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Secretion of glucagon-like peptide-1 induced by *Cynanchum* pregnane derivatives: preliminary hypotheses regarding key structural elements

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Abstract

In our continued efforts to explore the antidiabetic potential of pregnanes from Apocynaceae, especially from the Asclepiadoideae subfamily, sixteen derivatives **1-16** previously isolated from various *Cynanchum* species, have been evaluated for their ability to increase glucagon-like peptide-1 (GLP-1) release in a cell assay. As a result, otophyllaside A (**2**), wilfoside C1N (**13**), and wilfoside C3N (**16**) were found to stimulate significantly the secretion of GLP-1, doubling the extracellular content of this incretin. Caudatin 3-*O*- β -D-glucopyranosyl-(1 \rightarrow 4)- β -D-oleandropyranosyl-(1 \rightarrow 4)- β -D-cymaropyranosyl-(1 \rightarrow 4)- β -D-cymaropyranoside (**6**) was also bioactive, but to a lesser extent (133% of secretion compared to control cells). This study confirms the potential of certain pregnane derivatives from subfamily Asclepiadoideae to stimulate the release of GLP-1, reduced in patients with type 2 diabetes. In addition, this study outlines some putative structure-activity relationships: the nature of the sugar chain at C-3, as well as the ester moiety at C-12 position appear to be key elements to stimulate GLP-1 secretion.

Keywords: Pregnanes; *Cynanchum*; Apocynaceae; diabetes; glucagon-like peptide-1; structure-activity relationships

1. Introduction

Diabetes is a multifactorial metabolic disorder that has reached epidemic proportions, affecting 463 million people worldwide in 2019 (International Diabetes Federation, 2019). This disease is characterized by chronic high blood sugar levels, due to impaired ability of the body to produce or respond to insulin; the latter situation is called type 2 diabetes (T2D), which represents 90% of all diabetes cases. Often asymptomatic for years, T2D leads to several complications afterwards, both at the microvascular and macrovascular level. Two decades ago, glucagon-like peptide-1 (GLP-1), an intestinal hormone secreted after food intake, was identified as a key peptide in blood sugar regulation. Indeed, this peptide increases glucose-stimulated insulin secretion by pancreatic β cells, decreases glucagon production, delays gastric emptying, reduces food intake by promoting satiety, contributing thus to attenuate excessive postprandial glycemia (Drucker, 2018). This has led to the development of therapeutic strategies miming (GLP-1 receptor agonists) or prolonging (gliptins) this incretin effect. As T2D has been shown to be associated with an impairment of the intestinal secretion of GLP-1 (Vilsboll et al., 2001), we have decided to develop an alternative strategy, focusing on natural products that could stimulate endogenous GLP-1 secretion, and consequently restore healthy physiology.

In this context, we previously discovered that two pregnane glycosides from *Cynanchum marnierianum* (Apocynaceae), marnieranosides A and B, significantly stimulate GLP-1 secretion of intestinal secretin tumour cells (STC-1) (Tsoukalas et al., 2016). We also demonstrated that P57, a polyhydroxylated pregnane glycoside from *Hoodia gordonii*, possesses a similar activity in this bioassay. Another cell assay revealed that the ethanolic extract of *Cynanchum descoingsii* also increases the secretion of GLP-1 ($270 \pm 25\%$ when tested at $100 \mu\text{g/mL}$) (Tsoukalas, 2015); even if bioassay-guided fractionation has not been carried out, it is conceivable that the bioactivity could be due to pregnane glycosides, common in the Asclepiadoideae sub-family. On the other hand, we reported that some pregnane derivatives from *Cynanchum menarandrense* have no effect on STC-1 cells (Tsoukalas et al., 2017).

