3478.
Toland, W. G.; Ferstandig, L. L. *J. Org. Chem.* **1958**, *23*, 1350.
Ueda, T.; Fujita, Y. *Chem. Ind. (London)* **1962**, 1618.
Wilson, B. J.; Garst, J. E.; Linnabary, R. D.; Channell, R. B. *Science (Washington, DC)* **1977**, *197*, 573.
Wilson, B. J.; Garst, J. E.; Linnabary, R. D.; Doster, A. R. In "Effects of Poisonous Plants on Livestock"; Keeler, R. R.; Van Kampen, K. R.; James, L. F., Eds.; Academic Press: New York, 1978; p 311.
Zamojski, A.; Kozluk, T. *J. Org. Chem.* **1977**, *42*, 1089.

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Toxicity and Bitterness in Australian *Dioscorea bulbifera* L. and *Dioscorea hispida* Dennst. from Thailand

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Underground tubers of bitter yams (*Dioscorea bulbifera* L. commonly known as var. *rotunda*), which are eaten after complex processing by Australian aborigines, were analyzed for acute toxicity and for the toxic alkaloid dioscorine. Results demonstrated that these tubers were not toxic, so they were extracted and the extracts were analyzed by thin-layer chromatography in order to determine the cause of bitterness. A major bitter component was found to be diosbulbin D (0.07 mg/g), a furanoid norditerpene previously isolated by Ida et al. (1978). Traditional aboriginal food processing techniques were found to be very efficient in removing diosbulbin D, thus making the bitter yams palatable. The alkaloid and bitter principle content of raw samples of this variety was compared with that of a dioscorine-containing yam from Thailand (*Dioscorea hispida* Dennst.), which is also used as food.

Yams of the genus *Dioscorea* are a staple subsistence food in some tropical regions of the world. The dorman underground tubers and the aerial tubers of both wild and cultivated varieties are harvested as a starch source in West Africa, parts of southeast Asia, the Pacific Islands, India, and Central America (Coursey, 1967).

Some species and varieties, particularly wild forms, are toxic and/or unpalatable. Of the 59 species recorded from southeast Asia, 8 are known to be bitter and/or poisonous if eaten raw (Burkill, 1954). The local people prepare the tubers before consumption to make them edible. Toxic alkaloids previously isolated from varieties of *Dioscorea hispida* Dennst. and *Dioscorea dumetorum* (Kunth.) Pax. are dioscorine and dihydrodioscorine, respectively. To date, other classes of bitter compounds recorded are (1) saponins and sapogenins in Central American, South African, and Indian species, (2) tannins and polyphenols in Indo-Chinese varieties, and (3) furanoid norditerpenes (diosbulbins), which have been isolated only from varieties of *Dioscorea bulbifera* L. (Martin, 1979; Telek et al., 1974).

Three species of yam are indigenous to Australia, but their botanical nomenclature is currently under review (Yen, 1984). One of these, *D. bulbifera*, commonly known as var. *rotunda*, is a bush food of northern Australian aboriginal people. Only the underground tuber is eaten, not the small aerial tuber (bulbil). Other varieties of *D. bulbifera* occur widely as food plants, both in Africa and in other parts of Asia (Alexander and Coursey, 1969). In Australia today, it is mainly eaten by those who are returning to their traditional homelands. In the raw state, it is described in aboriginal English as "cheeky", by which is meant bitter or poisonous. Treatment practices vary but commonly involve baking, followed by leaching of the sliced tubers in running water, overnight. The resultant food is then eaten without further preparation. These Australian techniques are very similar to those used in other parts of the world for preparing bitter yams (Alexander and Coursey, 1969).

Since no prior analysis of the pharmacologically active components of Australian yam varieties have been carried out, the aims of the following experiments were to identify the nature of the toxic and/or bitter components of this yam variety and to examine the effectiveness of traditional food processing methods in removing these substances.

The composition of the extracts of the cheeky yam were compared throughout with that of an authentic dioscorine-containing yam, *D. hispida* Dennst. (from Thailand). This toxic yam is also prepared as a vegetable in southeast Asia (Burkill, 1954).

The findings are of interest not only to chemotaxonomists in tracing species differences but also to workers in Australian aboriginal communities.

EXPERIMENTAL SECTION

Plant Material. Underground tubers of *D. bulbifera* were collected by aboriginal people from two locations in northeast Arnhem Land, NT, Australia, during April–September (dry season and middle of dormant stage), in 1981, 1982, and 1983. Underground tubers of *D. hispida*

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were received in May 1982 and July 1983 from the Forest Products Research Division, Royal Forestry Department, Bangkok, Thailand, courtesy of Dr. Phichai Tovivich, South-East Asian Network for the Chemistry of Natural Products.

