Elucidation of a new oleanane glycoside from *Barringtonia asiatica*

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Dedicated to Professor Boris A. Trofimov on his 65th birthday

(received 27 Mar 03; accepted 05 Aug 03; published on the web 08 Aug 03)

Abstract

A new oleanane glycoside, ranuncoside VIII, 3-*O*-{[β-D-galactopyranosyl-(1→3)-2-β-D-glucopyranosyl-(1→2)]-β-D-glucuronopyranosyloxy}-21-*O*-{[(2*E*)-2-methyl-1-oxo-2-butenyl]oxy}-22-*O*-(2-methyl-1-oxobutoxy)-15,16,28-trihydroxy-(3β,15α,16α,22α)-olean-12-ene, was extracted from the seeds of the fish-poisoning plant, *Barringtonia asiatica.* This compound, whose structure was determined by HR-FAB-MS and NMR spectroscopy, demonstrated significant piscicidal activity in a model assay.

Keywords: *Barringtonia asiatica*, oleanane glycoside, piscicide, ranuncoside VIII

Introduction

Reports describing the utilization of plants as piscicides (fish poisons) by Polynesian cultures have appeared as early as 1862.[1] In a more recent ethnobotanical study, two plant species, *Tephrosia piscatoria* [Fabaceae] (Pers.) and *Barringtonia asiatica* [Lecythidaceae] (L.) have been specifically mentioned in this regard.[2] The *Tephrosia* species has been extensively studied, and it's piscicidal activity has been ascribed to various biologically active rotenoid compounds isolated from the roots of these plants.[3–5] The most well-known rotenoid natural product is rotenone, which has found commercial application both as a useful insecticide and as a piscicide for controlling unwanted fish species in recreational and other waterways[6] The biological activity of this compound has been ascribed to its ability to inhibit mitochondrial electron transport and consequently block oxidative phosphorylation.[7,8] There has been considerably less published on the *Barringtonia* species and no report on the principle compounds responsible for the reported piscicidal activity.

The seeds of the fruit of *B. asiatica,* (commonly known in Samoa as "futu" or "vutu") are still used by native Polynesian people for fishing purposes, while the use of *T. piscatoria* for fishing has largely been banned.[2] The futu seeds are ground on lava rocks and the resulting pulp in placed in the fishing waters. The bioactive material is leached out of the pulp into the water, and the poisoned fish float up to the surface and are harvested. Human consumption of fish poisoned by *B. asiatica* has no reported negative effects, however ingesting the seed itself, or drinking the poisoned water is reported to have adverse effects.[9] Although other related species *(Barringtonia* spp.) show antibacterial and antifungal activity,[10,11] there is very little work reported on compounds isolated from *Barringtonia asiatica,* except for a very recent report citing two new saponins isolated from the seeds of this plant, neither of which is credited with the piscicidal activity of the seeds.[12] We wish now to report the structure of the principle piscicidal compound present in this species.

Results and Discussion

Bioassay-guided isolation and structural determination of the 1-butanol extract of the *Barringtonia asiatica* seeds led to the discovery of a new compound, ranuncoside VIII, 3-*O*-{[β-D-galactopyranosyl-(1→3)-2-β-D-glucopyranosyl-(1→2)]- β-D-glucurono-pyranosyloxy}-21-*O*-{[(2*E*)-2-methyl-1-oxo-2-butenyl]oxy}-22-*O*-(2-methyl-1-oxobutoxy)-15,16,28-trihydroxy-(3β,15α,16α,22α)-olean-12-ene (Figure 1).

