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Article

New Pregnane Glycosides from *Gymnema sylvestre*

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Abstract: Four new pregnane glycosides **1**–**4** were isolated from the ethanol extract of the stem of *Gymnema sylvestre* and named gymsylvestrosides A–D. Hydrolysis of compound **1** under the catalysis of *Aspergilus niger* β-glucosidase afforded compound **5** (gymsylvestroside E). Their structures were determined by spectroscopic methods such as HRESIMS, 1D and 2D NMR, as well as HMQC-TOCSY experiment. Compounds **1**–**4** were screened for *Saccharomyces cerevisiae* α-glucosidase inhibitory activity.

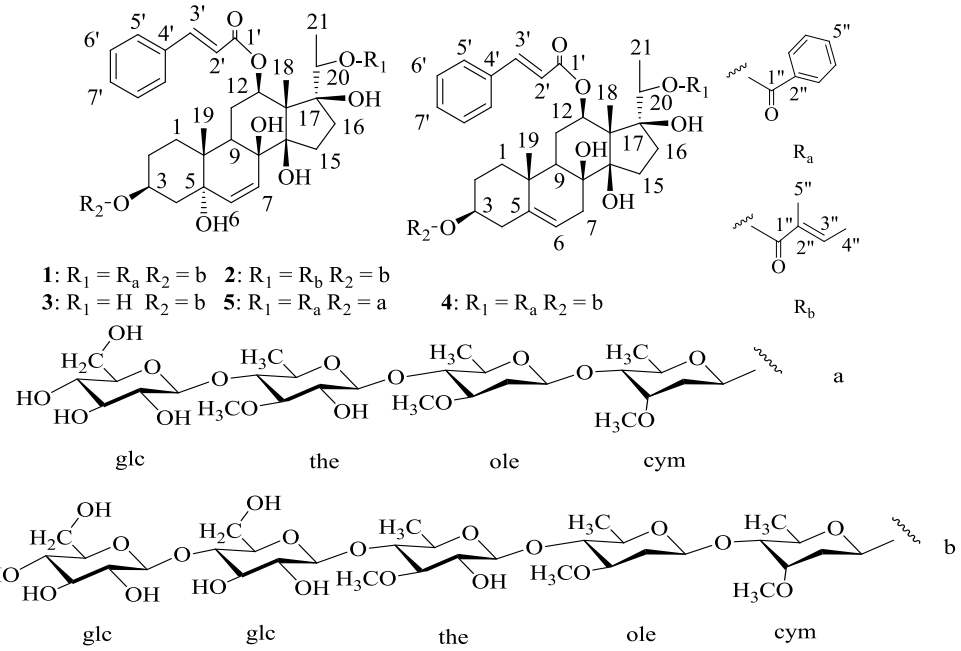
Keywords: *Gymnema sylvestre*; Asclepiadaceae; pregnane glycosides; α-glucosidase

1. Introduction

Gymnema sylvestre (Retz) Schult is a liana plant of the Asclepiadaceae family that grows in tropical and subtropical regions of the World. In China, it is distributed mainly in the provinces of Guangdong, Guangxi and Yunnan [1] and is traditionally used by local people as an anti-inflammatory and analgesic herbal medicine [2]. It is also a folklore medicine in India used for the treatment of malaria, hyperglycemia, mosquito and snake bites [3]. Pharmacological studies showed that *G. sylvestre* has hypoglycemic [4,5], anti-caries [6,7] and weight reducing [8–10] effects. Triterpenoids [11–18], cyclitols [19], flavonoids [20], peptides [21], pectin [22] and alkaloids [23,24] have been isolated from this plant. Some triterpene saponins from *G. sylvestre* were found to be able to attenuate hyperglycemia

induced by pituitary growth hormone or adrenocorticotropic hormone [25,26] and to inhibit intestinal glucose absorption in diabetic rats [27–30].

In the 1990s, Yoshikawa *et al*. reported the isolation of a series of pregnane glycosides from the species *G. alternifolium* [31,32]. In our study on the hypoglycemic constituents of *G. sylvestre*, four new pregnane glycosides **1**–**4** were isolated from the 50% ethanol extract of the dry stem of *G. sylvestre* and named as gymsylvestrosides A–D. The NMR spectra of these compounds showed the presence of a complex sugar moiety comprising deoxysugars and glucose in their structures. Serious overlapping of the NMR signals made it difficult to give a clear assignment of the glycosyl structure. Several enzymes were screened for the ability to hydrolize the glycosyl moiety. The β-glucosidase from *Aspergilus niger* was found to be able to remove a terminal glucose from the sugar moiety of compound **1**. Enzymatic hydrolysis of compound **1** catalyzed by *Aspergilus niger* β-glucosidase afforded compound **5** (gymsylvestroside E), which gave NMR spectra clear enough for the elucidation of the glycosyl structure. The present paper describes the isolation and structural elucidation of gymsylvestrosides A–E (**1**–**5**, Figure 1).



cym: *β*-D-cymaropyranosyl, ole: *β*-D-oleandropyranosyl, the: *β*-D-thevetopyranosyl, glc: *β*-D-glucopyranosyl.

Figure 1. Structures of compounds **1**–**5**.

2. Results and Discussion

2.1. Isolation and Structure Elucidation

The 50% ethanol extract of the dried stems of *G. sylvestre* was partitioned between water and *n*-butanol. The butanol portion was separated successively by column chromatography over D-101 macroporous resin, silica gel, MCI resin and preparative HPLC equipped with an ODS column. Four pregnane glycosides **1**–**4** were thus obtained. Hydrolysis of compound **1** catalyzed by β-glucosidase afforded compound **5**. The molecular mass of **5** was 162 units less than that of **1**, indicating the loss of

a hexose group from **1**. Positive results in the Libermann-Buchard and Keller-Kiliani reactions pointed out that compounds **1**–**5** were steroidal saponins and contained α-deoxy sugars.