Therefore, it was interesting to go deeper in order to figure out some structure-activity relationships (SAR) involved in this specific bioactivity. In this purpose, we have decided to evaluate the GLP-1 secretagogue effect of sixteen other pregnanes **1-16**, that we have previously isolated from various *Cynanchum* species (Fig. 1). These compounds share a similar $\Delta^{5,6}$ pregnene core corresponding to deacetylmetylxigenin, but they differ from the esterification at C-12 position and from the glycosidic chain at C-3 (nature, number, and sequence of sugar units): cynotophylloside E (**1**), otophyllside A (**2**), qingyangshengenin (**3**), qingyangshengenin 3-*O*- β -D-cymaropyranosyl-(1 \rightarrow 4)- β -D-digitoxopyranoside (**4**), caudatin 3-*O*- β -D-thevetopyranosyl-(1 \rightarrow 4)- β -D-cymaropyranosyl-(1 \rightarrow 4)- β -D-digitoxopyranoside (**5**), caudatin 3-*O*- β -D-glucopyranosyl-(1 \rightarrow 4)- β -D-oleandropyranosyl-(1 \rightarrow 4)- β -D-cymaropyranosyl-(1 \rightarrow 4)- β -D-cymaropyranoside (**6**), wilfoside M1N (**7**), cynotophylloside C (**8**), otophyllside T (**9**), caudatin (**10**), qingyangshengenin 3-*O*- β -D-oleandropyranosyl-(1 \rightarrow 4)- β -D-cymaropyranosyl-(1 \rightarrow 4)- β -D-digitoxopyranoside (**11**), cynotophylloside D (**12**), wilfoside C1N (**13**), wilfoside K1N (**14**), cynauricoside A (**15**), and wilfoside C3N (**16**). This study allows us to outline

preliminary hypotheses about structural elements involved in the GLP-1 secretagogue activity of polyhydroxylated pregnane derivatives from Asclepiadoideae species.

2. Results and discussion

The GLP-1 secretion assays on STC-1 cells revealed that the pregnane derivatives **2**, **16**, and **13** are significantly active (Table 1), doubling the secretion of GLP-1 compared to control cells (208, 204, and 194% of secretion, respectively). Compound **6** also stimulate GLP-1 secretion, but to a lesser extent (133%). Furthermore, none of these compounds was found to disrupt STC-1 cells membrane integrity (data not shown). Up to date, there is no consensual positive control for GLP-1 secretion in STC-1 cell trials, so we have decided to include in our bioassay the pregnane P57 from *H. gordonii*, for which we had already reported secretagogue properties (Tsoukalas et al., 2016); its activity is confirmed in the present study with a GLP-1 release of $146 \pm 3\%$ compared to control cells.

Although different mechanisms involved in the release of glucagon-like peptide-1 have been already proposed, little is known about the specific mechanisms induced by pregnane glycosides. Although it is difficult to establish accurate structure-activity relationships (SAR) in the absence of a clearly identified biological target, we have attempted to establish some SAR based on these results. This was made possible by the closely-related structures of compounds **1-16**, varying only slightly regarding substitutions at C-3 and C-12 positions. Careful study of this set of results have highlighted the following hypotheses: esterification at C-12 position seems to be a key feature for GLP-1 secretagogue activity. More than the nature of the substituent, it is its size that seem to be important; indeed, bioactivity can be observed with compounds esterified either by *p*-hydroxybenzoyl (**2**) or ikemaoyl (**6**, **13**, **16**) groups. On the other hand, there is no stimulation of GLP-1 secretion in the absence of esterification (**1**, **7**) or with a bigger substituent such as a cinnamoyl moiety (compounds **14** and **15**). The sole presence of a suitable esterification at C-12 is however not sufficient to observe bioactivity, it has to be linked with the presence of an appropriate glycosidic linkage at position 3. For example, cells treated with the aglycones **3** and **10**, although carrying a *p*-hydroxybenzoyl and an ikemaoyl group at C-12 respectively, secrete the same GLP-1 content than control cells (104% and 103% respectively). Results seem to indicate that, as for the substituent at position 12, the glycosidic chain should not be too short, and three or four saccharide moieties appears to be preferable. As there is no pregnane in this study with five or more sugars, it is impossible to conclude concerning long chains. Regarding more in details, all pregnane glycosides in this study contain a single linear glycosidic chain linked at C-3 position, consisting of one to four sugar units. As is often the case in the Asclepiadoideae sub-family (Vleggaar et al., 1993), most of these sugars are 2,6-dideoxy- or 6-deoxyhexoses, more rarely glucose (compound **6**). The sugar units are all in 1→4 linkage, and although the majority of them are in the β-D configuration, α-L-diginose can be encountered when linked at the second position, as well as α-L-cymarose when it is at the extremity of the glycosidic chain. The results tend to show that the presence of a β-D-cymaropyranose directly linked to the genin conditions also the activity. For example, compounds **2** and **11** display almost

