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# Steroidal alkaloids from *Cryptolepis obtusa*

Alexandra Paulo[a], M. Luisa Jimeno[b], Elsa T. Gomes[a], Peter J. Houghton[c,*]

[a]*CECF-Faculty of Pharmacy, University of Lisbon, Av. das Forças Armadas, 1649-019 Lisbon, Portugal*
[b]*Centro de Quimica Organica "Manuel Lora Tamayo", Calle Juan de la Cierva, 3, 28006 Madrid, Spain*
[c]*Pharmacognosy Research Laboratories, King's College London, Franklin-Wilkins Building, 150 Stamford Street, London SE1 8WA,UK*

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**Abstract**

Two novel diglycosylated steroidal alkaloids of 5Δ-pregnene nucleus, named obtusine-20(R)-*O*-[β-thevetopyranosyl-(1 → 4)-β-cymaropyranoside] and obtusolactam-20(R)-*O*-[β-thevetopyranosyl-(1 → 4)-β-cymaropyranoside], together with the known β-sitosteryl-3-*O*-β-glucopyranoside were isolated from the roots of *Cryptolepis obtusa* N. E. Br. 

*Keywords: Cryptolepis obtusa*; Periplocaceae or Asclepiadaceae; Saponins; 5Δ-pregnene steroidal alkaloids

## 1. Introduction

The genus *Cryptolepis* R. Br. includes about 20 species distributed throughout the tropical regions of Africa, Madagascar, Asia, Australia and Papua New Guinea. The genus is assigned either to the Periplocoideae subfamily of the Asclepiadaceae or to the newly created family Periplocaceae (Hutchinson & Dalziel, 1963; Cronquist, 1981), a taxon poorly investigated from the chemical point of view. Continuing our studies on the chemistry and biological activity of *Cryptolepis* species (Paulo, Pimentel, Viegas, Pires, Duarte & Cabrita, 1994; Paulo, Duarte & Gomes, 1994; Paulo, Gomes & Houghton, 1995; Paulo, Gomes, Duarte, Perrett & Houghton, 1997; Paulo, Gomes, Steele, Warhurst & Houghton, in press) the roots of *Cryptolepis obtusa* N. E. Br. were analysed for their major secondary metabolites. The aqueous extracts of roots of *C. obtusa* are traditionally used in Mozambique as an anti-abortive remedy, vermifuge and to treat abdominal pains (Mendes & Jansen, 1984). To our knowledge, no phytochemical work has been reported on the roots of this species. We describe herein the isolation and structural elucidation, on the basis of extensive high-field NMR studies, of two new diglycosylated steroidal alkaloids (**1** and **2**) with a novel 5Δ-pregnene skeleton.

## 2. Results and discussion

TLC using general spray reagents (Wagner, Bladt & Zgainski, 1984) of the chloroformic crude extract of the roots of *C. obtusa* revealed the presence of alkaloidal and non-alkaloidal saponins as major constituents. The chloroformic extract was chromatographed on a silica gel column and the alkaloid fraction obtained was further fractionated and purified by semi-preparative HPLC and prep. TLC to afford compounds **1** and **2**.

Compound **1** gave positive reaction with the Dragendorff spray reagent and for that reason it was suspected to be an alkaloid. The molecular formula of $C_{38}H_{63}O_{12}N$ was deduced by DEPT, $^{13}C$ NMR and FABMS data. The presence of two methyl singlets (0.69 and 0.96 ppm), one methyl doublet (1.17 ppm) and one olefinic proton signal at 5.31 ppm in the $^{1}H$ NMR spectrum of **1** indicated the presence of a pregnene molecule. The decoupled $^{13}C$ NMR and DEPT

* Corresponding author. Tel.: +44-207-848-4775.
*E-mail address:* peter.houghton@kcl.ac.uk (P.J. Houghton).