Compound **1** was isolated as a colorless powder. The positive HRESIMS gave a pseudo-molecular ion peak at *m/z* 1,422.6627 $[M+NH_4]^+$ (calcd. 1,422.6694), corresponding to a molecular formula of $C_{70}H_{100}O_{29}$. The ^{13}C-NMR and DEPT spectra displayed signals of 70 carbons, comprising six methyls, eight methylenes, a methine, three methoxyl groups, two hydroxylmethenes, twenty-six hydroxylmethines (including five anomeric sugar carbons), four oxygenated quaternary carbons and two ester carbonyls.

The carbon signals of two angular methyl groups (δ_C 12.4 and 21.5), a methyl group (δ_C 15.6) attached to a tertiary carbon (δ_C 75.3), four oxygenated quaternary carbons (δ_C 74.7, 74.0, 87.7 and 88.2) and a double bond (δ_C 136.7 and 127.3) exhibited the features of a polyhydroxypregnane fragment, which was analogous to the pregnane skeleton of the known compound prosapogenin reported in the literature [32,33]. The anomeric carbon signals at δ_C 97.7, 101.8, 103.9, 104.5 and 104.9 and the ester carbonyl signals at δ_C 165.6 and 166.8 indicated that compound **1** was a pregnane glycoside carrying two acyl groups.

The ^{1}H and ^{13}C-NMR (Tables 1 and 2) signals of the pregnane skeleton were assigned by analysis of the ^{1}H,^{1}H-COSY, TOCSY, HMQC and HMBC spectra. The ^{1}H,^{1}H-COSY and TOCSY spectra of **1** displayed a spin system from H-1 to H-4, H-9 to H-12, as well as correlations between H-6/H-7, H-15/H-16 and H-20/H-21, which could designated to the four ring skeleton of a pregnane derivative. The HMBC spectrum showed correlations from H-19 to C-1, C-5, C-9 and C-10, H-18 to C-12, C-13, C-14 and C-17, indicating the two angular methyl groups were connected to C-10 and C-13, respectively. The HMBC correlation from H-21 to C-17 and C-20 suggested the attachment of a side chain on C-17. The presence of a double bond between C-6 and C-7 was evident from the HMBC correlations from H-6 (δ_H 5.94, d, J = 10.4 Hz) to C-10 (δ_C 39.6) and C-8 (δ_C 74.0) and H-7 (δ_H 6.26, d, J = 10.4 Hz) to C-9 (δ_C 36. 6) and C-5 (δ_C 74.7). These data showed a structural feature similar to that of the known compound gymnepregoside E [33]. Further comparison of the NMR data of the two compounds revealed that the NMR signals of the aglycone part of **1** were nearly identical to those of gymnepregoside E. Full assignment of the ^{1}H and ^{13}C-NMR signals of the pregnane skeleton was realized by analysis of the ^{1}H,^{1}H-COSY, TOCSY, HMQC and HMBC spectra and comparing with those of gymnepregoside E. The aglycone structure of compound **1** was thus determined as pregn-6-ene-3,5,8,12,14,17,20-heptol.

The relative configuration of the pregnane skeleton was determined by a ROESY experiment carried out in DMSO-d_6. In the ROESY spectrum, the correlated signals between H-1a (δ_H 1.59)/H-3 (δ_H 3.06) and H-1a/C_5-OH (δ_H 3.59) indicated that the A ring has a chair-like configuration and the substitution on C-3 is β-oriented. The NOE correlation between H-1a/H-9 (δ_H 1.85) suggested an A/B trans junction for the A ring. The H-18 signal at δ_H 1.48 showed correlations with C_8-OH (δ_H 4.09), C_{14}-OH (δ_H 5.23) and C_{17}-OH (δ_H 5.30), indicating that these hydroxyl groups are β-oriented. The configuration of H-12 was confirmed to be α-oriented by the NOE between H-12 (δ_H 4.73) and H-9. Judging from the NOEs between H-12/H-20 (δ_H 4.61), H-20/H-16a (δ_H 1.96) and H-21(δ_H 1.21)/H-16b (δ_H 1.79), the C-20 was considered to have a *S* configuration.

Table 1. ^{1}H-NMR (400 MHz) of the aglycones of compounds **1**–**5** (in pyridine-d_5, J in Hz).

