Pregnane Glycosides from *Cynanchum marnierianum* Stimulate GLP-1 Secretion in STC-1 Cells*

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Key words
- *Cynanchum marnierianum*
- Apocynaceae
- marnieranosides
- pregnanes
- GLP-1
- cytotoxicity

Abstract

In the framework of the search for natural glucagon-like peptide-1 secretagogues, the bioassay-guided fractionation of the ethanolic extract from *Cynanchum marnierianum* led to the isolation of two new pregnane glycosides named marnieranosides A (**1**) and B (**2**). The structures were determined based on spectroscopic data and were established as 12*β*,20*S*-*O*-dibenzoyl-pregn-6-en-5*α*,8*β*,14*β*,17*β*-tetraol-3-*O*-*β*-D-oleandropyranosyl-(1 → 4)-*β*-D-cymaropyranoside (**1**) and 12*β*,20*R*-*O*-dibenzoyl-pregn-6-en-5*α*,8*β*,14*β*-triol-3-*O*-*β*-D-oleandropyranosyl-(1 → 4)-*β*-D-canaropyranosyl-(1 → 4)-*β*-D-cymaropyranoside (**2**). They present structural analogies to pregnanes previously described in species known for their appetite suppressant and antihyperglycemic effects, such as P57 from *Hoodia gordonii*. Lupeol (**3**), a known dipeptidyl peptidase-4 inhibitor, and the insulinomimetic kaempferol-3-*O*-neohesperidoside (**4**) were also identified in *C. marnierianum*. In an *in vitro* assay on secretin tumor cell line-1 cells, compounds **1, 2**, and P57 were found to stimulate the secretion of GLP-1 by 130 % (all tested at 100 µM). These results suggest that *C. marnierianum* could be of great interest in the treatment of type 2 diabetes, and that pregnane derivatives should be partly responsible via the stimulation of glucagon-like peptide-1 secretion.

Abbreviations

DPP-4:	dipeptidyl peptidase-4
GLP-1:	glucagon-like peptide-1
STC-1:	secretin tumour cell line
T2D:	type 2 diabetes

received February 26, 2016
revised April 15, 2016
accepted April 17, 2016

Bibliography
DOI http://dx.doi.org/10.1055/s-0042-107675
Published online May 25, 2016
Planta Med 2016; 82: 992–999

ISSN 0032-0943

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Introduction

GLP-1 is gradually becoming a suitable pharmaceutical target for the development of new drugs to treat T2D [1]. Indeed, this incretin is rapidly secreted in the gastrointestinal tract after food intake to induce glucose-dependent stimulation of insulin secretion, and to inhibit glucagon production. In addition to its hypoglycemic action, GLP-1 also decreases food intake by increasing satiety in the brain and delays gastric emptying, thus helping to lower postprandial glycemia [1, 2]. Previous reports have indicated that the secretion of GLP-1 is reduced in patients suffering from T2D [3]. Two therapeutic strategies are currently available involving the GLP-1 pathway in the treatment of diabetes: the first one involves the injection of GLP-1 receptor agonists (GLP-1 analogues) with an extended half-life, while the other one is associated with enhancing the half-life of GLP-1 by inhibiting the enzyme responsible for its inactivation, the DPP-4 enzyme [1]. However, these two approaches have drawbacks, and thus we decided to focus on another promising approach, which involves the use of selective secretagogues that can restore the natural production of GLP-1, and therefore regulate the postprandial glycemia.

Recently, some plant extracts have been shown to stimulate the secretion of GLP-1, proving the emerging potential of natural products for this new therapeutic approach [4–6]. In the search for the discovery of natural GLP-1 secretagogues, we focused our research on plant species that showed an appetite suppressant effect as well as blood sugar regulation with the hypothesis that

* Dedicated to Professor Dr. Dr. h.c. mult. Kurt Hostettmann in recognition of his outstanding contribution to natural product research.

it could be mediated via GLP-1 pathway [1,2]. In view of this stated hypothesis, our attention was drawn to *Hoodia gordonii* (Masson) Sweet ex Decne., an Apocynaceae from southern Africa, well known for its appetite suppressant and hypoglycemic effects. Despite the fact that its mechanisms of action and *in vivo* targets remain elusive, *H. gordonii* has been extensively exploited for the development of weight-loss dietary supplements [7]. However, due to the scarcity and legislation associated with the availability of *H. gordonii*, we decided to study species phylogenetically related to *H. gordonii*, with the assumption that they should have similar biosynthetic pathways and thus display potential anorectic and hypoglycemic effects. We therefore selected 16 different species belonging to the Asclepiadoideae subfamily (e.g., *Caralluma* and *Ceropegia* species) and screened their extracts in order to assess their ability to stimulate GLP-1 secretion in STC-1 cells. Out of the entire extracts screened, we found out that the ethanolic extract of *Cynanchum marnierianum* Rauh significantly stimulated GLP-1 secretion (130%) when compared to control cells. *C. marnierianum* is a leafless endemic species of Madagascar that belongs to the Apocynaceae family (subfamily Asclepiadoideae as *H. gordonii*) [8]. *C. marnierianum* presents an interest for ornamental purposes but no medicinal use has been recorded. Generally, there are few data as regards to the phytochemical or pharmacological properties of Madagascan *Cynanchum* species, although there has been a previous report on the wound healing, antitussive, and antimalarial properties of some species in this genus [9]. *Cynanchum aphyllum* L. is the only Madagascan species that has been studied in regards to the chemistry, reporting the presence of seco-pregnane-type derivatives [10].

