Published in final edited form as:

Steroids. 2017 September 01; 125: 27–32. doi:10.1016/j.steroids.2017.06.005.

Pregnane glycosides from *Cynanchum menarandrense*

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Abstract

Five new pregnane-type steroidal glycosides, named menarandrosides A-E (**1-2**, **5-7**) were isolated from the aerial parts of *Cynanchum menarandrense*, together with three known compounds, carumbelloside I (**3**), carumbelloside II (**4**), and pregnenolone-3-*O*-gentiobioside (**8**). Their structures were determined on the basis of spectroscopic analyses including NMR and mass spectrometry, reporting C-21 steroids glycosylated only by one or two glucose moieties. Compounds were then investigated for their potential to stimulate glucagon-like peptide-1 (GLP-1) secretion in intestinal cells; although none of the pure compounds had any influence, the fraction enriched in pregnanes exhibited a significant activity, suggesting a possible synergistic effect. Furthermore, none of the purified compounds affected cell viability.

Keywords

Cynanchum menarandrense; pregnanes; C-21 steroidal glycosides; menarandrosides; glucagon-like peptide-1; cytotoxicity

1 Introduction

During a previous search for antidiabetic natural compounds, the ethanolic extract of *Cynanchum marnierianum*, an endemic Apocynaceae from Madagascar, exhibited significant glucagon-like peptide 1 (GLP-1) secretagogue activity. A bio-guided fractionation was undertaken to isolate the active components, identified as pregnane glycosides [1]. These promising results have prompted us to investigate further *Cynanchum* species from Madagascar for the same activity. Despite its high diversity and peculiar morphology (stem-succulents with reduced leaves), the Madagascan clade is poorly studied compared to species growing in China, for which phytochemical data are abundant, describing mainly pregnane derivatives. The only other publication regarding a Madagascan

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Conflicts of Interest

The authors declare no conflict of interest.

species also reports the presence of pregnane derivatives [2], indicating that these compounds are a feature of this widespread genus. Even so, there are no data about biological activity of these endemic *Cynanchum* species. After a preliminary selection of various *Cynanchum* species endemic from Madagascar, an *in vitro* bioassay revealed that the ethanolic extract from *C. menarandrense* Jum. & H. Perrier also induced a stimulation of GLP-1 secretion in STC-1 intestinal cells in a dose-dependent manner from $204 \pm 4\%$ (at 30 $\mu\text{g/mL}$) to $245 \pm 7\%$ (at 100 $\mu\text{g/mL}$). Based on the results obtained from *C. marnierianum*, we decided to focus our investigation on the pregnane-containing fractions, leading to the isolation of eight C-21 steroidal glycosides (Fig. 1). The purified compounds were then assessed for their capacity to stimulate GLP-1 secretion on STC-1 and primary intestinal cells. Effects on cell viability were also evaluated. Two closely-related standard compounds, pregnenolone and its sulphate derivative, were also submitted to the aforementioned biological assays.

2 Experimental

2.1 General methods

Optical rotations were obtained on a JASCO P200 polarimeter. Melting points were determined using a Büchi® B-540 melting point apparatus. IR spectra were measured on a Perkin-Elmer FTIR Spectrum 2. NMR spectra were recorded on a Bruker Avance III - 500 MHz NMR spectrometer equipped with a multi-nucleus probe BBFO (5 mm). UV spectra were recorded on the PDA detector of the Varian LC-920 system. LC-HRESIMS data were recorded on an Agilent 6520 Accurate-Mass Q-TOF hyphenated to an Agilent 1200 system equipped with a Zorbax Agilent C18 column (50 mm $\times$ 2.1 mm, 1.8 μm). Compounds were purified by semi-preparative HPLC with a Gilson 322 system equipped with an Axia C18 column (21.2 mm $\times$ 100 mm, 5 μm). HPLC analyses were performed on a Varian LC-920 system with a Kinetex column C18 100 Å (100 $\times$ 3.0 mm, 2.6 μm). Solid-phase extractions were performed on Chromabond® SPE cartridges (Macherey-Nagel). The reading of the microplates was performed on an ELISA Versamax plus® (Molecular Devices) plate reader. Solvents for extraction, fractionation and HPLC were supplied by Sigma-Aldrich. The bioassay material was purchased as following: GLP-1 ELISA kit (Active GLP-1, EGLP-35K Millipore), DMEM and PBS (Gibco), BSA (Sigma-Aldrich) and MTS mother solution CellTiter 96 (Promega), compounds standards pregnenolone and pregnenolone sulphate (Sigma-Aldrich).

2.2 Plant material

Aerial parts of *Cynanchum menarandrense* were collected in September 2012 in the Antsokay Arboretum, Toliara, Madagascar, and authenticated by Andry Petignat, director of the arboretum.