NO.	δ_H (J in Hz)					
	1 [(a)]	**1** [(a),(b)]	**2**	**3**	**4**	**5** [(a)]
1a	1.68 (m)	1.59 (m)	1.71 (m)	1.73 (m)	2.44 (m)	1.69 (m)
1b	2.18 (m)	1.21 (m)	2.21 (m)	2.22 (m)	2.57 (m)	2.17 (m)
2a	2.01 (m)	1.63 (m)	2.01 (m)	2.03 (m)	1.79 (m)	2.02 (m)
2b	2.16 (m)	1.26 (m)	2.16 (m)	2.16 (m)	2.08 (m)	2.14 (m)
3	4.18 (m)	3.06 (m)	4.18 (m)	4.20 (m)	3.85 (m)	4.20 (m)
4a	2.07 (m)	1.79 (m)	2.08 (m)	2.08 (m)	2.41 (m)	2.07 (m)
4b	2.25 (m)	1.64 (m)	2.27 (m)	2.27 (m)	2.58 (m)	2.27 (m)
5-OH		3.59 (s)				
6	5.94 (d, 10.4 Hz)	5.40 (d, 10.4 Hz)	5.93 (d, 10.4 Hz)	5.92 (d, 10.4 Hz)	5.36 (m)	5.95 (d, 10.4 Hz)
7a	6.26 (d, 10.4 Hz)	5.63 (d,10.4 Hz)	6.26 (d, 10.4 Hz)	6.26 (d, 10.4 Hz)	2.41 (m)	6.25 (d, 10.4 Hz)
7b					2.53 (m)	
8-OH		4.09 (s)				
9	2.41 (m)	1.85 (m)	2.41 (m)	2.41 (m)	1.78 (m)	2.41 (m)
11a	2.20 (m)	1.72 (m)	2.23 (m)	2.24 (m)	2.05 (m)	2.22 (m)
11b	2.43 (m)	1.54 (m)	2.49 (m)	2.43 (m)	2.37 (m)	2.45 (m)
12	5.38 (m)	4.73 (m)	5.35 (m)	5.37 (m)	5.25 (m)	5.38 (m)
14-OH		5.23 (s)				
15a	2.00 (m)	1.70 (m)	1.48 (m)	1.48 (m)	1.80 (m)	2.00 (m)
15b	2.19 (m)	1.66 (m)	2.17 (m)	2.16 (m)	2.15 (m)	2.21 (m)
16a	2.01 (m)	1.96 (m)	1.48 (m)	1.48 (m)	1.81 (m)	2.02 (m)
16b	2.10 (m)	1.79 (m)	2.03 (m)	1.98 (m)	2.14 (m)	2.11 (m)
17-OH		5.30 (s)				
18	2.20 (s)	1.48 (s)	2.18 (s)	2.22 (s)	2.18 (s)	2.20 (s)
19	1.52 (s)	0.85 (s)	1.57 (s)	1.59 (s)	1.32 (s)	1.54 (s)
20	5.28 (br q, 6.0 Hz)	4.61 (br q, 6.0 Hz)	5.11 (br q, 6.1 Hz)	4.09 (br q, 6.4 Hz)	5.28 (m)	5.28 (br q, 6.2Hz)
21	1.54 (d, 6.0 Hz)	1.21 (d, 6.0 Hz)	1.48 (d, 6.1 Hz)	1.36 (d, 6.4 Hz)	1.57 (d, 5.8 Hz)	1.56 (d, 6.2 Hz)
Cinnamoyl moiety						
2'	6.51 (d, 16 Hz)	5.96 (d, 16 Hz)	6.74 (d, 16 Hz)	6.97 (d, 16 Hz)	6.50 (d, 16 Hz)	6.51 (d, 16 Hz)
3'	7.80 (d, 16 Hz)	7.30 (d, 16 Hz)	7.93 (d, 16 Hz)	8.14 (d, 16 Hz)	7.87 (d, 16 Hz)	7.80 (d, 16 Hz)
5', 9'	7.35 (m)	7.27 (m)	7.66 (m)	7.52 (m)	7.35 (m)	7.37 (m)
6', 8'	7.33 (m)	7.10 (m)	7.41 (m)	7.32 (m)	7.33 (m)	7.31 (m)
7'	7.35 (m)	7.27 (m)	7.40 (m)	7.33 (m)	7.35 (m)	7.37 (m)
(*E*)-2-Methyl-2-butenoyl or benzoyl moiety						
3''	8.21 (d, 7.6 Hz)	7.90 (d, 7.2 Hz)	7.00 (d, 7.3 Hz)		8.23 (d, 7.2 Hz)	8.21 (d, 7.2 Hz)
4''	7.30 (m)	7.35 (m)	1.51 (d, 7.3 Hz)		7.39 (m)	7.32 (m)
5''	7.50 (m)	7.60 (m)	1.78 (s)		7.56 (m)	7.53 (m)
6''	7.30 (m)	7.35 (m)			7.39 (m)	7.32 (m)
7''	8.21 (d, 7.6 Hz)	7.90 (d, 7.2 Hz)			8.23 (d, 7.2 Hz)	8.21 (d, 7.2 Hz)

[(a)] Measured at 500 MHz; [(b)] DMSO-d_6 as solvent.

Table 2. ^{13}C-NMR (100 MHz) of the aglycones of compounds **1**–**5** (in pyridine-d_5).

NO.	δ_C					
	1 [(a)]	**1** [(a),(b)]	**2**	**3**	**4**	**5** [(a)]
1	27.6 (t)	26.6 (t)	27.5 (t)	27.6 (t)	38.7 (t)	27.6 (t)
2	26.5 (t)	25.2 (t)	26.5 (t)	26.5 (t)	29.8 (t)	26.5 (t)
3	74.9 (d)	73.3 (d)	74.9 (d)	74.9 (d)	77.5 (d)	74.9 (d)
4	39.0 (t)	37.4 (t)	39.0 (t)	39.0 (t)	39.1 (t)	39.1 (t)
5	74.7 (s)	72.6 (s)	74.7 (s)	74.8 (s)	139.1 (s)	74.7 (s)
6	136.7 (d)	135.4 (d)	136.6 (d)	136.1 (d)	119.3 (d)	136.7 (d)
7	127.3 (d)	124.0 (d)	127.3 (d)	127.6 (d)	34.8 (t)	127.3 (d)
8	74.0 (s)	72.6 (s)	74.0 (s)	73.8 (s)	74.2 (s)	74.0 (s)
9	36.6 (d)	34.9 (d)	36.5 (d)	36.6 (d)	44.0 (d)	36.6 (d)
10	39.6 (s)	38.3 (s)	39.6 (s)	39.6 (s)	37.2 (s)	39.6 (s)
11	23.6 (t)	22.3 (t)	23.7 (t)	23.6 (t)	25.6 (t)	23.6 (t)
12	75.8 (d)	74.2 (d)	75.6 (d)	75.9 (d)	74.6 (d)	75.8 (d)
13	58.1 (s)	56.8 (s)	57.9 (s)	58.1 (s)	57.0 (s)	58.1 (s)
14	88.2 (s)	87.1 (s)	88.1 (s)	88.9 (s)	88.9 (s)	88.2 (s)
15	33.1 (t)	32.1 (t)	33.1 (t)	33.3 (t)	33.7 (t)	33.1 (t)
16	34.3 (t)	33.5 (t)	34.2 (t)	33.5 (t)	34.0 (t)	34.3 (t)
17	87.7 (s)	86.5 (s)	87.7 (s)	87.9 (s)	87.5 (s)	87.7 (s)
18	12.4 (q)	11.6 (q)	12.3 (q)	12.7 (q)	11.5 (q)	12.5 (q)
19	21.5 (q)	20.9 (q)	21.5 (q)	21.6 (q)	17.9 (q)	21.6 (q)
20	75.3 (d)	73.9 (d)	74.4 (d)	70.4 (d)	75.8 (d)	75.3 (d)
21	15.6 (q)	15.0 (q)	15.5 (q)	19.6 (q)	15.3 (q)	15.6 (q)
Cinnamoyl moiety						
1'	166.8 (s)	165.5 (s)	166.7 (s)	167.0 (s)	166.8 (s)	166.8 (s)
2'	120.1 (d)	118.9 (d)	120.3 (d)	119.6 (d)	120.3 (d)	120.2 (d)
3'	143.9 (d)	143.2 (d)	143.7 (d)	145.2 (d)	143.8 (d)	143.9 (d)
4'	134.8 (s)	133.9 (s)	134.8 (s)	134.9 (s)	134.9 (s)	134.8 (s)
5', 9'	128.5 (d)	128.0 (d)	128.5 (d)	128.6 (d)	128.5 (d)	128.5 (d)
6', 8'	129.1 (d)	128.8 (d)	129.2 (d)	129.2 (d)	129.1 (d)	129.1 (d)
7'	130.4 (d)	130.1 (d)	130.5 (d)	130.5 (d)	130.4 (d)	130.4 (d)
(*E*)-2-Methyl-2-butenoyl or benzoyl moiety						
1''	165.6 (s)	164.6 (s)	166.7 (s)		165.6 (s)	165.6 (s)
2''	131.2 (d)	130.3 (d)	129.4 (s)		131.2 (d)	131.2 (d)
3''	130.2 (d)	129.4 (d)	137.7 (d)		130.2 (d)	130.2 (d)
4''	128.7 (d)	128.5 (d)	14.1 (q)		128.7 (d)	128.7 (d)
5''	133.2 (d)	133.1 (d)	12.2 (q)		133.2 (d)	133.2 (d)
6''	128.7 (d)	128.5 (d)			128.7 (d)	128.7 (d)
7''	130.2 (d)	129.4 (d)			130.2 (d)	130.2 (d)

