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MOLLUSCICIDAL SAPONINS OF *PHYTOLACCA DODECANRA*: OLEANOGLYCOTOXIN-*A*

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Abstract—The structure of one of the major molluscicidal saponins of the fruit of *Phytolacca dodecandra* has been elucidated as 3-[2,4-di-*O*-(β-D-glucopyranosyl)-β-D-glucopyranosyl]-olean-12-ene-28-oic acid. The combined use of 300 Mc. PMR, MS and GC–MS led to this structural assignment.

INTRODUCTION

THE SHRUB *Phytolacca dodecandra*, commonly known in Ethiopia as Endod, produces large quantities of berries that, when dried, ground and suspended in water, may kill snails at as low a concentration as 10 ppm.[1–3] In view of the potential of the Endod plant for controlling schistosomiasis, a disease transmitted by aquatic snails, there have been several attempts to elucidate the structure of the active principle. Horton[4] speculated that the active material may be a glycoside or glucuronide of oleanolic acid. King *et al.*[5] and Powell and Whalley[6] found four different sugars, oleanolic acid, and bayogenin in the hydrolysis products of the crude active fraction. The work described here concerns the isolation and structure determination of one of the major biologically active saponins; future papers will be concerned with the structures of other components of the complex mixture.

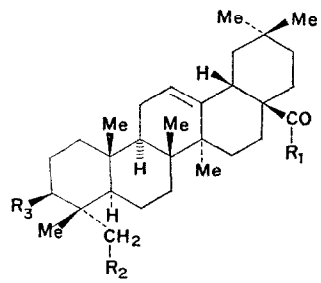
RESULTS

For isolation of the saponins, the dried finely ground Endod berries were defatted with light petroleum, then extracted with warm water. Partition with butanol provided a biologically active light tan powder, representing 20–25% of the initial ground berries.[7] This

[1] LEMMA, A. (1965) *Ethiop. Med. J.* **3**, 187.
[2] LEMMA, A. (1970) *Bull. World Health Organ.* **42**, 597.
[3] LEMMA, A. and DUNCAN, J. (1970) *J. Parasitol.* **56**(4), 213.
[4] HORTON, W. J. (1968) *World Health Organ. Molluscicide Information Series*, No. 24, V.
[5] KING, T. A., JERVERS, K., RICHARDSON, H. and FALSHAW, C. P. (1968) Abstract of 5th International Symposium on the Chemistry of Natural Products, F. 43, London.
[6] POWELL, J. W. and WHALLEY, W. B. (1969) *Phytochemistry* **8**, 2105.
[7] LEMMA, A., BRODY, G., NEWELL, G. W., PARKHURST, R. M. and SKINNER, W. A. (1972) *J. Parasitology* **58**, 104–107.

material was acetylated and examined by TLC, which showed a complex mixture of over ten distinct components, three of these appearing to be major. One of the major components, representing about 18% of the crude saponin acetates, was isolated by repetitive chromatography in sufficient quantity for further study. Deacetylation of this material yielded a biologically active substance (ED_{90}, 3 ppm, 24 hr, *Biomphalaria glabrata*) which was named oleanoglycotoxin-*A*. Reacetylation gave material identical with the originally isolated acetate.

Acid hydrolysis of oleanoglycotoxin-*A* gave a water-insoluble solid with the same R_f as oleanolic acid (I), which had previously been established as the major sapogenin of the Endod saponins.[4-6] Further examination of its permethyl derivative by GC–MS demonstrated that the major component (86%) had a retention time and MS identical with those of authentic methyl *O*-methyloleanolate (II). A lesser component (11%) gave a MS identical with that of authentic methyl di-*O*-methylhederagenin (III). This would indicate that oleanoglycotoxin-*A*, in spite of the chromatographic homogeneity of its acetate, contains a minor component derived from hederagenin (IV). The aqueous phase of the acid hydrolysis of oleanoglycotoxin-*A* contained only glucose, unambiguously identified by GLC of its trimethylsilyl derivative.[8]



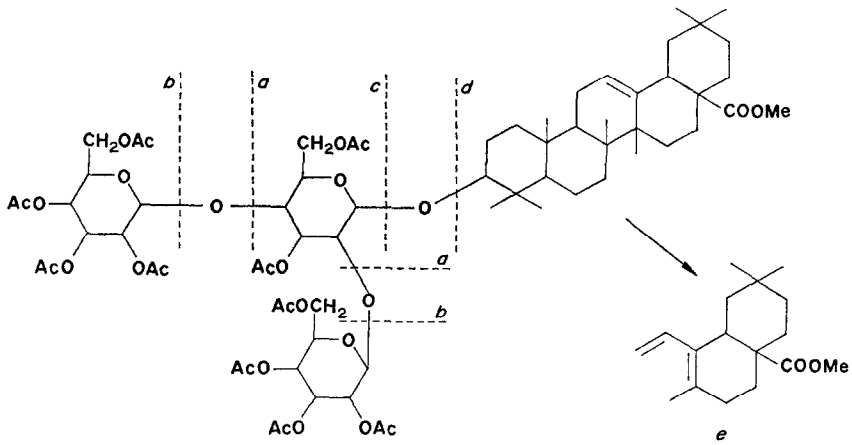
(I) $R_1 = R_3 = OH$; $R_2 = H$
(II) $R_1 = R_3 = OMe$; $R_2 = H$
(III) $R_1 = R_2 = R_3 = OMe$
(IV) $R_1 = R_2 = R_3 = OH$