Initial fish toxicity studies at the collection site in Western Samoa confirmed that the bioactivity was associated with the non-water-soluble, 1-butanol fraction. The results of this initial field-assay are shown in Figure 2. All of the fish exposed to the 1-butanol fraction were dead after 5 h. while the water fraction was only slightly toxic. Subsequent toxicity tests were carried out using brine shrimp. Initial preparative HPLC fractions were tested for toxicity on an equal weight basis. The most non-polar fraction again proved to be the most toxic. It appeared that the crude seed extract was considerably more toxic to fish than to brine shrimp. Piscicidal activity was found in several components separated by HPLC, however we carried out structural work only with the most active fraction. Ranuncoside VIII was isolated utilizing preparative HPLC from the fraction determined by the brine shrimp assay to be the most toxic (Figure 3). This compound is, in part, responsible for the observed piscicidal activity of these seeds. No attempt was made at this time to purify and identify any of the other compounds.

The low resolution FAB mass spectrum of ranuncoside VIII yielded two major peaks at approximately 1196 and 672 amu. The molecular ion peak $[M+Na]^+$ was identified at 1196 amu, which corresponds to a molecular weight of 1173. A high resolution MS spectrum established the molecular mass as 1172.5805 amu, corresponding to the molecular formula $C_{58}H_{92}O_{24}$ (calc. 1172.5952). The second, smaller peak which had an exact mass of 672.4527 amu was identical to the *m/z* ratio of a molecular fragment obtained upon acid hydrolysis of the compound. The mass difference between the two peaks, of approximately 501 amu, was attributed to the loss of

hydrolyzable sugar moieties. Gas chromatographic analysis of the silanized (TMS) acid hydrolysis product of ranuncoside VIII confirmed the presence of three sugars whose retention times corresponded to known samples of glucose, galactose, and glucuronic acid. The molecular formula of the aglycone portion of the molecule was then determined by HR-FAB-MS to be $C_{40}H_{64}O_8$ (calc. 672.4584).