[(a)] Measured at 125 MHz; [(b)] DMSO-d_6 as solvent.

The ^{13}C-NMR spectrum gave signals of an (*E*)-cinnamoyl at δ_C 166.8 (C-1'), 120.1 (C-2'), 143.9 (C-3'), 134.8 (C-4') 128.5 (C-5',9'), 129.1 (C-6',8') and 130.4 (C-7'), and a benzoyl group at δ_C 165.6 (C-1''). 131.2 (C-2''), 130.2 (C-3'',7''), 128.7 (C-4'',6'') and 133.2 (C-5''). The locations of the cinnamoyl on C-12 and the benzoyl on C-20 were confirmed by the HMBC experiment which was demonstrated

by correlations from δ_H 5.38 (H-12) to C-1' (δ_C 166.8) and δ_H 5.28 (H-20) to C-1" (δ_C 165.6). Based on above evidence, the aglycone part of compound **1** was determined to be 12-*O*-(*E*)-cinnamoyl-20-*O*-benzoyl-(20*S*)-pregn-6-ene-3β,5α,8β,12β,14β,17β,20-heptol.

The ^{1}H-NMR spectrum (Table 3) of **1** displayed five anomeric proton signals at δ_H 5.17 (brd, J = 10.9 Hz, 1H), 4.68 (brd, J = 9.6 Hz, 1H), 4.88 (d, J = 7.8 Hz, 1H), 5.09 (d, J = 7.8 Hz, 1H) and 5.20 (d, J = 7.8 Hz, 1H), indicating the existence of five β-configurated glycosyl linkages. The methyl signals at δ_H 1.39 (d, J = 6.0 Hz, 3H), 1.64 (d, J = 5.2 Hz, 3H) and 1.75 (d, J = 5.6 Hz, 3H) and the COSY data suggested the presence of three 6-deoxysugars. Since some of the sugar signals were seriously overlapped, compound **1** was subjected to enzymatic hydrolysis catalyzed by β-glucosidase, which afforded compound **5**.

Compound **5** was a colorless powder. Its HRESIMS spectrum showed a pseudo-molecular ion peak at *m/z* 1,260.6208 $[M+NH_4]^+$ (calcd. 1,260.6166), corresponding to a molecular weight of 1242, 162 mass number less than that of compound **1**. The ^{1}H- and ^{13}C-NMR data of **5** were nearly identical to that of **1** except the loss of the anomeric proton at δ_H 5.20 (1H, d, J = 7.8 Hz) and a set of oxymethine signals, which were assignable to a terminal glucose group.

Starting from the anomeric protons at δ_H 5.17, 4.68, 4.88 and 5.14, the signals of four sugar fragments (S_1–S_4) in the glycosyl moiety of **5** were assigned by analysis of the HMQC-TOCSY spectrum (Figure 2) combined with the HMQC and COSY data.

The glycosyl moiety was found being composed of a β-linked terminal glucose and three β-linked deoxy-sugars (see Tables 3 and 4). In the HMBC spectrum, three methoxy groups (δ_H 3.52, S_1-OCH_3; 3.51, S_2-OCH_3 and 3.94, S_3-OCH_3) were correlated with the carbon signals at δ_C 77.7 (S_1-C-3), 79.1 (S_2-C-3) and 86.2 (S_3-C-3), indicating that each of the deoxy-sugars bore a methoxy group on C-3. Identification of the deoxy-sugars was reached by inspection of the NOE spectra (Figure 3). Nuclear Overhauser Effects were observed between the anomeric proton at δ_H 5.17 (S_1-H-1) and the signals at δ_H 4.16 (S_1-H-5), 2.23 (S_1-H-2) and 3.52 (S_1-OCH_3), suggesting that S_1 is a β-cymaropyranose. Similarly S_2 and S_3 were identified as β-oleandropyranose and β-thevetopyranose. As regards the configuration of the deoxy-sugars, previous studies revealed that all the β-linked 2-deoxysugars have the D-configuration, whereas the α-linked sugars are mostly L-sugars [34]. Further, chemical shift values for C-2 of the 2-deoxysugars (cymarose, oleandrose and digitoxose) can be used as argument to determine its configuration [35]. The chemical shift of C-2 in the L-sugars is less than 35.0 ppm, but that of C-2 in the D-sugars appears above 36.0 ppm [36–39]. In the case of compound **5**, the two 2-deoxysugars were β-linked and their C-2 signals occurred at δ_C 36.7 and 37.5, respectively. The cymarose and oleandrose moieties had thus D-configuration. Configuration of the β-thevetopyranosyl unit was presumed to be D-form because its NMR data were close to those in the literature [40–42] and L-thevetopyranose [43] is rarely found in Nature.