2.3 Extraction and isolation

The aerial parts (17.67 g) were cut and extracted by maceration with ethanol (sonication for 10 min, 3 cycles) to yield 4.94 g of dry ethanolic extract. This extract was then suspended in 250 mL of water and submitted to thorough liquid/liquid extraction with EtOAc (4 $\times$ 250 mL) to give after solvent removal 3.15 g of EtOAc extract and 1.79 g of residual aqueous

fraction. The organic phase was further fractionated by solid-phase extraction. The SPE silica (50 g) cartridge was conditioned with cyclohexane, the sample was charged on the cartridge and eluted using the following gradient: 100% cyclohexane (200 mL), diethyl ether/cyclohexane (2-8, 3-7, 5-5 v/v ; 200 mL each), 100% diethyl ether (200 mL), EtOAc/diethyl ether (7-3, 5-5, 2-8 v/v ; 200 mL), EtOAc 100% (200 mL), methanol/EtOAc (2-8, 3-7, 5-5, 7-3, 9-1 v/v ; 300 mL), and finally methanol 100% (200 mL). The fraction eluted with methanol/EtOAc (3-7 v/v) was further separated by semi-preparative HPLC, using a mixture of A (acidified water, 0.1% v/v formic acid) and B (acidified ACN, 0.1% v/v formic acid), with the following gradient at a flow rate of 30 mL/min: 20 to 30% B, 0-10 min; 30 to 100% B, 10-13 min; 100% B 13-15 min, leading to the isolation of compounds **1** (4.9 mg), **3** (2.3 mg), **4** (1.0 mg), **5** (3.8 mg), and **7** (1.4 mg). The freeze-dried residual aqueous fraction was loaded on a SPE C18 silica (50g) cartridge and eluted using the following gradient: H₂O 100% (200 mL), ACN/H₂O (2-8, 3-7, 4-6, 5-5, 6-4, 8-2 v/v ; 200 mL) and ACN 100% (200 mL). The fraction eluted with ACN/H₂O 3-7 (30.2 mg) was further purified on a SPE silica (1 g) using chloroform/methanol 85-15 for elution, yielding compound **8** (3.4 mg). The fraction eluted with ACN/H₂O 3-7 (62.0 mg) was further fractionated by semi-preparative HPLC. The sample was eluted with a mobile phase consisting of a mixture of A (acidified water, 0.1% v/v formic acid) and B (acidified ACN, 0.1% v/v formic acid), using the following gradient at a flow rate of 30 mL/min: 13 to 20% B, 0-10 min; 20 to 70% B, 10-14 min; 100% B 14-16 min, leading to the isolation of compounds **2** (2.4 mg), **6** (1.8 mg), **4** (12.2 mg), and **7** (10.0 mg). All compounds were isolated in purity > 97%, as evaluated by HPLC-UV and confirmed by NMR analyses.

2.3.1 Menarandroside A (1): 12 α -hydroxy-pregn-5-en-20-one 3-O- β -D-glucopyranoside—White needle-like crystals; $[\alpha]_D^{20} + 14.7$ (c 0.4, MeOH); UV (nm): 199, 280 sh; IR $\nu_{\max}$ cm⁻¹: 3360, 2933, 1689, 1017; mp 230-236°C; HRESIMS m/z 517.2764 [M+Na]⁺, (calcd error 1.62 ppm for C₂₇H₄₂NaO₈); ¹H NMR (500 MHz, CD₃OD) and ¹³C NMR (125 MHz, CD₃OD): see Table 1 and Table 2.

2.3.2 Menarandroside B (2): 12 α -hydroxy-pregn-5-en-20-one 3-O- β -D-gentiobioside—White powder, $[\alpha]_D^{20} -4.4$ (c 0.4, MeOH); UV (nm): 199, 280 sh; mp 226-230°C; HRESIMS m/z 679.3296 [M+Na]⁺ (calcd error 0.69 ppm for C₃₃H₅₂NaO₁₃); ¹H NMR (500 MHz, CD₃OD) and ¹³C NMR (125 MHz, CD₃OD): see Table 1 and Table 2.

2.3.3 Carumbelloside I (3): 14 β -hydroxy-pregn-5-en-20-one 3-O- β -D-gentiobioside—White needle-shaped crystals; HRESIMS m/z 679.3296 [M+Na]⁺ (calcd error 0.33 ppm for C₃₃H₅₂NaO₁₃); ¹H NMR (500 MHz, CD₃OD): 5.42 (m, H6), 4.39 (d, 7.9, H1'), 4.38 (d, 7.8, H1''), 4.12 (d, 11.8, 1.9, H6'a), 3.87 (d, 11.8, 1.9, H6''a), 3.79 (d, 11.5, 5.5, H6'b), 3.67 (d, 11.8, 5.0, H6''b), 3.57 (m, H3), 3.45 (m, H5'), 3.35 (m, H3''), 3.34 (m, 2H H3' and H4'), 3.27 (m, 2H: H4'' and H5''), 3.27 (dd, 9.0, 8.2, H17), 3.21 (d, 8.7, 7.6, H2''), 3.16 (d, 8.9, 7.3, H2'), 2.44 (ddd, 13.7, 4.9, 2.0, H4 α), 2.27 (m, H4 β), 2.21 (m, H16 β), 2.13 (m, H7 β), 2.11 (s, H21), 2.1 (m, H12 α), 1.96 (m, H2 α), 1.89 (dt, 13.4, 3.9, H1 β), 1.87 (m, H8), 1.82 (m, H7 α), 1.75 (m, H16 α), 1.64 (m, 2H: H11 β and H12 β), 1.62 (m, H2 β), 1.60 (m, 2H: H15 α and H15 β), 1.52 (m, H9), 1.51 (m, H11 α), 1.16 (td, 13.4, 3.8, H1 α), 1.04 (s, H19), 0.75 (s, H18); ¹³C NMR (125 MHz, CD₃OD): see Table 1.