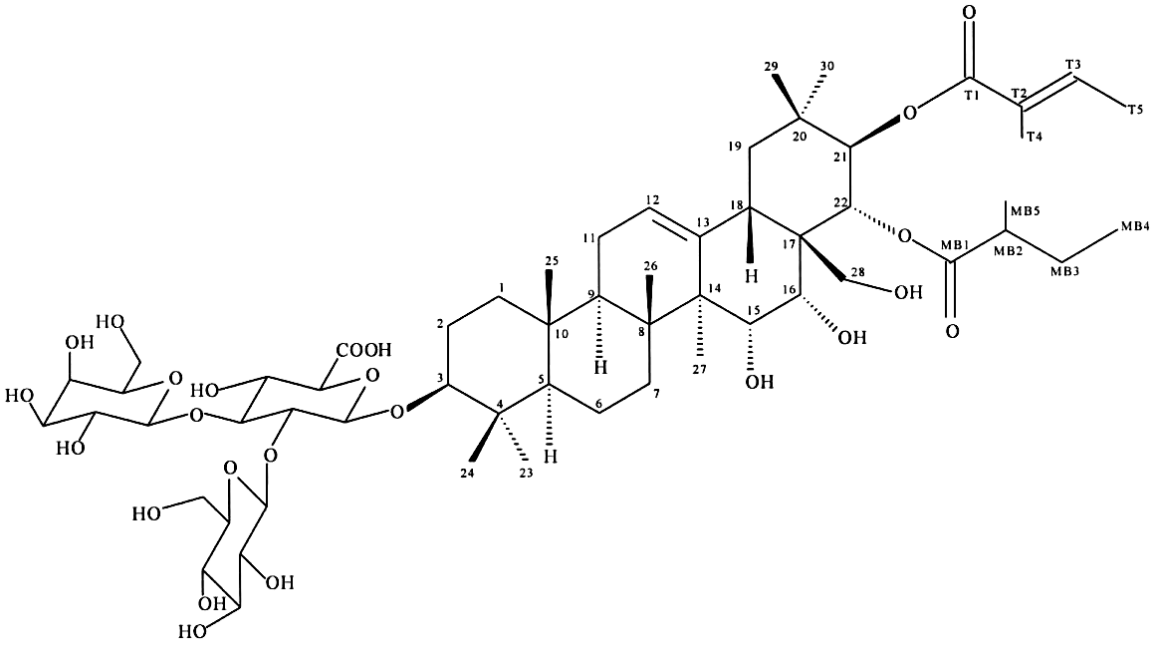

Figure 1

A ^{13}C- NMR spectrum of ranuncoside VIII revealed the presence of 58 resonances (Table 1). Among these are three resonances observed at 105.60, 105.58, and 104.27 ppm, which are consistent with the presence of three sugar anomeric carbons. Three additional resonances seen at 176.96, 172.24, and 168.36 ppm fall in the region normally associated with carbonyl groups of esters or carboxylic acids. Four slightly lower field signals (144.06, 137.52, 129.78, and 125.76 ppm) are indicative of two C=C bonded systems. Comparison of the ^{13}C resonances of the isolated compound with those of the known ranuncoside VI [13,14] which differs from the structure shown in figure 1 only in the side groups, revealed a very close correlation between the oleanane triterpenoid moieties; NMR resonances for carbon atoms 1-30 for the two ranuncosides (corresponding to those associated with the five six-membered rings) are all within 2 ppm of each other (Table 1).

A DEPT experiment showed 26 methine, 10 methylene, and 11 methyl carbon atoms, giving a total of 47 protonated atoms, and 11 quaternary carbons. A HETCOR experiment confirmed the DEPT data and enabled identification of all the respective proton–carbon attachments except for the methylene resonances at 37.06 and 37.28 ppm (Table 1). The ^{1}H signal at 7.11 ppm was

assigned to a proton on the β carbon atom of an αβ-unsaturated carbonyl system. This value, in conjunction with the HETCOR data, was consistent with the presence of a tiglic acid ester. The two relatively low-field proton signals at 6.63 and 6.29 ppm, on carbons- 21 and 22, were identified as methine carbons and were consistent with the presence of two ester moieties. The methine proton signal of H-5 resonating at 0.80 ppm was at an unusually high field position, indicative of an oleanane triterpenoid.[12,14] HMBC data enabled virtually all of the heteronuclear connectivities within the aglycone structure to be established, including the respective attachment sites of the tigloyl and 2-methylbutyryl groups. ROESY analysis confirmed the relative conformations of the agylcone stereogenic centers. Through-space interactions were observed between the following protons: 3 and 23; 5 and 23; 15 and 26,28; 16 and 28; 18 and 21,27; 21 and 30; 22 and 29; 25 and 26. Roesy interactions were also observed between the protons attached to the 1` carbon and the 5` carbon on each sugar thus indicating that each linkage was a β linkage. This was confirmed by the diaxial coupling constants of the three anomeric protons (8, 7.5 and 7.5 Hz)

Table1. NMR Spectral Data of Ranuncoside VIII

Carbon	^{13}C δ(ppm)	DEPT	HETCOR	COSY	Ranuncoside VI
1	37.06	CH_2			39.3
2	27.01	CH_2	2.13, 1.83	3	27.0
3	89.79	CH	3.30	2	89.6
4	39.92	C	-		39.7
5	55.82	CH	0.80		55.8
6	19.12	CH_2	1.83, 1.67		19.1
7	37.28	CH_2			36.8
8	41.79	C	-		41.7
9	47.42	CH	1.68		47.4
10	39.25	C	-		37.2
11	24.31	CH_2	1.87	12	24.3
12	125.76	CH	5.50	11	124.8
13	144.06	C	-		144.4
14	48.11	C	-		48.0
15	67.89	CH	4.20	16	67.6
16	73.46	CH	4.39	15	72.8
17	48.79	C	-		48.6
18	41.19	CH	3.10	19	41.2
19	47.20	CH_2	1.40, 3.06	18	47.1
20	36.85	C	-		36.3
21	79.48	CH	6.63	22	81.3
22	73.35	CH	6.29	21	71.8
23	28.18	CH_3	1.23		28.3
24	17.06	CH_3	1.08		17.1
25	16.12	CH_3	0.82		16.1
26	17.90	CH_3	1.00		17.8
27	21.51	CH_3	1.87		21.4
28	63.22	CH_2	3.51, 3.76	28	63.5
29	29.81	CH_3	1.10		29.8
30	20.39	CH_3	1.31		20.3
tigloyl					(ranuncosideVI)
1	168.36	C	-		168.1
2	129.78	C	-		129.5
3	139.52	CH	7.11	T4	136.2
4	14.55	CH_3	1.68	T3, T54	14.2
5	12.74	CH_3	1.97	T3	12.5
2-methylbutyryl					
1	179.96	C	-		
2	41.88	CH	2.08		
3	27.25	CH_2	1.23, 1.57		
4	12.19	CH_2	0.68		
5	17.52	CH_3	1.00		

