An aphid repellent glycoside from *Solanum laxum*

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Abstract

A spirostanic saponin was isolated from the ethanolic extract of the aerial parts of *Solanum laxum* Steud. The compound, named luciamin, was characterised by NMR spectroscopy, mass spectrometry and chemical methods, as (22*R*, 25*S*)-spirost-5-en-3 β, 15 α-diol 3-*O*-{β-D-glucopyranosyl (1→2)-β-D-glucopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-galactopyranoside}. The compound was tested against the aphid *Schizaphis graminum* by incorporation in artificial diets. It showed a deterrent (toxic) activity against the insect and is the first spirostane glycoside reported to have this activity. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Solanum laxum; Solanaceae; Bioassay; Glycosides; *Schizaphis graminum*

1. Introduction

Several classes of compounds, including alkaloids (Zúñiga et al., 1985), flavonoids and related compounds (Dreyer and Jones, 1981), and diterpenes (Rose et al., 1981), are toxic for and/or deter feeding of herbivores and insects. The Solanaceae family is a rich source of active secondary metabolites. In particular, the genus *Solanum* produces a great variety of steroidal saponins and glycoalkaloids of importance in the natural resistance of these plants against several pests (Friedman et al., 1991). *Solanum* glycosides and glycoalkaloids affect the aphid *Schizaphis graminum* (Rondani) (Homoptera: Aphididae) (Soulé et al., 1999). This aphid is of economic interest, since it is considered the worst cereal aphid pest (Anonymous, 1981), yet it does not normally settle on *Solanum* plants.

In our 5-year continuous study of the structure and activity of the glycosides produced by Uruguayan *Solanum* plants (Ferreira et al., 1994, 1996; Günter et al., 1997; Soulé et al., 1999) we now report the isolation, characterisation and biological activity against *Schizaphis graminum* of a new spirostanic saponin named luciamin isolated from *Solanum laxum* Steud, a native

bindweed that grows in association with riverside vegetation.

2. Results and discussion

The material obtained after ethanolic extraction of the aerial parts of *S. laxum* and further evaporation, was partitioned between water and chloroform. During this procedure, a layer formed between the water and the chloroform. It was separated and the residue after concentration was fractionated by medium pressure liquid chromatography (MPLC) using first reversed and then normal stationary phases. Two known saponins, laxumine A and laxumine B (Ferreira et al., 1996), and a third new saponin, named luciamin (**1**), were isolated by this method.

Luciamin (**1**) was obtained as a pale yellow powder, $[\alpha]_D^{20} = -65°$ (MeOH, *c* 0.3). The IR spectrum exhibited, among other signals, absorption bands indicating presence of hydroxyl groups (3417 and 1069 cm^{-1}) (Parikh, 1974). The ^{1}H NMR spectrum and the typical steroidal coloration produced by H$_2$SO$_4$ aspersion and charring on TLC plates suggested a glycosidic structure.

The sugars obtained after acid hydrolysis of luciamin (Fig. 1, **1**) were analysed as their alditol acetates by gas chromatography–mass spectrometry (GC–MS) using
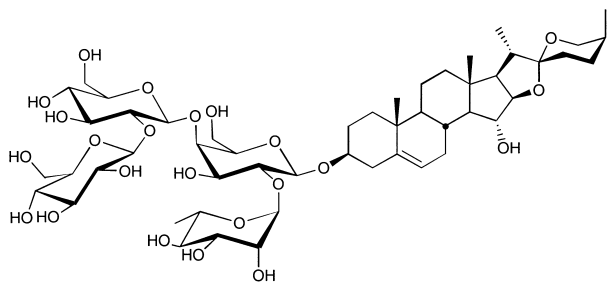
Fig. 1. Chemical structure of luciamin.