Based on the aforementioned screening, an extensive bioassay-guided fractionation was performed to isolate and identify the metabolites responsible for the observed secretagogue effect, and to assess their GLP-1 stimulating activity. Furthermore, the cytotoxicity of the isolated compounds was also evaluated.

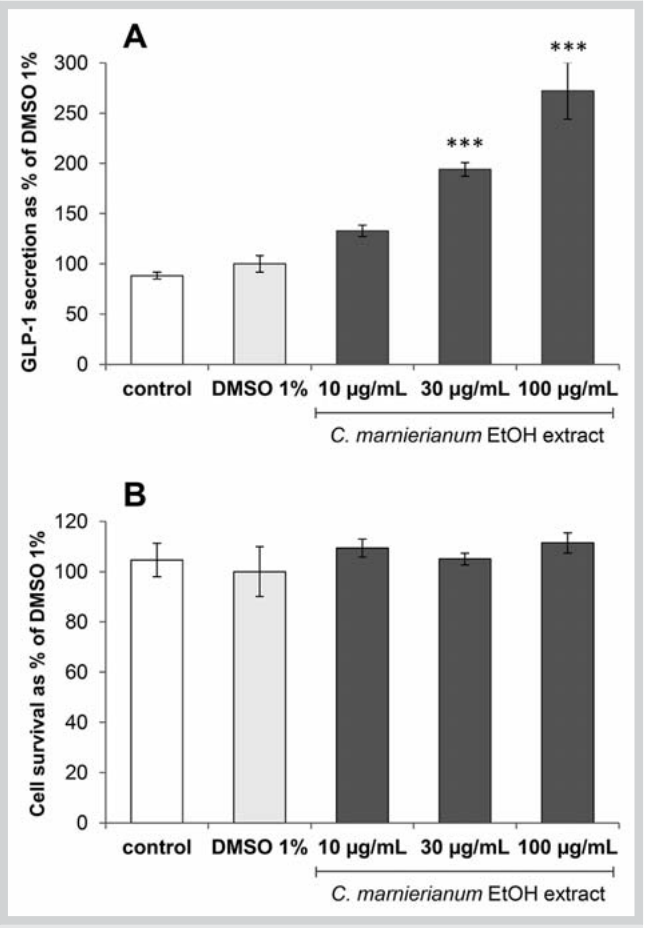

Fig. 1 **A** GLP-1 secretion in STC-1 cells (***$p < 0.05$). **B** Cell survival measured with the MTT assay.

Results and Discussion

The preliminary *in vitro* assay indicated that the ethanolic extract from the aerial parts of *C. marnierianum* could stimulate the secretion of GLP-1 in a dose-dependent manner (from 133 to 272% according to the tested concentration) compared to cells only treated with the vehicle (**Fig. 1 A**). The MTT assay on the residual cells also indicated the absence of toxicity (**Fig. 1 B**). The bioassay-guided fractionation of this crude extract led to the isolation of compounds **1** (3.2 mg) and **2** (0.9 mg). Lupeol (**3**) and kaempferol-3-*O*-neohesperidoside (**4**) were also identified in this extract.

Compound **1** was isolated as a white amorphous powder. Its UV spectrum presenting a maximum of absorption at 229 nm as well as a shoulder at 274 nm indicated the presence of an aromatic system. Its molecular formula was determined to be $C_{49}H_{66}O_{15}$ (17 degrees of unsaturation) on the basis of the observed pseudo-molecular peak at *m/z* 917.4283 $[M + Na]^+$ in the HR-ESI-MS spectrum. An additional peak could be observed at *m/z* 773.3469, and could be attributed to the loss of a methoxylated dideoxy sugar moiety, which is a feature of the Asclepiadoideae species [11]. The ^{13}C NMR and DEPT spectra indicated the presence of 49 carbons signals, which included 10 quaternary carbons, 8 methylenes, 24 methines, and 7 methyls (**Table 1**). Twenty-one of these carbon resonances were attributed to a pregnane moiety by means of the observed COSY, HSQC, and HMBC correlations, as well as the comparison of the NMR data with those of the previously reported structurally related compounds [10,12–15]. The two *cis* olefinic protons at δ_H 5.55 (d, $J = 10.3$ Hz) and 5.83 (d, $J = 10.3$ Hz) were attributed to the methines at positions 6 and 7, which showed HMBC correlations to the oxygenated carbon at C-8 (δ_C 74.8) and C-5 (δ_C 76.1 ppm), respectively. Therefore, the pregnane skeleton of compound **1** consists of a 5*α*,8*β*,12*β*,14*β*,17*β*,20*β*-hexahydroxy-pregn-6-ene, previously reported in the genera *Araujia, Caralluma, Gymnema*, and *Stephanotis* from the Asclepiadoideae subfamily [12–14,16–17]. The report of a $\Delta^{6,7}$ pregnene structure is in accordance with a previous work on *C. aphyllum* revealing the presence of $\Delta^{6,7}$-8,14-*seco*-pregnene glycosides [10]. Additional signals observed in the ^1H NMR spectrum indicated the presence of two aromatic moieties in the structure, as indicated by resonances attributed to a ten aromatic proton $(AB)_2X$ system, indicating the presence of two monosubstituted aromatic rings. In the HMBC spectrum, the proton at positions 3*/7* (δ_H 7.60, dd, $J = 7.8$, 1.5 Hz) and 3**/7** (δ_H 7.65, dd, $J = 7.8$, 1.5 Hz) correlated to the carbonyl of the ester moiety at C-1* (δ_C 166.7) and C-1** (δ_C 167.9), respectively, indicating that the two aromatic systems are two benzoyl moieties esterified on the skeleton of compound **1**. Furthermore, two anomeric protons could be observed in the ^1H NMR spectrum resonating at δ_H 4.65 (dd, $J = 9.6$, 1.9 Hz) and 4.70 (dd, $J = 9.6$, 1.7 Hz). The spin system observed in the ^1H-^1H COSY spec-

Table 1 ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) data of marnieranosides A (**1**) and B (**2**) in methanol-d_4.

