Triterpene Saponins in the Defensive Secretion of a Chrysomelid Beetle, *Platyphora ligata*

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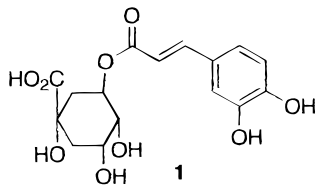
The secretion of the defensive glands of adults of the chrysomelid beetle *Platyphora ligata* from Panama has been shown to contain, besides chlorogenic acid (**1**) and a mixture of phosphatidylcholines, two new oleanane triterpene saponins, named ligatosides A and B. Their structures were established as 3-*O*-*β*-D-glucuronopyranosyl-16α,23-dihydroxyoleanolic acid-28-*O*-2-(3,4-dimethoxybenzoyl)-*β*-D-glucopyranoside (**2**) and 3-*O*-*β*-D-glucopyranosyl-(1→2)-*β*-D-glucuronopyranosyl-16α,23-dihydroxyoleanolic acid-28-*O*-2-(3,4-dimethoxybenzoyl)-*β*-D-glucopyranoside (**3**), respectively, by a combination of extensive 1D and 2D NMR methods (COSY, HMQC, HMBC, and TOCSY) and FABMS. This is the first report of triterpene saponins in the defensive secretion of an insect.

Chrysomelids, or leaf-beetles, are one of the major families of Coleoptera, comprising about 25 000 species. Defensive mechanisms are prominent and diverse in these insects, ranging from crypsis, mechanical devices such as spines, or sudden escape, to chemical defense in many brightly colored, aposematic species.[1,2] Defensive glands are found in both larvae and adults, but their occurrence is restricted to some taxa. In the adults of Chrysomelinae, after disturbance, the secretion oozes from the gland pores and accumulates in marginal grooves of the elytra and pronotum. The defensive chemicals identified so far in adult chrysomelines comprise a variety of toxins, such as cardenolides,[3] polyoxygenated steroids,[4] amino acid derivatives,[5] 3-nitropropanoates of isoxazolinone *N*-glucosides,[6] and pyrrolizidine alkaloids.[7] Some of these derivatives (e.g., the cardenolides[8] and the isoxazolinone glucosides[9]) have been shown to be biosynthesized by the insects, whereas others, such as the pyrrolizidine alkaloids,[10] are sequestered from their food plant.

We have now undertaken the chemical examination of the defensive secretion of adult beetles of the as yet unexplored genus *Platyphora* common in Central and South America. We report here the isolation and structure determination of two new triterpene saponins, ligatosides A (**2**) and B (**3**), together with the identification of a mixture of phosphatidylcholines and chlorogenic acid (**1**) from the defensive secretion of *Platyphora ligata* (Stål).

Results and Discussion

The defensive secretion of about 1400 adults of *P. ligata* were obtained, as described in the Experimental Section, on small pieces of filter paper and stored in CH_3OH.[11] A TLC analysis (*n*-BuOH−HOAc−H_2O, 8:2:2) showed the presence of three spots. As shown by the subsequent separations, some of these spots comprised more than one compoud. The components of the mixture were separated as described in the Experimental Section using a combination of Sephadex LH-20 chromatography and reversed-phase HPLC. The most polar component (R_f 0.22) was easily identified as a mixture of phosphatidylcholine derivatives by comparison of the ^{1}H NMR spectra and FABMS with those reported in the literature.[12] Negative-ion FABMS indicated that the major fatty acid constituents of the mixture were C16:0, C18:0, C18:1, C18:2, C20:0, C20:1, and C24:1. The less polar compound (R_f 0.75) was identified as chlorogenic acid (**1**) by comparison of its spectral properties with literature data.[13,14]