2.3.4 Carumbelloside II (4): 14 β -hydroxy-pregn-5-en-20-one 3-O- β -D-

glucopyranoside—White needle-shaped crystals; mp 268-270°C (lit [5] 274-276°C); IR $\nu_{\max}$ cm⁻¹: 3336, 2935, 1689, 1035 (lit [5] 3400, 2920, 1670, 1050); HRESIMS m/z 517.2764 [M+Na]⁺ (calcd error 1.75 ppm for C₂₇H₄₂NaO₈); ¹H NMR (500 MHz, CD₃OD) : 5.41 (m, H₆), 4.38 (d, 7.8, H_{1'}), 3.86 (dd, 12.0, 1.5, H_{6'a}), 3.60 (m, H₃), 3.60 (dd, 11.5, 6.0, H_{6'b}), 3.35 (m, H_{3'}), 3.28 (m, H_{4'}), 3.26 (m, H_{5'}), 3.26 (br. t, 8.5, H₁₇), 3.14 (dd, 9.2, 7.8, H_{2'}), 2.44 (ddd, 13.3, 4.9, 2.2, H_{4 α}), 2.27 (td, 12.4, 2.0, H_{4 β}), 2.21 (m, H_{16 β}), 2.13 (m, H_{7 β}), 2.12 (s, H₂₁), 2.08 (m, H_{12 α}), 1.95 (m, H_{2 α}), 1.90 (m, H_{1 β}), 1.87 (m, H₈), 1.83 (m, H_{7 α}), 1.73 (m, H_{16 α}), 1.70 (m, H_{12 β}), 1.60 (m, 2H: H_{2 β} and H_{11 β}), 1.59 (m, 2H: H_{15 α} and H_{15 β}), 1.52 (m, 2H: H₉ and H_{11 α}), 1.15 (td, 13.6, 3.2, H_{1 α}), 1.04 (s, H₁₉), 0.75 (s, H₁₈); ¹³C NMR (125 MHz, CD₃OD): see Table 1.

2.3.5 Menarandroside C (5): 12 α ,14 β -dihydroxy-pregn-5-en-20-one 3-O- β -D-

glucopyranoside—White needle-shaped crystals; [α]_D²⁰ + 20.1 (c 0.4, MeOH); UV (nm): 199, 280 sh; mp 212-216°C; IR $\nu_{\max}$ cm⁻¹: 3341, 2932, 1689, 1022; HRESIMS m/z 533.2704 [M+Na]⁺, (calcd error 2.81 ppm for C₂₇H₄₂NaO₉); ¹H NMR (500 MHz, CD₃OD) and ¹³C NMR (125 MHz, CD₃OD): see Table 1 and Table 2.

2.3.6 Menarandroside D (6): 12 α ,14 β -dihydroxy-pregn-5-en-20-one 3-O- β -D-

gentiobioside—White powder; [α]_D²⁰ + 20.07 (c 0.1, MeOH); UV (nm): 199, 280 sh; HRESIMS m/z 695.3250 [M+Na]⁺ (calcd error 0.11 ppm for C₃₃H₅₂NaO₁₄); ¹H NMR (500 MHz, CD₃OD) and ¹³C NMR (125 MHz, CD₃OD): see Table 1 and Table 2.

2.3.7 Menarandroside E (7): 14 β ,19-dihydroxy-17 α -pregn-5-en-20-one 3-O- β -D-

D-glucopyranoside—White needle-shaped crystals; [α]_D²⁰ -7.6 (c 0.4, MeOH); UVmax (nm): 199, 280 sh; mp 225-230°C; IR $\nu_{\max}$ cm⁻¹: 3379, 2921, 1686, 1019; HRESIMS m/z 533.2704 [M+Na]⁺ (calcd error 1.27 ppm for C₂₇H₄₂NaO₉); ¹H NMR (500 MHz, CD₃OD) and ¹³C NMR (125 MHz, CD₃OD): see Table 1 and Table 2.