Tubers were stored at room temperature on the laboratory bench. A small sample that underwent long-term (18-month) freezing in the freezer compartment of a domestic refrigerator (−5 °C) did not give different results.

Equipment and Methods. Mass spectra (MS) were obtained with a JEOL JMS D100 mass spectrometer and IR spectra on a Perkin-Elmer 257 grating infrared spectrometer. UV absorption was recorded on a Varian Series 634 UV–vis spectrometer, and NMR spectra were observed by using a JEOL JNM-FX-200 Fourier transform NMR spectrometer.

Investigation of Toxicity. Preliminary screening for toxic components of fresh, uncooked specimens of both species was carried out, using a field alkaloid test (Culvenor and Fitzgerald, 1963) and a test for cyanide (Everist, 1981). A sample of uncooked peeled yam (*D. bulbifera*) (fresh weight 107 g) was dried for 4 days at room temperature under vacuum. The resultant material (33 g) was powdered with a mortar and pestle and submitted for LD_{50} determinations (ip in the mouse) to Pharmaceutical Consulting Services, Sydney.

An extraction process for dioscorine (Leete and Pinder, 1972) was performed on both species, with the structure of any dioscorine isolated confirmed by mass spectrometry (MS) (*m/e* 221 amu) and thin-layer chromatography (TLC) on Merck silica gel 60 F_{254} plates developed in chloroform–ethanol–ammonia, 100:10:0.5, and compared with literature R_f [Leete and Pinder (1972): $R_f = 0.3$], and carbon-13 nuclear magnetic spectrometry (NMR). The efficiency of this extraction was checked by enriching a blank sample with pure dioscorine and measuring the proportion recovered, which was found to be 80%.

Investigation of Bitter Compound. Raw tuber of cheeky yam was chewed by a series of tasters to determine bitterness, while subsequent yam preparations were tasted by chewing dried filter paper strips, which had previously been dipped in the various extracts and then dried. *D. hispida* was not tasted, for obvious reasons.

Chromatographic tests for known bitter compounds were attempted on a *D. bulbifera* extract. Preparation of the extract involved macerating *D. bulbifera* tuber (425 g) in 0.5 M hydrochloric acid and leaving it to stand for 2 days. The filtrate was basified (pH 8.0) with potassium carbonate and extracted with ether for 4 days (Leete and Pinder, 1972). After evaporation under vacuum, the ether extract was chromatographed on Merck TLC silica gel 60F_{254} plates by using chloroform–ethanol–ammonia (100:10:0.5). TLC plates were sprayed independently with each of the following reagents: Dragendorff reagent for alkaloids [E. Merck (1971): 41], sulfuric acid reagent for alkaloids and steroids [E. Merck (1971): 95], antimony(V) chloride for terpenes and saponins [E. Merck (1971): 9], tin(VI) chloride for polyphenols [E. Merck (1971): 99], zinc chloride for saponins [E. Merck (1971): 103], Erhlich's reagent for terpenes (Ida et al., 1978), and vanillin–hydrochloric acid for polyphenols (Martin and Ruberte, 1975).

Further qualitative tests were performed on both yam species in order to identify saponins and polyphenols. Saponin identification involved frothing and a Liebermann-Burchard test (Griffin et al., 1968) and an attempted sapogenin (diosgenin) isolation (Julian, 1962). An oxidative browning effect (Martin and Ruberte, 1975) was observed on freshly cut surfaces of *D. bulbifera* but not on *D. hispida*. Since polyphenols were though to be responsible for this, extraction of polyphenolic substances (Martin and Ruberte, 1975) was also attempted from *D. bulbifera*.

Table I. Ethyl Acetate Fraction

R_f	color (366 nm)	
0.90	lemon	
0.85	bone	section α (not bitter)
0.82	yellow	
0.73	purple	
0.61	purple	
0.55	yellow	
0.50	bone	section β (bitter)
0.46	brown	
0.44	blue	
0.36	brown	
0.32	blue	
0.29	brown	
0.27	blue	section γ (not bitter)
0.22	brown	
0.19	yellow	
0.15	lemon	
0.13	yellow	
0.00	brown	section δ (not bitter)