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The two remaining constituents ligatosides A (**2**) (R_f 0.57) and B (**3**) (R_f 0.75), were shown to be new triterpene saponins. Ligatoside A (**2**), an amorphous solid, has a molecular formula of $C_{51}H_{74}O_{19}$, determined from its negative- and positive-ion FABMS (quasi-molecular ions at *m*/*z* 989 $[M - H]^-$, 991 $[M + H]^+$, and 1013 $[M + Na]^+$, respectively). The negative-ion FABMS displayed prominent fragment ions at *m*/*z* 663 ($C_{36}H_{55}O_{11}$) and 487 ($C_{30}H_{47}O_5$), thus showing that the aglycon contained 30 carbon and five oxygen atoms. The complete assignment of the hydrogen and carbon atom signals of the aglycon moiety was performed by 2D NMR methods (COSY, HMQC, and HMBC) and is reported in Table 1. These data support the identification of the aglycon as 16α,23-dihydroxyoleanolic acid, an oleanane triterpene already found in several plants.[15] Also, the presence in the molecule of a *β*-D-glucopyranoside moiety, which esterified the C-28 carboxyl group of the aglycon (H-1 of glucose at δ_H 5.62, giving an HMBC correlation with the aglycon C-28 signal at δ_C 176.7), could also be deduced from the NMR data. Moreover, this *β*-glucopyranoside moiety was itself esterified at C-2 (H-2 at δ_H 5.10 giving an HMBC cross-peak with a CO signal at δ_C 166.8) by a 3,4-dimethoxybenzoate

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Table 1. ^{1}H and ^{13}C NMR Data of **2** and **3** (600 and 150.87 MHz, CD_3OD, δ, J in Hz)

	2[a]		**3**	
carbon	^{13}C	^{1}H	^{13}C	^{1}H
		Aglycon part (A)		
1	39.4	0.87, m; 1.52, m	39.7	0.88, m; 1.52, m
2	26.2	1.68, m; 1.80, m	26.6	1.71, br q, 11.3; 1.80, m
3	83.4	3.57, dd, 12.0, 4.2	83.3	3.59, m
4	44.2		44.2	
5	48.1	1.05, m	48.5	1.06, br d, 12.0
6	19.1	0.95, m; 1.14, m	19.2	0.96, m; 1.14, m
7	32.6	−0.01, br d, 12.6; 1.12, m	32.9	0.01, br d, 11.4; 1.12, m
8	40.5		40.5	
9	48.0	1.52, m	48.2	1.52, m
10	37.9		37.9	
11	24.4	1.80, m; 1.80, m	24.7	1.79, m; 1.79, m
12	123.8	5.20, br s	123.8	5.20, br s
13	145.0		145.0	
14	42.8		42.8	
15	36.3	0.86, m; 1.08, m	36.8	0.86, m; 1.08, m
16	74.8	4.40, br s	74.8	4.40, br s
17	49.9		49.9	
18	41.7	2.89, dd, 13.8, 4.6	41.9	2.89, dd, 13.8, 4.2
19	47.9	0.97, br d, 13.8	48.0	0.97, m
		2.22, t, 13.8		2.22, t, 13.8
20	31.6		31.6	
21	36.3	1.10, m; 1.91, dd, 12.6, 4.8	36.8	1.08, m; 1.91, dd, 13.2, 4.8
22	32.7	1.64, m; 1.95, br d, 13.2	32.7	1.66, m; 1.95, m
23	64.9	3.23, d, 11.0; 3.58, d, 11.0	65.1	3.23, d, 12.6; 3.70, d, 12.6
24	13.4	0.69, s	13.7	0.70, s
25	16.6	0.87, s	16.8	0.88, s
26	17.3	0.35, s	17.6	0.35, s
27	27.1	1.20, s	27.3	1.20, s
28	176.7		176.7	
29	33.5	0.83, s	33.6	0.83, s
30	24.8	0.89, s	25.0	0.89, s
		3-*O*-Glucuronic acid (GluA)		
1	106.6	4.43, d, 7.8	105.0	4.55, d, 6.2
2	75.3	3.20, dd, 9.6, 7.8	81.4	3.59, m
3	77.4	3.34, dd, 9.6, 9.0	78.1	3.53, m
4	73.3	3.48, dd, 9.6, 9.0	71.5	3.45, m
5	76.8	3.82, d, 9.6	76.3	3.76, d, 9.5
6	172.0		172.0	
		28-*O*-Glucose (Glu′)		
1	93.7	5.62, d, 8.4	93.7	5.62, d, 8.4
2	74.4	5.10, dd, 9.0, 8.4	74.4	5.11, dd, 9.0, 8.4
3	76.1	3.76, t, 9.0	76.3	3.76, t, 9.0
4	71.2	3.49, dd, 9.6, 9.0	71.5	3.50, dd, 9.6, 9.0
5	79.1	3.45, m	79.2	3.45, m
6	62.5	3.73, dd, 12.0, 4.8	62.5	3.73, dd, 11.4, 4.2
		3.87, dd, 12.0, 1.2		3.87, dd, 11.4, 2.4
		2′-*O*-Glucose (Glc″)		
1			104.9	4.69, d, 7.8
2			76.6	3.23, m
3			77.9	3.36, t, 9.0
4			72.0	3.25, m
5			78.3	3.25, m
6			63.3	3.65, dd, 11.4, 4.2; 3.85, br d, 11.4
		Aromatic Moiety		
1	166.8		166.8	
2	123.5		123.5	
3	114.2	7.58, d, 1.8	114.2	7.58, d, 1.8
4	155.6		155.6	
5	150.5		150.5	
6	111.9	7.04, d, 8.4	111.9	7.03, d, 8.4
7	125.9	7.72, dd, 8.4, 1.8	125.9	7.72, dd, 8.4, 1.8
8	56.8	3.92, s	56.8	3.93, s
9	56.8	3.89, s	56.8	3.89, s