2.3.8 Pregnenolone-3-O-gentiobioside (8): pregn-5-en-20-one 3-O- β -D-

gentiobioside—White powder; mp 230-233°C (lit [7] 231-235°C); IR $\nu_{\max}$ cm⁻¹: 3379, 2921, 1687, 1018; HRESIMS m/z 663.3367 [M+Na]⁺, (calcd error 1.27 ppm for C₃₃H₅₂NaO₁₂); ¹H NMR (500 MHz, CD₃OD): 5.37 (br. d, 5.3, H₆), 4.38 (d, 8.0, H_{1'}), 4.36 (d, 7.9, H_{1''}), 4.12 (dd, 11.5, 1.9, H_{6'b}), 3.86 (dd, 11.9, 2.1, H_{6'a}), 3.79 (dd, 11.5, 4.9, H_{6'a}), 3.67 (dd, 11.8, 5.3, H_{6'b}), 3.56 (tt, 6.9, 4.6, H₃), 3.44 (m, H_{5'}), 3.37 (m, H_{4'}), 3.35 (m, H_{3'}), 3.30 (m, H_{4''}), 3.27 (m, 2H : H_{3''} and H_{5''}), 3.23 (dd, 7.9, 9.0, H_{2''}), 3.17 (dd, 9.0, 8.0, H_{2'}), 2.62 (br. t, 8.9, H₁₇), 2.42 (td, 12.4, 2.5, H_{4 α}), 2.26 (ddd, 13.3, 4.7, 2.1, H_{4 β}), 2.13 (s, H₂₁), 2.12 (m, H_{16 α}), 2.06 (m, H_{12 α}), 1.99 (m, H_{7 β}), 1.95 (m, H_{2 α}), 1.88 (dt, 13.3, 3.3, H_{1 β}), 1.70 (m, H_{15 α}), 1.65 (m, 2H: H_{11 α} and H_{16 β}), 1.61 (m, H_{2 β}), 1.58 (m, H_{7 α}), 1.49 (m, 3H: H₈, H_{11 β} and H_{12 β}), 1.22 (m, H_{15 β}), 1.20 (m, H₁₄), 1.11 (td, 13.6, 3.6, H_{1 α}), 1.02 (s, H₁₉), 1.02 (m, H₉), 0.63 (s, H₁₈); ¹³C NMR (125 MHz, CD₃OD): see Table 1.

2.4 Enzymatic hydrolysis

Pure compounds were solubilised in pH 5.0 acetate buffer (concentration 1 mg/mL); an aliquot of 1 mL of each solution was incubated at 37°C during 24 hours with 1 mg of β -D-glucosidase from almonds (Roth). The hydrolysis has been monitored by TLC analysis (Silicagel 60 F254, Merck) using dichloromethane-methanol-water (85:15:1, v/v) as mobile phase.

2.5 Cell culture, GLP-1 secretagogue assays, and cell viability

The GLP-1 secretion assay was carried out according to the previously published protocol [1]. Briefly, STC-1 cells were cultured in DMEM medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 μ g/L streptomycin, at 37°C in 5% CO₂. Then 100 μ L of a 5×10^5 cells/mL suspension was seeded onto 96-well cell culture plates and allowed to adhere overnight. Cells were then exposed to each compound at the concentration of 100 μ M, and the GLP-1 levels in the supernatant were measured after 2 h of incubation according to the ELISA kit protocol. Results were expressed as a percentage of the GLP-1 content (pM) secreted by the treated cells compared to the basal GLP-1 content (pM) released from the cells treated with DMSO 1%. The remaining cells were submitted to the MTS assay: after addition of 100 μ L of a 317 μ g/mL MTS solution in PBS in each well, incubation for 1 hour and subsequent dissolution of the formazan in DMSO, plates were read at 480 nm.

Processing and culture of primary intestinal cells was done as described previously [3]. Briefly, mice aged 10-22 weeks were killed by cervical dislocation and duodenal tissue was taken (10 cm region adjacent to stomach). Intestinal tissue was cleaned thoroughly with PBS and the outer muscle layer removed. Tissue was digested using collagenase type XI (Sigma) and cell suspensions were plated onto 24-well plates. After 24 hours, wells were washed three times with standard extracellular buffer supplemented with 0.1% BSA (fatty-acid free, Sigma) and 10 mM glucose. Test compounds were diluted to appropriate concentration in the same extracellular buffer and added to each well (250 μ L). Cells were incubated at 37°C for 2 hours; solutions were then removed and centrifuged at 2000 RPM for 5 minutes. Supernatants were snap frozen on dry ice, supernatant and lysate samples were collected. GLP-1 contents were measured using a total GLP-1 assay (Meso ScaleDiscovery, Gaithersburg, MD, USA). GLP-1 secretion was calculated as a percentage of total GLP-1 content per well. Percentage GLP-1 secretion in response to treatments was compared to % GLP-1 secretion from control wells (extracellular buffer alone) measured in parallel on the same day.

3 Results and discussion

Compound **1** was isolated as white needle-like crystals. Its UV spectrum presented a maximum of absorption at 199 nm as well as a shoulder at 280 nm indicating its terpenic nature. Its molecular formula was determined to be C₂₇H₄₂O₈ on the basis of the observed pseudo-molecular peak at m/z 517.2764 [M+Na]⁺. An additional major peak at m/z 315.2316 [M+H-162-H₂O]⁺ could be attributed to the loss of an hexose moiety [4]. This was confirmed with NMR by a signal corresponding to an anomeric proton at δ H 4.39 (d, J = 7.8

