A new steroidal saponin with antiinflammatory and antiulcerogenic properties from the bulbs of *Allium ampeloprasum* var. *porrum*

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**Abstract**

A new steroidal saponin was isolated from the bulbs of *Allium ampeloprasum* var. *porrum* L. On the basis of chemical evidence, comprehensive spectroscopic analyses and comparison of known compounds, its structure was established as (3β,5α,6β,25R)-6-[(β-D-glucopyranosyl)oxy]-spirostan-3-yl O-β-D-glucopyranosyl-(1→2)-O-[β-D-glucopyranosyl-(1→3)]-β-D-galactopyranoside (1). Results of the present study indicated that the steroidal saponin showed haemolytic effects in the *in vitro* assays and demonstrated antiinflammatory activity and gastroprotective property using *in vivo* models.

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*Allium ampeloprasum* var. *porrum*  
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1. **Introduction**

*Allium ampeloprasum* var. *porrum* L. (Liliaceae), a bulbous perennial plant, is one of the daily edible green vegetables for Brazilian people. It is widely cultivated and used as food in Brazil. This plant is used, not only as food, but also as medicine. The bulbs have been reputedly used in the traditional Brazilian medicine for treating inflammatory symptoms. The crushed bulb is used to treat initial stages of cough, mucous secretion and sore throat. The fresh juice is taken orally as a stomachic and antispasmodic and is also reputed to possess digestive properties [1]. While chemical studies on saponins of *Allium* specimens have been reported [2–4], no chemical investigation of *A. ampeloprasum* var. *porrum* has been reported as yet. In this paper, we report the structural elucidation and biological activities of the new steroidal saponin 1 (Fig. 1).

2. **Experimental**

2.1. **General**

Melting points were determined by an Electrothermal 9200 micro-melting point apparatus and are uncorrected. The optical rotations were measured with a Perkin Elmer 243B polarimeter. IR spectra were measured with a Perkin Elmer 599B spectrometer. The MALDI-TOFMS was obtained using a Perseptive Voyager RP mass spectrometer. GC-EIMS was carried out with FID using a ZB glass capillary column (0.25×50 m). Mass spectra were taken with a VG Auto SpecQ spectrometer. NMR experiments were performed with a Mercury-300 spectrometer at 308.1 K. All the 2D NMR spectra were acquired in pyridine-*d*<sub>5</sub> with tetramethylsilane (δ = 0.00) used as internal standard. <sup>1</sup>H NMR spectra were recorded at 300 MHz and <sup>13</sup>C NMR spectra at 75 MHz. Silica gel columns (200–400 mesh ASTM, Merck) and Sephadex LH-20 (Pharma-  
cia) were used for column chromatography (CC). Thin layer chromatography (TLC) was performed on silica gel plates (Kieselgel 60F<sub>254</sub>, Merck) using the following solvent systems: (A) CHCl<sub>3</sub>/MeOH/H<sub>2</sub>O (65:35:10, v/v/v, lower phase) for steroidal saponin 1, (B) CHCl<sub>3</sub>/MeOH (95:5, v/v) for sapogenin 1a, and (C) *n*-BuOH/Me<sub>2</sub>CO/H<sub>2</sub>O (4:5:1, v/v/v) for...
monosaccharides. Spray reagents were orcinol/H₂SO₄ for steroidal saponin 1 and monosaccharides and CeSO₄ for sapogenin 1a.

2.2. Plant material

The bulbs of *A. ampeloprasum* var. *porrum* were purchased from a nursery in Rio de Janeiro, Brazil, in September 2007. The bulbs were cultivated, and a voucher specimen (LQPM 60) of the plant was deposited at the Laboratory of Medicinal Plant Chemistry, Federal University of Rio de Janeiro.