pure reference samples (Sawardeker et al., 1965), and the absolute configurations were determined by GC of the glycosides obtained by reaction with (+)-2-butanol and trimethylsilylation (Gerwig et al., 1979). D-Glucose, L-rhamnose and D-galactose in the relative proportions 2:1:1 were the only sugars detected. The linkages by which the sugars are connected were determined by methylation analysis according to Jansson et al. (Jansson et al., 1976). Analysis of the products by GC–MS and comparison with pure reference samples yielded the acetates of 2,3,4-tri-*O*-methyl-L-rhamnitol, 2,3,4,6-tetra-*O*-methyl-D-glucitol, 3,4,6-tri-*O*-methyl-D-glucitol and 3,6-di-*O*-methyl-D-galactitol. These results indicated the presence of terminal rhamnose and glucose groups, a 2-substituted glucose and a 2,4-disubstituted galactose residue.

From the ^{1}H and ^{13}C NMR spectra of luciamin information on the sugar residues and their anomeric configurations were obtained. Using different 1D and 2D experiments (H–H COSY, relay and double relay H–H COSY), most of the ^{1}H NMR signals could be assigned and the $^3J_{H1,H2}$ values determined (Table 1).

The ^{1}H NMR spectrum of luciamin in methanol-d_4 at 30°C showed signals corresponding to four anomeric protons at δ 5.14 ($J = 1.6$ Hz), 4.80 ($J = 7.9$ Hz), 4.58 ($J = 7.6$ Hz) and 4.48 ($J = 7.6$ Hz), confirming the presence of a tetrasaccharide moiety consistent with the sugar and methylation analyses. Proton decoupled HMQC experiments allowed the assignment of the corresponding carbon signals (Table 1).

This information, together with the values of $^3J_{H1,H2}$ for the anomeric protons and published chemical shift data for each sugar residue (Jansson et al., 1989) allowed the assignment of the different spin systems to specific sugar residues and the determination of their respective anomeric configurations. The data showed that the rhamnose residue was an α-pyranoside whereas the other residues were β-pyranosides.

The FAB mass spectrum of luciamin (**1**) gave a $[M + Na]^+$ ion at m/z 1085 and a $[M + H]^+$ ion at m/z 1063 indicating that the molecular weight was 1062.

The high energy collision induced dissociation mass spectrum (CID-MS) of the luciamin $[M + H]^+$ ion at m/z 1063 using He as the collision gas, showed among others, fragments at m/z 901, 739, 593 and 431 corresponding to

the successive loss of two hexose residues, the rhamnose residue and a further loss of an another hexose, respectively.

The ^{1}H NMR spectrum of luciamin showed, in addition to the signals of the oligosaccharide residue, signals corresponding to one olefinic proton at δ 5.38, two methyl groups substituted at quaternary carbons at δ 0.85 (*s*) and δ 1.06 (*s*) and three methyl groups substituted at methine carbons at δ 0.99 (*d*, $J = 6.8$ Hz), δ 1.09 (*d*, $J = 7.2$ Hz) and δ 1.30 (*d*, $J = 7.0$ Hz).

The ^{13}C NMR spectrum showed signals for 51 carbons, 27 of which arose from the aglycone moiety. The spectrum displayed, among other, signals corresponding to two olefinic carbons at δ 141.4 and 123.2, and a spirostanic carbon atom at δ 110.7 (Agrawal et al., 1985). These data, and comparison with published data (Agrawal et al., 1985, 1998; Barbosa Filho et al., 1989; Ferreira et al., 1994, 1996), suggested that luciamin consisted of a Δ^5-spirosten aglycone, which is a structure commonly found in *Solanum* plants, substituted with a tetrasaccharide.

The CID mass spectrum of the $[M + H]^+$ ion at m/z 1063 produced by FAB ionisation of luciamin, showed an ion at m/z 431 corresponding to the protonated aglycone. This indicated the molecular weight of the aglycone to be 430 mass units, which corresponds to a molecular formula of C27H$_{42}$O$_4$, suggesting the presence of a Δ^5-spirosten skeleton substituted with two hydroxyl groups.