the same structure: they are both esterified at position 12 by a *p*-hydroxybenzoyl group and possess a tetrasaccharidic linear chain at C-3; they just differ by the nature of the first sugar: β -D-cymaropyranose for compound **2** and β -D-digitopyranose for compound **11**. While **2** is one of the most active pregnane derivatives in this study, compound **11** has no effect on GLP-1 secretion. In the same way, pregnane glycoside **16** (with a β -D-cymarose as first sugar moiety) is bioactive while **5** (having a β -D-digitose at this position) is not. Finally, the rather weak activity of compound **6** could be due to the nature of the sugar at the extremity of the moiety, i.e. a hexose and not a deoxy- or dideoxyhexose, but this hypothesis would require, to be reinforced, the evaluation of other similar compounds.

To summarize, all bioactive compounds in this study (**2**, **6**, **13**, and **16**) share similar features:

- i. they are esterified at C-12 by an organic monocarboxylic acid of moderate size;
- ii. this esterification is completed by the presence of a glycosidic linear fragment at C-3, consisting of 3-4 sugar units, preferably 2,6-dideoxysaccharides, with β -D-cymaropyranose directly attached to the pregnane skeleton.

These observations are in accordance with previously published data, reporting the GLP-1 secretagogue activity of P57 and of marnieranosides A and B (Tsoukalas et al., 2016). Interestingly, marnieranosides and P57 display structural elements similar to those aforementioned (Supplem. S1): P57 also exhibits a $\Delta^{5,6}$ pregnene core with a high degree of hydroxylation (hoodigogenin), esterified at C-12 by a tigloyl moiety, while marnieranosides are $\Delta^{6,7}$ pregnenes esterified by a benzoyl group at C-12 (and C-20). These three compounds also possess a linear saccharide chain at C-3, consisting of several 2,6-dideoxy- or 6-deoxyhexoses, with β -D-cymaropyranose directly linked to the steroid core. On the contrary, menarandosides A-E and carumbellosides I and II had no effect on GLP-1 secretion (Tsoukalas et al., 2017); these $\Delta^{5,6}$ pregnenes, isolated from *Cynanchum menarandrense*, exhibit a rather rudimentary structure (Supplem. S1), with an unesterified genin and a sugar linkage at C-3 made up of one to two glucose moieties exclusively. All these data reinforce the above-mentioned SAR hypotheses.

The high degree of oxidation and esterification of the genin, as well as the peculiar nature of encountered sugars, makes pregnanes from Asclepiadoideae remarkable natural products. The present study highlights the importance of these specific features for bioactivity: both the nature of the sugar chain at C-3 and the ester at C-12 are key elements to increase the release of GLP-1, a peptide involved in blood glucose regulation. This work provides a starting point for further in-depth medicinal chemistry studies to identify a lead compound. Then, mechanistic studies and *in vivo* confirmation of the hypoglycemic potential of such molecule could be undertaken.

3. Experimental

3.1. Compounds isolation

~~The isolation and identification of each~~ Each pregnane evaluated in this study was provided by the Department of Natural Product Chemistry of Shanghai Institute of Materia Medica. Their isolation and identification have been previously reported as following (> 91% of relative purity at 220 nm);