The sequential lingkage of the four sugars shown in Figure 3 was deduced from the NOE correlations between S_2-H-1/S_1-H-4, S_3-H-1/S_2-H-4 and S_4-H-1/S_3-H-4 and the HMBC (Figure 4) cross peaks between S_2-H-1/S_1-C-4 (δ_C 83.0), S_3-H-1/S_2-C-4 (δ_C 83.1) and S_4-H-1/S_3-C-4 (δ_C 83.2). This was confirmed by comparison of the NMR data of the sugar moiety with that of verticilloside A [44], which carried the same glycosyl moiety. Based on above evidences, the structure of compound **5** is elucidated as 12-*O*-(*E*)-cinnamoyl-20-*O*-benzoyl-(20*S*)-pregn-6-ene-3β,5α,8β,12β,14β,17β,20-heptol,

3-*O*-β-D-glucopyranosyl-(1→4)-β-D-thevetopyranosyl-(1→4)-β-D-oleandropyranosyl-(1→4)-β-D-cymaropyranoside, named gymsylvestroside E.

Table 3. ^{1}H-NMR (400 MHz) of the glycosyl part of compounds **1**–**5** (in pyridine-d_5, *J* in Hz).

Sugar	NO.	δ_H (*J* in Hz)				
		1 [(a)]	**2**	**3**	**4**	**5** [(a)]
Cym	**1**	5.17 (brd, 10.9 Hz)	5.17 (brd, 10.9 Hz)	5.17 (brd, 10.9 Hz)	5.29 (brd, 10.9 Hz)	5.17 (brd, 10.5 Hz)
	2a	1.73 (m)	1.73 (m)	1.73 (m)	1.89 (m)	1.73 (m)
	2b	2.20 (m)	2.20 (m)	2.23 (m)	2.31 (m)	2.23 (m)
	3	3.98 (m)	3.98 (m)	3.98 (m)	3.98 (m)	3.98 (m)
	4	3.43 (m)	3.43 (m)	3.43 (m)	3.43 (m)	3.42 (m)
	5	4.16 (m)	4.16 (m)	4.16 (m)	4.16 (m)	4.16 (m)
	6	1.39 (d, 6.0 Hz)	1.39 (d, 6.0 Hz)	1.39 (d, 6.0 Hz)	1.46 (d, 6.0 Hz)	1.39 (d, 6.0 Hz)
	OMe	3.52 (s)	3.52 (s)	3.52 (s)	3.59 (s)	3.52 (s)
Ole	**1**	4.68 (brd, 9.6 Hz)	4.68 (brd, 9.6 Hz)	4.68 (brd, 9.6 Hz)	4.70 (brd, 9.6 Hz)	4.68 (brd, 9.6 Hz)
	2a	2.47 (m)	2.47 (m)	2.46 (m)	2.49 (m)	2.48 (m)
	2b	1.73 (m)	1.73 (m)	1.73 (m)	1.76 (m)	1.73 (m)
	3	3.57 (m)	3.57 (m)	3.57 (m)	3.57 (m)	3.57 (m)
	4	3.59 (m)	3.59 (m)	3.59 (m)	3.59 (m)	3.52 (m)
	5	3.55 (m)	3.58 (m)	3.55 (m)	3.51 (m)	3.51 (m)
	6	1.64 (d, 5.2 Hz)	1.64 (d, 5.2 Hz)	1.64 (d, 5.2 Hz)	1.68 (d, 5.2 Hz)	1.64 (d, 5.2 Hz)
	OMe	3.50 (s)	3.51 (s)	3.51 (s)	3.51 (s)	3.51 (s)
The	**1**	4.88 (d, 7.8 Hz)	4.88 (d, 7.6 Hz)	4.88 (d, 7.6 Hz)	4.88 (d, 7.6 Hz)	4.88 (d, 7.6 Hz)
	2	3.90 (m)	3.90 (m)	3.90 (m)	3.90 (m)	3.90 (m)
	3	3.67 (m)	3.68 (m)	3.67 (m)	3.67 (m)	3.69 (m)
	4	3.83 (m)	3.83 (m)	3.83 (m)	3.83 (m)	3.88 (m)
	5	3.75 (m)	3.77 (m)	3.75 (m)	3.75 (m)	3.75 (m)
	6	1.75 (d, 5.6 Hz)	1.76 (d, 5.6 Hz)	1.75 (d, 5.6 Hz)	1.75 (d, 5.6 Hz)	1.75 (d, 5.6 Hz)
	OMe	3.91 (s)	3.82 (s)	3.82 (s)	3.91 (s)	3.94 (s)
Glc	**1**	5.09 (d, 7.8 Hz)	5.09 (d, 7.8 Hz)	5.09 (d, 7.8 Hz)	5.09 (d, 7.8 Hz)	5.14 (d, 7.8 Hz)
	2	4.02 (m)	4.02 (m)	4.02 (m)	4.02 (m)	4.04 (m)
	3	4.28 (m)	4.28 (m)	4.28 (m)	4.28 (m)	4.25 (m)
	4	4.31 (m)	4.31 (m)	4.30 (m)	4.31 (m)	4.23 (m)
	5	3.93 (m)	3.93 (m)	3.93 (m)	3.93 (m)	3.98 (m)
	6a	4.30 (m)	4.30 (m)	4.30 (m)	4.30 (m)	4.32 (m)
	6b	4.50 (m)	4.50 (m)	4.50 (m)	4.50 (m)	4.54 (m)
Glc	**1**	5.20 (d, 7.8 Hz)	5.20 (d, 8.5 Hz)	5.20 (d, 8.5 Hz)	5.20 (d, 8.5 Hz)	
	2	4.10 (m)	4.10 (m)	4.10 (m)	4.10 (m)	
	3	4.23 (m)	4.23 (m)	4.23 (m)	4.23 (m)	
	4	4.19 (m)	4.16 (m)	4.19 (m)	4.19 (m)	
	5	4.04 (m)	4.04 (m)	4.04 (m)	4.04 (m)	
	6a	4.30 (m)	4.32 (m)	4.30 (m)	4.31 (m)	
	6b	4.53 (m)	4.53 (m)	4.53 (m)	4.53 (m)	

[(a)] Measured at 500 MHz; Cym: β-D-cymaropyranosyl, Ole: β-D-oleandropyranosyl, The: β-D-thevetopyranosyl, Glc: β-D-glucopyranosyl.