The proton spin system of the A, B and C rings were assigned by different H–H correlation experiments (H–H double quantum filtered phase sensitive COSY and relay and double relay COSY). These data, together with the proton-carbon correlation from a ^{1}H-decoupled HMQC experiment and comparison with the literature, allowed the assignment of the carbon signals. H-3 (δ 23.59) was readily identified by its coupling with H-4α (δ 2.45), H-4β (δ 2.29), H-2α (δ 1.91) and H-2β (δ 1.60). The C-2, C-3 and C-4 signals (δ 30.5, 80.3 and 38.3, respectively) are similar to the corresponding values for diosgenin 3-*O*-β-D-galactoside (δ 30.5, 78.4 and 39.6 respectively) (Agrawal et al., 1985).

The observed n.O.e. between the anomeric proton of the galactose residue (δ 4.48) and H-3 (δ 3.59) confirmed that the tetrasacharride was linked to the 3-OH of the aglycone. The signals for H-1α (δ 1.08) and H-1β (δ 1.87) were assigned by their coupling with H-2α and H-2β.

The coupling between the signal for the vinylic H-6 (δ 5.38) and H-7β (δ 2.36) and H-7α (δ 1.75) allowed their identification. H-8 (δ 1.70) was identified by its coupling with both H-7 and a cross peak between its signal and the H-6 signal in the relay H–H COSY spectrum. The H-9 signal at 1.07 was readily assigned by its coupling with H-8. Similarly, all the ^{1}H and ^{13}C NMR signals for the A, B and C rings were assigned (Table 1) and were

Table 1
^{1}H and ^{13}C chemical shifts of luciamin in CD$_3$OD solutions at 30°C

	Aglycone				Sugar residues	
	δ_H		δ_C		δ_H	δ_C
C$_{number}$	α	β		C$_{number}$		
					α-L-Rhamnopyranosyl (1→2	
1	1.08	1.87	38.7	1	5.14 (1.6)	102.5
2	1.91	1.60	30.5	2	3.92	72.0
3		3.59	80.3	3	3.62	71.9
4	2.45	2.29	38.3	4	3.40	72.9
5			141.4	5	4.13	69.9
6	5.38		123.2	6	1.30	18.1
7	1.75	2.36	33.2	→2,4)-β-D-Galactopyranosyl (1→		
8		1.70	30.7	1	4.48 (7.6)	100.8
9	1.07		51.6	2	3.76	78.8
10			37.9	3	3.68	71.5
11	1.47	1.57	21.8	4	4.02	79.2
12	1.26^a	1.72^a	41.4	5	3.52	74.2
13			41.4	6/6'	3.57/3.91	63.2
14	1.25		61.7	→2)-β-D-Glucopyranosyl (1→4		
15		3.93	79.3	1	4.58 (7.6)	104.4
16	4.18		91.1	2	3.65	81.6
17	1.90		60.9	3	3.60	79.1
18		0.85	17.8	4	3.24	72.0
19		1.06	19.8	5	3.38	78.5
20		2.56	38.3	6/6''	3.60/3.90	63.2
21	0.99		14.7	β-D-Glucopyranosyl (1→2		
22			110.7	1	4.80 (7.9)	104.4
23	1.88	1.38	27.3	2	3.22	72.0
24	1.36	2.04	25.7	3	3.69	76.8
25	1.65		27.9	4	3.38	66.8
26	3.26	3.95	66.5	5	3.52	75.2
27		1.09	16.4	6/6'	3.63^a/3.90	61.2

^{a} Approximate values.

characteristic for a Δ^5-spirosten system (Agrawal et al., 1985, 1998; Barbosa Filho et al., 1989; Ferreira et al., 1994, 1996). The signals for the C-18 and C-19 methyl groups and the quaternary carbons C-5, C-10 and C-13 were assigned by comparison with published data for similar compounds (Agrawal et al., 1985; Ferreira et al., 1996). The observed NOE between the C-19 methyl protons (δ 1.06) and H-1β (δ 1.87), H-2β (δ 1.60), H-4β (δ 2.29), H-8 (δ 1.70) and H-11β (δ 1.57) established their β-position.