Hz) in the ^1H NMR spectrum. Several aliphatic protons, one olefinic proton, as well as several protons carried by oxygenated carbons were also identified. The ^{13}C NMR and DEPT spectra confirmed the presence of 27 carbons, including 4 quaternary carbons, 8 methylenes, 12 methines, and 3 methyls (Table 1). The characteristic chemical shift of the quaternary carbon at 212.9 ppm revealed the presence of a ketone moiety. Twenty-one of these carbon resonances were attributed to a pregnane structure by means of the observed COSY, HSQC, HMBC correlations, as well as the comparison with the literature [5]. The olefinic proton at δH 5.39 (dd, $J = 4.9, 2.3$ Hz) was attributed to H-6, which showed HMBC 3J correlations with C-4 (δC 39.9), C-8 (δC 33.3), and C-10 (δC 37.7). The ketone was proven to be on the C-20 by the HMBC 2J correlations with H-17 (δH 3.23, t, $J = 9.0$ Hz) and H-21 (δH 2.14, s). The H-17 orientation was found to be α by its NOE correlation with H-14 that correlated itself with H-9[6]. Two oxygenated methines were attributed to the positions 3 and 12. The orientation of H-12 resonating at δH 4.01 was proven to be β by its coupling constant (br. t, $J = 2.7$ Hz) and its NOE correlation with CH_3 -18. Therefore, the pregnane skeleton of compound **1** was found to be the $3\beta, 12\alpha$ -dihydroxy-pregn-5-en-20-one, in accordance with the HRMS fragment observed at m/z 315.2316. The hexose was found to be linked at position 3, and was identified as β -D-glucose based on NMR data and enzymatic hydrolysis by β -D-glucosidase. Therefore, compound **1** was identified as the 12α -hydroxy-pregn-5-en-20-one 3- O - β -D-glucopyranoside, a new compound for which we propose the trivial name menarandroside A.

Compound **2** was isolated as a white powder. Its UV spectrum was identical to the one of compound **1** and its molecular formula was determined to be $\text{C}_{33}\text{H}_{52}\text{O}_{13}$ on the basis of the observed pseudo-molecular peak at m/z 679.3296 $[\text{M}+\text{Na}]^+$ in the HR-ESI-MS spectrum. The peaks observed at m/z 477.2849 $[\text{M}+\text{H}-162-\text{H}_2\text{O}]^+$ and 315.2321 $[\text{M}+\text{H}-2\times 162-\text{H}_2\text{O}]^+$ suggested the presence of an additional hexose, which was confirmed by an additional anomeric proton and further osidic signals in the ^1H NMR spectrum, while the remaining signals were identical to those of **1**. The complete enzymatic hydrolysis indicated that the glycosidic chain was composed of two β -D-glucopyranose moieties. In the HMBC spectrum, the anomeric proton of the terminal β -D-glucopyranose H-1'' (δH 4.38, d, $J = 7.8$ Hz) correlated with the C-6' (δC 69.8) of the first β -D-glucopyranose, indicating a β -D-glucopyranosyl-(1 $\rightarrow$ 6)-D-glucopyranose linkage, namely a gentiobiose. Consequently, compound **2** was identified as the 12α -hydroxy-pregn-5-en-20-one 3- O - β -D-gentiobioside, a new pregnane derivative named menarandroside B.

Compound **3** was isolated as a white powder, and its molecular formula was determined to be identical to that of compound **2** on the basis of the observed pseudo-molecular peak at m/z 679.3296 $[\text{M}+\text{Na}]^+$. Compound **3** also exhibited the same pattern of fragmentation with major fragments observed at m/z 477.2845 $[\text{M}+\text{H}-162-\text{H}_2\text{O}]^+$ and 315.2320 $[\text{M}+\text{H}-2\times 162-\text{H}_2\text{O}]^+$. However, the ^1H NMR spectrum revealed a methylene (δH 2.10 and 1.64, m) instead of an hydroxylated methine on the position 12. Though, the ^{13}C NMR spectrum indicated the presence of an hydroxylated quaternary carbon which was attributed to the position 14 by its HMBC 3J correlations with H-12, H16, and CH_3 -18. The orientation of the hydroxyl moiety carried by the C-14 (δC 85.8) was shown to be β after the acquisition of a NOESY spectrum in $\text{DMSO}-d_6$, and the observation of its correlations with the CH_3 -18 and CH_3 -21

above the pregnane plane. These data were in accordance with literature for the 14 β -hydroxy-pregn-5-en-20-one 3-*O*- β -D-gentiobioside, known as carumbelloside I, previously isolated from another Apocynaceae, *Caralluma umbellata* [5].

Compound **4** presented many similarities with compound **1**. It was isolated as white needle-shaped crystals, its molecular formula was determined to be also C₂₇H₄₂O₈ on the basis of the observed pseudo-molecular peak at m/z 517.2764 [M+Na]⁺ and it presented the same pattern of fragmentation than **1** with a major fragment at m/z 315.2312 [M+H-162-H₂O]⁺. However, regarding the NMR data for the pregnane core, they were identical to those of **3**. After comparison with the published data [5], **4** was identified as carumbelloside II, possessing one single glucosyl moiety compared to carumbelloside I, and previously obtained from *Caralluma umbellata* as well [5].

The molecular formula of compound **5** was determined to be C₂₇H₄₂O₉ on the basis of the observed pseudo-molecular peak at m/z 533.2704 [M+Na]⁺, suggesting the eventuality of an isomer of compounds **1** and **4** with an additional hydroxyl group. The ¹H and ¹³C NMR data showed both positions 12 and 14 being hydroxylated by the observation of the deshielded methine H-12 (δ H 4.16, br. t, J = 2.6 Hz) and C-14 resonating at 87.3 ppm. The aforementioned data along with the 2D NMR spectra interpretation led to the determination of compound **5** structure as 12 α ,14 β -dihydroxy-pregn-5-en-20-one 3-*O*- β -D-glucopyranoside. Compound **5**, exhibiting a structure similar to **1** with an additional β -hydroxyl group at position 14, was named menarandroside C.