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Table 1
$^1$H and $^{13}$C NMR data ($\delta$ in ppm from TMS) of compounds **1**, **2** and **3**

| Position | **1**[a] | | | | | **2**[b] | | **3**[c] | |
|---|---|---|---|---|---|---|---|---|---|
| | $\delta$ $^{13}$C | $\alpha$ | $\delta$ $^1$H | $\beta$ | $J$ (Hz) | $\delta$ $^{13}$C | $\delta$ $^1$H $J$ (Hz) | $\delta$ $^{13}$C | $\delta$ $^1$H $J$ (Hz) |
| 1 | 37.22 | 1.03 | | 1.82 | | 37.8 | | 38.4 | 0.98, 1.71 |
| 2 | 30.91 | 1.77 | | 1.48 | | 31.9 | | 31.1 | 1.77, 2.14 |
| 3 | 70.35 | 3.54 | | | *m* | 70.8 | | 79.0 | 3.97 |
| 4 | 38.26 | 2.20 | | 1.90 | | 39.0 | | 40.2 | 2.48, 2.75 |
| 5 | 140.50 | | | | | 141.0 | | 141.8 | |
| 6 | 121.73 | | 5.31 | | *br s* | 122.2 | 5.37 *br s* | 122.8 | 5.36 *dd* (4.8, 2.5) |
| 7 | 29.45 | 1.42 | | 1.94 | | 29.9 | | 33.1 | 1.55, 1.89 |
| 8 | 31.85 | | | 1.24 | | 32.4 | | 32.9 | 1.37 |
| 9 | 49.62 | 0.95 | | | *m* | 50.3 | | 51.2 | 0.89 |
| 10 | 36.66 | | | | | 37.2 | | 37.8 | |
| 11 | 20.48 | | 1.51, 1.44 | | | 20.7 | | 22.2 | 1.43 |
| 12 | 38.72 | | 2.23, 2.34 | | | 39.0 | | 40.8 | 1.13, 1.99 |
| 13 | 45.26 | | | | | 46.0 | | 43.4 | |
| 14 | 51.02 | 1.80 | | | | 51.6 | | 57.7 | 0.91 |
| 15 | 78.37 | | | 3.50 | | 75.1 | | 24.3 | 1.30 |
| 16 | 68.13 | 3.28 | | | | 68.2 | | 29.4 | 1.25, 1.84 |
| 17 | 85.35 | | | | | 86.2 | | 57.1 | 1.10 |
| 18 | 14.07 | | | 0.69 | *s* | 14.7 | 0.76 *s* | 12.9 | 0.67 *s* |
| 19 | 19.29 | | | 0.96 | *s* | 19.6 | 1.02 *s* | 20.3 | 0.95 *s* |
| 20 | 69.01 | | 3.84 | | *q* (6.4) | 69.8 | 3.85 *q* (6.6) | 37.3 | 1.37 |
| 21 | 16.35 | | 1.17 | | *d* (6.4) | 16.6 | 1.22 *d* (6.6) | 19.9 | 1.00 *d* (6.4) |
| 22 | 113.62 | | | | | 169.0 | | 35.1 | 1.10, 1.40 |
| 23 | 86.33 | 4.71 | | 5.10 | *d* (7.5) | 46.7 | | 27.2 | 1.26 |
| 24 | 17.90 | | 1.23 | | *s* | 23.1 | | 46.9 | 1.01 |
| 25 | | | | | | | | 30.3 | 1.69 |
| 26 | | | | | | | | 20.1 | 0.88 *d* (6.4) |
| 27 | | | | | | | | 20.9 | 0.90 *d* (6.4) |
| 28 | | | | | | | | 25.4 | 1.05, 1.52 |
| 29 | | | | | | | | 13.1 | 0.91 *t* (6.7) |
| Cym | | | | | | | | | |
| 1′ | 99.1 | | 4.79 | | *dd*(9.7, 1.8) | 99.6 | 4.83 *dd* (8.0, 1.8) | | |
| 2′ | 29.8 | | 1.63, 2.04 | | | 30.1 | 2.05, 1.70 | | |
| 3′ | 77.6 | | 3.53 | | *q* (3.3) | 79.4 | 3.53 | | |
| 4′ | 83.0 | | 3.70 | | | 83.3 | 3.75 | | |
| 5′ | 70.5 | | 3.25 | | | 71.7 | 3.30 | | |
| 6′ | 18.1 | | 1.28 | | | 18.4 | 1.28 | | |
| 3′-$OCH_3$ | 56.6 | | 3.32 | | *s* | 56.9 | 3.35 *s* | | |
| Thev | | | | | | | | | |
| 1″ | 101.3 | | 4.26 | | *d* (7.7) | 102.0 | 4.32 *d* (7.3) | | |
| 2″ | 69.7 | | 3.62 | | | 70.8 | 3.55 | | |
| 3″ | 84.1 | | 3.12 | | | 84.7 | 3.15 | | |
| 4″ | 78.6 | | 3.30 | | | 79.6 | 3.40 | | |
| 5″ | 73.1 | | 3.92 | | | 73.9 | 4.00 | | |
| 6″ | 16.9 | | 1.27 | | | 17.1 | 1.27 | | |
| 3″-$OCH_3$ | 58.1 | | 3.46 | | *s* | 58.3 | 3.50 *s* | | |
| Glu | | | | | | | | | |
| 1′ | | | | | | | | 103.4 | 5.08 |
| 2′ | | | | | | | | 76.2 | 4.08 *dd* (8.2, 8.0) |
| 3′ | | | | | | | | 79.5 | 4.31 |
| 4′ | | | | | | | | 72.5 | 4.29 |
| 5′ | | | | | | | | 79.4 | 4.01 |
| 6′ | | | | | | | | 63.7 | 4.44 *ddd* (11.7, 5.2, 2.0) 4.59 *dd*(11.7, 2.0) |