The proton spin system of the D and E rings was assigned in a similar way. The signal at δ 1.25 was assigned to H-14 because of its coupling with H-8. The coupling between H-14 and a proton at δ 3.93 identified the latter as H-15. The chemical shifts for the H-15 and C-15 (δ 79.3) signals indicated that C-15 was hydroxylated. The signal at δ 4.18 (dd, J = 9.2 and 6.4 Hz) was assigned to H-16. This proton is coupled with the protons at δ 3.93 and 1.90 corresponding to H-15 and H-17, respectively. The multiplet at δ 2.56 corresponds to H-20, according to its coupling with H-17 and the C-21 methyl groups at δ 0.99.

The continuity of the proton spin system is broken between rings E and F in the spirostan system due to the presence of the quaternary C-22 (δ 110.7). The proton and carbon chemical shifts of the methyl group at C-27 (δ$_H$ 1.09 and δ$_C$ 16.4, respectively), indicated an S configuration at C-25 (Barbosa Filho et al., 1989; Agrawal et al., 1998). The use of several 1D and 2D NMR methods allowed the assignment of all the signals of the F ring. The chemical shifts of the resonance signals of C-22 (δ 110.7), H-23β (δ 1.38), H-23α (δ 1.88), and C-23 (δ 27.3) confirmed the 22α-O configuration at C-22 and the axial configuration of the methyl group at C-27.

The CID mass spectrum of the ion at m/z 431 which corresponds to the protonated aglycone, showed ions at m/z 413 and 395, formed by loss of one and two molecules of water, respectively. The ion at m/z 269 formed by the loss of a fragment of 126 mass units from the pseudomolecular ion of the protonated aglycone, indicated the absence of any substitution on ring F. (Faul and Djerassi, 1970).

All the above data identified luciamin (1) as (22R, 25S)-spirost-5-en-3 β, 15 α-diol 3-O-{β-D-glucopyranosyl (1→2)-β-D-glucopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-galactopyranoside}.

Luciamin was tested against the greenbug, Schizaphis graminum Rondani, by addition of the tested compound to artificial diets. Previous work has shown a structure-dependent activity for both Solanum glycosides and glycoalkaloids against this insect (Soulé et al., 1999).

The toxicity was tested feeding aphids on artificial diets containing variable amounts of luciamin (Experiment 1). Survival was read after 24 h (Table 2). A constant decrease of the survival down to ca. 45% was observed for all tested concentrations. This plateau effect could be attributed either to a toxic or a deterrent effect (Argandoña et al., 1983; Rovirosa et al., 1994). To discriminate among these two possibilities, a second bioassay was designed. In experiment 2, aphids were fed on diets containing different concentrations of luciamin (1). After 12 h, live aphids were removed, and allowed to feed for another 12 h on diet only. After this second period, aphid survival was similar for all the tested concentrations of luciamin (1). If the compound were toxic, the expected result would be an inverse relationship between the concentration of the tested compound and the aphid survival. However, aphid survival in this experiment was almost equal for all the luciamin (1) concentrations assayed. The decrease of aphid survival down to ca 70% at 24 h in experiment 2 for all luciamin (1) concentrations (Table 3) indicated the repellent effect of luciamin against the aphid S. graminum.

Structurally related compounds, laxumin A and laxumin B, have been assayed previously against the same aphid (Soulé et al., 1999). These compounds have been shown to be toxic. The toxicity data from both laxumins A and B, and the toxicity data for luciamin, revealed a

Table 2
Experiment 1: percentage of relative survival (%RS) of aphids fed on diet with various concentrations of luciamin after 24 h[a]

Concentration of luciamin (vM)				
0 (control)	50	100	250	500
100±0	47±2[b]	49±4[b]	48±3[b]	40±4[b]

[a] Data are expressed as mean + SEM.
[b] Statistically significant differences from the control ($\alpha \leqslant 0.05$).