Table1. Continue

GlcA				
1′	105.60	CH	4.96	2″
2′	79.36	CH	4.46	1′, 3′
3′	88.07	CH	4.37	2′, 4′
4′	72.16	CH	4.53	3′, 5′
5′	77.56	CH	4.57	4′
6′	172.24	C	-	-
Glc				
1′	104.27	CH	5.67	2′
2′	76.69	CH	4.09	1′, 3′
3′	78.89	CH	4.25	2′, 4′
4′	72.87	CH	4.16	3′, 5′
5′	78.12	CH	3.83	4′, 6′
6′	63.31	CH_2	4.34, 4.43	5′
Gal				
1′	105.58	CH	5.32	2′
2′	73.20	CH	4.52	1′, 3′
3′	75.66	CH	4.14	2′, 4′
4′	70.51	CH	4.46	3′3′
5′	77.69	CH	4.16	3′, 5′
6′	63.70	CH_2	4.35, 4.44	5′

HMBC data were also crucial in establishing the connectivity of the sugar moieties. The HMBC cross peaks clearly showed the connectivity of the three sugar residues in the glycone moiety to be 3-*O*-β-galactopyranosyl(1→3)-[β-glucopyranosyl(1→2)]-β-glucuronopyranosyl. All of the sugars in ranuncoside VIII showed the same relative stereochemistry which was assumed to be the common D form. This result corresponds to the findings recently reported by Herlt *et al*. for two other saponins found in *Barringtonia asiatica*.[12] Furthermore, the ^{13}C- NMR signals of these reported glycones show very close agreement with the sugar portion of ranuncoside VIII.

Experimental Section

General Procedures. All separations were performed on a Rainin HPLC system with Dynamax SD-200 pumps (25 ss) and a UV-DII Dynamax UV/Vis detector. Reverse-phase C-18 analytical (4.6 x 250 mm) and preparative (21.4 x 250 mm) Microsorb columns (5μm particle size, 100 Å pore size), with guard, were used, and the mobile phase consisted of HPLC- grade water and acetonitrile, both acidified with 0.1% trifluoroacetic acid. All mass spectroscopic data were obtained on a JEOL SX 102A double focusing, reverse geometry, high resolution mass spectrometer, with standard software, using FAB-Xenon methodology and a standard sodiated

thioglycerol matrix. Gas chromatographic sugar analysis was performed on a Hewlett Packard 5890A gas chromatograph with a 7673A autosampler, 3393A integrator, and ChromPerfect software, using a Restek Rt_x-1 100% dimethyl polysiloxane column (0.25 μm, 3.0 m, 0.32 mm ID). The samples were silylated and compared with silylated known standards. All NMR spectra were obtained using a Varian VXR-500S spectrometer with standard Varian pulse sequences and parameters, and standard 5 mm tubes and 20 mg samples at ambient temperature in pyridine-d_5.

Materials. Plant material (*Barringtonia asiatica*) used for this study was collected on the island of Upolu near Soluafata, Western Samoa. The seeds were removed from the fibrous mesocarp, placed in 70% ethanol, stored under refrigeration, and shipped to Brigham Young University. Upon arrival at BYU, the seeds were stored at 10°C in 70% ethanol to inhibit fungal growth. A voucher specimen is maintained by the L.D.S. Church College of Western Samoa, Sauniatu, Western Samoa.