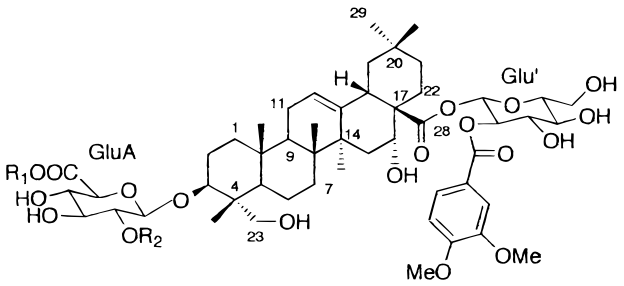
[a] Recorded after addition of 0.2 μL of TFA.

residue (see Table 1). The quite unusual proton chemical shifts of the H_3C-26 (δ_H 0.35) and of the H-7eq (δ_H −0.01) indicated that the molecule adopts a conformation in which the aromatic ring must be close to C-7 and C-26 of the aglycon. Surprisingly, the interpretation of the NMR signals due to the second sugar moiety proved to be much less straightforward. The first NMR spectrum taken on a 1.5-mg sample showed broad, ill-resolved signals for the H atoms of this part of the molecule. COSY, HMQC, and HMBC correlations were generally missing. The same NMR experiments, performed on 5.0 mg of **2**, instead of 1.5 mg, led to even worse results, as most of the ^{1}H signals

of this sugar moiety disappeared completely. Reasoning that this behavior could be due either to intermolecular associations or to equilibria between different species in solution, the spectra were acquired in the presence of 0.2 μL of trifluoroacetic acid. Under these conditions, the hydrogen atoms of the second sugar moiety gave well-resolved signals. Particularly telling was the appearance of a doublet at δ_H 3.82 (J = 9.6 Hz), which was conspicuously absent in the previous NMR spectra. This signal gave a COSY correlation with a double doublet at δ_H 3.48 (J = 9.6, 9.0 Hz) and an HMBC cross-peak with a carboxyl carbon signal at δ_C 172.0. These data allowed us to identify the second sugar as β-D-glucuronic acid, which was attached at C-3 of the aglycon on the basis of the chemical shift of C-3 (δ_C 83.4) and the observation of an HMBC cross-peak between H-1 of glucuronic acid and C-3 of the triterpene. The complete assignment of the ^{1}H signals of the two sugar moieties (Table 1) was confirmed by selective 1D TOCSY experiments, and the presence of glucuronic acid was substantiated by diazomethane methylation of **2**, affording the corresponding methyl ester derivative **2a**, whose spectral properties were nearly identical with those of **2**. In the proton NMR spectrum of **2a**, the only significant differences were the appearance of a methyl singlet at δ_H 3.75, and the well-resolved shape of the protons belonging to the methyl glucuronate moiety. Ligatoside A (**2**) is 3-*O*-β-D-glucuronopyranosyl-16α,23-dihydroxyoleanolic acid-28-*O*-2-(3,4-dimethoxybenzoyl)-β-D-glucopyranoside.