	Marnieranoside A (1)		Marnieranoside B (2)	
Position	**δ_H (*J* in Hz)**	**δ_C**	**δ_H (*J* in Hz)**	**δ_C**
1	1.78, m	28.0	1.31, m	30.2
	1.42, m		1.59, m	
2	1.85, m	27.0	1.96, m	27.0
	1.67, m		1.60, m	
3	4.11, m	75.9	4.13, m	76.0
4	1.99, m	39.4	2.44, dd (11.8, 4.3)	34.1
	1.76, m		1.43, dd (11.8, 9.6)	
5	–	76.1	–	83.8
6	5.55, d (10.3)	136.5	5.75, d (10.1)	133.3
7	5.83, d (10.3)	127.6	6.03, d (10.1)	132.6
8	–	74.8	–	75.5
9	1.98, m	37.1	2.30 dd (12.7, 1.5)	38.4
10	–	40.1		38.6
11	2.00, m	24.1	1.57, m	23.5
	1.71, m		1.92, m	
12	5.01, dd (10.2, 4.3)	76.8	5.06, dd (11.2, 4.8)	75.5
13	–	59.1	–	55.7
14		88.9	–	87.9
15	1.93, m	33.5	2.06, m	33.3
	1.85, m		1.66, m	
16	2.10, m	35.0	1.57, m	26.3
	1.98, m		1.96, m	
17	–	88.8	2.53, dt (11.0, 8.5)	54.0
18	1.68, s	12.4	1.68, s	17.4
19	0.99, s	21.3	0.99, s	18.7
20	4.84, q (6.0)	75.7	4.84, q (6.0)	74.3
21	1.29, d (6.0)	15.6	1.29, d (6.0)	20.9
12-*O*-benzoyl				
1*	–	166.7	–	166.8
2*	–	131.7	–	131.8
3*/7*	7.60, dd (7.8, 1.5)	130.9	7.60, dd (7.8, 1.5)	130.6
4*/6*	7.32 [a]	129.3	7.32 [b]	129.4
5*	7.53 [a]	134.1	7.53 [b]	134.1
20-*O*-benzoyl				
1**	–	167.9	–	167.8
2**	–	132.3	–	132.2
3**/7**	7.65 dd (7.8, 1.5)	130.6	7.65 dd (7.8, 1.5)	130.6
4**/6**	7.10 [a]	129.3	7.10 [b]	129.3
5**	7.42 [a]	133.9	7.42 [b]	133.9
Cymaropyranosyl				
1′	4.65, dd (9.6, 1.9)	100.0	4.66, dd (9.8, 1.8)	100.0
2′	2.29, br dd (12.5, 5.5)	37.8	2.25, m	38.3
	1.41, dt (11.5, 9.0)		1.38, m	
3′	3.34, dt (12.7, 6.3)	80.3	3.37, m	80.3
4′	3.19, dt (12.9, 6.0)	84.2	3.16, t (8.9)	84.4
5′	3.35, dq (11.0, 5.4)	72.5	3.35, m	72.0
6′	1.27, d (6.2)	18.8	1.26, d (6.0)	18.7
OMe′	3.43, s	57.6	3.42, s	57.8
Canaropyranosyl				
1″			4.71, dd (9.6, 1.8)	101.5
2″			2.19, m	37.9
			1.45, m	
3″			3.59, ddd (8.5, 5.3, 1.9)	81.9
4″			3.00, t (8.9)	77.2
5″			3.35, m	73.5
6″			1.30, d (6.0)	18.5
Oleandropyranosyl				
1‴	4.70, dd (9.6, 1.7)	101.5	4.62, dd (9.8, 1.8)	101.5
2‴	2.30, br dd (12.3, 5.1)	37.9	2.39, m	37.9
	1.44, dt (12.3, 9.6)		1.39, m	
3‴	3.20, ddd (11.6, 8.7, 4.9)	81.9	3.24, m	81.9
4‴	2.98, br t (9.1)	77.2	3.01, t (9.0)	77.2
5‴	3.24, dq (9.5, 6.2)	73.5	3.36, m	73.5
6‴	1.31, d (6.0)	18.5	1.28, d (6.0)	18.5
OMe‴	3.41, s	57.8	3.44, s	57.8