[a] Spectra taken in $CDCl_3$ + 2 drops of $CD_3OD$ at 500 MHz ($^1$H) and 125 MHz ($^{13}$C).
[b] Spectra taken in $CDCl_3$:$CD_3OD$ (1:1) at 400 MHz ($^1$H) and 100 MHz ($^{13}$C).
[c] Spectra taken in pyridine-$d_5$ at 400 MHz ($^1$H) and 100 MHz ($^{13}$C).

spectra confirmed the presence of a double bond by showing a quaternary carbon signal at 140.5 ppm and a methine signal at 121.7 ppm. The $^{13}C$ NMR and HSQC spectra also showed two methine carbons at 101.3 and 99.1 ppm which were one bond correlated with the doublets resonating downfield at 4.26 and 4.79 ppm, respectively. These observations were consistent with a diglycosylated 5Δ-pregnene molecule. The decoupled $^{13}C$ NMR and DEPT spectra showed the presence of 5 quaternary carbons, 8 methyl, 8 methylene and 17 methine carbons. The HSQC spectrum showed the correlation of directly bonded protons and carbons. The next step was to analyse the HMBC spectrum, which allowed to correlate protons and carbons through two or three bond coupling. The singlet methyls resonating at 14.1/0.69 ppm and 19.3/0.96 ppm were assigned to C-18 and C-19, respectively, by comparison with literature data (Itokawa, Xu & Takeya, 1988; Puri, Wong & Puri, 1994) and the long-range correlation between the methyl C-19 and the quaternary carbon at 140.5 ppm established the double bond at C-5. Proton and carbon chemical shifts were assigned for almost all positions of the 5Δ-pregnene nucleus (Table 1) based on long-range correlations observed between those signals and C/H-18, C/H-19 and C/H-9α signals, HSQC data, homo-correlations observed in the $^1H$–$^1H$ COSY spectrum and confirmed with literature (Itokawa et al., 1988; Puri et al., 1994). The quaternary carbon resonating at 85.4 ppm was assigned to C-17β-OH due to long-range connectivities with H-18 and by comparison with literature data (Warashina & Noro, 1996).