Compound **6** was isolated as a white powder. Its molecular formula was determined to be C₃₃H₅₂O₁₄ on the basis of the observed pseudo-molecular peak at m/z 695.3250 [M+Na]⁺ in the HR-ESI-MS spectrum. Again, the difference with **5** led to the deduction that compound **6** bore an additional hexose. This was confirmed by major fragments observed in the MS spectrum and by the 1D and 2D NMR data. Compound **6** was thus identified as the 12 α ,14 β -dihydroxy-pregn-5-en-20-one 3-*O*- β -D-gentiobioside, a new natural product named menarandroside D.

Compound **7** was isolated as white needle-shaped crystals, and its molecular formula was determined to be C₂₇H₄₂O₉ – like compound **5** – on the basis of the observed pseudo-molecular peak at m/z 533.2704 [M+Na]⁺. Compared to the previous compounds, no signal corresponding to the CH₃-19 was detected in the ¹H spectrum. Instead, it revealed the resonance of a second hydroxymethyl moiety (H-19a: δ H 3.84, d, J = 11.8 Hz; H-19b: δ H 3.54, d, J = 11.8 Hz), partially overlapping with the signals of the glucopyranose hydroxymethyl moiety (H-6'a: δ H 3.85, dd, J = 11.5, 1.1 Hz; H-6'b: δ H 3.64, dd, J = 12.1, 5.0 Hz). The HMBC ³ J correlations of CH₂-19 with C-5 (δ C 136.7) and C-9 (δ C 47.8) confirmed the assignment of the hydroxyl group on the position 19. The second hydroxyl group was attributed, as in compounds **3-6**, on C-14 (δ C 87.5). The H-17 (δ H 2.97, dd, J = 9.6, 4.6 Hz) was more shielded than in compounds **1-6** and showed a NOE correlation with CH₃-18, indicating its beta orientation. Consequently, compound **7** was determined as the 14 β ,19-dihydroxy-17 α -pregn-5-en-20-one 3-*O*- β -D-glucopyranoside, a new natural compound named menarandroside E.

Compound **8** was isolated as a white powder. On the basis of the observed pseudo-molecular peak at m/z 663.3367 $[M+Na]^+$, its molecular formula was determined to be $C_{33}H_{52}O_{12}$. The comparison with the molecular formula of **2** and **3** suggested a similar structure but with an hydroxyl group less. This assumption was corroborated by the fragments at m/z 461.2890 $[M+H-162-H_2O]^+$ and 299.2383 $[M+H-2\times 162-H_2O]^+$, and the NMR data indicating the presence of a sole hydroxylation position on C-3, involved in the glycosidic linkage. The comparison of the NMR data with the ones of a commercial standard of pregnenolone established the structure of the aglycone. The carbohydrate NMR signals were identical to the ones of compounds **2**, **3**, and **6**, suggesting the glycosylation of the pregnenolone by a gentiobiose moiety. The 2D NMR correlations confirmed this observation and led to the determination of compound **8** structure as the pregn-5-en-20-one 3-*O*- β -D-gentiobioside, which has been previously isolated from the bark and the roots of *Nerium oleander* (syn. *Nerium odorum*), also belonging to the Apocynaceae family [7].

Compared to pregnane glycosides identified in *C. marnierianum*, compounds **1-8** exhibit a rather simple structure, either regarding the unsubstituted aglycone core, either considering the glycosidic chain, made only of one or two glucose moieties. This is also quite unusual compared to pregnane glycosides occurring in *Cynanchum* species, where sugar moieties often consist of a linear chain of 2,6-dideoxyhexoses such as cymarose or oleandrose, linked at C-3 and involving up to six sugars [8]. The pregnane skeleton is also often esterified by monocarboxylic acids, such as tiglic, benzoic or cinnamic acids, which is not the case for pregnanes **1-8**, for which all hydroxyl groups are free [2,9,10].

Compounds **1-8** and standards of pregnenolone and pregnenolone sulphate were assessed for their GLP-1 secretagogue effect on STC-1 cells at the concentration of 100 μ M, as well as for their effect on cell viability. All compounds were found to be non-cytotoxic according to the MTS assay, with a cell viability superior to 90%. Regarding the GLP-1 secretagogue effect, none of the compounds stimulated the secretion of GLP-1 in STC-1 cells (values similar to the vehicle), as opposed to the previously assessed 12,20-*O*-dibenzoyl or 12-*O*-tigloyl 2,6-dideoxyhexose pregnane glycosides [1]. This observation could indicate the necessity of the 12-*O* acylation and the presence of a dideoxyhexose chain linkage to the pregnane structure to stimulate the secretion of GLP-1. Nevertheless, this observation is yet to be confirmed by further studies with other pregnanes to establish a thorough structure-activity relationship evaluation. In order to avoid a false-negative result, the isolated steroids were submitted to a GLP-1 secretagogue assay using the more responsive primary cells (same concentration of 100 μ M) [11]; the remaining aliquot of the fraction that yielded compounds **1-8** was also investigated (50 μ g/mL).