Bioassays. The freshwater fish bioassays were conducted using small native fish (mosquito fish) collected from the Sauniatu river (Western Samoa). Each well of a standard 12-well sample plate was filled with river water (5 mL), and three fish were added to each well. Initial biological evaluation of the crude fraction at a concentration of 250 μL mL^{-1} showed that the non-water-soluble, 1-butanol fraction was responsible for most of the activity (Figure 2).

Brine shrimp bioassays were conducted in triplicate.[15] Following the hatching of brine shrimp eggs, a solution (5 mL) containing the brine shrimp was pipetted into individual test tubes and the number of shrimp in each tube was determined. Following removal of the solvent a 100 μg sample of each isolated HPLC peak (1, 2, 3, 4, 5, 6, and 7) was added to each tube. A control set (also in triplicate) was used, in which only water (5 mL) was added to the brine shrimp. The total number of living shrimp in each tube was again measured after 24 hr and compared to the initial number to determine toxicity. Although activity was found in each of the HPLC peaks, peak 7 was the most active (Figure 3).

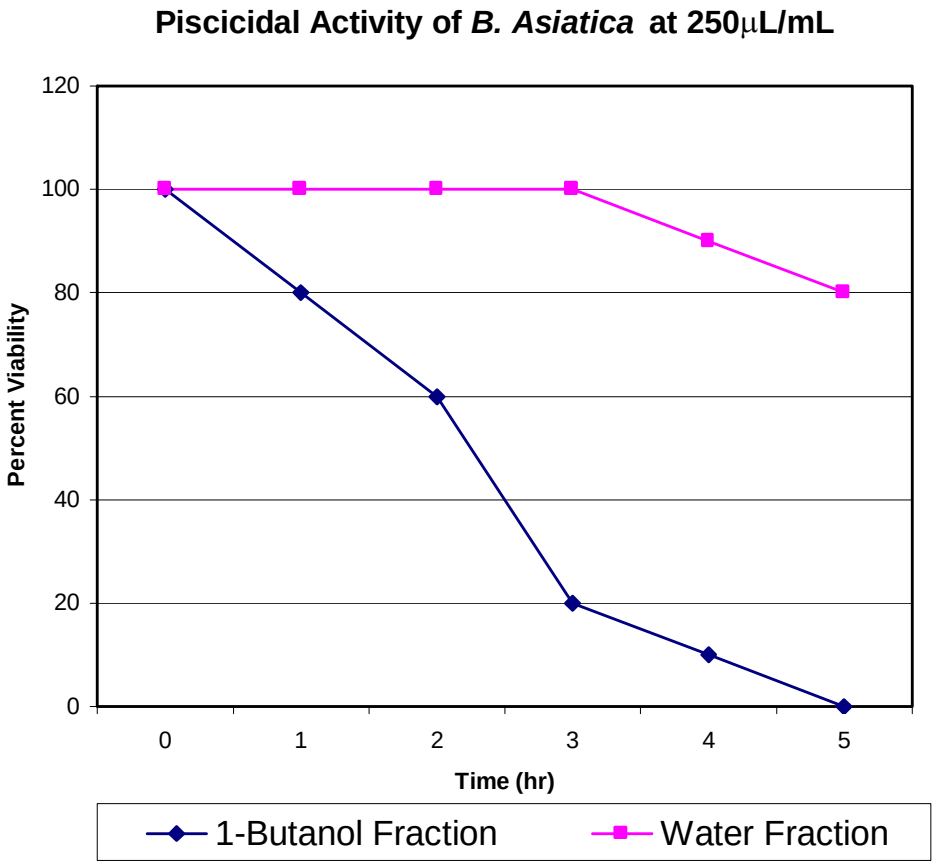

Figure 2

Isolation of Ranuncoside VIII. Five seeds (400 g) of *B. asiatica*, together with the 70% ethanol storage solution (1L) and water (1L) were mixed until homogenized in a Waring blender. The pulp and extraneous plant material were removed by