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FOETIDISSIMOSIDE A, A NEW 3,28-BIDESMOSIDIC TRITERPENOID SAPONIN, AND CUCURBITACINS FROM *CUCURBITA FOETIDISSIMA*

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Key Word Index—*Cucurbita foetidissima*; Cucurbitaceae; 3,28-bidesmosidic triterpene saponin; cucurbitacins; structure analysis.

Abstract—A new 3,28-bidesmosidic triterpenoid saponin, foetidissimoside A, was isolated from the roots of *Cucurbita foetidissima* and its structure established as echinocystic acid 3-*O*-β-D-glucuropyranoside 28-*O*-[β-D-xylopyranosyl (1→4)α-L-rhamnopyranosyl(1→2)]-α-L-arabinopyranoside mainly by ^{1}H NMR, ^{13}C NMR, GC/MS and FABMS analyses. Cucurbitacin B and E with their glucosides, cucurbitacin D and isocucurbitacin B were identified by HPLC analysis.

INTRODUCTION

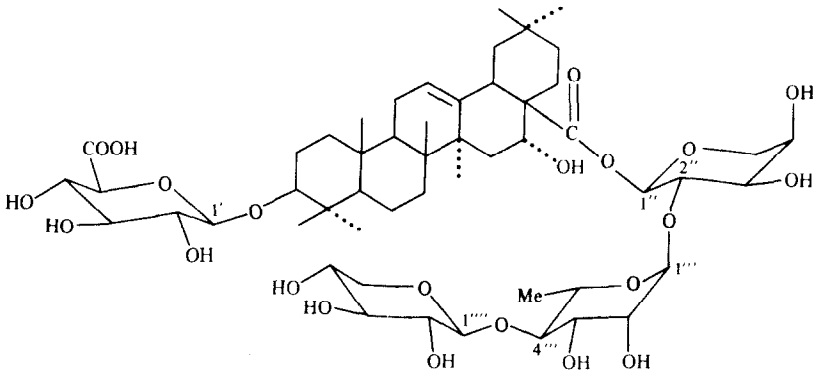
Cucurbita foetidissima H. B. K. (*C. perrenis*, A. Gray, *Cucumis perrenis* E. James) commonly called calabazilla or buffalo gourd is an indigenous plant to Texas, Arizona, New Mexico and California. Cultivation of the plant is of interest because the seeds contain a high amount of protein and oil [1]. It is also characterised by a huge root weighing up to 50 kg which contains up to 50% starch. The root was used as a soap substitute and as a fish poison by the Indians and Spanish Californians. This led to the assumption that these properties are due to the presence of saponins [2]. A preliminary investigation of an alcoholic extract of the roots exhibited some pharmacological properties: oxytocic action, spasmolytic effect on the isolated intestine of rat and mouse, constriction of the coronary vessels of the rat heart and irritating effects [3]. The literature however reveals very little on the chemical constituents of the root except that it contains starch and cucurbitacins [4, 5]. In the present paper, we report on the isolation and structural elucidation of the major saponin from the root of *Cucurbita foetidissima* and on the identification of the cucurbitacins by HPLC analysis [6].

RESULTS AND DISCUSSION

A methanol extract of the roots of *Cucurbita foetidissima* was extracted successively with chloroform and *n*-butanol. The latter extract purified by precipitation with ether showed the presence of three main spots in the R_f range of 0.25–0.6 on silica gel TLC (developed with $CHCl_3$–MeOH–AcOH–H_2O, (60:32:12:8) [7]) and detected with Komarowsky- and Nile blue reagents. This extract was resolved by analytical HPLC into three main peaks [7]. A combination of flash chromatography on normal phase silicagel and reversed phase (RP-8) material and centrifugal thin layer chromatography [8] resulted in the isolation of three saponins one of them in major amount which has been assigned the name foetidissimoside A. The isolated saponins analysed by HPLC showed the same R_t as the three peaks mentioned above.