[a, b] Overlapping signals

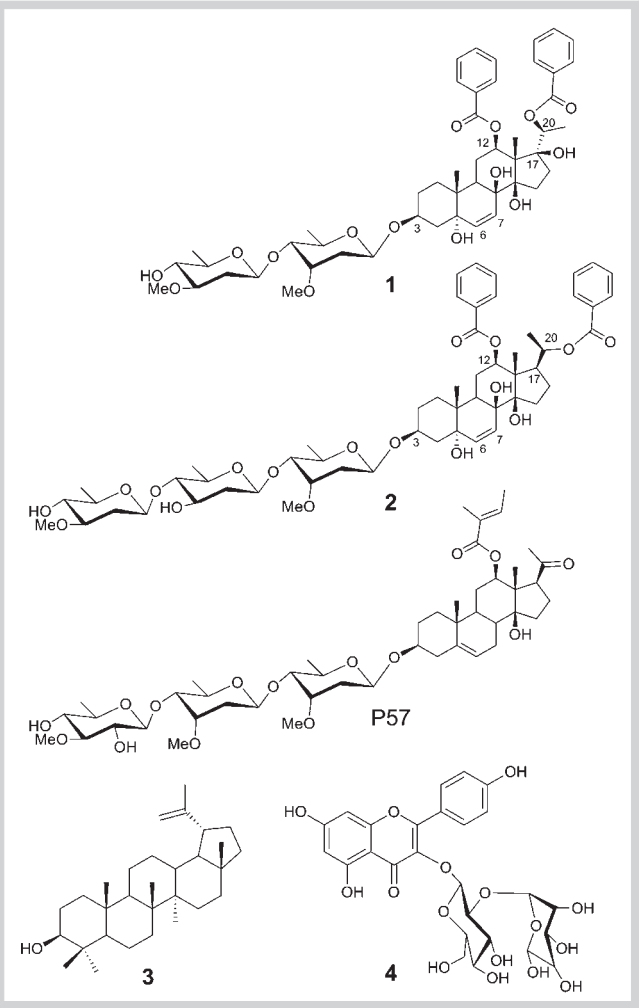

Fig. 2 Chemical structures of marnieranoside A (**1**), marnieranoside B (**2**), P57, lupeol (**3**), and kaempferol-3-*O*-neohesperidoside (**4**).

trum along with the coupling constants, the ROESY, and the HMBC correlations permitted the identification of a sugar chain composed of two 2,6-dideoxy-hexoses, each bearing a methoxy group at position 3. In the ROESY spectrum, the anomeric proton H-1″ (δ_H 4.70, dd, J = 9.6, 1.7 Hz) correlates with H-3″ (δ_H 3.20, ddd, J = 11.6, 8.7, 4.9 Hz) and H-5″ (δ_H 3.24 dq, J = 9.5, 6.2 Hz), indicating an α orientation of these protons. In addition, a ROE signal between H-4″ (δ_H 2.98 br t, J = 9.1 Hz) and CH_3-6″ (δ_H 1.31, d, J = 6.0 Hz) was also observed, indicating the planar orientation of the hexose, which led us to identify the terminal sugar as an oleandropyranose, which is in accordance with the ion observed at *m/z* 773.3469, thus assigned as [M + Na-oleandrose]$^+$. The anomeric proton of the oleandrose also displayed an HMBC ^3J correlation to C-4′ (δ_C 84.2) of the first sugar moiety, which was identified as a cymaropyranose based on the correlations observed in the ROESY spectra between H-1′ (δ_H 4.65, dd, J = 9.6, 1.9 Hz) and H-5′ (δ_H 3.35, dq, J = 9.5, 6.2 Hz), as well as between H-3′ (δ_H 3.34, dt, J = 12.7, 6.3 Hz) with H-4′ (δ_H 3.19 dd, J = 12.9, 6.0 Hz) and H-6′ (δ_H 1.27, d, J = 6.2 Hz). Furthermore, the configuration of sugars was assessed as beta-D based on the coupling constants of both anomeric protons and chemical shifts of the C-2 carbons [18]. The HMBC ^3J correlation between the carbonyl of the benzoyl ester at δ_C 167.7 (C-1*) and H-20 (δ_H 4.84, q, J = 6.3 Hz) proved the acylation of the aglycone by the benzoyl group at position C-20. The chemical shift of the proton and carbon at position 12 (δ_C 76.8 and δ_H 5.01) also supports the esterification of its hydroxyl with the latter benzoyl group. The glycosidic chain was shown to be linked to position 3 of the aglycone by the HMBC ^3J correlation between the anomeric proton of the cymaropyranose at δ_H 4.65 and the C-3 at δ_C 75.9. Literature data have shown that in 20-hydroxy-C/D-*cis*-pregnane-type steroids, steric hindrance between methylene-16 (H-16), the substituent at position 20, and the methyl groups at positions 18 and 21 of the cyclopentane ring can lead to chemical shifts indicative of the absolute configuration of the carbon 20 (C-20). The transposition ($\Delta\delta_C < 0.7$) of the carbon chemical shifts of these positions between our experimental data and the closest structures published in the literature led to the deduction of the 20*S* configuration [12–14, 19, 20]. Consequently, compound **1** was identified as 12*β*,20*S*-*O*-dibenzoyl-pregn-6-en-5α,8*β*,14*β*,17*β*-tetraol-3-*O*-*β*-D-oleandropyranosyl-(1 → 4)-*β*-D-cymaropyranoside and named marnieranoside A (**Fig. 2**).