The first indication of the total composition of oleanoglycotoxin-*A* was given by the NMR spectrum of its peracetyl derivative. In particular, the presence of ten acetate methyls (ppm, 1·73 (3 × Me); 1·76; 1·80 (2 × Me); 1·91; 1·93; 2·06 and 2·10) indicated 3 mol of glucose present to 1 mol of oleanolic acid, whose seven methyl groups are clearly apparent at 0·87, 0·92, 0·96, 1·00, 1·05, 1·19 and 1·24 ppm. Because of its low abundance and structural similarity to oleanolic acid, the hederagenin component of oleanoglycotoxin-*A* is not expected to make an observable contribution to the NMR spectrum.

MS not only confirms this conclusion, but gave additional structural information. Peracetyl oleanoglycotoxin-*A* was methylated with diazomethane, yielding a derivative which gave a MS with a group of peaks at *m/e* 1374, 1375 and 1376. The peracetate methyl ester of a triglucoside of oleanolic acid required a MW of 1376. Evidently, in this spectrum *m/e* 1374 and 1375 are due to pronounced losses of H_2 and H, respectively, from the molecular ion. This behavior has been observed also for permethyl and peracetyl oleanoglycotoxin-*A*, and numerous other related saponins, including a synthetic peracetyl monoglucoside of oleanolic acid.

[8] SWEELEY, C. C., BENTLEY, R., MAKITA, M and WELLS, W. W (1963) *J. Am. Chem Soc.* **85**, 2497.

The MW (1376) of the major component could be confirmed by further examination of this MS. Loss of a terminal peracetyl glucose unit (mass 347) (Scheme I) is expected to give rise to an intense 'M minus 347' peak, observed here as fragment *a* at *m/e* 1029. This peracetyl glucose is itself observed without the glucosidic oxygen as ion *b* at *m/e* 331. That all three glucose units are contained within one single saccharide moiety, as opposed to being attached independently at different points to the oleanolic acid unit, is demonstrated by the appearance of a peracetyl triglucose unit in the MS at *m/e* 907 (*c*). This saccharide is clearly attached at the hydroxyl of oleanolic acid; MS loss of the trisaccharide unit with the linking oxygen gives one of the most intense peaks of the spectrum at *m/e* 453 (*d*). The appearance of fragment *e* at *m/e* 262, originating by a mechanism typical of Δ^{12}-unsaturated triterpenes, confirms that the carboxyl is present as a methyl ester, ruling out the possibility of the saccharide being bound to the carboxyl group.



SCHEME 1. MS FRAGMENTATION OF PERACETYL OLEANOGLYCOTOXIN-*A* METHYL ESTER.

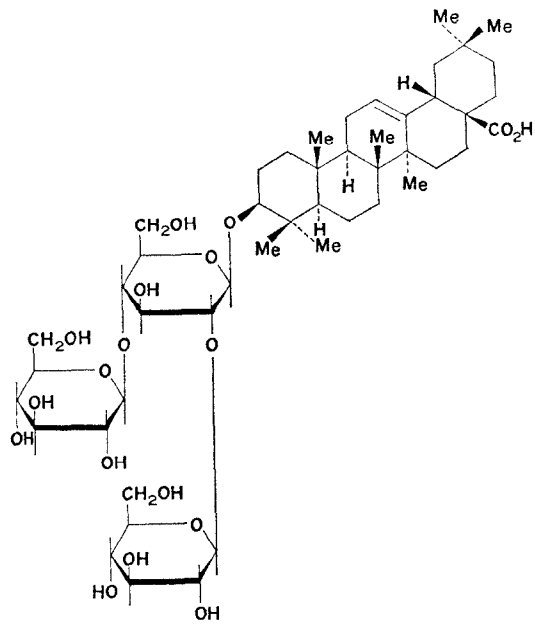
A mass spectrum of peracetyl oleanoglycotoxin-*A* (i.e. not treated with diazomethane) was similar to that of the methyl derivative, except that all fragments which contain the carboxyl group appeared 14 m.u. lower. That is, fragments *M*, *a*, *d* and *e* appeared at *m/e* 1362, 1015, 439 and 248, respectively. Those fragments which had lost the carboxyl function (i.e. M — COOMe, *d* — HCOOMe and *e* — COOMe) remained unchanged. Thus, the methyl ester of the derivative illustrated in Scheme 1 was introduced by treatment with diazomethane, and oleanoglycotoxin-*A* therefore contains a free carboxylic acid.