Furanoid Norditerpene Isolation. *D. bulbifera* tuber (133.5 g) was peeled and cut into cubes (2 cm^2), which were boiled for 10 min. They were blotted dry and macerated in a blender (Sunbeam) with ether (150 mL). The solid was extracted in a Soxhlet apparatus with ether (600 mL) for 2 h. The solid remaining after the ether extraction was reextracted with 95% ethanol (800 mL) for 2 h. After extraction, the tuber was essentially free of bitterness and was palatable. The ether extract was discarded, while the ethanol was removed by evaporation to leave a small aqueous residue (40 mL). This aqueous residue was then extracted with an equal quantity of ethyl acetate. The bitterness was located in the ethyl acetate fraction (2 mL), and subsequent preparative chromatography of this fraction on Merck PLC silica gel 60F_{254} plates developed in chloroform–ethanol–ammonia (100:10:0.5) revealed 18 compounds when visualized at 366 nm (Table I).

Sections α–δ were removed from the PTLC plate and then extracted with hot methanol (40 mL). Each of the four extracts was evaporated under vacuum (≃3 mL), tasted, and left overnight. Section β yielded off-white colored crystals (20 mg) that were recrystallized from ethyl acetate and were bitter.

A melting point of 222–224 °C and $R_f = 0.50$ were recorded for these bitter crystals. The trimethylsilylated derivative was prepared by using Tri-Sil "Z" (Pierce Chemical Co.), while the methyl derivative was prepared with diazomethane (Aldrich). Mass spectra (MS) were obtained of these derivatives, which indicated a molecular weight of 344 amu for the underivatized compound. An ultraviolet maximum (λ_{max}) at 212 nm was observed. Anal. Calcd for $C_{19}H_{20}O_6$: C, 66.27; H, 5.85; O, 27.88. Found: C, 65.5; H, 6.59; O, 27.91.

The infrared spectrum was as follows (KBr, cm^{-1}): 3650–3200 (H_2O?), 3145 (furan), 3000–2830 (aliphatic CH), 1778 (C=O lactone), 1755 (C=O lactone), 1720 (C=O), 1610 (furan), 1515 (C=C), 1465 (-CH_2-), 1395, 1375 (Me), 1345, 1315, 1275, 1250, 1227, 1205, 1190, 1170, 1160, 1145, 1135, 1105, 1082, 1075, 1025 (C—O), 1007, 992 (CK), 965 (CH), 942 (CH), 920 (CH), 905 (CH), 880 (furan), 830 (CH), 810, 800, 780, 755, 720, 680.

Effect of Food Processing. Effects of traditional leaching, boiling, and baking processes on the bitter principles were also investigated. Four small tubers (*D. bulbifera*, 580 g) were each quartered, and a piece was taken from each tuber to give four equivalent batches of

Table II. Results of Investigations

test applied	species: *D. bulbifera* (Australia)	species: *D. hispida* (Thailand)
toxicity		
LD_{50} mice (ip)	nontoxic max dose (4.5 g/kg of body wt)	not tested
alkaloids	negative	positive
cyanide	negative	negative
dioscorine	negative	(1.2 mg of crude/g)
bitterness		
taste	bitter	not tested
alkaloids[a]	negative	positive
steroids[a]	positive	not tested
terpenes[a]	positive	not tested
sapogenins[a]	negative	not tested
polyphenols[a]	positive	not tested
frothing (saponins)	negative	negative
Liberman-Buchard (saponins)	negative	negative
diosgenin	negative	negative
oxidative browning (polyphenols)	positive	negative
polyphenol extraction	negative	not tested
diosbulbin D	(0.07 mg/g)	negative

[a] Chromatographic spray reagents.

tuber (145 g). One batch was baked whole for 2 h at 150 °C in an oven, while another was grated (Sunbeam blender, water), placed in a nylon stocking, and leached with tap water (300 mL/min) overnight. A third batch was boiled whole in distilled water (800 mL) for 15 min. The last batch was kept as a control. After processing, each batch was extracted for furanoid norditerpenes as above, yielding an ethyl acetate fraction (5 mL). Each of these fractions was tasted and chromatographed on Merck TLC silica gel $60F_{254}$ plates in chloroform–ethanol–water (70:30:5), producing brown spots, R_f = 0.88 (authentic diosbulbin D, R_f = 0.88), after visualization with Ehrlich's reagent.

RESULTS AND DISCUSSION

Toxicity Investigations. Screening tests on *D. bulbifera* proved negative for alkaloids and cyanide, and when administered to mice at a maximum dose of 4.5 g (dry weight)/kg of body weight, it was not acutely toxic. *D. hispida*, however, gave a positive result for alkaloids and a negative one for cyanide. The toxicity of *D. hispida* in a wide range of animals has been demonstrated by Leyva and Gutierrez (1937) and so was not tested here.