Table 3
Experiment 2 (deterrency test): percentage of relative survival (%RS) of aphids fed for 12 h on sachets with diet containing different concentrations of luciamin (%RS at 12 h), and non-cumulative percentage of relative survival of live aphids transferred to new sachets and fed for another 12 h only with holidic diet (%RS at 24 h)[a]

	Concentration of luciamin (μM)				
	0 (control)	50	100	250	500
%RS at 12 h	100±0	93±5	80±7[b]	85±3[b]	70±4[b]
%RS at 24 h	100±0	64±9[b]	70±13[b]	72±6[b]	76±4[b]

[a] Data are expressed as mean + SEM.
[b] Statistically significant differences from the control ($\alpha \leqslant 0.05$).

similar pattern of biological activity. The decrease in aphid survival to a constant level, which was found for all three compounds, may mean that laxumins A and B, like luciamin, also exert a deterrent effect on aphids.

Aphids are sap-feeding insects. They are agriculturally important pests, not only because of the direct damage they cause, but most importantly, because of their role as virus vectors (Harris and Maramorosch, 1977; Raman and Radcliffe, 1992).

Aphid feeding is a complex process that includes superficial recognition of the host plant, stylet penetration of the vegetal tissue, probing of the sap, and finally, feeding (Dixon, 1978). It has been demostrated that virus propagation by aphids occurs during the probing, and especially, the feeding processes. Deterrent compounds decrease probing and inhibit feeding, probably affecting the transmission of some types of virus by aphids.

Luciamin is the first steroidal glycoside reported to be an aphid repellent. This property of luciamin, together with the role of aphids as virus vectors, deserves further investigation in order to establish wether a similar activity exists in other members of this family of compounds, and to explore their potential biological and economic benefits.

3. Experimental

3.1. General

Solns were concd under red. pres. at temps not exceeding 40°C. For GC, a Shimadzu GC 14B instrument fitted with a FID detector was used. Sepn of the alditol acetates and the partially methylated alditol acetates was performed on a SE-52 fused-silica capillary column, using a temp. programme from 140°C (3 min) to 240°C at 3°C/min. Electron impact ionisation GC–MS was performed using the column and conditions mentioned above on a Shimadzu QP 5050.

FAB–MS was recorded on a Jeol JMS-SX/SX-102A tandem mass spectrometer by bombardment of samples (dissolved in a glycerol matrix) with Xe atoms of average translational energy 6 keV. The instrument was operated at an accelerating voltage of 10 kV. MS/MS was conducted using the first two-sector (B_1E_1) to select the precursor ions and the second mass spectrometer (B_2E_2) to analyse product ions. In the collision-induced dissociation (CID) experiment, the pressure in the collision chamber was adjusted in order to reduce the intensity of the precursor ion to approximately one half.

The ^1H and ^{13}C NMR spectra were obtained at 400 and 100 MHz, respectively, at 30°C on a Brüker Avance DPX 400 NMR instrument with CD_3OD used as solvent. Chemical shifts are reported in ppm, using TMS (δ_H 0.00) and Me_2CO (δ_C 31.00) as int. references. 2D (COSY, relay COSY, and HMQC) experiments were performed according to standard pulse sequences in the Brüker software.

3.2. Plant material

Aerial parts of *S. laxum* Steud were collected on the Santa Lucía riverbanks in December 1992. A voucher specimen was deposited at the Facultad de Química Herbarium, Montevideo, under catalogue no. MVFQ 1057 leg. E. Alonso Paz.

3.3. Extraction and isolation of the plant material

Aerial parts of *S. laxum* (1.5 kg) were chopped and extracted with EtOH (5 l) at room temperature for 7 days. After filtration and evaporation of the solvent to near dryness, a dark green extract (92 g) was obtained. Part of this extract (70 g) was suspended in H_2O (150 ml), filtered and defatted with $CHCl_3$ (3×100 ml). At the H_2O-$CHCl_3$ interface a third layer was detected, which was separated and evaporated to dryness.

