Bioactivity-Directed Isolation and Characterization of Quinoside A: One of the Toxic/Bitter Principles of Quinoa Seeds (*Chenopodium quinoa* Willd.)

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Quinoa is a hardy and nutritious South American food crop. Before consumption, aqueous extraction of quinoa seeds is necessary to remove bitter principles that have never been completely characterized. Guided by a brine shrimp lethality bioassay and a simple taste for bitterness, aqueous extracts of the whole seeds were fractionated chromatographically in an attempt to isolate the toxic/bitter principles. These undesirable compounds appear to be a mixture of saponins whose acidic hydrolysis gave oleanolic acid and hederagenin (3:1) as the only detectable saponin aglycons by GC/MS analysis. One of these saponins (quinoside A) has been identified as a tetraglycoside of hederagenin; the proposed structure (1) is named olean-12-ene-28-oic acid, 3,23-bis(*O*-β-D-glucopyranosyloxy)-*O*-β-D-glucopyranosyl-(1→3)-*O*-α-L-arabinopyranosyl ester (3β,4α).

Quinoa, *Chenopodium quinoa* Willd. (Chenopodiaceae), has been called one of the "wonder plants of the Incas" (Mahoney et al., 1975). Few crops are so resistant to harsh conditions such as drought, moderate frost, high altitude, poor soil, salinity, and pests; it requires minimal care during its growth. The seeds are used in making soups or are ground into flour to prepare breads, cakes, and beer; younger leaves are also consumed in salads (Simmonds, 1965; Weber, 1978; Cardoza and Tapia, 1979). Quinoa seeds have a higher nutritive value than most cereal grains; they average 12–14% in protein rich in essential amino acids, resulting in a protein quality equivalent to that of whole dry milk (White et al., 1955; Van Etten et al., 1963; Mahoney et al., 1975). Yet, other than among the Andean Indians, quinoa is practically unknown and is not utilized as a food grain.

Many varieties have been developed (White et al., 1955; *Plant Genet. Res. Lett.*, 1979), but almost all possess a single major disadvantage—bitterness. The seed coats contain, likely as a protection against insects and birds, unknown principles that have a very bitter taste and must be removed by aqueous washing prior to consumption. Most often the bitterness of quinoa has been attributed to saponins, and a method of saponin analysis, based on hemolysis of red blood cells, has been reported (Aguilar et al., 1979). The common triterpene oleanolic acid has been identified by GC in acidic hydrolysates of quinoa seed extracts (Augusto and Amaya, 1979), and more recently in roots as well as in vitro tissue cultures of Real de Puno and Blanca de Junin varieties (Burnouf-Radosevich and Paupardin, 1983).

This paper reports the isolation and characterization of one of the bitter principles, quinoside A, through bioactivity-directed fractionation of the aqueous seed extracts. A bioassay for lethality in brine shrimp (Meyer et al., 1982), as well as a simple taste test for bitterness, was employed to assist in monitoring the partitionings and chromatographic separations.

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EXPERIMENTAL SECTION

Plant Material. A large quantity (100 lb) of commercial *C. quinoa* seeds (assigned USDA Accession No. 63651) was obtained from Luis Levy, Inexa Industria Extracta, C.A., Quito, Ecuador; this material was used for the fractionation studies; this material and the variety Real de Puno from southern Peru were used for total hydrolysis of the corresponding crude saponins to identify the aglycons with GC/MS.

Brine Shrimp Lethality Bioassay. Samples were prepared in brine at 1000, 100, and 10 μg/mL (or intermediate dilutions, as needed), and the bioassay was performed, essentially as described previously (Meyer et al., 1982), to give LC_{50} values expressed in micrograms per milliliter.

Tests for Bitterness. Samples were solutions or suspensions of about 500 μg/mL of the extracts in double-distilled water. The tongue was rinsed briefly before each test to provide a control for the taste of the water. Approximately 0.1 mL of sample was dripped onto the tongue and expelled after 3 s. Liberal rinsing with tap water served to remove traces of sample. A waiting period between tests was sometimes necessary to allow a taste to dissipate. Samples were scored subjectively according to the following scale: B–, not bitter; B±, slightly bitter; B+, definitely bitter; B++, intensely bitter.