cynotophyllosides C (**8**), D (**12**), E (**1**), and caudatin 3-*O*- β -D-thevetopyranosyl-(1 \rightarrow 4)- β -D-cymaropyranosyl-(1 \rightarrow 4)- β -D-digitoxopyranoside (**5**) have been obtained from *Cynanchum otophyllum* C.K. Schneid. (Li et al., 2015), as well as otophyllside A (**2**) (Mu et al., 1986), otophyllside T (**9**) (Shen et al., 2014), qingyangshengenin (**3**) and caudatin (**10**) (Ma et al., 2007). Caudatin 3-*O*- β -D-glucopyranosyl-(1 \rightarrow 4)- β -D-oleandropyranosyl-(1 \rightarrow 4)- β -D-cymaropyranosyl-(1 \rightarrow 4)- β -D-cymaropyranoside (**6**) has been purified from *Cynanchum caudatum* roots (Warashina and Noro, 1995). Roots of *Cynanchum wilfordii* (Maxim.) Hemsl. have afforded qingyangshengenin 3-*O*- β -D-cymaropyranosyl-(1 \rightarrow 4)- β -D-digitoxopyranoside (**4**) (Li et al., 2016) and qingyangshengenin 3-*O*- β -D-oleandropyranosyl-(1 \rightarrow 4)- β -D-cymaropyranosyl-(1 \rightarrow 4)- β -D-digitoxopyranoside (**11**) (Xiang et al., 2009). Wilfosides M1N (**7**), C1N (**13**), K1N (**14**) (Tsukamoto et al., 1985a), and C3N (**16**) (Tsukamoto et al., 1985b) have been also obtained from *C. wilfordii* roots. Cynauricoside A (**15**) (Liu et al., 2013) have been isolated from *Cynanchum auriculatum* Royle ex Wight roots.

3.2. Materials, chemicals and reagents

STC-1 cells (ATCC® CRL-3254™) were purchased from LGC standards. The cell-culture material was purchased as following: GLP-1 active ELISA kit (Merck Millipore), Dulbecco's modified Eagle's medium (DMEM, Gibco), Hanks' Balanced Salt Solution (HBSS, Thermo Fisher Scientific), fetal bovine serum (FBS, Gibco Thermo Fisher Scientific), penicillin, and streptomycin (Lonza). The pregnane P57 was purchased from LGC Standards. The reading of the 96-well plates was performed with a SAFAS Monaco plate reader.

3.3. Bioassays

GLP-1 secretion assays and cytotoxicity assays were carried out as described previously (Huguet et al., 2019). Briefly, compounds **1-16** and P57 were dissolved in HBSS containing 1% DMSO for a final concentration of 100 μ M, and 125 μ L of these solutions were added to the cells in 96-well plates for 2 hours. HBSS containing 1% DMSO was used as control, and P57 as positive control. Afterwards, the GLP-1 content of 100 μ L of supernatant was quantified with an ELISA kit. The assay was performed in two distinct experiments for a total of six replicates (n=6), except for P57 tested twice in both experiments (n=4). Results were expressed as the percentage of secreted GLP-1 compared to the basal secretion of cells treated with the vehicle only (considered as 100% of secretion). For the statistical analysis, a one-way ANOVA with post hoc Dunnett test was performed (***) $p_{adj} \leq 0.001$.

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Conflicts of Interest

The authors declare no conflict of interest.