Table 4. ^{13}C (100 MHz) NMR of the glycosyl part of compounds **1**–**5** (in pyridine-d_5).

Sugar	NO.	δ_C				
		1 [(a)]	**2**	**3**	**4**	**5** [(a)]
Cym	**1**	97.7 (d)	97.6 (d)	97.7 (d)	96.3 (d)	97.7 (d)
	2	36.7 (t)	36.7 (t)	36.7 (t)	37.2 (t)	36.7 (t)
	3	77.7 (d)	77.6 (d)	77.7 (d)	77.8 (d)	77.7 (d)
	4	83.0 (d)	83.0 (d)	83.0 (d)	83.2 (d)	83.0 (d)
	5	68.9 (d)	68.9 (d)	68.9 (d)	68.8 (d)	68.9 (d)
	6	18.5 (q)	18.4 (q)	18.5 (q)	18.4 (q)	18.5 (q)
	OMe	58.7 (q)	58.6 (q)	58.7 (q)	58.8 (q)	58.7 (q)
Ole	**1**	101.8 (d)	101.8 (d)	101.8 (d)	101.8 (d)	101.8 (d)
	2	37.5 (t)	37.5 (t)	37.5 (t)	37.6 (t)	37.5 (t)
	3	79.1 (d)	79.1 (d)	79.2 (d)	79.2 (d)	79.1 (d)
	4	83.2 (d)	83.2 (d)	83.2 (d)	83.4 (d)	83.1 (d)
	5	71.9 (d)	71.8 (d)	71.8 (d)	71.8 (d)	71.9 (d)
	6	18.6 (q)	18.6 (q)	18.6 (q)	18.6 (q)	18.6 (q)
	OMe	57.3 (q)	57.3 (q)	57.3 (q)	57.3 (q)	57.4 (q)
The	**1**	103.9 (d)	104.0 (d)	103.9 (d)	103.9 (d)	104.0 (d)
	2	74.9 (d)	74.9 (d)	74.8 (d)	74.9 (d)	74.8 (d)
	3	86.3 (d)	86.3 (d)	86.3 (d)	86.3 (d)	86.2 (d)
	4	83.3 (d)	83.3 (d)	83.4 (d)	83.4 (d)	83.2 (d)
	5	71.9 (d)	71.8 (d)	71.9 (d)	71.9 (d)	71.9 (d)
	6	18.7 (q)	18.7 (q)	18.7 (q)	18.7 (q)	18.7 (q)
	OMe	60.6 (q)	60.6 (q)	60.6 (q)	60.6 (q)	60.6 (q)
Glc	**1**	104.5 (d)	104.5 (d)	104.6 (d)	104.6 (d)	104.8 (d)
	2	75.3 (d)	75.3 (d)	75.3 (d)	75.3 (d)	75.8 (d)
	3	76.8 (d)	76.8 (d)	76.8 (d)	76.8 (d)	78.6 (d)
	4	81.5 (d)	81.5 (d)	81.5 (d)	81.5 (d)	71.9 (d)
	5	76.2 (d)	76.2 (d)	76.2 (d)	76.2 (d)	78.1 (d)
	6	62.3 (t)	62.2 (t)	62.3 (t)	62.3 (t)	63.0 (t)
Glc	**1**	104.9 (d)	104.9 (d)	104.9 (d)	104.9 (d)	
	2	74.7 (d)	74.7 (d)	74.9 (d)	74.7 (d)	
	3	78.2 (d)	78.2 (d)	78.2 (d)	78.2 (d)	
	4	71.5 (d)	71.5 (d)	71.5 (d)	71.4 (d)	
	5	78.4 (d)	78.4 (d)	78.4 (d)	78.4 (d)	
	6	62.4 (t)	62.3 (t)	62.4 (t)	62.4 (t)	

[(a)] Measured at 125 MHz; Cym: β-D-cymaropyranosyl, Ole: β-D-oleandropyranosyl, The: β-D-thevetopyranosyl, Glc: β-D-glucopyranosyl.

As mentioned above, compound **5** was derived from **1** by removing a terminal glucose. In the HMBC spectrum of **1**, the anomeric proton (δ_H 5.20, d, J = 7.8 Hz, 1H) of the terminal glucose was correlated with S_4-C-4 (δ_C 81.5). The signals of S_4-C-3, S_4-C-4 and S_4-C-5 exhibited glycosylation shifts of Δ −1.8 ppm, +9.6 ppm and −1.9 ppm, respectively. The terminal glucose was therefore determined to locate on C-4 of another glucose moiety. These evidences allowed to elucidate the structure of compound **1** as 12-*O*-(*E*)-cinnamoyl-20-*O*-benzoyl-(20*S*)-pregn-6-ene-3β,5α,8β,12β,14β,17β,20-heptol,

3-*O*-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-β-D-thevetopyranosyl-(1→4)-β-D-oleandropyranosyl-(1→4)-β-D-cymaropyranoside, named gymsylvestroside A.