The 1D-TOCSY and 2D-HSQC-TOCSY spectra allowed the establishment of the carbon and proton pairs of each sugar in the glycosidic chain of **1** and the identification of them as monomethoxy-2,6-dideoxy-hexose and monomethoxy-6-deoxyhexose. Having as a starting point the anomeric proton/carbon, the HMBC, $^1H$–$^1H$ COSY spectra, coupling constants and literature data (Aquino, Peluso, Tommasi, Simone & Pizza, 1996) allowed the complete assignments of chemical shifts and the identification of the two sugars as β-cymaropyranosyl and β-thevetopyranosyl to be made (Table 1). The downfield shift of C-4 of the β-cymaropyranosyl moiety (83.0 ppm) indicated that C4-OH was not free. A three-bond heterocorrelation was observed in the HMBC spectrum between the C-4 of the β-cymaropyranosyl moiety and the anomeric carbon of the β-thevetopyranosyl. This final observation led to the conclusion that the diglycosidic chain of **1** was β-thevetopyranosyl-(1 → 4)-β-cymaropyranoside.

The anomeric carbon of the first sugar of the glycosidic chain (99.1 ppm) showed long-range connectivities with a methine proton at 3.84 ppm which was three-bond correlated ($^1H$–$^1H$ COSY) with the methyl doublet resonating at 1.17 ppm. These observations

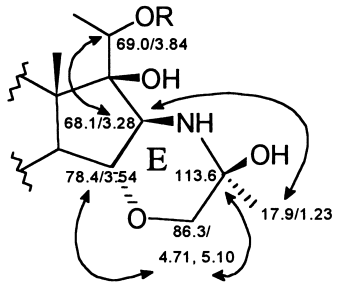



Fig. 1. Long-range connectivities observed between carbon/proton signals in the HMBC spectrum that allowed completion of the structure of the genin moiety of **1**.

indicated that the glycosidic chain was linked to C-20 of the pregnene molecule and assigned the carbon and proton chemical shifts to positions 20 (69.0/3.84 ppm) and 21 (16.4/1.17 ppm) (Warashina & Noro, 1996). Considering the work of Itokawa et al. (1988) who assigned all carbons of 5Δ-pregnene-3β, 16α, 20(S)-triol and 5Δ-pregnene-3β, 16β, 20(R)-triol isolated from the related species *Periploca sepium*, a carbon chemical shift of 69.0 ppm (C-20 of **1**) was in complete agreement with a 20(R)-*O*-glycosylated carbon, since a C-20(R)-OH shows a $\delta = 66.7$ ppm and a C-20(S)-OH a $\delta = 70.5$ ppm. Moreover, Warashina & Noro (1996) reported a $\delta = 70.9$ ppm for a C-20(S)-OH and a $\delta =$ 75.7 ppm for a C-20(S)-OR. Carbon and proton at position 20 (69.0/3.84 ppm) showed connectivities in the HMBC spectrum with a methine resonating at 68.1/3.28 ppm which was assigned to C-16. Based on literature (Itokawa et al., 1988), a $\delta = 68.1$ ppm was too upfield for either a C-16β-OH (73.2 ppm) or a C-16α-OH (77.6 ppm). For this reason, it was concluded that a less electrophilic atom like a nitrogen should be linked to C-16 instead of an oxygen. Since a $^1H$–$^1H$ NOESY correlation between H-20(R) and H-16 was observed, and that was only possible with H-16α, the nitrogen atom was assigned to the 16β position. Finally, the correlations shown in Fig. 1 were observed in the HMBC spectrum of **1**. These data and the $^1H$–$^1H$ COSY correlation observed between H-16α and the methine proton resonating at 3.54 ppm allowed the assignment of C-15, whose carbon chemical shift (78.4 ppm) strongly implied that it was linked to an oxygen atom. Given the above mentioned HMBC correlations, C-16 and C-15 had to be part of another hexane ring (E-ring) which included the 16β-NH group, a quaternary carbon C-22 (113.6 ppm) linked to a methyl group (17.9/1.23 ppm, singlet) and to an hydroxyl group, a methylene carbon C-23 (86.3 ppm) and finally, the 15-*O*- group (Fig. 1). Knowing that the NH group was linked to 16β position and assuming that E-ring of **1** would adopt a chair conformation, the 15-*O* group was considered to be α. Proton 23β (axial) was assigned to 5.10 ppm due to the $^1H$–$^1H$ NOESY corre-

lation observed with H-15β (axial). The methyl group C-24 was assigned to the 22α position (axial) because a four-bond correlation between this singlet and the doublet resonating at 5.10 ppm (H-23β, axial) was observed in the $^1H$–$^1H$ COSY spectrum. Consequently, the 22-OH was assigned to the β (equatorial) position. Compound **1** was then concluded to be the novel steroidal alkaloid named obtusine-20(R)-*O*-[β-thevetopyranosyl-(1 → 4)-β-cymaropyranoside].