The brown oily solid isolated from *D. hispida* was examined by MS, TLC, and ^{13}C NMR and shown to be dioscorine after comparison with the literature data (Leete, 1977). The yield of crude dioscorine previously reported from fresh tubers of *D. hispida* ranges from 0.17 mg/g (Leete and Pinder, 1972) to 0.60 mg/g (Leyva and Gutierrez, 1937). Our results (1.2 mg/g) therefore show a relatively high yield.

Bitterness Investigation. Raw *D. bulbifera* tuber was intensely bitter to our palates. A concentrated ether extract of *D. bulbifera* was prepared that contained the bitter component, leaving the residual tissue only faintly bitter. After chromatography of the ether extract, qualitative tests were carried out with the results shown in Table II. Dragendorff reagent failed to identify any alkaloids, confirming the previous field test result. Several steroidal compounds were indicated by the sulfuric acid reagent, while both Ehrlich and antimony (V) chloride reagents suggest the presence of a terpenoid structure. Saponins or sapogenins did not appear to be present in any appreciable quantity as the zinc chloride reagent, frothing, Lieberman-Burchard Test, and diosgenin extraction showed. Working with a Japanese form of *D. bulbifera*, Kawasaki et al. (1968) also found that saponins were not present.

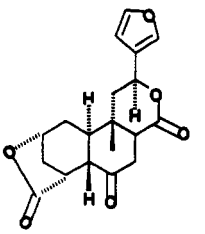

Figure 1. Structure of diosbulbin D.

Table III. Effects of Processing on Diosbulbin D Content

process	tates	amount of diosbulbin D, mg/g of fresh tuber	% diosbulbin D remaining rel to control
control	very bitter	0.068	100
boiling	very bitter	0.068	100
baking	very bitter	0.067	100
leaching	slightly bitter	0.009	14

Some polyphenols were indicated by tin(IV) chloride, vanillin–HCl reagents, and the browning of freshly cut tissue. However, no polyphenols were isolated by the extraction, implying that they occurred in relatively small quantities. Overall, there appeared strong evidence for the existence of a bitter terpene in *D. bulbifera*, possibly small amounts of steroids and polyphenols, but no alkaloids or saponins. Saponins were also not found in *D. hispida*, which agrees with literature findings of Pinder (1953), who analyzed Malayan material.

Preparative TLC of the ethyl acetate fraction yielded a bitter section of five bands at R_f = 0.61, 0.55, 0.50, 0.46, and 0.44 (see Table I). Only one of these compounds (R_f = 0.50) could be isolated in crystalline form. Proton decoupled and coupled ^{13}C NMR spectrum ($CDCl_3$) yielded spectra identical with that of an authentic sample of diosbulbin D. (courtesy of Prof. Tetsuya Komori, Faculty of Pharmaceutical Sciences, Kyushu University, Japan). The structure of the bitter crystals thus isolated was confirmed by MS, IR, UV, and NMR to be the furanoid norditerpene diosbulbin D previously isolated by Ida et al. (1978) (see Figure 1).

Thus, diosbulbin D was found to be a major bitter compound present (yield 0.07 mg/g). Other workers (Ida et al., 1978) have found that the content of various diosbulbins in bitter Japanese *D. bulbifera* ranges from 0.6 μg/g to 0.15 mg/g. Our results are within this range.

Once the presence of a major bitter compound, diosbulbin D, in *D. bulbifera* had been established, our next task was to investigate the effects of aboriginal processing (Table III). These were simulated according to field observations. After processing, each of the batches were assayed for diosbulbin D content, which was monitored by TLC and Ehrlich reagent, relative to an authentic diosbulbin D sample.

Neither the boiling nor baking processes appeared to alter the diosbulbin D content. Each probably served only to soften and open up the tissue and cellular structure, aiding subsequent leaching and increasing palatability. Leaching seemed to be the only process to alter the bitter content, decreasing it to a very low level (under the taste threshold), thereby effectively removing most of the diosbulbin D after 12 h of leaching and rendering the final food palatable.

The implications of these findings are that the cheeky yam (*D. bulbifera* var *rotunda*) is not toxic, but due to its diosbulbin content, it is bitter. This is in contrast to the toxic alkaloid-containing yam (*D. hispida*), which did not contain any of the bitter components detected by our tests. Traditional Australian processing techniques effectively remove the bitter compound. Further research on the distribution of bitter varieties of *Dioscorea* may be useful not only for chemotaxonomy but also for ethnabotanical studies.