Compound **2** was also isolated as a white amorphous powder, exhibiting the same UV spectrum as compound **1**. Its molecular formula was determined as $C_{55}H_{76}O_{17}$ (18 degrees of unsaturation) from the observed pseudo-molecular ion at *m/z* 1031.4960 [M + Na]$^+$ in the HR-ESI-MS spectrum. This difference of 114 amu ($C_6H_{10}O_2$) compared to the molecular formula of compound **1** could suggest the presence of an additional dideoxy-hexose in the sugar chain. The fragments observed at *m/z* 865.4355, 735.3728, and 591.2943 as well as their dehydrated derivatives reinforce this hypothesis. The resemblance in the ^{1}H NMR spectrum of compounds **1** and **2** was remarkable, indicating that they both possess similar structural features. Additional signals attributable to three anomeric protons at δ_H 4.66, 4.71, and 4.62 were observed in the spectra of **2** (**Table 1**). The spin system observed in the ^{1}H-^{1}H COSY spectrum along with the coupling constants, ROESY, and the HMBC correlations led to the identification of the sugar chain composed of a cymaropyranose, canaropyranose, and a terminal oleandropyranose linked with 1 → 4 glycosidic bonds. This is in accordance with the fragments previously observed in the HR-ESI-MS spectrum at *m/z* 865.4355 [M + H-oleandrose]$^+$, 735.3728 [M + H-oleandrose-canarose]$^+$, and 591.2943 [M + H-oleandrose-canarose-cymarose]$^+$. Careful comparison of the spectral data with those published in the literature confirmed the unambiguous identification of this chain [16, 21]. As in compound **1**, the coupling constants of the anomeric protons as well as the distinctive chemical shift of the carbons at position 2 of each hexose resonating a chemical shift greater than 35.0 ppm showed that these hexoses possess the beta-D configuration [18]. The proton resonating at δ_H 2.53 (td, J = 11.0, 8.5 Hz), which showed an HSQC correlation to the carbon at δ_C 54.0 (C-17), was found to be in position 17 based on HMBC ^3J correlations from H-18 (δ_H 1.68, s) and H-21 (δ_H 1.29, d, J = 6.0 Hz) to C-17. Its orientation was found to be under the plane of the cyclopentane ring D based on the ROESY correlations observed with H-15α (δ_H 2.06, m) and H-16α (δ_H 1.57, m), both orientated below the plane, while their respective germinal protons H-15*β* (δ_H 1.66, m) and H-16β (δ_H 1.96, m) correlated with the *β* orientated CH_3-18 (δ_H 1.68, s). As cited previously, 20-hydroxy-C/D-*cis*-pregnane-type steroids present characteristic C-16 and C-20 ^{13}C NMR chemical shifts depending on the absolute configuration of C-20. In the case of 20*R* pregnanes, C-20 and C-16 have chemical shifts around 75 ppm and 26 ppm, respectively, which is in accordance with values for compound **2**, since C-20 and C-

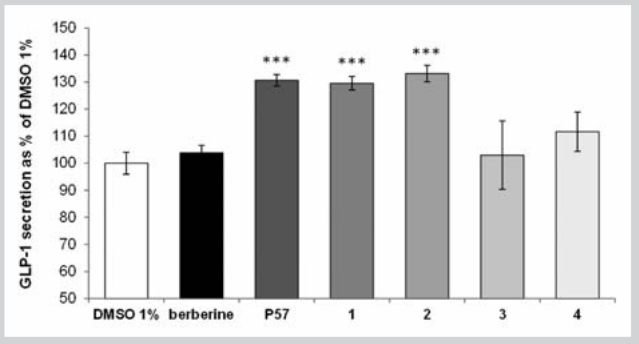

Fig. 3 GLP-1 secretion induced by berberine, P57, and isolated compounds (100 µM) on STC-1 cells (***$p < 0.05$).

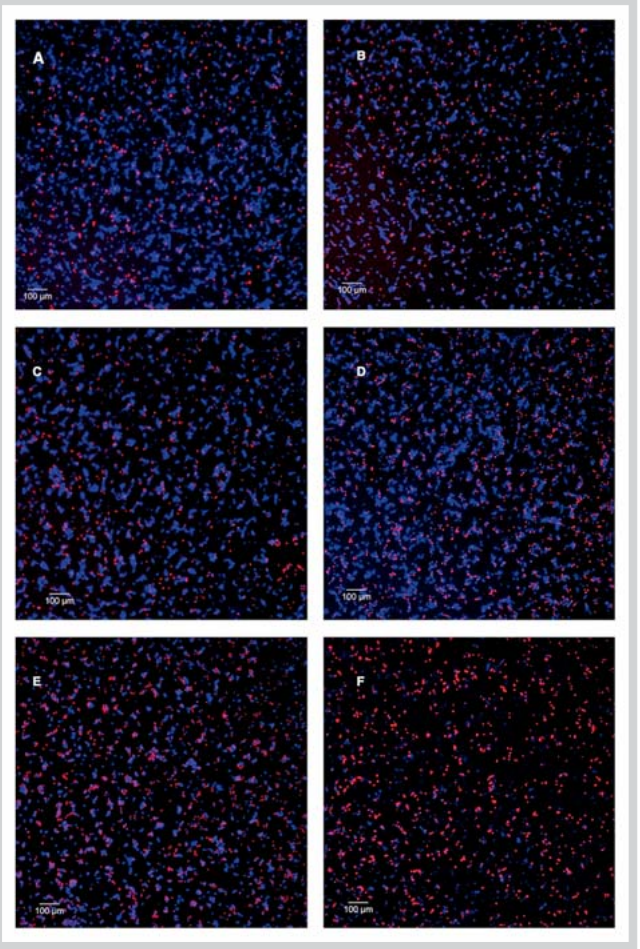

Fig. 4 Cytometric images of STC-1 cells after treatment with **A** glucose 4.5 g/L, **B** DMSO 1%, **C** marnieranoside A (**1**), **D** marnieranoside B (**2**), **E** P57, and **F** berberine. Cellular nuclei are revealed by a blue fluorescence (Hoechst staining); when cellular integrity is compromised, nuclei show a red fluorescence (PI staining). Scale bar = 100 µm. (Color figure available online only.)