Foetidissimoside A was a white, amorphous compound which exhibited an intense molecular ion peak $[M-H]^-$ at *m/z* 1057 indicating a M_r of 1058. Signals at *m/z* 925 $[M-H-132]^-$, 779 $[M-H-132-146]^-$, 647 $[M-H-132-146-132]^-$ and 471 $[M-H-132-146-132-176]^-$ corresponded to the consecutive losses of the pentosyl, rhamnosyl, pentosyl and glucuronic acid units. Broad IR absorption bands at 3400, 1725 and 1610 cm^{-1} showed that foetidissimoside A was an ester glycoside bearing also a free carboxylic group. After acid hydrolysis, foetidissimoside A yielded echinocystic acid (IR, MS and co-TLC with an authentic sample) and rhamnose, xylose, arabinose, (co-TLC with authentic samples) in a molar ratio of 1:1:1 as estimated by GLC after conversion into their alditol acetates and glucuronic acid (co-TLC with an authentic sample). Hydrolysis with 0.5 N KOH yielded a prosapogenin which furnished by further acid hydrolysis glucuronic acid and echinocystic acid (co-TLC with an authentic sample).

These data indicate that the glucuronic acid is linked by its hemiacetalic group to the aglycone at C-3 or C-16 whilst the other sugars must be bound to the genine by a glycosidic ester linkage at C-28. The presence of a signal at δ89, 1 in the ^{13}C NMR spectrum agreed with the glycosylation at the C-3 hydroxyl in accordance with published data of 3-*O*-glucosyl echinocystic acid [9]. Therefore the compound had to be a 3,28-bidesmosidic saponin. The interglycosidic linkage was established by the permethylation/sodium borodeuteride/GC/MS method [9, 10] and by partial acidic and enzymatic hydrolysis. The saponin, methylated by Hakomori's method [11] was hydrolysed with 2N trifluoroacetic acid. The resulting mixture of methylated monosaccharides was subjected to reduction with sodium borodeuteride followed by acetylation. The GC/MS analysis of the product [12] indicated the formation of 1,2,5-tri-*O*-acetyl 3,4-di-*O*-methylpentitol, 1,4,5-tri-*O*-acetyl-6-deoxy-2,3-di-*O*-methylhexitol, 1,5-di-



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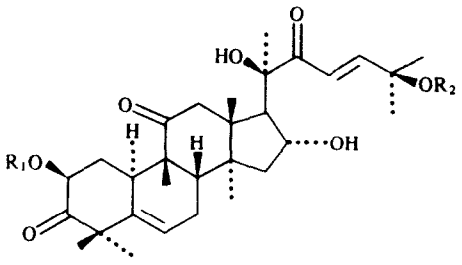
O-acetyl 2,3,4-tri-*O*-methylpentitol and 2,6-di-*O*-acetyl 3,4,5-tri-*O*-methyl gulonic acid methylester which correspond to 1→2 linked pentose, 1→4 linked rhamnose, terminal pentose and terminal glucuronic acid respectively (Table 1). These results precluded the possibility of a branched saccharide moiety and confirmed that glucuronic acid is bound to the aglycone with the hemiacetalic group.

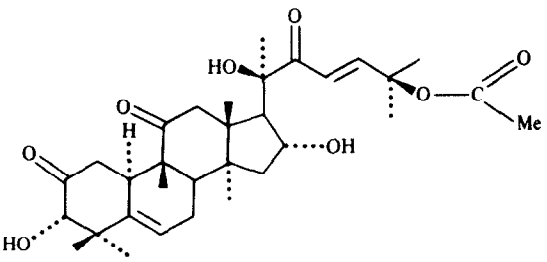
Enzymatic hydrolysis of the saponin with β-D-glucuronidase for 24 hr at 37° yielded a saponin A-1 which after acid hydrolysis gave arabinose and rhamnose only (TLC comparison with authentic sample). These results showed that enzymatic hydrolysis eliminated β-D-glucuronic acid and the terminal β-D-xylose. The discrimination between the linkage types 28-CO-arabinosyl (1→2) rhamnosyl (1→4) xylose and 28-CO-rhamnosyl (1→4) arabinosyl (1→2) xylose was possible by identification of the anomeric carbons in the ^{13}C NMR spectrum of foetidissimoside A (Table 2) by comparison with data of the literature. The signal of the anomeric carbon at δ93, 7 was assigned to the disubstituted arabinose attached to the C-28 carboxyl group of echinocystic acid [13]. The other anomeric carbon signals at δ101.1, 106.7 and 106.9 were assignable to those of a disubstituted rhamnopyranose, a terminal glucuropyranose and a terminal xylopyranose, respectively. In the ^1H NMR spectrum of the saponin in DMSO-d_6 + CF_3COOD, the anomeric proton signals δ4.27 (1H, *d*, *J* = 7 Hz), 4.46 (2H, *d*, *J* = 5 Hz) and 5.55 (1H, *s*) led to the assignments of the anomeric configuration of its glucuropyranoside and xylopyranoside units as β and that of its arabinopyranoside and rhamnopyranoside unit as α. This was supported by the shifts of its carbon signals (Table 2). Therefore the structure proposed for the foetidissimoside A is echinocystic acid 3-*O*-β-D-glucuropyranoside 28-*O*-[β-D-xylopyranosyl (1→4) α-L-rhamnopyranosyl (1→2)] α-L-arabinopyranoside (**1**). Several publications have reported the distribution of echinocystic acid glycosides in plants [14–17].