2.3. Extraction and isolation

The fresh bulbs (1.16 kg) were cut into small pieces and extracted with MeOH (5 l) for 72 h at room temperature. The extract was concentrated under reduced pressure to remove the MeOH (500 ml) and roughly chromatographed. The crude saponin (930 mg) was subjected to column chromatography on silica gel (2.8×90 cm) eluted with CHCl₃/MeOH (10:5:1:4, v/v/v/v; 2 l; fraction of 25 ml) to afford a TLC homogeneous compound 1 (fractions 59–62; 250 mg; *Rf* = 0.41) which gave a dark blue color with orcinol/H₂SO₄.

2.4. Spectrometric identification of compound 1

\[ (3\beta,5\alpha,6\beta,25R)-6-\{[\beta-\beta-D-glucopyranosyl]oxy\}-spirostan-3-yl \ 0-\beta-D-glucopyranosyl-(1→2)-\beta-\beta-D-glucopyranosyl-(1→3)-\beta-D-galactopyranoside \] (1): amorphous powder, mp 273–275 °C; [α]D = 58° (c 0.1, CHCl₃/MeOH (1:1)). IR νmax (KBr): 3380 (OH), 2935 (CH), 1445, 1375, 1235, 1150, 1050, 915, 898, 860, 745 cm⁻¹. ¹H NMR (pyridine-d₅): δ 3.93 (1 H, m, H-3), 0.95 (1 H, m, H-5), 3.88 (1 H, m, H-6), 4.45 (1 H, m, H-16), 0.86 (3 H, s, H-18), 1.20 (3 H, s, H-19), 1.84 (1 H, m, H-20), 1.17 (3 H, d, J = 6.7 Hz, H-21), 1.46 (1 H, m, H-25), 3.38 (1 H, dd, J = 10.7, 10.7 Hz, H-26ax), 3.46 (1 H, dd, J = 2.8, 10.7 Hz, H-26eq), 0.68 (3 H, d, J = 5.6 Hz, H-27), 4.92 (1 H, d, J = 7.3 Hz, Gal H-1), 5.12 (1 H, d, J = 8.1 Hz, Glc H-1), 5.16 (1 H, d, J = 7.6 Hz, Glc H-1), 5.20 (1 H, d, J = 7.6 Hz, Glc H-1). ¹³C NMR (pyridine-d₅) data: see Table 1. MALDI-TOFMS: m/z 1104.2065 [M+Na]⁺ (calcd. 1104.2135).

2.5. Acid hydrolysis of compound 1

A solution of compound 1 (100 mg) in 1 M HCl/1,4-dioxane (1:1, v/v; 10 ml) was heated in a sealed tube for 1 h at 100 °C. After cooling, the reaction mixture was neutralized with 1 M NaOH in MeOH and evaporated to dryness. The salts that were extracted at room temperature with MeOH (5 l) for 72 h at room temperature. The resulting organic phase was evaporated in vacuo and the crude saponin (fractions 19–23; 5 × 186 mg) was subjected to column chromatography on silica gel (2.8×90 cm) eluted with CHCl₃/MeOH/n-ButOH/H₂O (10:5:1:4, v/v/v/v; 2 l; fraction of 25 ml) to afford a TLC homogeneous compound 1 (fractions 59–62; 250 mg; *Rf* = 0.41) which gave a dark blue color with orcinol/H₂SO₄.

![Fig. 1. Structure of compound 1.](image-url)
2.7. Determination of D, L configurations of monosaccharides as their trimethylsilylated derivatives

The hydrolysates (fractions 10–13) were combined on the basis of TLC behaviors, concentrated under vacuum and the dried residue was chromatographed on silica gel column (1.6×40 cm) with gradient CHCl3/MeOH (19:1 to 7:3; 1 l; fraction of 10 ml) to afford the sapogenin 1a (30 mg) and sugar mixture (48 mg). The sapogenin 1a (Rf=0.40) and the sugar mixture were obtained from the fractions analyzed by silica gel-TLC using the solvent systems (B) CHCl3/MeOH (95:5, v/v) and (C) n-BuOH/Me2CO/H2O (4:5:1, v/v/v), respectively. A sample of the sugar mixture (1 mg) was dissolved in pyridine (100 μl) and analyzed by silica gel-TLC in the above described solvent (C). After spraying with orcinol/H2SO4, glucose and galactose gave blue spots at Rf values of 0.38 and 0.35, respectively.