16 resonated at δ_C 74.3 and 26.3, respectively [19,20]. Thus, compound **2** was identified as 12*β*,20*R*-*O*-dibenzoyl-pregn-6-en-5*α*,8*β*,14*β*-triol-3-*O*-*β*-D-oleandropyranosyl-(1 → 4)-*β*-D-canaropyranosyl-(1 → 4)-*β*-D-cymaropyranoside, a new pregnane derivative named marnieranoside B (**Fig. 2**).

In addition to these two new pregnane derivatives, the phytochemical investigation of *C. marnierianum* led to the isolation of two known compounds, lupeol (**3**) and kaempferol-3-*O*-neohesperidoside (**4**; **Fig. 2**). The structures were confirmed by spectral comparison with standards and previously published literature [22]. Interestingly, both compounds were previously reported as potential antidiabetic molecules; compound **3** has been described as a DPP-4 inhibitor, increasing GLP-1 plasma half-life [23], and compound **4** itself has shown insulin-mimetic effects *in vitro* [24].

Marnieranosides present structural features of benzoyl-substituted $\Delta^{6,7}$ pregnene glycosides with a sugar chain consisting of 2,6-dideoxy-hexoses, which displays structural analogy to compounds that have been previously reported as appetite suppressants and antihyperglycemic agents from species in the Asclepiadoideae subfamily. For example, similar structures have been reported from *Caralluma tuberculata* N.E.Br. and *Caralluma quadrangula* (Forssk.) N.E.Br., both known to possess hypoglycemic and anorectic effects [16,25]. Furthermore, common structural features can be observed with the hypoglycemic and anorectic compounds from *H. gordonii*, notably with the pregnane P57AS3 (P57, **Fig. 2**), the putative active compound of hoodia, but also with gordonoside F [7]. These acylated $\Delta^{5,6}$ pregnanes bear a glycosidic chain in position 3 composed of three to four 2,6-dideoxy-hexoses (oleandrose and cymarose) as seen in marnieranosides A and B.

The isolated compounds were assessed for their inherent capacity to stimulate GLP-1 secretion in STC-1 cells, as well as for their cytoxicity. Berberine, which has been previously established as a GLP-1 secretagogue, was used as a positive control [4]. We decided to compare the activity of compounds **1** and **2** to a commercial standard of P57 due to their close structures and botanical source origins. Only one study has been previously carried out about the GLP-1 secretagogue activity of P57, but reported a non-significant effect [26]. Tested at 100 µM, compounds **1** and **2** increased the release of GLP-1 by 129.6% ± 2.5% and 133.1% ± 3.0%, respectively, when compared to the control cells (**Fig. 3**). P57 exhibited a similar effect (130.6% ± 2.0%). Compounds **3** and **4** did not show any significant GLP-1 secretagogue effect. Unexpectedly, berberine did not stimulate the secretion of GLP-1 (103.9% ± 2.7% at 100 µM), but also showed a significant reduction in cell viability as observed with the MTT assay (data not shown). We also tested chlorogenic acid at 40 mM, another natural product shown to stimulate GLP-1 secretion *in vitro* [5], and found it to be cytotoxic to the cells. Interestingly, most of the papers dealing with natural GLP-1 secretagogues did not report the cytotoxic effect of the tested compounds. This is, however, essential since cell toxicity could manifest as a disruption of the cell membrane integrity, leading to a passive excretion of GLP-1 that could be mistakenly interpreted as a physiological secretion. In order to exclude the hypothesis of false positive results often encountered with natural products, and to go deeper on the evaluation of cytotoxicity, the effects of compounds **1, 2**, P57, and berberine were evaluated by means of image cytometry. Compounds **1** and **2** did not significantly affect the viability of STC-1 cells (**Fig. 4**). This was not the case for P57 and, especially, berberine, which induced a significant rise in the number of dead cells, indicating a possible proapoptotic effect on the STC-1 line. Further flow cytometry monitoring (PS labelling by AnnexinV) confirmed that P57 at 100 µM led to a decrease of 20% of cell viability, and that berberine at 30 µM induced a drastic reduction of STC-1 cell viability of 75%, mainly due to apoptosis but also, to a

lesser extent, necrosis (data not shown). However, it is interesting to note that whereas P57 appears to be moderately toxic on STC-1 cells, it is nevertheless considered one of the main active principles of a plant traditionally consumed for centuries. Furthermore, P57 has been shown to activate different receptors, leading to hormone release, notably GLP-1 [26]. Such experiments should be conducted on marnieranosides to definitely prove the activation of metabolic pathways leading to GLP-1 secretion. In addition, animal *in vivo* assays could confirm the efficacy and safety of *C. marnierianum* extract, as it has been done for the Chinese *Cynanchum auriculatum* Royle ex Wight [15].

The identification of two pregnanes glycosides stimulating the GLP-1 secretion validated our approach based on ethnopharmacological data and phylogenetic considerations. Our result strongly suggests that the appetite suppressant and antihyperglycemic properties observed with various Asclepiadoideae could be possibly mediated via the GLP-1 pathway, and that pregnane derivatives are responsible for these effects. Furthermore, the identification of lupeol, which is known to inhibit DPP-4 – the enzyme inactivating GLP-1 – together with the insulinomimetic kaempferol-3-*O*-neohesperidoside reinforces the potential of *C. marnierianum* as a potent antihyperglycemic species.

Materials and Methods

Plant material and extraction

C. marnierianum grown in a greenhouse was supplied in October 2012 by a horticulturist ("La Ferme aux Cactus", Tieste-Uragnoux, Gers, France), and identified by Paul Saint Pie from the botanical garden of Asson, France. A voucher specimen (n° STR-60522) is deposited at the Herbarium of the University of Strasbourg, France.