The presence of echinocystic acid in Cucurbitaceous plants was previously reported in *Echinocystis fabacea* [2] and in *Marah macrocarpus* [18]. The foetidissimoside A however seems to be the first example of a saponin which contains an echinocystic acid unit with glucuronic acid attached to it. The other two saponins B and C are also derivatives of echinocystic acid with glucuronic acid, glucose, rhamnose, xylose and arabinose as sugars moities.

Other oleanane-saponins have been reported in the *Cucurbitaceae* family, in particular in *Momordica* sp. [14, 19], *Luffa* sp. [14, 16] and *Hemsleya* sp. [20]. From *Gymnostemma* damarane saponins have been isolated which are closely related to the major saponins of *Panax ginseng* (*Araliaceae*) [20].



2 R_1 = H; R_2 = H
3 R_1 = H; R_2 = Ac
4 R_1 = Glucose; R_2 = Ac



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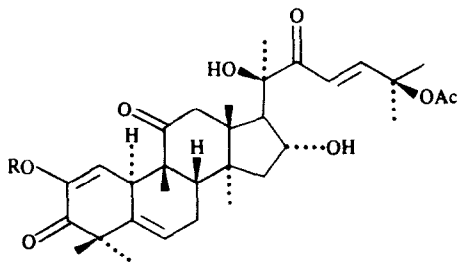

6 R = H
7 R = Glucose

Table 1. Mass spectral data for partially methylated alditol acetates of **1**

	R_t(min)	MS fragments (*m/z*)
1,5-di-*O*-Acetyl-2,3,4-tri-*O*-methylpentitol	11.45	59, 71, 101, 117, 130, 162
1,4,5-tri-*O*-Acetyl-6-deoxy-2,3-di-*O*-methylhexitol	14.04	59, 71, 87, 102, 118, 143, 162, 203
1,2,5-tri-*O*-Acetyl-3,4-di-*O*-methylpentitol	15.08	57, 71, 88, 101, 117, 161, 190
2,6-di-*O*-Acetyl-3,4,5-tri-*O*-methyl gulonic acid methylester	24.10	59, 72, 85, 102, 133, 175, 204, 232

Table 2. ^{13}C NMR spectral data of foetidissimoside A (**1**) measured in pyridine-d_5 (TMS as internal standard)

Echinocystic acid C	δ		Sugars C	δ
1	39.6	glucuronic acid	1′	106.7
2	27.3		2′	75.6
3	89.1		3′	78.6
4	40.2		4′	71.1
5	56.1		5′	78.4
6	18.7		6′	171.8
7	33.3[a]	arabinosyl 1,2	1″	93.7
8	39.6	disubstituted	2″	76.1
9	47.3		3″	70.1
10	37.1		4″	70.3
11	24.9		5″	62.8
12	122.8	rhamnosyl 1,4	1‴	101.1
13	144.5	disubstituted	2‴	72.2
14	41.4		3‴	72.7
15	36.1[b]		4‴	83.7
16	74.1		5‴	68.7
17	49.7		6‴	18.4
18	42.2	terminal xyloxyl	1⁗	106.9
19	47.3		2⁗	75.2
20	31.0		3⁗	78.1
21	36.3[b]		4⁗	71.0
22	32.1		5⁗	67.5
23	28.4			
24	15.8			
25	17.1[c]			
26	17.7[c]			
27	28.4			
28	175.9			
29	33.5[a]			
30	24.9			

[a, b, c] Chemical shifts may be interchanged.