Compound **2** also reacted with Dragendorff reagent and was considered to be an alkaloid. The peak at *m/z* 730 $[M + Na]^+$ in the FABMS spectrum, the DEPT and $^{13}C$ NMR data indicated the molecular formula $C_{38}H_{61}O_{11}N$ for **2**. The careful analysis of the $^1H$ NMR spectrum of **2** revealed the presence of two methyl singlets at 0.76 and 1.02 ppm, one methyl doublet at 1.22 ppm, two anomeric protons at 4.32 and 4.83 ppm and one broad multiplet olefinic proton signal at 5.37 ppm indicating that **2** was also a pregnene diglycosylated compound. The $^{13}C$ NMR and DEPT spectra of **2** were analysed by comparison with $^{13}C$ data of **1** (Table 1). The aglycone of **2** was confirmed to be a 5Δ-pregnene derivative due to the presence of a quaternary carbon signal at 141.0 ppm (C-5), a methine carbon at 122.2 ppm (C-6), three methyl carbons at 14.7 ppm (C-18), 19.6 ppm (C-19) and 16.6 ppm (C-21), two quaternary carbons at 37.2 ppm (C-10) and 46.0 ppm (C-13) and finally, three methine carbons at 32.4 ppm (C-8), 50.3 ppm (C-9) and 51.6 ppm (C-14). The quaternary carbon resonating at 86.2 ppm was assigned to C-17β-OH, the methine carbon at 69.8 ppm to the 20(R) position of the aglycone and the methine carbon at 68.2 ppm to a C-16β-NH also by comparison with **1** NMR data. However, the $^{13}C$ NMR spectrum of **2** did not show the quaternary carbon at 113.6 ppm nor the methylene carbon at 86.3 ppm, assigned to C-22 and C-23 of **1**. Instead of the above mentioned signals, it showed a quaternary carbon at 169.0 ppm that could be assigned to a carbonyl carbon of an amide or lactam group (Pretsh, Clerc, Seibl & Seimon, 1983), a methylene carbon at 46.7 ppm that could be assigned to a $CH_2$ linked to the carbonyl carbon of the amide/lactam group (Pretsh et al., 1983), a quaternary carbon at 75.1 ppm and a methyl group at 23.1 ppm. Once again, by comparison with **1**, taken in consideration the chemical shifts of the carbons involved and with the help of models and tables, the carbonyl carbon was assigned to position 22, the $CH_2$ to position 23, the quaternary carbon to position 15 to which a methyl carbon would be linked (C-24). This methyl group is proposed to be at C-15α because at this position it avoids repulsions with the C-18 methyl group and so the 15α-methyl isomer is more stable than the 15β-methyl one. The aglycone of **2** is proposed to be 5Δ-pregnene-15α-methyl-3β, 17β, 20(R)-triol-[16β, 15β]-pentalactam.

The $^1H$ chemical shifts of sugars were obtained and assigned by the analysis of $^1H$–$^1H$ COSY spectrum having the anomeric protons as starting points. By comparison of $^1H$ and $^{13}C$ chemical shifts of sugar moiety with those moiety obtained for **1** and referred to literature (Aquino et al., 1996), it was concluded that the sugar chain of **2** was identical to that of **1**, i.e., β-thevetopyranosyl-(1 → 4)-β-cymaropyranoside (Table 1). When the $^{13}C$ and $^1H$ chemical shifts of CH-20 and $CH_3$-21, similar to the correspondent ones of **1**, were considered it was concluded that the sugar chain in **2** was also linked to C-20 of the aglycone. Compound **2** was named obtusolactam-20(R)-*O*-[β-thevetopyranosyl-(1 → 4)-β-cymaropyranoside].