Although pure steroids did not significantly enhance GLP-1 secretion, it is noteworthy that the pregnane-rich fraction led to a two-fold stimulation of GLP-1 secretion compared to the untreated cells ($6.8 \pm 1.1\%$ vs. $3.7 \pm 0.5\%$, $P < 0.05$, $n = 9$ wells, 3 independent experiments), suggesting a possible synergistic effect.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We want to thank the Region Alsace and the University of Strasbourg Foundation for financial support. We are grateful to Andry Petignat, from the Antsokay arboretum, for providing plant specimen. Denis Weldin, Régis Saladin, CD Muller and Sarah Ali Azouaou are acknowledged for the precious help with the STC-1 cell culture, as well as the PACSI team for the MS and NMR analyses.

Funding

This work was supported in Strasbourg by the Region Alsace [n° D11RREG4] and the University of Strasbourg Foundation [n° CONV/2011/01/FUDS 18], and in Cambridge by the Wellcome Trust (106262/Z/14/Z, 106263/Z/14/Z) and the Medical Research Council (MRC_MC_UU_12012/3).

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Compound	R ₁	R ₂	R ₃	H-17	R ₄
1 Menarandroside A	OH	α H	CH ₃	α	H
2 Menarandroside B	OH	α H	CH ₃	α	β-D-Glc
3 Carumbelloside I	H	β OH	CH ₃	α	β-D-Glc
4 Carumbelloside II	H	β OH	CH ₃	α	H
5 Menarandroside C	OH	β OH	CH ₃	α	H
6 Menarandroside D	OH	β OH	CH ₃	α	β-D-Glc
7 Menarandroside E	H	β OH	CH ₂ OH	β	H
8 Pregnenolone-3- <i>O</i> -gentiobioside	H	α H	CH ₃	α	β-D-Glc

Figure 1.
Structures of compounds **1-8**

Table 1¹³C NMR (125 MHz) data of compounds **1-8** in MeOD.

Position	Compound							
	1	2	3	4	5	6	7	8
	δ C							
1	38.5	38.6	38.8	38.8	38.4	38.4	34.6	38.4
2	30.8	31.1	31.0	30.9	30.5	31.5	31.2	30.6
3	79.9	80.1	80.3	79.9	79.7	80.1	79.7	80.1
4	39.9	40.0	39.9	39.8	39.6	39.6	39.9	39.7
5	142.1	142.1	141.5	141.5	140.7	140.9	136.7	142.0
6	122.8	122.8	123.1	123.0	123.0	122.3	127.1	122.6
7	32.9	32.7	27.2	27.2	26.4	26.4	28.2	32.8
8	33.3	32.8	36.2	36.2	35.8	35.8	38.9	33.1
9	45.4	45.1	44.5	44.6	40.0	40.0	47.8	51.3
10	37.7	37.7	38.2	38.2	37.5	37.7	43.1	37.4
11	29.8	29.6	21.3	21.3	29.8	29.8	22.9	22.1
12	72.4	72.2	32.1	32.1	74.2	74.2	40.3	39.8
13	49.2	49.2	49.4	49.4	50.9	51.0	50.6	45.2
14	49.4	49.7	85.8	85.8	87.3	87.5	87.5	58.0
15	25.1	24.9	33.4	33.4	33.3	33.3	34.6	25.4
16	23.2	23.3	22.6	22.6	21.9	21.9	25.5	23.7
17	56.8	56.1	61.3	61.3	54.6	54.6	64.0	64.7
18	14.5	14.0	17.6	17.6	18.0	18.1	15.8	13.7
19	19.8	19.4	19.8	19.8	19.9	19.9	63.5	19.9
20	212.9	212.9	213.7	213.7	213.2	213.3	220.1	211.5
21	31.1	31.1	31.8	31.8	31.1	31.1	32.8	31.9
3- <i>O</i> -glycopyranosyl								
1'	102.6	102.7	102.8	102.6	102.3	102.7	102.6	102.6
2'	75.3	74.9	75.2	75.3	74.8	74.9	75.3	74.9
3'	78.2	77.7	78.1	78.0	77.8	77.7	71.8	77.8
4'	71.8	71.2	71.6	71.8	71.4	71.2	78.2	71.2
5'	78.0	76.8	77.1	78.2	77.5	76.8	78.0	76.8
6'	62.9	69.8	69.8	62.9	62.7	69.8	63.0	69.6
6'- <i>O</i> -glycopyranosyl								
1''	-	104.6	104.9	-	-	105.0	-	104.6
2''	-	74.7	75.2	-	-	74.7	-	74.9
3''	-	77.8	78.2	-	-	77.8	-	77.8
4''	-	71.6	71.7	-	-	71.6	-	71.4

Position	Compound							
	1	2	3	4	5	6	7	8
	δ C							
5''	-	77.8	78.2	-	-	77.8	-	77.8
6''	-	62.2	62.9	-	-	62.2	-	62.7

Table 2¹H NMR (500 MHz) data of menarandrosides A, B (compounds **1-2**) and C, D, E (compounds **5-7**) in MeOD.