All the MS data thus far discussed referred to derivatives of the major component of oleanoglycotoxin-*A*, i.e. a triglucoside of oleanolic acid. Also visible in the MS are peaks which may be attributed to a triglucoside of hederagenin, the minor component already mentioned. MS fragments containing the sapogenin portion of the molecule appear to a minor extent shifted 58 units toward higher mass; e.g. note in particular the pair of peaks for fragments *a* at *m/e* 1029 and 1087. This is indicative of the additional *O*-acetyl group in the hederagenin moiety.

In order to determine the structure of the trisaccharide present, oleanoglycotoxin-*A* was permethylated and then hydrolyzed to give a mixture of partially methylated glucose derivatives. The mixture thus obtained was pertrimethylsilylated and analyzed by GC–MS. The resulting gas chromatogram, revealed the presence of two pairs of glucose derivatives,

each pair representing an equilibrium of α- and β-anomers of a single derivative. A comparison of the MS of these sugars with those previously reported[9] for trimethylsilylated 2,3,4,6-tetra-*O*-methylglucopyranose and trimethylsilylated 3,6-di-*O*-methylglucopyranose established their identity. Oleanoglycotoxin-*A* thus contains 2 mol of 1-substituted glucose to 1 mol of 1,2,4-trisubstituted glucose, bound to each other in a single saccharide unit. Only one structure is possible (excluding assignment of anomeric configurations) for the major component of oleanoglycotoxin-*A*, namely that indicated by formula V.

The anomeric assignments presented here are derived from a more detailed analysis of the NMR spectrum of the acetate. Three 1-proton doublets at 4·23, 4·35 and 4·82 ppm, each with coupling J 8 Hz, are due to the C-1 protons of the three glucose units. The coupling constants imply a β-pyranoside in each case, since an α-pyranoside or either of the anomeric furanosides would give splittings of 4·2 Hz or less.[10]



(V)

The remainder of the saccharide signals in the NMR spectrum may be classified in three groups: (a) all C-H adjacent to an acetate appear higher than 5·0 ppm; (b) C-H_2 adjacent to an acetate (e.g. all C-6 protons) appear somewhat upfield, from 3·9 to 4·6 ppm; (c) C-H adjacent to –OR functions appear even further upfield, from 3·2 to 3·9 ppm. Included within the last group are the C-5 protons at 3·26 and 3·42 ppm (two and one protons, respectively), and the protons adjacent to the saccharide linkage positions, i e. C-2 and C-4 of the central glucose at 3·82 ppm at 3·54 ppm, respectively.

These assignments were verified by spin decoupling experiments. Thus, irradiation of the C-5 protons at 3·26 and 3·42 ppm caused partial collapse of the signals assigned to the C-6

[9] PETERSON, G. and SAMUELSON, O. (1968) *Svensk Papperstid* **71** (20), 731.
[10] CAPON, B. and THACKER, D. (1964) *Proc. Chem. Soc.* 369.

protons, and irradiation of a C-3 proton (i.e. at 5·4 ppm) caused simultaneous collapse of the central glucose C-2 and C-4 signals to doublets.

Thus, the major constituent of oleanoglycotoxin-*A* is 3-[2,4-di-*O*-(β-D-glucopyranosyl)-β-D-glucopyranosyl]-olean-12-ene-28-oic acid (V). The minor hederagenin-containing constituent probably has a similar structure, although small amounts of trimethylglucoses identified in the acid hydrolysis product of permethyl oleanoglycotoxin-*A* leaves open the possibility that hederagenin and/or a small part of the total oleanolic acid may be associated with a linear trisaccharide.

EXPERIMENTAL

All GLC analyses were carried out with a 3 m × 3·2 mm column of 1% SE-30 on 100–120 mesh Gas Chrom Q, at a flow rate of 23 ml/min. Temperatures required were 280° for the methylated sapogenins, 180° for the pertrimethylsilylated glucose. MNR spectra were determined with a Varian HR 300 nuclear magnetic resonance spectrometer employing a superconducting magnet at 70·5 kG. The sample was examined in a 5-mm sample tube at 35°. Mass spectra were determined with an LKB model 9000 combination gas chromatograph–mass spectrometer.