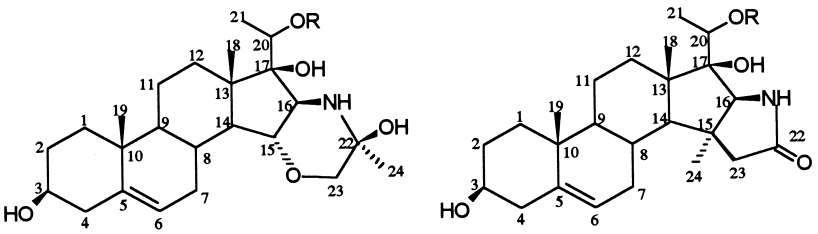
Compound **3** was obtained from the non-alkaloidal fraction of the chloroformic crude extract. The FABMS and NMR data of **3** indicated that it was a C29 steroid linked to one hexose and it was then identified as β-sitosteryl-3-*O*-β-glucopyranoside by comparison of $^{13}C$ chemical shifts with those reported in the literature (Sakakibara, Kaiya, Fukuda & Ohki, 1983). Once $\delta$ $^{13}C$ were assigned the $\delta$ $^1H$ were easily assigned by analysis of HETCOR and $^1H$–$^1H$ COSY spectra. Complete assignments of $\delta$ $^1H$ are reported for the first time (Table 1).

A chemotaxonomic analysis revealed that these novel steroidal alkaloids (**1** and **2**) isolated from *C. obtusa* differ markedly from those identified in Asclepiadaceae, where they are basically polyhydroxysteroids esterified with nicotinic acid at C-12 or C-20 (Hegnauer, 1964; Summons, Ellis & Gellert, 1972; Hegnauer, 1989; Aquino et al., 1996; Ma & Fang, 1997), and also from those of pregnane type identified in Apocynaceae, since in these steroidal alkaloids the transamination occurs at C-3 or C-20 (Hegnauer, 1964). However, if one considers the theory of Hegnauer (1964) that pregnane alkaloids in Apocynaceae evolved from cardenolides, it can be concluded that steroidal alkaloids **1** and **2** may have evolved from 16-acetyl-gitoxigenin, a cardenolide isolated from a related species of *Cryptostegia* (Periplocaceae or Periplocoideae) (Sanduja, Lo, Euler, Alam & Morton, 1984).

## 3. Experimental

### 3.1. Plant material

The roots of *Cryptolepis obtusa* N. E. Br. were collected in April 1996 in Maputo, Mozambique by Mr. Daniel Zunguza of the Faculty of Biology, Eduardo Mondlane University, Mozambique. Plant material was identified by comparison with the voucher specimen PJ 7314 deposited at Eduardo MondlaneUniversity Herbarium (LMU), Maputo. The identification



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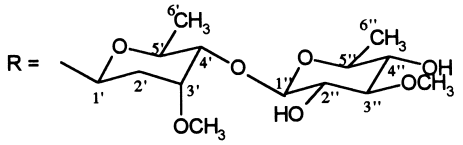

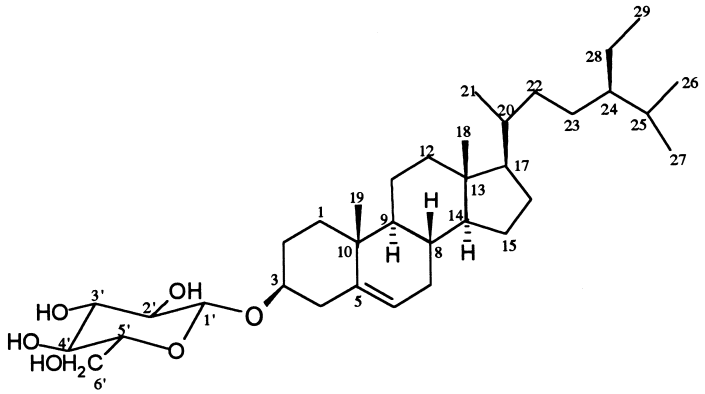

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was confirmed by specialists of the Royal Botanic Gardens Herbarium, Kew, UK.