Position	Compound				
	1	2	5	6	7
δ H (J in Hz)					
1	1.13 (td, 13.7, 3.4) 1.85 (dt, 13.2, 3.1)	1.14 (td, 13.7, 3.6) 1.85 (dt, 13.3, 3.3)	1.17 (td, 14.2, 3.9) 1.89 (m)	1.18 (td, 13.4, 4.5) 1.89 (m)	1.13 (td, 12.5, 3.3) 2.08 (m)
2	1.94 (br. dt, 12.6, 2.2) 1.62 (m)	1.98 (m) 1.61 (m)	1.96 (m) 1.62 (m)	1.96 (m) 1.62 (m)	1.91 (m) 1.52 (dd, 11.2, 3.6)
3	3.60 (m)	3.58 (tt, 11.3, 4.6)	3.62 (m)	3.59 (tt, 11.0, 4.6)	3.64 (m)
4	2.44 (ddd, 13.5, 4.5, 2.0) 2.26 (ddd, 13.4, 12.0, 2.0)	2.43 (ddd, 13.5, 4.7, 2.2) 2.27 (m)	2.46 (ddd, 13.2, 4.8, 1.6) 2.29 (m)	2.46 (ddd, 12.9, 4.5, 2.4) 2.28 (m)	2.52 (ddd, 13.1, 4.5, 1.6) 2.27 (m)
6	5.39 (dt, 4.9, 2.3)	5.39 (br. d, 5.9)	5.42 (br. dd, 3.0, 2.8)	5.43 (br. dd, 3.0, 2.8)	5.68 (br. d, 4.4)
7	1.61 (m) 2.00 (dtd, 17.3, 5.4, 2.9)	1.67 (m) 2.03 (m)	2.19 (m) 1.81 (m)	2.19 (m) 1.80 (m)	1.81 (m) 2.30 (m)
8	1.52 (dd, 10.5, 4.9)	1.54 (dd, 10.5, 4.9)	1.90 (m)	1.90 (m)	2.16 (ddd, 13.1, 4.5, 1.6)
9	1.4 (ddd, 11.5, 10.9, 6.9)	1.41 (m)	1.70 (m)	1.70 (m)	1.15 (td, 12.5, 3.3)
11	1.75 (td, 13.2, 3.1) 1.72 (m)	1.77 (m) 1.66 (m)	1.86 (m) 1.8 (m)	1.84 (m) 1.75 (m)	1.59 (m) 1.69 (m)
12	- 4.01 (br. t, 2.7)	- 4.01 (br. t, 2.8)	- 4.16 (br. t, 2.6)	- 4.16 (br. t, 2.4)	1.59 (m) 1.40 (m)
14	1.70 (m)	1.72 (m)	-	-	-
15	1.72 (td, 9.3, 2.7) 1.25 (m)	1.73 (m) 1.25 (m)	1.55 (m) 1.54 (m)	1.55 (m) 1.54 (m)	2.09 (m) 1.70 (m)
16	2.10 (m) 1.64 (m)	2.11 (m) 1.70 (m)	2.21 (m) 1.83 (m)	2.21 (m) 1.84 (m)	2.03 (td, 9.6, 1.9) 1.90 (m)
17	3.23 (t, 9.0)	3.24 (dd, 9.0, 7.8)	3.79 (t, 8.8)	3.79 (m)	2.97 (dd, 9.6, 4.6)
18	0.65 (s)	0.65 (s)	0.72 (s)	0.71 (s)	1.03 (m)
19	1.02 (s)	1.02 (s)	1.05 (s)	1.05 (s)	3.84 (d, 11.8)
	-	-	-	-	3.54 (d, 11.8)
21	2.14 (s)	2.14 (s)	2.18 (s)	2.19 (s)	2.25 (s)
3- <i>O</i> -glycopyranosyl					
1'	4.39 (d, 7.8)	4.39 (d, 7.7)	4.39 (d, 7.8)	4.40 (d, 8)	4.39 (d, 7.7)
2'	3.14 (dd, 8.9, 7.7)	3.16 (dd, 9.3, 7.7)	3.17 (dd, 9.0, 7.8)	3.15 (dd, 8.9, 7.7)	3.14 (dd, 9.1, 7.9)
3'	3.35 (m)	3.35 (m)	3.37 (m)	3.34 (m)	3.26 (dd, 9.1, 7.9)
4'	3.26 (m)	3.37 (m)	3.31 (m)	3.35 (m)	3.35 (m)
5'	3.27 (m)	3.45 (m)	3.28 (m)	3.46 (m)	3.27 (m)
6'	3.85 (dd, 12.0, 1.6) 3.65 (dd, 12.0, 5.1)	3.79 (dd, 11.6, 5.5) 4.12 (dd, 11.6, 2.0)	3.86 (dd, 11.8, 1.5) 3.68 (dd, 11.8, 5.4)	4.12 (m) 3.79 (dd, 11.6, 1.9)	3.85 (dd, 11.5, 1.1) 3.64 (dd, 12.1, 5.0)

Compound					
Position	1	2	5	6	7
δH (J in Hz)					
6'- <i>O</i> -glycopyranosyl					
1''	-	4.38 (d, 7.8)	-	4.39 (d, 7.7)	-
2''	-	3.23 (dd, 9.1, 7.8)	-	3.21 (dd, 9.0, 7.9)	-
3''	-	3.27 (m)	-	3.35 (m)	-
4''	-	3.30 (m)	-	3.27 (m)	-
5''	-	3.27 (m)	-	3.29 (m)	-
6''	-	3.87 (dd, 11.6, 5.5)	-	3.87 (dd, 11.8, 5.3)	-
	-	3.67 (dd, 11.8, 5.4)	-	3.67 (dd, 11.6, 5.5)	-