Analytical Techniques. Gas chromatographic conditions were as follows: (a) 8 ft, 15% butanediol succinate on Supelcoport 100/120, oven temperature 195 °C, injector temperature 240 °C, detector temperature 300 °C, flow rate 22 mL/min N_2; (b) 8 ft, 25% diethylene glycol succinate on Supelcoport 100/120, oven temperature 190 °C, injector temperature 240 °C, detector temperature 300 °C, flow rate 22 mL/min N_2; (c) 6 ft, 3% OV-101 on Gas Chrom Q 100/120, oven temperature initially 250 °C increasing at 4 °C/min to 300 °C, injector temperature 260 °C, detector temperature 340 °C, flow rate 26 mL/min He. GC experiments were performed on a Varian Aerograph Series 1400 (conditions a and b) or a Bendix Model 2600 (condition c) with flame ionization detectors.

GC/MS experiments were performed on a Finnigan 4000 (condition b except 6-ft column, 20 mL/min He) with chemical ionization (isobutane) or on a Kratos MS 30 (condition c except 3-ft column) with electron impact ionization. Additional mass spectra were obtained on a Finnigan 4000. Field desorption mass spectra were measured at Eli Lilly and Co. on a Varian-MAT Model 731 using carbon dendrite emitters with an emitter current of 21 mA. The droplet countercurrent chromatograph (DCCC) was a Rikakikai Model S (Tokyo, Japan); the

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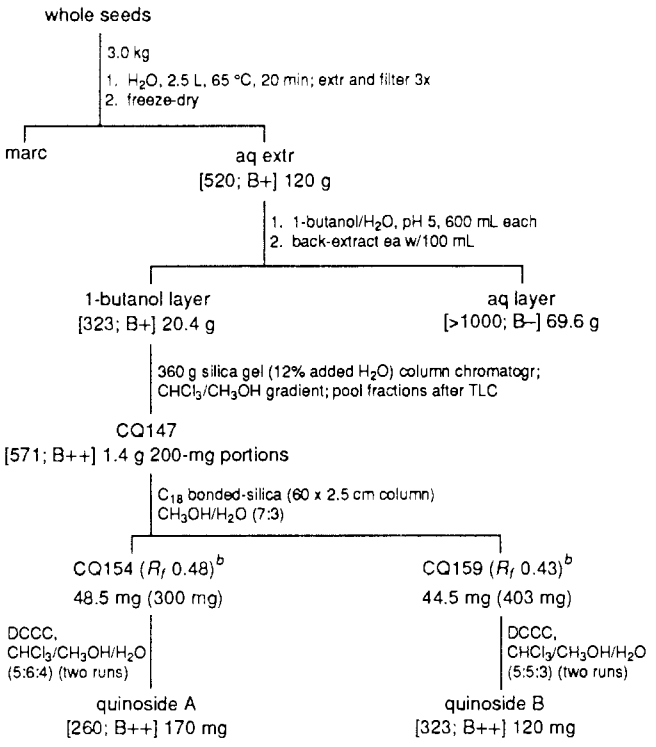
Scheme I. Fractionation of Quinoa Extracts To Yield Toxic/Bitter Saponins[a]



[a] Brine shrimp LC_{50} values (Meyer et al., 1982) and relative bitter tastes (see the Experimental Section) are given in brackets and were used to guide the fractionation. [b] TLC R_f on silica gel; ethyl acetate/CH_3OH/H_2O (8:2:1).

ascending mode was utilized with chloroform/methanol/water (5:6:4, 5:5:3).

^{13}C NMR spectra of hederagenin were measured on a Nicolet NTC-200 in pyridine-d_5.

TLC analysis of the saponins were performed on (A) silica gel plates with ethyl acetate/methanol/water (8:2:1) and (B) Whatman K C_{18} (reversed-phase) plates with methanol/water (7:3), sprayed with acetic anhydride/H_2SO_4. Sapogenins were chromatographed on (C) silica plates developed with toluene/ethyl acetate/formic acid (57:40:3). Spots were visualized with acetic anhydride/H_2SO_4. TLC systems used to identify sugars were (D) acetone/methanol/chloroform/water (15:2:2:1) on silica gel, (E) 2-butanone/acetic acid/methanol (3:1:1) on silica gel, (F) ethyl acetate/acetic acid/water/methanol (15:3:2:3) on silica gel, (G) 2-propanol/water (17:1) on silica gel, (H) 1-butanol/pyridine/water (2:2:1) on cellulose, and (I) formic acid-2-butanone/*tert*-butyl alcohol/water (3:6:8:3) on cellulose, sprayed with α-naphthol/H_2SO_4 or aniline phthalate.