\[(3β,5α,6β,25R)-Spirostan-3,6-diol (1a): Colorless needles from benzene, mp 239–240 °C; [α]D^25 = -72.5° (c 0.5, CHCl3); IR ν max (KBr): 3520, 3250 (OH), 2955, 2930, 2875, 2850 (CH), 1660, 1540, 1450, 1375, 1340, 1240, 1175, 1075, 1055, 1015, 1005, 980, 955, 915, 900, 860 cm^{-1} (intensity 915–900, 25R-3α,5β,25R-spiroketone); EIMS: m/z 432 [M]+, 139 [C_9H_{15}O_2]^+; The 1H and 13C NMR (pyridine-d_5) data agreed well with those of literature data [5].

2.6. Molar carbohydrate composition of compound 1

Dried compound 1 (1 mg) was dissolved in 0.5 ml of methanolic 0.5 M HCl. Nitrogen was bubbled through the solution for 30 s and then the ampoule was sealed. After methanolysis for 24 h at 80 °C, the solution was concentrated under reduced pressure at 45 °C. The residue was dried for 12 h in a vacuum desiccator over P₂O₅. The dried sample was trimethylsilylated with 0.1 ml of silylating agent pyridine/hexamethyldisilazane/trimethylchlorosilane (5:1:1, v/v/v) for 30 min at room temperature. The molar carbohydrate composition of the glycosidic moiety of 1 was determined by GC-EIMS analyses of its monosaccharides as their trimethylsilylated methylglycosides [6].

2.7. Determination of d, l configurations of monosaccharides of compound 1

A sample of the sugar mixture (0.5 mg), obtained after acid hydrolysis of 1 (see Section 2.5.), was dissolved in (−)-2-butanol containing 1 M HCl (0.5 ml). The solution was flushed with nitrogen and heated for 6 h at 60 °C. The reaction solution was treated with an excess amount of Ag₂CO₃ and the mixture was centrifuged. The supernatant was evaporated to dryness at 45 °C, and the residue was dried in a desiccator containing P₂O₅ [7]. The resultant syrup was trimethylsilylated according to the procedure of Kamerling et al. [6]. The configurations of monosaccharides of the glycosidic moiety of 1 were determined by capillary GC and GC-EIMS of their trimethylsilylated (−)-2-butyxl glycosides [7].

2.8. Methylation analysis of compound 1

Compound 1 (1 mg) was dissolved in dimethylsulfoxide (200 μl) in a Teflon-lined screw-cap tube. Lithium methylsulfinyl carbanion (200 μl), prepared according to the procedure of Parente et al. [8], was added to the solution under an inert atmosphere and the mixture was sonicated for 60 min. After cooling to −4 °C, cold methyl iodide (400 μl) was added. Sonication was conducted in a sonication bath (20 °C) for 45 min. The methylation was terminated by the addition of water (4 ml) containing sodium thiosulfate, and the permethylated product extracted with chloroform (3×2 ml) and evaporated [8]. The methyl ethers were obtained after hydrolysis (4 N TFA, 2 h, 100 °C) and analyzed as alditol acetates by GC-EIMS [9].

2.9. Haemolytic activity

Normal human red blood cell suspension (0.5 ml of 0.5%) was mixed with 0.5 ml diluent containing 5, 10, 20, 30, 40, 50, 100, 250 and 500 μg/ml of compound 1 or the commercial saponin isolated from Quillaja saponaria (QS-21) in saline solution. Mixtures were incubated for 30 min at 37 °C and centrifuged at 70×g for 10 min. Free haemoglobin in the supernatant was measured by absorbance at 412 nm. Saline and distilled water were included as minimal and maximal haemolytic controls. The haemolytic percents developed by the saline control were subtracted from all groups. The adjacent concentration inducing 50% of the maximum haemolysis was considered the HD₅₀ (graphical interpolation). Experiments included triplicate at each concentration [10].