Part of this extract (1.0 g) was fractionated by MPLC on a reverse phase column (100×5.0 cm, C_{18}; Merck), using the following H_2O–MeOH step gradients: 2:3 (1 l, frs 1–60), 3:7 (1.5 l, frs 61–161) and 100% MeOH (1 l, frs 162–230). After evaporation of solvent, the frs were analysed by TLC and ^1H NMR spectroscopy, where frs 48–81 gave different mixtures of saponins. Frs 81–83 (40 mg) were mixed and redissolved in MeOH, and applied to a MPLC silica gel column (30×2 cm) eluted with $CHCl_3$–MeOH–2%NH_4OH (70:30:5). In pure saponin luciamin (**1**) (25 mg), $[\alpha]_D = -65°$ (MeOH, *c* 0.3) was isolated.

3.4. Hydrolysis of the glycoside and sugar analysis

Saponin (**1**) (1–3 mg) was hydrolysed with 2 N TFA at 120°C for 1 h, with the aqueous layer then freeze-dried with part of the lyophilised material next reduced with NaBH$_4$ (10 mg) in 1 M NH$_4$OH (1 ml) for 30 min at room temperature. Excess NaBH$_4$ was quenched with HOAc, and the boric acid removed by co-distillation with MeOH (3×1ml). The resulting alditols were acetylated with Ac$_2$O–pyridine (1:1, 1 ml) at 120°C for 20 min and analysed by GC–MS using pure reference samples as standards (Sawardeker et al., 1965). The remainder was treated with 1 M HCl in (+)-2-BuOH (200 μl) at 80°C for 8 h in a scale tube. The mix was then evaporated to dryness, trimethylsilylated with SIL A (Supelco, 0.2 ml) for 30 min at room temp., the solution evaporated with a stream of nitrogen and the sample redissolved in *n*-hexane, filtered, and analysed by GC as described by Gerwig et al. (1979).

3.5. Methylation analysis

Samples (1 mg) dissolved in DMSO were methylated using Na methylsulphinyl anion and CH$_3$I according to Hakomori (1964), with the procedure suggested in Jansson et al. (1976). The permethylated products were isolated using a Sep-Pak C$_{18}$ cartridge (Waeghe et al., 1983) and analysed as described in Jansson et al. (1976).

3.6. Aphids

S. graminum (Rondani) were reared on *Hordeum distichum* cv. Aramir in a phytotron at 25±4°C with a photoperiod of 24 h light.

3.7. Bioassays

Aphid toxicity and feeding deterrency assays were conducted using a holidic diet (Argandoña et al., 1982) placed between two layers of Parafilm M, to make a sachet, fitted over a glass cylinder. The test compound was added to the diet at different concentrations. Control sachets were filled only with holidic diet. Non-choice assays were performed under ambient light. Five replicates containing 10 aphids each were used for each treatment.

3.8. Experiment (1) — toxicity test

To test for toxicity, the mortality of aphids fed on diet with various concentrations of luciamin (**1**) was determined at 24 h and the rate of survival calculated (%RS 24 h, exp 1).

3.9. Experiment (2) – deterrency test

After fasting for half a day, the aphids were fed on sachets with diet containing different concentrations of luciamin (**1**) for 12 h. Aphid mortality was measured and the relative survival again calculated (%RS 12 h, experiment 2). Then the live aphids were transferred onto new sachets and fed for 12 h with a holidic diet only. The non-cumulative mortality of aphids was measured and the relative survival calculated as before (%RS 24 h, experiment 2).

3.10. Data analysis

The percentage of relative survival (%RS) was calculated as follows:

$$\%RS = \frac{(100 - \% \text{ mortality})^{*}_{\text{treatment}} 100}{(100 - \% \text{ mortality})_{\text{control}}}$$

Student's *t*-test was used to identify significant differences between data from control and test experiments at the $\alpha = 0.05$ level (Snedecor and Cochran, 1977).

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