Bioactivity-Guided Fractionation. The fractionation procedure is summarized in Scheme I. Warm (65 °C) aqueous extracts of whole quinoa seeds (3 kg) produced, after freeze-drying, a residue that was bitter and significantly toxic to brine shrimp. Partitioning of the residue between 600 mL each of 1-butanol and water (pH 5) concentrated the activity in the butanol. Column chromatography on deactivated silica gel with a 2–40% stepwise gradient of methanol in chloroform yielded over 1500 8-mL fractions, which were pooled after TLC analysis (system A). Pools of fractions 181–820 were lethal to the shrimp (LC_{50} < 600 μg/mL); the largest of these pools (CQ147, eluted with ca. 12.5% methanol in chloroform) was subjected, in 200-mg portions, to a low-pressure C_{18} bonded-phase silica gel column (60 × 2.5 cm) and eluted with methanol/water (7:3). Again, 100 8-mL fractions were collected, and two pools of these (CQ154, CQ159, fractions 14–24 and 58–70, respectively) concentrated the bitterness and the brine shrimp toxicity. Each of these contained one major component that was purified by DCCC in the ascending mode with chloroform/methanol/water (respectively, 5:6:4 and 5:5:3). Analysis of each of the purified compounds gave a single spot on normal-phase (system A) as well as reversed-phase (system B) TLC.

Total Saponin Extraction and Hydrolysis. Ground seeds were defatted with ethyl ether in a Soxhlet apparatus for 6 h. Crude saponins were extracted from the marc in a Soxhlet apparatus with three successive extractions using 80% butanol/80% ethanol in the concentration order 1:2, 1:1, and 2:1, respectively. The combined extracts were evaporated to dryness and redissolved in 80% butanol/80% ethanol (1:1). The saponins were precipitated with ethyl ether.

The saponin mixture was hydrolyzed by refluxing in 2 N HCl for 5 h. The insoluble material was recovered by filtration, rinsed with H_2O, dried at 60 °C, and extracted with methanol in a Soxhlet apparatus for 3 h. The resulting sapogenin mixture and reference compounds were derivatized by dissolving in pyridine and adding bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane. Derivatized samples were analyzed by GC or GC/MS using condition c.

Acid Hydrolysis. Quinoside A (54 mg) was acid-hydrolyzed by refluxing in 5 mL of 4 N HCl for 2 h. The aqueous solution, after filtering, was neutralized with Amberlite IRA (OH^-) ion-exchange resin (15 g) and examined by TLC (systems D–I) for the presence of sugars. The aglycon (6 mg) was acetylated in acetic anhydride–pyridine (1:2) (1.5 mL) or trimethylsilylated with bis(trimethylsilyl)trifluoroacetamide/chloroform/pyridine (2:1:2) (1.25 mL), and mass spectra were obtained.

Alkaline Hydrolysis. Quinoside A (15 mg) was heated to 90 °C for 40 min in 2 mL of 5% aqueous NaOH. The mixture was cooled and extracted with 1-butanol saturated with water. This extract was evaporated to dryness, redissolved in methanol, and examined by TLC (system A). The aqueous layer was neutralized using Dowex 50W (H^+) ion-exchange resin (10 g), evaporated to dryness, and hydrolyzed by refluxing for 1 h in 2 mL of 4 N HCl. After neutralization with Amberlite and reduction of the volume, the solution was examined by TLC (systems D–I) for the presence of sugars.

Enzymatic Hydrolysis. An 8-mg portion of quinoside A was shaken at 37 °C in 2.5 mL of H_2O and 2.5 mL of pH 6.8 buffer (Fisher pH 7.0 buffer adjusted with H_3PO_4) with 20 units of α-glucosidase (maltase, Sigma Type III). Toluene was added to each mixture to inhibit microbial involvement. Incubations lasted 4 days.

At 24-h intervals, 0.5-mL aliquots were withdrawn and extracted three times with 0.5 mL of 1-butanol saturated with water. The butanol layer was evaporated to dryness and analyzed for sapogenins by TLC (system C). The aqueous layer was reduced in volume and examined by TLC (systems D–I) for the presence of sugars.

Permethylation and Methanolysis. Quinoside A (40 mg) was permethylated by the Hakomori method (Hakomori, 1964; Conrad, 1972). The methylated saponin was refluxed in 2 mL of 5% hydrogen chloride in methanol for 2 h. The resulting solution was neutralized with Amberlite 401S (OH^-), evaporated to dryness, and redissolved in absolute ethanol for GC examination (conditions b and c).