The aerial parts (356.4 g) were cut and freeze-dried, giving 154.6 g of dry plant material, which was extracted by maceration with ethanol (sonication for 10 min, 3 cycles). The three extracts were combined and dried under vacuum to give 23.86 g of dry ethanolic extract.

Apparatus, materials, chemicals, and reagents

HPLC analyses were performed on a Varian LC-920 system equipped with a PDA detector and a column oven set at 40 °C. A Kinetex® Core-Shell column C18 100 Å (2.6 µm, 100 × 3.0 mm, i.d., Phenomenex) was used for analytical purposes. The mobile phase consisted 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 0.7 mL/min: 30 to 50% B, 0–10 min; 50 to 100% B, 10–13 min. For isolation, a Sunfire® C18 column (3.5 µm, 150 × 4.6 mm, i.d., Waters) was used on the same HPLC module, and fractions of interest were collected manually based on the UV acquisition. The mobile phase used consisted of A (acidified water, 0.05% v/v formic acid) and B (acidified ACN, 0.05% v/v, formic acid): 60 to 70% B, 0–10 min; 70 to 100% B, 10–13 min, at a flow rate of 1.0 mL/min, and at 40 °C. The separation by centrifugal partition chromatography (CPC) was performed on a Spot Prep II 250 module equipped with a 10-mL injection loop and coupled with a CPC column SCPC-250 (Armen) rotating at 2000 rpm, while the mobile phase was pumped at a flow rate of 4.0 mL/min. Fractions of 15 mL were collected by the Spot Prep II module automatic collector. HPLC-HRMS analyses were performed on an Agilent 1200 system equipped with a Zorbax RRHT SB-C18 (5 µm, 50 × 2.1 mm, i.d., Agilent) and a column oven set at 35 °C. The gradient consisted of a mixture of A (acidified water, 0.05% v/v formic acid) and B (acidified ACN, 0.05% v/v, formic acid): 2 to 100% B, 0–8 min; 100% B for 8–13 min, flow rate of 0.5 mL/min. The chromatograph was coupled to an Agilent mass spectrometer 6520 using an ESI source and a qTOF analyzer (desolvation gas: N_2 at a flow rate of 8 L/h, capillary voltage: 9000 V, source temperature: 340 °C). The mass spectra (50–3000 amu) were recorded at a frequency of 3 spectra/sec. NMR spectra were recorded on a Bruker Avance III – 500 MHz NMR spectrometer equipped with a multi-nucleus probe BBFO (5 mm); samples were dissolved in methanol-d_4 (Sigma). Optical rotations were measured on a P200 (Jasco) apparatus equipped with a sodium lamp and a 10-cm long cell kept at 25 °C. Specific rotation was calculated based on the mean of 20 measurements. Solid-phase extractions were performed on Chromabond® SPE cartridges packed with 50 g of silica (Macherey-Nagel). The stationary phase for the VLC separation was silica gel 60 (0.015–0.040 mm, Merck). Solvents for extraction, fractionation, and HPLC were supplied by Sigma-Aldrich. The phytochemical standards were purchased as follows: chlorogenic acid (Roth), lupeol (Extrasynthese, purity > 99%), berberine (Sigma-Aldrich, purity ≥ 98%), and P57 (LGC Standards). The cell culture materials were purchased as follows: GLP-1 ELISA kit (Active GLP-1, EGLP-35 K Millipore), DMEM and PBS (Gibco), MTT and BSA (Sigma-Aldrich), propidium iodide (Miltenyi Biotec SAS), and Hoechst reagent (Thermo Fisher Scientific). The reading of the plates was performed on an ELISA Versamax plus® (Molecular Devices) plate reader and the cytometry images were taken on a Celigo® S (Nexcelom Bioscience) apparatus treated with the Celigo® program.

Compound isolation

The ethanolic extract (13.65 g) was fractionated by liquid partitioning between 300 mL of water and 300 mL of cyclohexane thrice, yielding 8.10 g of dry aqueous phase, 0.35 g of precipitate, and 4.65 g of dry organic phase. The organic phase was further fractionated by solid-phase extraction. The SPE cartridge was conditioned with cyclohexane, and the sample dissolved in cyclohexane was eluted using the following gradient: 100% cyclohexane (200 mL), diethyl ether/cyclohexane (1–99, 2–98, 3–97, 5–95, 8–92, 15–85, 20–80, 30–70, and 50–50; 200 mL each), 100% diethyl ether (200 mL), EtOAc/diethyl ether (50–50, 200 mL), EtOAc 100% (200 mL), methanol/EtOAc (50–50, 200 mL), and methanol 100% (200 mL). The fraction eluted with diethyl ether/cyclohexane (8–92) was further separated by CPC using the ternary biphasic system. The fractionation was performed with the ternary system heptane-ACN-methanol (5 : 5 : 3, v/v/v). The coil was first entirely filled with the upper phase and rotation was set to the desired speed (1200 rpm). The lower phase was then pumped into the column at a flow rate of 6 mL/min in descending mode (mobile phase: lower phase; stationary phase: upper phase). After the equilibrium between the two phases (mobile phase 80 mL), the sample (340 mg) solubilized in 10 mL of an emulsion of the two phases (1–1, v/v) was injected through the injector and the effluent was collected from the outlet of the column while being monitored at 210 nm. Forty-three fractions of 15 mL were collected and analyzed by HPLC-UV. The fraction eluted at 90 min afforded compound **3** (26.6 mg). The bioactive fraction eluted with 100% EtOAc (162.2 mg) was further fractionated by centrifugal partition chromatography using the quaternary system cyclohexane-EtOAc-methanol-water (6 : 5 : 6 : 5, v/v/v/v). The experiment was performed with the quaternary system of cyclohexane-EtOAc-Methanol-H_2O (6 : 5 : 6 : 5) (v/v/v/v). The coil