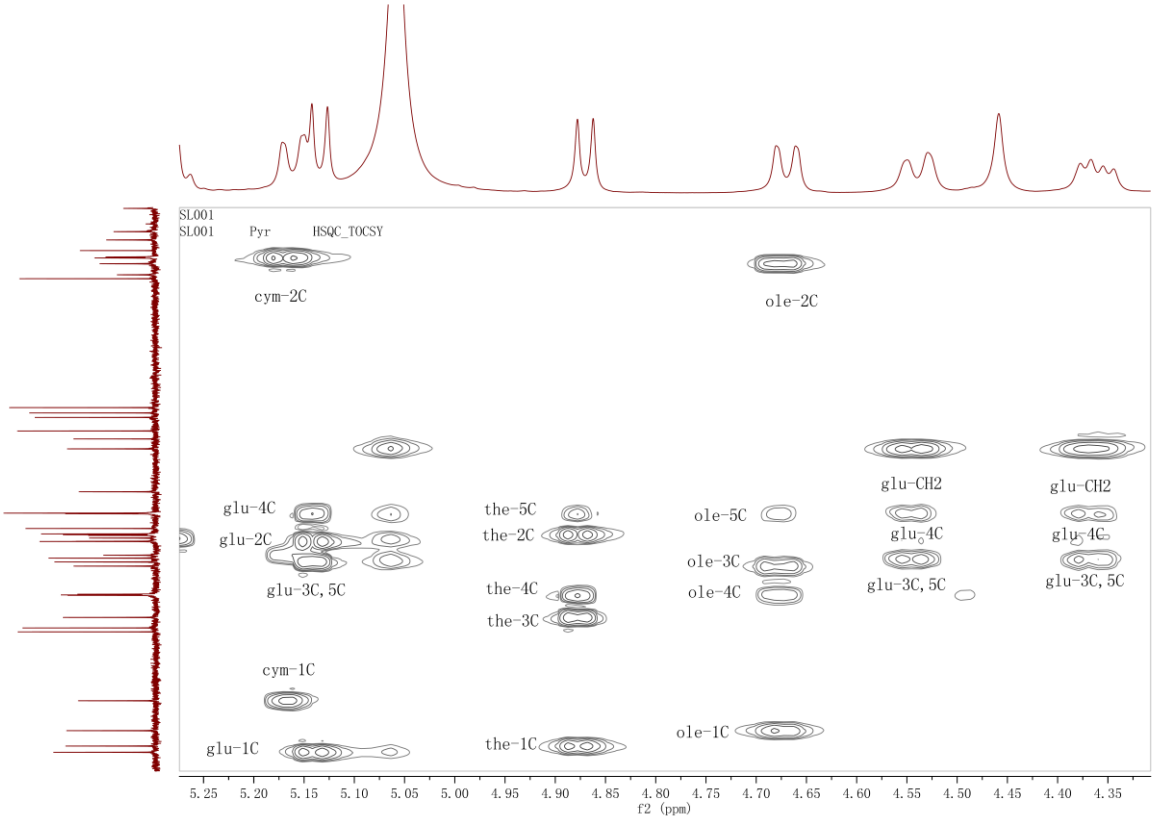


Figure 2. The HMQC-TOCSY spectrum of the glycosyl part of **5**.

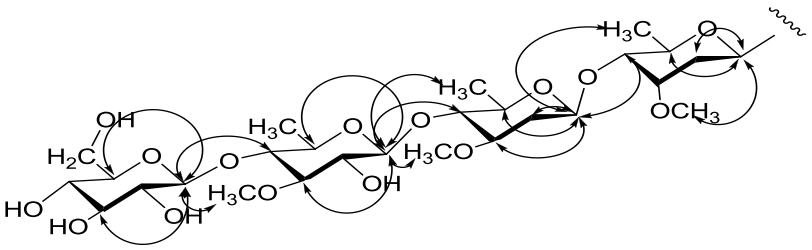


Figure 3. Key NOE of the sugars of **5**.

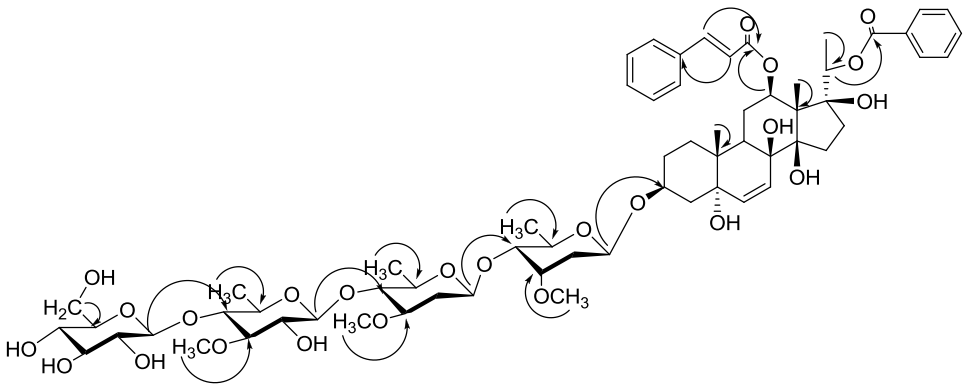


Figure 4. Key HMBC correlations of **5**.

Compound **2** was isolated as a colorless powder. Its molecular formula was determined to be $C_{68}H_{102}O_{29}$ by HRESIMS *m/z* 1,383.6593 $[M+H]^+$ (calcd. 1,383.6585). Comparing to the ^{13}C-NMR data of **1**, the benzoyl signals at δ_C 133.2, 128.7 (overlapped), 130.2 (overlapped), 131.2 and 165.6 disappeared in the spectrum of **2**, while signals for another acyl group were observed at δ_C 12.2, 14.1, 129.4, 137.7 and 166.7. This group was identified as 2-methyl-2-butenoyl by inspection of the ^{1}H,^{1}H-COSY, HMQC and HMBC spectra and comparison with reported data [31–33]. The HMBC coupling between H-20 (δ_H 5.11) and the carbonyl carbon (δ_C 166.7) of the 2-methyl-2-butenoyl group confirmed the connection of this group to C-20. The ^{1}H-NMR showed five anomeric proton signals at δ_H 5.17 (brd, J = 10.9 Hz), 4.68 (brd, J = 9.6 Hz), 4.88 (d, J = 7.8 Hz), 5.09 (d, J = 7.8 Hz) and 5.20 (d, J = 7.8 Hz). The nearly identical NMR data of the sugars and the HMBC correlations between δ_H 5.17/δ_C 74.9 (C-3), δ_H 4.68/δ_C 83.0 (cym-C-4), δ_H 4.88/δ_C 83.2 (ole-C-4), δ_H 5.09/δ_C 83.3 (the-C-4) and δ_H 5.20/δ_C 81.5 (glu-C-4) indicated that compound **2** carried the same sugar moiety as that of **1**. The structure of **2** was thus established as 12-*O*-(*E*)-cinnamoyl-(20*S*)-*O*-(*E*)-2-methyl-2-butenoyl-(20*S*)-pregn-6-ene-3β,5α,8β,12β,14β,17β,20-heptol, 3-*O*-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-β-D-thevetopyranosyl-(1→4)-β-D-oleandropyranosyl-(1→4)-β-D-cymaropyranoside, named gymsylvestroside B.