Reference Methyl *O*-Methyl-D-glucosides. Maltose (300 mg) was methylated by the Hakomori method. Following methanolysis and neutralization, the four major products were separated by column chromatography on silica gel (20 g) in a gradient of ethyl ether in toluene. Mass spectra distinguished the anomers of methyl 2,3,4,6-tetra-*O*-methyl-D-glucoside from those of methyl 2,3,6-tri-*O*-methyl-D-glucoside. Compounds with the higher R_f values on normal-phase TLC (systems D–G) were presumed to be the β-anomers (Wing and BeMiller, 1972).

Gentiobiose was similarly methylated and methanolyzed, but the products were not separated. The identities of the anomers of methyl 2,3,4,6-tetra-*O*-methyl-D-glucoside were established by TLC comparisons (systems D–I) with the previously prepared material. The two remaining major components were presumed to be the anomers of methyl 2,3,4-tri-*O*-methyl-D-glucoside.

RESULTS AND DISCUSSION

The aglycon of quinoside A was identified as hederagenin on the basis of mass spectra of the diacetate and

Table I. ^{13}C Chemical Shifts (ppm) of Quinoside A Aglycon (Hederagenin)

carbon no.[a]	pyridine-d_5
1	38.8
2	27.7
3	73.6
4	42.9
5	48.7
6	18.6
7	33.3
8	39.8
9	48.2
10	37.3
11	23.8
12	122.61
13	144.9
14	42.2
15	28.4
16	23.8
17	46.7
18	42.0
19	48.3
20	31.0
21	34.3
22	33.0
23	68.1
24	13.12
25	16.0
26	17.5
27	26.2
28	180.2
29	33.3
30	23.8

[a] Assignments in $CDCl_3$ have been made by Tori et al. (1974). Assignments in pyridine-d_5 are tentative and are based on chemical shift considerations.

trimethylsilyl derivatives and comparison of the ^{13}C NMR chemical shifts (see Table I) with published values (Tori et al., 1974). This identification was supported when a total saponin hydrolysate was analyzed by GC/MS. This mixture, in both quinoa accessions, consisted entirely of oleanolic acid and hederagenin in a ratio of approximately 3 to 1. Other sapogenins were not present in sufficient quantity to be detected.

Field desorption mass spectrometry gave the molecular weight of quinoside A as 1090 after subtraction of the weight of a sodium atom (23). Two losses of a hexose (M + 23 − 162; M + 23 − 324) were explicit. The remaining mass could be accounted for by hederagenin, one hexose, and one pentose. This was consistent with the observation of glucose and arabinose detected by TLC of the acid hydrolysate and suggested the presence of three glucose units and one arabinose.

Following permethylation and methanolysis of quinoside A, GC/MS (condition b) showed four sugar species to be present. Two of these (RRt = 0.72, 1.00) were the anomers of methyl 2,3,4,6-tetra-*O*-methylglucoside as shown by comparison with synthetic material. The remaining two peaks (RRt = 1.75, 1.84) had identical mass spectra (M − 31 = 161) and were apparently due to the anomers of methyl di-*O*-methylarabinoside; authentic material was not available, but the retention times (condition a, RRt = 1.75, 1.84) were significantly different from those reported for methyl 3,4-di-*O*-methylarabinoside (RRt = 1.39, 2.78) (Kizu and Tomimori, 1979) under similar conditions. Retention times (RRt) reported here were relative to methyl α-2,3,4,6-tetra-*O*-methyl-D-glucoside.

There is evidence from studies using trideuteriomethyl derivatives of permethylated arabinose (Kochetkov and Chizhov, 1965) that 60% of the ion intensity at *m/z* 143 (M − 63) is due to the loss of 1-OCH_3 followed by

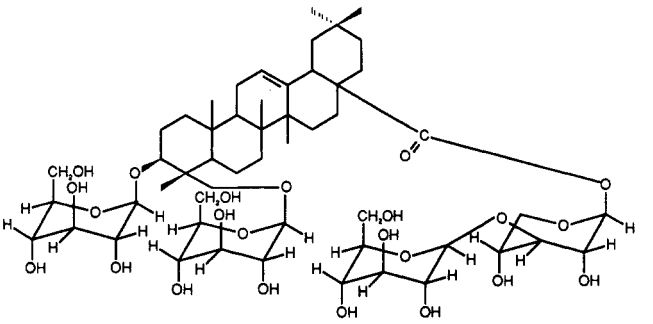


Figure 1. Quinoside A (1).