The saponin extract of *Cucurbita foetidissima* was screened for its molluscicidal activity on the snail *Biomphalaria glabrata* (200 ppm) [21] and for the sedative effect of mice (10 mg/kg [22]. Furthermore pure foetidissimoside A was subjected to the *in vitro* prostaglandin-cyclooxygenase (10^{-4} M) and 12-lipoxygenase (10^{-4} M) inhibition-tests [23]. No significant effect could be found in any of these bioassays.

Extraction of the roots with chloroform yielded a very bitter tasting fraction which could be separated by reverse phase HPLC into seven main peaks. By comparison with reference compounds in terms of retention time and UV spectra (recorded on line by the photodiode array detection system) the main constituents were identified as cucurbitacin D (**2**), B(**3**), Iso-B (**5**), E (**6**), cucurbitacin B-2-*O*-glucoside (**4**) and cucurbitacin-E-2-*O*-glucoside (**7**). The amount of total cucurbitacins was about 0.5% (w/w).

EXPERIMENTAL

Plant material. Roots of *Cucurbita foetidissima* were collected and provided by Dr M. R. Cagiotti (Perugia, Italy). A sample is deposited in the herbarium of the Institute of Pharmaceutical Biology (Munich).

Reference compounds. Cucurbitacin E (Roth, Karlsruhe); cucurbitacin B and D (Prof. Dr R. W. Doskotch, The Ohio State University, Columbus, U.S.A.); cucurbitacin E-glucoside (Prof. Dr M. Wichtl, Institut für Pharmazeutische Biologie der Universität Marburg); cucurbitacin B-glucoside (Dr K. Seifert, Institut für Biochemie der Pflanzen, Halle/Saale, D.D.R.); Iso-cucurbitacin B (isolated from *Cayaponia tayuya*) [24].

Instruments and general methods. Mps: uncorr. IR spectra (KBr disc) were recorded on a Perkin Elmer 727 B spectrophotometer. MS, FABMS: ZAB-HP, glycerol matrix. GC/MS: Kratos MS 80, R.F.A. on a fused silica capillary column (25 m × 0.25 mm) with OV 225. Gas flow rate for He: 0.8 ml/min. Temp. program: from 150 to 260° with 5°/min. EI 200°, 4 KV, 70 eV, 50 μA. GC analysis: Perkin Elmer 900 B, glass column (200 × 0.3 cm) packed with 3% OV 225 on chromosorb W-HP, 80/100 mesh, 210° for alditol acetates, carrier gas: Ar, 30 ml/min. ^{13}C NMR spectra were recorded in pyridine-d_5 with a Brucker WM-400 (100 MHz) spectrometer, and ^{1}H NMR spectra in DMSO-d_6 + CF_3COOD with a Brucker HX-270 spectrometer. Chemical shifts (δ) are given in ppm from TMS and coupling constants (J) in Hz. Centrifugal thin layer chromatography was carried out on a Chromatotron-apparatus (Model 7924T, Harrison research, Palo Alto, California). TLC and HPTLC employed precoated silica gel plates 60 F_{254} (Merck). The following TLC solvent systems were used: for saponins: (*a*) $CHCl_3$–MeOH–H_2O (64:40:8); (b) $CHCl_3$–MeOH–AcOH–H_2O (60:32:12:8); (c) EtOAc–AcOH–HCOOH–H_2O (100:11:11:26); for sapogenins, (d) toluene–Me_2CO (4:1); for monosaccharides, eluents (a) and (b). Spray reagents were for the saponins: Komarowsky reagent, a mixture (5:1) of *p*-hydroxy benzaldehyde (2% in MeOH) and H_2SO_4 50%; Godin reagent (vanillin–sulphuric acid) and Nile blue reagent; for the sugars: aniline phthalate and diphenylamine phosphoric acid reagent. HPLC; Analytical scale: HP 1090A Liquid chromatograph with 1090 photodiode array detection system (Hewlett Packard, Waldbronn).