centrifugation, and the supernatant liquid was passed through cheesecloth. The solution was then acidified to pH 2 with trifluoroacetic acid and partitioned three times with equal volumes of 1-butanol. The 1-butanol was removed from the combined fractions, and the resulting residue was exhaustively triturated with hexane. The remaining solid material (5 g) was separated by preparative HPLC (47% isocratic gradient). The sample was introduced onto the column in a 30% isopropanol/water mixture to avoid solubility problems. Seven fractions were obtained, corresponding to seven major chromatographic peaks. The brine shrimp assay revealed that most of the activity was in fraction 7, and this peak was further purified through multiple preparative HPLC runs to yield ranuncoside VIII (20 mg).

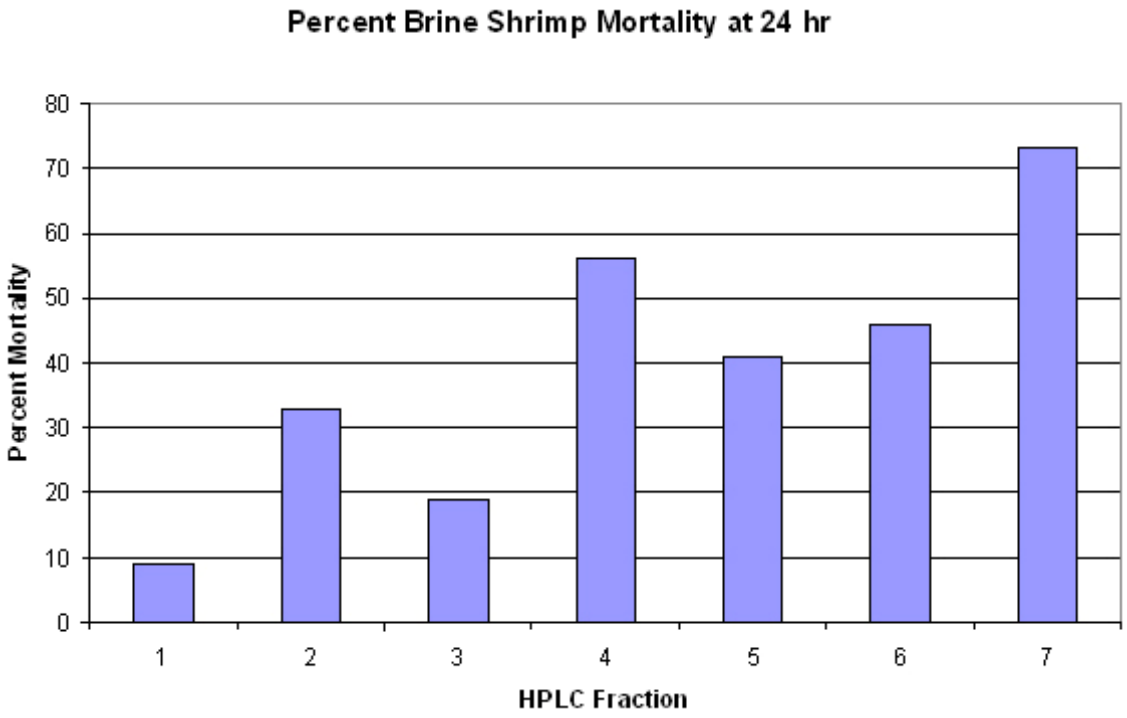

Figure 3

Sugar analysis. A sample of ranuncoside VIII (0.5 mg) was hydrolyzed in 3*M* HCl (0.5 mL) at 100 ^{o}C for 4 hr. The sample was taken to dryness, and the trimethylsilyl sugar derivatives were prepared for GC analysis by adding 50 μL dimethylformamide and 50 μL bis(trimethylsilyl) trifluoroacetamide containing 1% chlorotrimethylsilane to the dry powder, followed by heating at 70 ^{o}C for 15 min.

Acknowledgments

We are grateful to Brigham Young University for financial support and to Du Li and Bruce Jackson for help with the NMR and the MS experiments, respectively.

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