2.10. Animals

Male Swiss mice (three months old, 25–35 g) were obtained from the central animal care facilities, Health Sciences Centre, Federal University of Rio de Janeiro, Brazil. The mice were maintained under standard laboratory conditions (12 h light/dark cycle, at 22±2 °C). Standard pellet food and water were available ad libitum. The experimental protocol was performed according to the “Principles of Laboratory Animal Care” (NIH Publication 85-23, revised 1985).

2.11. Antiinflammatory activity

Antiinflammatory activity was evaluated by the carrageenan-induced oedema method [11]. Male Swiss mice (three months old, 25–35 g) in groups of five were administered orally with 1 ml of saline solution as the negative control or compound 1 (100 mg/kg) or the reference compound dexamethasone (25 mg/kg) dissolved in saline solution as positive controls. Acute inflammation was produced by subplantar injection of 50 μl of 1% freshly prepared colloidal suspension of carrageenan in physiological saline injected into the subplantar region of the right hind paw of the mice, 1 h after the oral administration of test sample as well as the negative and positive controls. The footpad thickness were measured with a spring-loaded dial gauge (Mitutoyo Corp., Tokyo, Japan) before and every 1 h during 5 h after induction of inflammation. Percent inhibition of the inflammation was determined by applying statistical methods followed by the calculation of percent inhibition for each group by comparing with control group.
2.12. Antiulcerogenic activity

Antiulcerogenic activity was evaluated by measuring acute gastric lesions induced by acidified ethanol [12]. Male Swiss mice (three months old, 25–35 g) in groups of five were fasted for 24 h before the experiment and administered orally with 1 ml of pure water as the negative control, or compound 1 (100 mg/kg), or the reference compound cimetidine (100 mg/kg) dissolved in vehicle as positive control. One hour after the treatments, all animals received orally 200 μL of acidified ethanol solution (0.3 M HCl/EtOH) to induce gastric lesions. The animals were killed 1 h after treatment with the ulcerogenic agent and the stomachs removed, opened along the greater curvature and rinsed with physiological saline to determine the lesion damage. The degree of gastric mucosal damage was evaluated from digital pictures using a computerized image analysis system. The percentage of the total lesion area (haemorrhagic lesions) to the total surface area of the stomach was defined as the ulcer index [13].

3. Results and discussion

Compound 1 was obtained as amorphous solid and gave a positive Liebermann–Burchard test for a steroidal saponin. It revealed a quasi-molecular weight ion peak at m/z 1104.2065 [M + Na]+ in the MALDI-TOFMS. In the 13C NMR spectrum, of the fifty-one carbon signals observed, there are four methyl groups, fourteen methylene groups (five of which were oxygenated), thirty methane groups (twenty-three of which were oxygenated) and three quaternary carbon atoms (one of which was oxygenated). The number of hydrogens attached to each individual carbon atom was determined by the DEPT spectrum. On the basis of the above mentioned MS and NMR spectral data (Table 1), compound 1 was assumed to be a saponin with the molecular formula C51H84O24, bearing four monosaccharide moieties.

In addition to this, the spirostanol glycosidic nature of compound 1 was indicated by the strong absorption bands at 3380 cm−1 for the hydroxy group and the characteristic 25 (R)-spiroketal absorption bands 898 and 915 cm−1 (intensity, 915<898 cm−1) in the IR spectrum [5,14]. The 25R stereochemistry of the Me-27 group was confirmed by protons and carbons resonances at positions 25, 26 and 27 in comparison with the stereochemical carbohydrate moieties.