### 3.2. Extraction and isolation of compounds

The dried roots were powdered and 250 g were defatted with hexane ($2 \times 1.5$ l). The powdered plant material was then extracted with $10 \times 1.5$ l of $CHCl_3$ to obtain 2 g of crude chloroformic extract. This extract was chromatographed on a silica gel column with a $CHCl_3$:MeOH gradient. The fractions were bulked according to their TLC profile on silica gel and six fractions were obtained: CoR-F1 to CoR-F6. Fraction CoR-F2 (51 mg) afforded a white precipitate after being treated three times with 1 ml of MeOH 80% and centrifuged. The precipitate obtained (10 mg) was concluded to be a pure compound (**3**), after control on TLC with three different systems. Fraction CoR-F3 revealed the presence of alkaloidal saponins after treatment of the TLC plates with Dragendorff and anisaldehyde-$H_2SO_4$ spray reagents [9]. The 80% MeOH soluble part of CoR-F3 (100 mg) was evaporated to dryness and resuspended in 1 ml of $CH_3CN$:$H_2O$ (40:60). The non-soluble fraction was separated after centrifugation and the solution injected, several times, in a HPLC RP-18 column ($250 \times 10$ mm) with the help of a 200 μl loop. Separation was performed for 30 min with $CH_3CN$:$H_2O$ (35:65) as the mobile phase and the flow rate was adjusted to 2.5 ml/min. Fractions were collected every 20 s and a refractive index detector was used. Fractions were controlled by TLC on RP-18 plates, developing three times with $CH_3CN$:$H_2O$ (45:55) and bulked according to $R_f$ (TLC) and $RR_t$ (HPLC). Fractions collected between 18 and 23 min showed only one spot in the RP-18 TLC system mentioned above. These fractions were combined, the acetonitrile evaporated and the aqueous solution freeze-dried. The 35 mg of the white powder obtained gave positive reaction with Dragendorff reagent and was then further purified by prep. TLC (0.25 mm pre-coated silica gel plates) with developing system Butanone:MeOH (60:5) to afford two compounds: **1** (17 mg) and **2** (6 mg) after detection with anisaldehyde-$H_2SO_4$ spray reagent sprayed to one side edge of the plate and eluted from silica gel with $CHCl_3$:MeOH (1:1) dried with $Na_2SO_4$.

*Obtusine-20(R)-O-[β-thevetopyranosyl-(1 → 4)-β-cymaropyranoside]* (**1**), white powder. FABMS (sodium *meta*-nitrobenzylalcohol) *m/z* (rel. int): 748 $[M + Na]^+$ (2), 726 $[M + H]^+$ (2), 663 $[M + 4H\text{-E-ring } (C_3H_7O_2N) + Na]^+$ (8), 647 $[M + 2H\text{-E-ring-}H_2O + Na]^+$ (10), 426 [M − OH-glycosidic chain + $Na]^+$

(100), 338 [glycosidic chain + Na]$^+$ (12), 279 (6). $^1$H and $^{13}$C NMR: see Table 1.

*Obtusolactam-20(R)-O-[β-thevetopyranosyl-(1 → 4)-β-cymaropyranoside]* (**2**), white powder. FABMS (sodium *meta*-nitrobenzylalcohol) *m/z* (rel. int): 730 [M + Na]$^+$ (6), 706 [M − H]$^+$ (10), 530 [M − O-thevetoside ($C_7H_{13}O_5$)]$^+$ (55), 338 [glycosidic chain + Na]$^+$ (40), 242 (100). $^1$H and $^{13}$C NMR: see Table 1.

*β-sitosteryl-3-O-β-glucopiranoside* (**3**), white powder. FABMS (sodium *meta*-nitrobenzylalcohol) *m/z* (rel. int): 599 [M + Na]$^+$ (55). $^1$H and $^{13}$C NMR: see Table 1.

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