HPLC of saponins: LiChrospher (10 g) CH-18 (5 μm) column (125 × 4 mm ID; Merck); eluent: linear gradient from 20 to 40% MeCN–H_2O with 1% 0.1 N phosphoric acid during 20 min, flow rate 1 ml/min; detection wavelength 210 nm.

HPLC of cucurbitacins: LiChrospher 100/CH-18 (5 μm) column (125 × 4 mm ID); eluent: linear gradient from 20 to 50% MeCN–H_2O; flow rate 1 ml/min; detection wavelength 230 nm.

Isolation of saponins. Powdered roots (500 g) were defatted with 3 l *n*-hexane for 24 hr in a Soxhlet. The air-dried plant

material was successively extracted with 3 l $CHCl_3$ for 24 hr yielding after evaporation 5 g of a brown residue and with 4 l MeOH for 68 hr yielding after evapn 30 g of a syrupy brown residue. The MeOH extract was dissolved in 400 ml H_2O and extracted with cyclohexane (3 × 200 ml). The aq. soln was extracted (3 × 200 ml) with water-saturated *n*-BuOH. On evapn of the solvent, 10 g residue was obtained. It was dissolved in a small quantity of MeOH and poured into 500 ml Et_2O. After filtration the residue was dissolved again in a little MeOH and pptd by adding Et_2O. This procedure was repeated 2 × in Et_2O and one time in Me_2CO. After filtration, a pale yellow powder (saponin mixture, 7 g) was obtained. It was dissolved in MeOH, the soln treated with charcoal, centrifugated and filtered to give 4 g of a white powder. It was first fractionated by flash chromatography on silica gel $<63\ \mu m$, Merck, using as eluent EtOAc–MeOH–H_2O (63:25:9). The further separations were performed by flash chromatography on reversed phase material, Lichroprep RP-8, Merck (40–63 μm), eluting with MeOH–H_2O, (65 - 35), by prep. TLC and CTLC on silica gel (layer thickness 1 mm) with $CHCl_3$–MeOH–H_2O (64:40:8) to give three saponins: foetidissimoside A 20 mg, B 10 mg and C 8 mg. Final purification of all compounds was carried out on a Sephadex LH-20 column with MeOH.

Foetidissimoside A (**1**). White amorphous powder. TLC: R_f 0.40 (a), 0.43 (b) 0.35 (c). Violet spots by spraying with Komarowsky reagent; IR ν_{max}^{KBr} cm^{-1}: 3500–3300 (OH), 2930 (CH), 1725 (CO ester), 1610, 1450, 1390, 1360; ^{13}C NMR: see Table 2. Negative FABMS *m/z* (rel. int.): 1057 $[M-H]^-$, (100), 925 (6), 779 (6), 647 (38), 601 (30), 585 (14), 471 (9), 453 (13) 337 (35). ^1H NMR: δ5.55 (1H, *s*, anomeric H of rhamnopyranose, 5.24 (1H, *br, s*, olefinic H), 4.27 (1H, *d*, $J=7$ Hz, anomeric proton of glucuronic acid), 4.46 (2H, *d*, $J=5$ Hz, anomeric proton of a disubstituted α-L-arabinopyranose and a terminal β-D-xylopyranose.

Basic hydrolysis. The saponin (**1**) (2 mg) was refluxed in 5 ml 0.5 N aq. KOH for 1.5 hr. The reaction mixture was adjusted to pH 6 with 0.5 aq. HCl, and then extracted with EtOAc. Removal of the solvent gave a saponin which was analysed by TLC: R_f 0.58 (a), violet spot with Komarowsky reagent. This saponin was submitted to acid hydrolysis with 10% aq. HCl for 2 hr. After repeated evapns of the solvent by adding MeOH at 40° to remove the acid, the residue was analysed by TLC on silica gel. Echinocystic acid, R_f 0.32 (d) and glucuronic acid, R_f 0.15–0.60 (a) were identified by comparison with an authentic sample.