References

- Drucker, D.J., 2018. Mechanisms of Action and Therapeutic Application of Glucagon-like Peptide-1. *Cell Metab.* 27, 740–756. <https://doi.org/10.1016/j.cmet.2018.03.001>
- Huguet, C., Olatunji, O.J., Real, E., Raharivelomanana, P., Urbain, A., 2019. Glucagon-like peptide-1 secretagogues from the leaves of *Oparanthus teikiteetini*. *Phytochem. Lett.* 30, 201–204. <https://doi.org/10.1016/j.phytol.2019.02.020>
- International Diabetes Federation, 2019. IDF Diabetes Atlas 9th Edition. Brussels, Belgium.
- Li, J.-L., Gao, Z.-B., Zhao, W.-M., 2016. Identification and Evaluation of Antiepileptic Activity of C21 Steroidal Glycosides from the Roots of *Cynanchum wilfordii*. *J. Nat. Prod.* 79, 89–97. <https://doi.org/10.1021/acs.jnatprod.5b00766>
- Li, J.-L., Zhou, J., Chen, Z.-H., Guo, S.-Y., Li, C.-Q., Zhao, W.-M., 2015. Bioactive C-21 Steroidal Glycosides from the Roots of *Cynanchum otophyllum* That Suppress the Seizure-like Locomotor Activity of Zebrafish Caused by Pentylentetrazole. *J. Nat. Prod.* 78, 1548–1555. <https://doi.org/10.1021/np501058b>
- Liu, S., Chen, Z., Wu, J., Wang, L., Wang, H., Zhao, W., 2013. Appetite suppressing pregnane glycosides from the roots of *Cynanchum auriculatum*. *Phytochemistry* 93, 144–153. <https://doi.org/10.1016/j.phytochem.2013.03.010>
- Ma, X.-X., Jiang, F.-T., Yang, Q.-X., Liu, X.-H., Zhang, Y.-J., Yang, C.-R., 2007. New pregnane glycosides from the roots of *Cynanchum otophyllum*. *Steroids* 72, 778–786. <https://doi.org/10.1016/j.steroids.2007.06.001>
- Mu, Q.Z., Lu, J.R., Zhou, Q.L., 1986. Two new antiepilepsy compounds - otophyllsides A and B. *Sci. Sin., Ser. B, Chem. Biol. Agric. Med. Earth Sci.* 29, 295–301.
- Shen, D.-Y., Wei, J.-C., Wan, J.-B., Huang, X.-J., Xiang, C., Li, B.-C., Zhang, Q.-W., Wang, Y.-T., Li, P., 2014. Four new C21 steroidal glycosides from *Cynanchum otophyllum* Schneid. *Phytochem. Lett.* 9, 86–91. <https://doi.org/10.1016/j.phytol.2014.04.017>
- Tsoukalas, M., 2015. Recherche de secrétagogues naturels du GLP-1 : exploration du potentiel antidiabétique d'espèces du genre *Cynanchum* (Apocynaceae) (thèse de Doctorat). Université de Strasbourg, Strasbourg.
- Tsoukalas, M., Muller, C.D., Lobstein, A., Urbain, A., 2016. Pregnane Glycosides from *Cynanchum marnierianum* Stimulate GLP-1 Secretion in STC-1 Cells. *Planta Med.* 82, 992–999. <https://doi.org/10.1055/s-0042-107675>
- Tsoukalas, M., Psichas, A., Reimann, F., Gribble, F.M., Lobstein, A., Urbain, A., 2017. Pregnane glycosides from *Cynanchum menarandrense*. *Steroids* 125, 27–32. <https://doi.org/10.1016/j.steroids.2017.06.005>

- Tsukamoto, S., Hayashi, K., Mitsuhashi, Hiroshi., 1985a. Studies on the constituents of Asclepiadaceae plants. LX. Further studies on glycosides with a novel sugar chain containing a pair of optically isomeric sugars, D- and L-cymarose, from *Cynanchum wilfordii*. Chem. Pharm. Bull. 33, 2294–2304.
- Tsukamoto, S., Hayashi, K., Mitsuhashi, Hiroshi., 1985b. Studies on the constituents of Asclepiadaceae plant - LVII. The structures of six glycosides, wilfoside C1N, C2N, C3N, C1G, C2G and C3G, with novel sugar chain containing a pair of optically isomeric sugars. Tetrahedron 41, 927–34. [https://doi.org/10.1016/S0040-4020\(01\)96411-7](https://doi.org/10.1016/S0040-4020(01)96411-7)
- Vilsboll, T., Krarup, T., Deacon, C.F., Madsbad, S., Holst, J.J., 2001. Reduced postprandial concentrations of intact biologically active glucagon-like peptide 1 in type 2 diabetic patients. Diabetes 50, 609–613. <https://doi.org/10.2337/diabetes.50.3.609>
- Vleggaar, R., Heerden, F.R. van, Anderson, L.A.P., Erasmus, G.L., 1993. Toxic constituents of the Asclepiadaceae. Structure elucidation of sarcovimisine A-C, pregnane glycosides of *Sarcostemma viminale*. J. Chem. Soc., Perkin Trans. 1 483–487. <https://doi.org/10.1039/P19930000483>
- Warashina, T., Noro, T., 1995. Steroidal glycosides from *Cynanchum caudatum*. Phytochemistry 39, 199–204. [https://doi.org/10.1016/0031-9422\(94\)00867-S](https://doi.org/10.1016/0031-9422(94)00867-S)
- Xiang, W.-J., Ma, L., Hu, L.-H., 2009. C21 Steroidal Glycosides from *Cynanchum wilfordii*. Helv. Chim. Acta 92, 2659–2674. <https://doi.org/10.1002/hlca.200900057>