Extraction of crude saponins. The dried fruit of *Phytolacca dodecandra* (Endod) was ground to a fine powder and defatted with light petrol. The defatted material was extracted with warm H_2O–BuOH. Evaporation of the BuOH gave a brown gum which upon trituration with Et_2O solidified to a tan powder representing 20–25% of the wt of the dried fruit.[7]

Acetylation of crude saponins. 20 g with Ac_2O–pyridine gave a brown gummy product which was dissolved in tetrahydrofuran, passed through a short Florisil column, and again evaporated *in vacuo* giving 19·55 g of almost colorless amorphous saponin acetate mixture.

*Isolation of oleanoglycotoxin-*A *acetate.* 2 g of the crude saponin acetates was chromatographed on a column (18 × 3 cm, 50 g Mallinckrodt SilicAR-CC7, 100–200 mesh) using $CHCl_3$–Et_2O gradient elution and following the progress of the fractions with TLC on SilicAR-7GF plates. One fraction, 348 mg, eluted with 5% Et_2O in $CHCl_3$, was further purified by repetitive chromatography on thick plates (SilicAR-7GF, $CHCl_3$–Et_2O, 1:1) finally giving 41·6 mg of the acetate as a colorless glassy solid, R_f 38–46 (SilicAR-7GH, Et_2O), $[\alpha]^{23}_D$ +17·37 ± 4·35° (*c* = 0·576, $CHCl_3$). (Found: C, 59·4; H, 7·19. Calc. for $C_{68}H_{98}O_{28}$: C, 59·89; H, 7·24%.) NMR spectrum, see text; MS, *m/e* 203, 248, 331, 393, 438, 439, 907, 1015, 1073, 1314, 1316, 1360, 1372, 1374, 1418. Comparison of the area of the TLC spots of the pure and crude materials allowed an estimate of the weight percent of oleanoglycotoxin-*A* acetate in the crude mixed saponin acetates to be 18%.

*Deacetylation of oleanoglycotoxin-*A *acetate.* The acetate (2·3 mg) was treated with an excess of MeOH–conc. NH_4OH (1:1) at 50° for 12 hr, then evaporated. Extraction with *n*-BuOH, followed by evaporation, gave the biologically active saponin as a colorless powder.

*Methylation of oleanoglycotoxin-*A *acetate.* The acetate (0·6 mg) in 10 ml of MeOH was methylated with excess CH_2N_2 in Et_2O. TLC indicated only a single product was formed. MS, *m/e* 203, 262, 331, 393, 452, 453, 907, 1029, 1087, 1316, 1317, 1360, 1374, 1375, 1376.

Acid hydrolysis. Oleanoglycotoxin-*A* (1·3 mg) was heated on the steam bath for 24 hr in a sealed tube containing 0·2 ml of 1 N HCl. The solid precipitate was centrifuged to one end, the liquid decanted to the other end and the tube cut in half. The solution was lyophilized and the residue was gas chromatographed as its pertrimethylsilyl derivative,[8] prepared by dissolving in HMDS–TMCS–pyridine. Only glucose was found to be present. The solid precipitate, crystd from EtOH–H_2O, was identical with an authentic sample of oleanolic acid on TLC in two solvents, R_f 70 (SiGF–EtAc); R_f 90 (SiGF acetone), m.p. 306–307·5°, m.m.p., no depression. (Found: C, 78·46; H, 10·54. Calc. for $C_{30}H_{48}O_3$: C, 78·90; H, 10·59%.) This material was permethylated[11,12] with MeI and NaH in $HCONMe_2$. Examination of the product by GC–MS revealed the presence of 86% methyl *O*-methyloleanolate, 11% of methyl di-*O*-methylhederagenin, and 3% of two unidentified compounds.

Permethylation. Oleanoglycotoxin-*A* (9·8 mg) was methylated twice with 50 mg NaH and 0·1 ml Me I in 0·2 ml dry $HCONMe_2$[11,12] and the product was purified by TLC. MS, *m/e* 187, 203, 219, 262, 391, 395, 407, 423, 453, 509, 861, 1094.

*Acid hydrolysis of permethyl oleanoglycotoxin-*A. The material (3·3 mg), 5 ml dioxane (distilled from sodium

*Acid hydrolysis of permethyl oleanoglycotoxin-*A. The material (3·3 mg), 5 ml dioxane (distilled from sodium), 4 ml of dist. H_2O and 1 ml of conc. HCl were refluxed for 15 hr. The solution was evaporated *in vacuo*, one drop of pyridine added and the sides washed down with Et_2O. Evaporation gave a yellow gum, which was derivatized by dissolving in 100 μl HMDS–TMCS–pyridine. GC–MS showed α- and β-anomers

[11] BRIMACOMBE, J. S., JONES, B. D., STACEY, M. and WILLARD, J. J. (1966) *Carbohyd. Res.* **2**, 167.
[12] THOMAS, D. W. (1969) *FEBS Letters* **5**, 53.

of trimethylsilylated-2,3,4,6-tetra-*O*-methylglucopyranose and trimethylsilylated-3,6-di-*O*-methylgluco pyranose.

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