Acid hydrolysis. Saponin **1** (5 mg) was hydrolysed with 2 N CF_3COOH (5 ml) at 110° for 6 hr in a sealed tube. The aglycone was extracted with CH_2Cl_2 and identified as echinocystic acid by co-TLC with an authentic sample, R_f 0.32 (d). After repeated evapns of the solvent of the aq. layer by adding MeOH to remove the acid, the residue was analysed by TLC. The presence of glucuronic acid, rhamnose, xylose and arabinose was established by comparison with an authentic sample; R_f 0.57–0.1, 0.51, 0.45, 0.37 respectively in system (a). Saponin A (3 mg) was hydrolysed with 2 N CF_3COOH at 110° for 6 hr and the soln evapd to dryness with MeOH. The residue was dissolved in 10 ml H_2O, and the mixture was reduced with $NaBH_4$ at 24° for 3 hr. After neutralization by adding 96% MeCOOH, the mixture was evapd to dryness by repeated co-distillation with MeOH. The resulting alditol mixture was refluxed with Ac_2O for 1 hr and the soln after removal of toluene evaporated to dryness. A sample was subjected to GLC to give the alditol acetates of rhamnose, xylose, arabinose in a molar ratio 1:1:1.

Enzymatic hydrolysis. Saponin **1** (2 mg) was dissolved in 1.3 ml, 0.1 M HOAc–NaOAc, pH 5 and incubated with β-D-glucuronidase (0.7 ml) from *Helix pomatia* (Sigma Chem. Co., Nr. G-0876) at 39° for 2 days. After adding hot EtOH the suspension was centrifuged and the supernatant evapd to dryness. This residue was dissolved in H_2O and extracted with *n*-BuOH. After evapn of the solvent this extract was analysed by TLC on silica gel. A new saponin spot appeared at R_f 0.77 (a) and R_f 0.83 (b). This saponin, after acid hydrolysis on the HPTLC-plate in an HCl-chamber (30 min. at 100°) and subsequent chromatography with the system (a) yielded rhamnose and arabinose.

Permethylation of saponins and preparation of the partially methylated alditol acetates. In a 10 ml injection bottle containing an atmosphere of N_2, 5 mg saponin **1** was dissolved in DMSO and Na-methylsulphinylmethanide in DMSO (2 ml) added dropwise. The mixture was sonicated for 30 min at 25° in a Bransonic 12 ultrasound bath (60 W, 50 kHz) and kept overnight at room temp. CH_3I (1.5 ml) was added to this soln under cooling, and the soln was further sonicated at room temp. for 1 hr. The excess of CH_3I was distilled off at 40°, 5 ml H_2O added and the soln extracted (5 ml × 4) with CH_2Cl_2. The CH_2Cl_2-phases were washed with H_2O and then evapd to dryness. The residue was hydrolysed with 90% HCOOH (1 ml) and 1 M CF_3COOH (1 ml) for 2 hr at 100°, the soln evapd to remove the acid, the residue dissolved in H_2O (2 ml) containing 2 drops of NH_4OH [22], and 25 mg $NaBD_4$ were added. After standing at room temp. for 2 hr the mixture was acidified with MeCOOH to pH 3,5 and the solvent evapd to dryness. H_3BO_3 in the residue was removed by three cycles of co-distillation with MeOH. The partially methylated alditols were acetylated with Ac_2O (1 ml) for 1 hr at 100°. The Ac_2O was removed by codistillation in the presence of toluene, and the residue dissolved in CH_2Cl_2 (3 ml), washed with H_2O (3 × 1 ml) and then evapd to dryness. The mixture thus obtained was subjected to GC/MS using the same system as that used for the alditol acetates. The peaks assigned to the saponin **1** are reported in Table 1.

Bioassays. The molluscicidal activity was measured with snails of the species *Biomphalaria glabrata* (for methodology, see ref. [21]). The motility test was carried out as described in ref. [22] with 4 groups of 5 Swiss male mice. The cyclooxygenase and lipoxygenase assays were performed as described in ref. [23]. The cyclooxygenase was obtained from bull seminal vesicle microsomes and human platelets and 12-lipoxygenase from human platelets.

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