3-OCH_3 as methanol. In the spectra of the unknown methyl di-*O*-methylarabinoside, *m/z* 143 was twice as intense as *m/z* 129. These peaks were interpreted as being due to the loss of 1-OCH_3 followed by 3-OH as water (*m/z* 143) or by 2/4-OCH_3 as methanol (*m/z* 129). Thus, arabinose in quinoside A would have to be attached to glucose through position 3.

The ratio of the combined areas of GC peaks due to methyl tetra-*O*-methylglucoside to those of methyl di-*O*-methylarabinoside was about 3 to 1. These data suggested the presence of three terminal glucose units and one substituted arabinose. Distribution of these sugars about the hederagenin aglycon requires glycosylation of all three available sites, i.e., the 3- and 23-hydroxyl and the 17-carboxyl groups.

Alkaline hydrolysis of quinoside A resulted in a prosapogenin. This butanol-soluble hydrolysis product reacted positively with α-naphthol–H_2SO_4 spray reagent, indicating that the compound still possessed sugar moieties. The water-soluble fraction of the hydrolysis product in which no monosaccharides were detected could be further hydrolyzed in acid to yield glucose and arabinose, according to TLC (systems D–I).

Incubation of quinoside A with maltase produced no new compounds. Incubation with emulsin produced a compound identical with the base-hydrolyzed saponin plus glucose and arabinose. This result established that D-glucose was in β-linkage to the arabinose, but the cleavage of arabinose was unexpected. The glycon specificity of emulsin is well-known (Barman, 1969), but reports on the behavior of an acylglycoside in the presence of this enzyme are not evident in the literature.

The configuration of the remaining glucose linkages and that of the arabinose are not established, nor are the absolute configurations of these sugars proven. The enzyme experiments were repeated with 20% dimethyl sulfoxide since this was found not to affect the activities of the enzymes with standard compounds; the results were the same, the enzyme apparently failing to attack two of the glucose units. In the absence of other evidence, the glucose units are tentatively proposed to be β-D. Likewise, the arabinose is proposed to be α-L.

Based on the above results, the proposed structure of quinoside A is olean-12-ene-28-oic acid, 3,23-bis(*O*-β-D-glucopyranosyloxy)-*O*-β-D-glucopyranosyl-(1→3)-*O*-α-L-arabinopyranosyl ester (3β,4α) (Figure 1) (1). According to reviews (Hiller and Voight, 1977; Hiller and Adler, 1982) only monodesmoside and bisdesmoside triterpenes are common; however, astragaloside VII (Kitagawa et al., 1983) set the precedent for triterpene tridesmosides. Data presented here support the fact that quinoside A is trisubstituted at the 3-, 23-, and 28-positions of hederagenin.

A glycoside of hederagenin was previously reported in only one other member of the Chenopodiaceae, *Spinacia*

oleracea L., which also contains a glycoside of oleanolic acid (Tschesche et al., 1969). Two glycosides of echincystic acid from *Chenopodium anthelminticum* have been characterized: one is an acyl glycoside (Chirva et al., 1971). No evidence was found for the presence of echinocystic acid, ursolic acid, or erythrodiol in the seeds of *C. quinoa*.

The unidentified saponin, quinoside B, is yet to be characterized, having been isolated in insufficient quantity for complete structural elucidation. Work on quinoside B and on additional toxic/bitter saponins is continuing. The presence of uncharacterized saponins in *Chenopodium* has been reported previously (Gonnerman, 1919; Dafert et al., 1934; Greifinger, 1934; Roberg, 1937). It is believed that the bitter taste of the seeds can now be confidently attributed to the saponins, one of which has been characterized. Additional bitter saponins are present and are probably glycosides of oleanolic acid.

The brine shrimp assay (Meyer et al., 1982) is expected to detect physiologically active compounds; its sensitivity to the bitter compounds of *Chenopodium* was not surprising since saponins are well-known to be toxic to cold-blooded aquatic animals. The establishment of isolation methods and the determination of the chemical identities of the quinosides will, hopefully, permit their toxicologic evaluation in higher animals and man. In addition, chemical identification of the quinosides will facilitate development of specific assays for selection and development of additional low saponin containing varieties of the grain.

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