Ligatoside B (**3**) has a molecular formula of $C_{57}H_{84}O_{24}$, determined from its negative- and positive-ion FABMS (quasi-molecular ions at m/z 1151 $[M - H]^-$ and 1175 $[M + Na]^+$, respectively). The negative-ion FABMS displayed prominent fragment ions at m/z 989 ($C_{51}H_{73}O_{19}$ $[M - H - C_6H_{10}O_5]^-$, 663 ($C_{36}H_{55}O_{11}$), and 179 ($[C_6H_{11}O_6]^-$), suggesting that **3** contained one hexose unit more than **2**. The ^{1}H and ^{13}C NMR spectra of **3** in CD_3OD (see Table 1) clearly indicated that **3** possesses basically the same structure as **2**, except for the presence of a third hexose unit, identified as β-D-glucopyranose. The latter is attached at C-2 of the glucuronic acid moiety on the basis of an HMBC cross-peak between H-1 of the second glucose moiety at δ_H 4.69 (J = 7.8 Hz) and the C-2 of glucuronic acid (δ_C 81.4). The broadening problems encountered with the H signals of the glucuronic acid moiety of **2** were again present in the case of **3**, but the assignment of signals could be made by comparison with the data of **2**. As for **2**, treatment of compound **3** with diazomethane afforded methyl ester **3a**. A complete 2D NMR study allowed assignment of all carbon and proton atom signals of the molecule. Thus, ligatoside B (**3**) is 3-*O*-β-D-glucopyranosyl-(1→2)-β-D-glucuronopyranosyl-16α,23-dihydroxyoleanolic acid-28-*O*-2-(3,4-dimethoxybenzoyl)-β-D-glucopyranoside.

To the best of our knowledge, this is the first report of the occurrence of triterpene saponins and chlorogenic acid in the defensive secretion of an insect. Phosphatidylcholines are ubiquitous primary metabolites but have never been found before in an insect defensive secretion. The simplest hypothesis to explain the presence of triterpenes and chlorogenic acid, which are typical plant compounds, in this chrysomelid beetle is that the beetle sequesters them from its food plant, *Mikania micrantha*. However, three arguments militate against this hypothesis: (a) a careful chemical examination of *M. micrantha* showed the absence of **1**, **2**, and **3**, and a literature search on the genus *Mikania* revealed[16,17] that no oleanane triterpenes have been reported from this genus to date; (b) the TLC patterns of secretions from *P. ligata* that were collected in the field on



2: R_1 = H; R_2 = H
2a: R_1 = Me; R_2 = H
3: R_1 = H; R_2 = β-D-Glucopyranose (Glc")
3a: R_1 = Me; R_2 = β-D-Glucopyranose (Glc")

Ayapana elata and *Neurolaena lobata* were identical to those observed for secretions of beetles fed on *M. micrantha*; and (c) we have found that the defensive secretions of several species of chrysomelids belonging to the genera *Platyphora*, *Desmogramma*, and *Leptinotarsa* contain oleanane saponins closely related to **2** and **3**.[18] These beetles feed on different host plants (in the families Asteraceae, Asclepiadaceae, and Convolvulaceae), which, according to the literature, seem to be devoid of oleanane triterpenes.[16] The origin of these compounds and their biological activities remain unknown and are the subject of continuing studies in our laboratories.

Experimental Section

General Experimental Procedures. UV spectra were obtained on a Philips PU 8700 UV/vis spectrophotometer in CH_3OH. IR spectra were recorded on a Bruker IFS 25 instrument as a film on a NaCl disk. FABMS measurements were obtained on a Micromass Autospec 3F instrument. The NMR spectra were recorded in CD_3OD at 25 °C on a Varian UNITY 600 spectrometer (^{13}C nominal frequency of 150.87 MHz). The chemical shifts (δ) are reported in parts per million from the solvent, and the coupling constants are given in hertz. The optical rotations were measured on a Perkin-Elmer 141 polarimeter (Na-vapor lamp) in a 10-cm cell at room temperature. TLC analyses were performed on Polygram SilG/UV_{254} precoated plates (0.25 mm). The compounds were visualized under UV_{254} light, and/or by spraying with a 2% ethanolic solution of phosphomolybdic acid, followed by a 3% ceric sulfate solution in 2 N H_2SO_4, and heating at 120 °C for 5 min. HPLC separations were performed on a Waters LCM1 plus apparatus coupled to a Waters 996 photodiode array detector set at 220 nm, equipped with a Symmetry RP C_{18} column (4.6 × 250 mm, 5μm), using 60% CH_3OH−0.1% TFA in H_2O and a flow of 4 mL/min.

Biological Material. Adults of *P. ligata* were collected in Panama in Cerro Campana and Cerro Azul on four Asteraceae: *M. micrantha* H. B. K., *Mikania guaco* H. & B. (Eupatoriae), *A. elata* (Steetz) King & H. Robinson (Eupatoriae), and *N. lobata* (L.) R. Br. (Heliantheae, Neurolaeninae). Identifications were made by comparison to specimens in the Smithsonian Tropical Research Institute insect collections and herbarium, Panama City. Vouchers of these species are stored there.