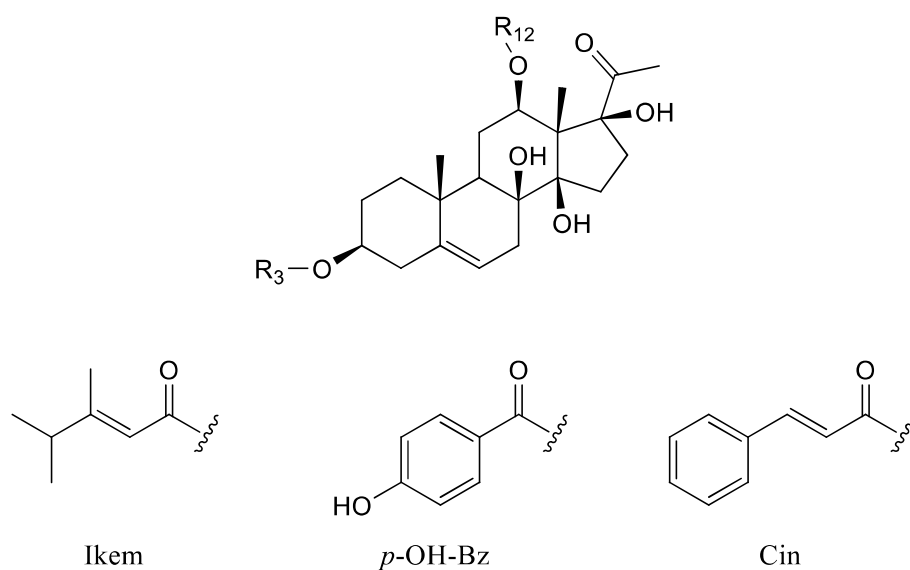
Table 1. Glucagon-like peptide-1 secretagogue activity induced by compounds **1-16** and by P57, used as positive control (tested at 100 μ M on STC-1 cells).

Compound	GLP-1 secretion stimulation (%)
control	100 \pm 19
P57	146 \pm 3
1	118 \pm 16
2^{***}	208 \pm 17
3	104 \pm 20
4	95 \pm 11
5	92 \pm 8
6	133 \pm 13
7	98 \pm 18
8	87 \pm 5
9	92 \pm 17
10	104 \pm 19
11	118 \pm 13
12	85 \pm 14
13^{***}	194 \pm 30
14	95 \pm 12
15	114 \pm 24
16^a	204 \pm 18

Data are expressed as the mean \pm SD of six experiments, except for P57 (n = 4).

^{***} $p_{\text{adj}} < 0.001$.

Figure 1. Structure of compounds **1-16**.



compound	R_3^a				R_{12}^b	bioactivity ^c
	ose 4	ose 3	ose 2	ose 1		
1		β D-Ole	β D-Cym	β D-Cym	H	-
7	α L-Cym	β D-Cym	α L-Dig	β D-Cym	H	-
10					Ikem	-
5		β D-The	β D-Cym	β D-Dit	Ikem	-
16		β D-Cym	α L-Dig	β D-Cym	Ikem	+
13	α L-Cym	β D-Cym	α L-Dig	β D-Cym	Ikem	+
6	β D-Glc	β D-Ole	β D-Cym	β D-Cym	Ikem	+
3					<i>p</i> -OH-Bz	-
8				β D-Cym	<i>p</i> -OH-Bz	-
12			β D-Cym	β D-Cym	<i>p</i> -OH-Bz	-
4			β D-Cym	β D-Dig	<i>p</i> -OH-Bz	-
9		β D-The	β D-Cym	β D-Dig	<i>p</i> -OH-Bz	-
11		β D-Ole	β D-Cym	β D-Dig	<i>p</i> -OH-Bz	-
2		β D-Ole	β D-Cym	β D-Cym	<i>p</i> -OH-Bz	+
15		β D-Cym	α L-Dig	β D-Cym	Cin	-
14	α L-Cym	β D-Cym	α L-Dig	β D-Cym	Cin	-

^a Cym: cymarose; Glc: glucose; Ole: oleandrose; Dig: digitose; Dit: digitoxose; The: thevetose. All sugars are (1→4)-linked.

^b Ikem: ikemaoyl; *p*-OH-Bz: *p*-hydroxybenzoyl; Cin: cinnamoyl.

^c Stimulation of GLP-1 secretion in STC-1 cells when tested at 100 μ M.