was first entirely filled with the upper phase and rotation was set to 2000 rpm. The lower phase was pumped into the column at a flow rate of 4 mL/min in descending mode (mobile phase: lower phase; stationary phase: upper phase). After the equilibrium between the two phases (mobile phase 10 mL), the sample solution of the reaction mixture (162.2 mg) in 10 mL of an emulsion of the two phases (1–1, v/v) was injected through the injector and the effluent was monitored at 210 nm and collected from the outlet of the column. During the elution, 48 fractions of 15 mL were collected in addition to 16 fractions collected during the extrusion of the stationary phase from the column. The obtained fractions were analyzed by HPLC-UV. The 32nd fraction eluted at 70 min afforded compound **1** (3.2 mg, purity > 96% by HPLC). The residual precipitate from the initial liquid partitioning (0.35 g) was dissolved in methanol and fractionated by size exclusion chromatography on a Sephadex LH-20 column (h = 64 cm, d = 2.4 cm). Fractions of 2 mL were collected and pulled into five final fractions based on their TLC and HPLC profile. The last fraction gave compound **4** (2.3 mg).

Furthermore, 10.18 g of the ethanolic extract were dissolved in 300 mL of water, and partitioned three times with 300 mL of EtOAc. The residue (5.49 g of dry EtOAc extract) was fractionated on an SPE cartridge as described above. The fraction eluted with 100% EtOAc (dry weight 114.7 mg) was further fractionated on a silica gel VLC column (53 g, Merck silica 0.015–0.0400 mm, column height and internal diameter = 6 cm) and eluted with mixtures of chloroform/methanol of increasing polarity. The fraction eluted with chloroform-methanol 15 : 1 v/v (10.3 mg) was subjected to HPLC. The fraction collected at 13.70 min afforded compound **2** (0.9 mg, purity > 96% by HPLC).

Cell culture, cell viability, and glucagon-like peptide-1 secretagogue assays

STC-1 cells were generously donated by Pr. Johan Auwerx (EPFL) and maintained in DMEM (4.5 g/L glucose) medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/L streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. Bioassays were performed between the 6th and 17th subculture when 70% of cell confluence was attained. One hundred µL of a 5×10^5 cells/mL suspension in DMEM (1 g/L glucose) were incubated overnight in each well of an ELISA 96-well plate. The samples were dissolved in DMEM medium (1 g/L glucose, 0.1% fat-free BSA, 2% DMSO). One hundred µL of the diluted treatment were added to the corresponding cells in order to obtain the desired final concentration of the treatment. After 2 h of incubation, the GLP-1 levels in the supernatant were measured according to the ELISA kit protocol. Evaluation was carried out at the concentration of 100 µM and results are 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 MTT assay by adding 100 µL of a 5 mg/mL MTT solution in PBS, 1 h incubation, dissolution of the formazan in DMSO, and reading of the plate at 570 nm. For the image cytometry assay, the remaining cells of the GLP-1 secretion evaluation were incubated with medium containing propidium iodide (1.5 µM) and Hoechst reagent (5.0 µM), and after 1 h incubation, photos of each well were taken at visible and fluorescent light [($\lambda_{excitation}$ = 377 nm, $\lambda_{emission}$ = 470 nm) and ($\lambda_{excitation}$ = 531 nm, $\lambda_{emission}$ = 629 nm)] and processed with the Celigo® program. Experiments were done in triplicate. Statistical calculations were carried out with one-way ANOVA followed by Dunnett's multiple comparison test using Prism 5.0 GraphPad software. Results are expressed as the mean ± S.E.M. of three independent experiments. P values < 0.05 were considered to be significant.

Marnieranoside A (**1**): white amorphous powder; $[\alpha]_D^{20}$ +29.6 (*c* 0.1, methanol); HR-ESIMS *m/z* 917.4283 $[M + Na]^+$ (calcd. error 0.17 ppm for $C_{49}H_{66}NaO_{15}$); UV: see Results and Discussion; ^{1}H-NMR and ^{13}C-NMR: see **Table 1**.

Marnieranoside B (**2**): white amorphous powder; $[\alpha]_D^{20}$ +11.2 (*c* 0.1, methanol); HR-ESIMS *m/z* 1031.4960 $[M + Na]^+$ (calcd. error 2.16 ppm for $C_{55}H_{76}NaO_{17}$); UV: see Results and Discussion; ^{1}H-NMR and ^{13}C-NMR: see **Table 1**.

Acknowledgments

We want to thank Jean-Marie Solichon and Andry Petignat for providing plant samples for the initial biological screening. Drs. Denis Weltin and Régis Saladin are acknowledged for their suggestions regarding the secretion assay, as well as Dr. Sarah Ali Azouaou for the precious help with the STC-1 cell culture. Dr. Joshua Olatunji is also gratefully acknowledged for careful proofreading and relevant comments.

Fundings

This research was supported by grants from the Region Alsace (n° D11RREG4) and the University of Strasbourg Foundation (n° CONV/2011/01/FUDS 18).

Conflict of Interest

The authors declare no conflict of interest.

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