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Registry No. Diosbulbin D, 66756-57-8; dioscorine, 3329-91-7.

LITERATURE CITED

Alexander, J.; Coursey, D. G. In "The domestication and exploitation of plants and animals"; Ucko, P. J.; Dimbleby, G. W., Ed.; Duckworth: London, 1969; pp 409–411.
Burkill, I. H. In "Flora Melesiana"; Van Steenis, C., Ed.; Noordhoff: Indonesia, 1954; Vol. 4 (Ser. 1), pp 293–335.
Coursey, D. G. "Yams"; Longmans: London, 1967; pp 6–11.
Culvenor, C. C. J.; Fitzgerald, J. S. *J. Pharm. Sci.* **1963**, *52*, 303–304.
E. Merck. "Dyeing Reagents for Thin Layer and Paper Chromatography"; E. Merck: Darmstadt. West Germany, 1971.
Everist, S. L. "Poisonous Plants of Australia"; Angus and Robertson: Sydney, Australia, 1981; p 29.
Griffin, W.; Owen, W.; Parkin, J. E. *Planta Med.* **1968**, *Feb.*
Ida, Y.; Kubo, S.; Fugita, M.; Komori, T.; Kawasaki, T. *Justus Leibigs Ann. Chem.* **1978**, 818–833.
Julian, P. U.S. Patent 3 019 22, 1962.
Kawasaki, T.; Komori, T.; Setoguchi, S. *Chem. Pharm. Bull.* **1968**, *16*, 2430.
Leete, E. *Phytochemistry* **1977**, *16*, 1705.
Leete, E.; Pinder, A. R. *Phytochemistry* **1972**, *11*, 3219.
Leyva, J. F.; Gutierrez, E. *Phillipp. Med. Assoc. J.* **1937**, *17*, 349.
Martin, F. W. In "Tropical Foods: Chemistry and Nutrition"; Inglett, G. E.; Charalambous, G., Eds.; Academic Press: New York, 1979; pp 249–263.
Martin, F. W.; Ruberte, R. *J. Agric. Food Chem.* **1975**, *23*, 1218.
Pinder, A. R. *J. Chem. Soc.* **1953**, 1825–1828.
Telek, L.; Martin, F. W.; Ruberte, R. *J. Agric. Food Chem.* **1974**, *22*, 334.
Yen, D. E., Department of Prehistory, Research School of Pacific Studies, Australian National University, Canberra, personal communication, 1984.

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High-Performance Liquid Chromatographic Determination and Hydrolysis Studies of Phenyl Phosphorodiamidate, a Urease Inhibitor

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A high-performance liquid chromatographic (HPLC) method was developed for the determination of phenyl phosphorodiamidate (PPDA). This method was employed in a kinetic study of the hydrolysis of PPDA in the pH range of 2–12 at temperatures of 25, 35, and 45 °C. Two parallel competing reactions were observed: The acid-catalyzed reaction produced ammonia and phenyl phosphoramidate; the base-catalyzed reaction yielded phenol and phosphorodiamidic acid. Phenol and phenyl phosphoramidate were also determined by HPLC. Apparent first-order rate constants were determined for the hydrolysis of PPDA as a function of pH and temperature. Activation energies of 9.4 and 18.0 kcal/mol were obtained for the acid- and base-catalyzed reactions, respectively.

Improving the efficiency of urea as a source of fertilizer nitrogen in the developing countries is a major objective of the International Fertilizer Development Center (IFDC). One aspect of this work that has received much attention recently is the addition of a urease inhibitor, such as phenyl phosphorodiamidate (PPDA), to urea applied to flooded rice to reduce the rate of urea hydrolysis and subsequent ammonia volatilization loss. Extensive field trials are presently being conducted in Asia and elsewhere. As part of the support program for the agronomic research currently being conducted, a high-performance liquid chromatographic method has been developed both to determine PPDA and to study its hydrolysis.

Urea hydrolysis to ammonia and carbon dioxide is catalyzed by the enzyme urease present in the soil. It has been shown (Vlek and Craswell, 1979) that rapid hydrolysis of urea applied to flooded rice can cause ammonia volatilization losses of up to 50% of the applied nitrogen. Vlek and Craswell (1981) discussed the factors influencing ammonia volatilization from flooded systems and possible approaches for preventing such losses. One such approach is the use of an additive that would inhibit the urease-catalyzed hydrolysis of urea. Heber et al. (1979) described the use of PPDA as a urease inhibitor for upland crops

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