According to the literature, steroidal saponins are shown to possess several physiological properties depending on their chemical structures, such as haemolytic activity and capacity for alteration of membrane permeability. Additionally, these compounds isolated from medicinal plants have been reported to have important biological activities, such as stimulation of lymphocyte proliferation [17], promotion of antibody production [18], radical scavenging capacity [19], and inhibition of the production of inflammatory cytokines by activated macrophages [20]. In order to confirm the utilization of this species in the traditional medicine and to evaluate the biological properties of compound 1 (Fig. 1), the antiinflammatory activity and gastroprotective property were investigated using in vivo models. Additionally, it was screened for haemolytic activity in the in vitro assays.

Generally, steroidal saponins possess powerful haemolytic capacity because steroids have higher affinities for cholesterol on erythrocyte membranes [21]. The ability of compound 1 to induce haemolysis of human red blood cells was investigated and compared with the saponin QS-21 isolated from Q. saponaria, a substance commonly used in animal and human experimental models. According to the results obtained, compound 1 was shown to possess potent haemolytic capacity (HD50 6.5 μg/mL), which is of the same order of magnitude as the reference commercial compound (HD50 5 μg/mL). This can be explained by the amphipathic characteristic of its structure, containing a hydrophobic steroidal nucleus and two hydrophilic carbohydrate moieties.

In addition to this, the antiinflammatory activity of compound 1 was investigated using an acute inflammation model. The results were measured by inhibition of carrageenan-induced mouse paw oedema [11]. The carrageenan-induced inflammation is a biphasic phenomenon. The early phase of oedema is attributed to the release of histamine,
serotonin and similar substances. The later phase results mainly from the potentiating effects of prostaglandins on mediator release [22]. Compound 1 showed significant antiinflammatory potential, promptly controlling both phases of inflammation and provoking an inhibition of oedema formation similar to the reference compound dexamethasone (Fig. 2). In comparison with the literature reports, compound 1 showed antiedematous properties with potency similar to that of bioactive compounds isolated from other medicinal plants used against inflammatory disorders [20,23].

Generally, commercially available antiinflammatory drugs are associated with unwanted side effects especially ulceration, which is the most common and serious problem [24]. Since there are popular information about the use of this plant for the treatment of digestive disorders, with the aim to confirm its utilization in the traditional medicine, the gastroprotective property of compound 1 was investigated. The antiulcerogenic activity was evaluated by measuring the inhibition of acute gastric lesions induced by acidified ethanol [12].

Acidified ethanol tends to dissolve the components of the mucous membrane of the stomach, bringing gastric blood flow to a standstill that contributes to the development of the haemorrhage and necrotic aspects of tissue injury [25]. By macroscopic observations, in the control animals that received only water before acidified ethanol administration, intense and widespread gastric hyperemia and thickened lesions were evident. In contrast, the stomachs of the animals which received compound 1 showed an aspect close to normality, with significant reduction in gastric hyperemia and in number and severity of lesions. This protective action is closely related to the reference compound cimetidine at the same dosage [24]. The intensity of gastric ulcers was quantified by the percentage of the injury area in relation to the control group (Fig. 3).

This result suggests that compound 1 probably interfere with the ulcerogenic mechanism, showing a cytoprotective property. The mechanisms which protect the gastric mucosa against acute attack by necrotic agents involve a variety of events [26]. Among these, a crucial role is played by mucus production, which is an important protective factor for the gastric mucosa and consists of a viscous, elastic and adherent barrier formed by water and glycoproteins that covers the entire gastrointestinal mucosa. The protective properties of the mucus barrier depend not only on its structure but also on the amount or thickness of the layer covering the mucosal surface [27]. Since literature reports indicated that steroidal saponins possess the ability to increase the mucosal defensive factors, inducing the turnover of glycoproteins in the mucosal cells, thus increasing the quantity of cellular mucus, probably this can be the mode of action of compound 1, preventing the penetration of the necrotizing agent or interacting with the macromolecules of the gastric mucosa [28,29].

In conclusion, the investigation of the biological properties of compound 1 indicated that this substance may be the potential therapeutic agent involved in the gastroprotective property and the treatment of inflammatory conditions, justifying the use of this plant as a food source and in the traditional medicine.

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References