The beetles were stimulated with forceps; the secretions oozing from pores along the elytra and pronotum were collected on bits of filter paper and stored in methanol. Secretions of beetles fed on the different host plants were collected separately. The bulk of secretions on which most of the analyses were performed came from beetles reared in Belgium on *M. micrantha* grown in a greenhouse. Beetles were remilked at about 15-day intervals, and secretions were pooled until 1400 secretions were accumulated.

Extraction and Isolation. After filtration of the methanol solution, the remaining filter papers were exhaustively extracted with a 1:1 CH_2Cl_2−CH_3OH mixture. Evaporation of the pooled extracts under reduced pressure afforded 76.5 mg of a yellowish oil, the TLC of which (eluent: *n*-BuOH−HOAc−H_2O, 8:2:2) showed the presence of three major spots (R_f 0.22, 0.57, and 0.75). The crude extract was submitted to chromatography on Sephadex LH-20 using CH_3OH as eluent. This furnished three fractions: F1 (R_f 0.22, 5.5 mg), F2 (R_f 0.57 and 0.75, 27.0 mg), and F3 (R_f 0.75, 23.5 mg), thus showing that under the TLC conditions used, two of the components had the same R_f (0.75). The ^{1}H NMR and FABMS analyses of fraction F1 showed that it was a mixture of phosphatidylcholines, differing by the nature of the acyl residues. No attempt was made to fully characterize this fraction. Fraction F3 contained one compound that was identified as chlorogenic acid (**1**) on the basis of its spectral data.[13,14]

Further HPLC purification of fraction F2 afforded ligatoside A (**2**) (4.7 mg, t_R 14.3 min) and ligatoside B (**3**) (5.4 mg, t_R 12.3 min).

Ligatoside A (2): UV (MeOH) λ_{max} (log ϵ) 220 (4.11), 264 (3.85), 294 (3.58) nm; IR (NaCl, film) ν_{max} 3376, 2916, 1680, 1212, 1136 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 1; FABMS (glycerol, negative mode) *m*/*z* 989 (47, [M − H]$^-$), 663 (22), 617 (11), 487 (6), 469 (3), FABMS (glycerol, positive mode) *m*/*z* 1013 (0.4, [M + Na]$^+$), 837 (0.1), 626 (0.1), 471 (0.15), 453 (0.4), 435 (0.2), 407 (0.6), 327 (36), 165 (100); HRFABMS (nitrobenzyl alcohol, positive mode) *m*/*z* 991.4949 ([M + H]$^+$, calcd for $C_{51}H_{75}O_{19}$, 991.4902).

Ligatoside B (3): UV (MeOH) λ_{max} (log ϵ) 219 (4.15), 257 (3.94), 262 (3.94), 292 (3.63) nm; IR (NaCl, film) ν_{max} 3397, 2937, 1680, 1203, 1136 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 1; FABMS (glycerol, negative mode) *m*/*z* 1151 (100, [M − H]$^-$), 989 (8), 825 (8), 779 (18), 663 (3), 179 (87); FABMS (glycerol, positive mode) *m*/*z* 1191 (0.5, [M + K]$^+$), 1175 (1.2, [M + Na]$^+$), 1153 (2.0, [M + H]$^+$), 973 (0.02), 827 (0.5), 471 (1.2), 327 (75), 165 (100); HRFABMS (nitrobenzyl alcohol, positive mode) *m*/*z* 1175.5439 ([M + Na]$^+$, calcd for $C_{57}H_{84}O_{24}Na$, 1175.5245).

Ligatoside A methyl ester (2a): $[\alpha]^{20}_D$ +11 (*c* 0.126, MeOH); FABMS (glycerol, negative mode) *m*/*z* 677 (9, [M − H − ArGlu]$^-$); FABMS (glycerol, positive mode) *m*/*z* 1027 (1, [M + Na]$^+$); ^{1}H and ^{13}C NMR, nearly identical to those of **2**, except for the COO*CH*$_3$ signal at δ_H 3.75, δ_C 52.8.

Ligatoside B methyl ester (3a): $[\alpha]^{20}_D$ +24 (*c* 0.18, MeOH); FABMS (glycerol, negative mode) *m*/*z* 839 ([M − H − ArGlu]$^-$); FABMS (glycerol, positive mode) 1189 ([M + Na]$^+$); ^{1}H and ^{13}C, NMR: nearly identical to those of **3**, except for the COO*CH*$_3$ signal at δ_H 3.75, δ_C 52.8.

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