Compound **3**, a colorless powder, was designated a molecular formula of $C_{63}H_{96}O_{28}$, based on the pseudo-molecular ion peak at *m/z* 1,323.5978 $[M+Na]^+$ (calcd. 1,323.5986) in its HRESIMS spectrum. The NMR data of **3** were almost identical to those of **1** (Tables 1–4), except for the loss of the signals of the benzoyl group. Comparing to the data of **1**, the C-21 signal (δ_C 19.6) in the spectrum of **3** moved 3 ppm downfield and the C-20 (δ_C 70.4) shifted 4.9 ppm upfield, showing the presence of a free hydroxyl group on C-20. Further analysis of the 2D NMR data elucidated the structure of compound **3** as 12-*O*-(*E*)-cinnamoyl-(20*S*)-pregn-6-ene-3β,5α,8β,12β,14β,17β,20-heptol, 3-*O*-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-β-D-thevetopyranosyl-(1→4)-β-D-oleandropyranosyl-(1→4)-β-D-cymaropyranoside, named gymsylvestroside C.

Compound **4** was obtained as a colorless powder. Its HRESIMS data (*m/z* 1,411.6318 $[M+Na]^+$, calcd. 1411.6299) suggested a molecular formula of $C_{70}H_{100}O_{28}$, which has one oxygen less than that of compound **1**. The ^{1}H- and ^{13}C-NMR data of **4** were only slightly different from that of **1**. The C-5 signal (δ_C 74.7) of **1** disappeared in the ^{13}C-NMR spectrum of **4**, while an additional methene signal occured at δ_C 34.8 (C-7). The signals of the double bond in the pregnane skeleton shifted to δ_C 119.3 and 139.1. The ^{1}H-NMR of **4** displayed an olefinic proton at δ_H 5.36 (H-6), which correlated in the HMBC spectrum with the methyl carbon at δ_C 37.2 (C-10). The signal of Me-19 (δ_H 1.32) showed HMBC correlation with a disubstituted vinyl carbon at δ_C 139.1. These facts concluded the presence of a double bond between C-5 and C-6. Comparing to the reported data for gymnpregoside F [33], the pregnane skeleton of **4** was determined to be (20*S*)-pregn-5-ene-3β,17β,14β,12β,5α,8β,20-heptol. Detailed investigation of the 1D and 2D NMR spectra of **4** revealed the presence of signals of a cinnamoyl, a benzoyl group and the same sugar moiety as in **1**. The location of the cinnamoyl at C-12, the benzoyl at C-20 and the sugar moiety at C-3 was confirmed by the HMBC correlations from δ_H 5.25 (H-12) to δ_C 166.8, δ_H 5.28 (H-20) to δ_C 165.6 and δ_H 5.29 (S1-H-1) to δ_C 77.5. The structure of **4** could thus be determined as 12-*O*-(*E*)-cinnamoyl-20-*O*-benzoyl-(20*S*)-pregn-5-ene-3β,17β,14β,12β,5α,8β,20-heptol, 3-*O*-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-β-D-thevetopyranosyl-(1→4)-β-D-oleandropyranosyl-(1→4)-β-D-cymaropyranoside, named gymsylvestroside D.

2.2. Biological Activity Assay

The *Saccharomces cerevisiae* α-glucosidase inhibition activities of compounds **1**–**4** were assayed using *p*-nitrophenyl-α-D-glucopyranoside (*p*NPG) as substrate and acarbose (J&K) as positive control. Compounds **1**–**4** did not show significant inhibitory effect (Table 5).

Table 5. The inhibitory activity of the compounds **1**–**4**.

Sample	Concentration	OD	Inhibition Ratio
	(mg/mL)	Average ± RSD	(%)
Negetive control	0	1.8573 ± 0.0129	
Compound **1**	1.02	1.7661 ± 0.0077	4.9
Compound **2**	1.07	1.7860 ± 0.0033	3.8
Compound **3**	1.11	1.7356 ± 0.0025	6.6
Compound **4**	1.16	1.7901 ± 0.0029	3.6
Acarbose	0.50	1.3654 ± 0.0026	26

3. Experimental

3.1. General Procedures

Optical rotations were measured on an Optical Activity Limited polAAr 3005 spectropolarimeter (Optical Activity Limited, Ramsey, UK). IR and UV spectra were taken out on a Nicolet-Is5 infrared spectrometer (Thermo Fisher, Boston, MA, USA) and a Cintra-20 UV-Vis spectrometer (GBC, Melbourne, Australia), respectively. HRESIMS was obtained with Waters G2 Q-TOF (Waters, Milford, MA, USA) or Agilent 6520 Q-TOF (Agilent Technologies, Santa Clara, CA, USA) mass spectrometer. 1D and 2D NMR spectra were recorded with JNM-ECA-400 (JEOL Ltd., Tokyo, Japan) or Bruker AVANCE III 500 (Bruker Biospin, Switzerland) superconducting NMR spectrometer. TMS and pyridine-d_5 were used as internal standards for ^{1}H- and ^{13}C-NMR measurements, respectively. Preparative HPLC was carried out with Waters Autopurification System (Waters). YMC-PACK ODS-A (S-5 um, 20 × 250 mm; YMC Co. Ltd., Kyoto, Japan) column was used in preparative HPLC. Microplate reader-MaxM5 (Molecular Devices, Santa Clara, CA, USA) was used to determine the absorbance of the enzymatic reaction. Silica gel (SiO_2; 200–300 mesh; Qingdao Marine Chemical Inc., Qingdao, China), MCI GEL resin (50 um; Mitsubishi Plastics, Tokyo, Japan) and D-101 macroporous resin (16–60 mesh; Nankai University Chemical Plant, Tianjin, China) were used for column chromatography. Acarbose (lot No. 298087) was purchased from J&K Scientific Co. Ltd. α-Glucosidases (lot No. 1001604919) and *p*-nitrophenyl-α-D-glucopyranodase (*p*NPG) (lot No. 101381642) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). β-Glucosidases (*Aspergillus niger*) was provided by Baiping Ma (Institute of Radiation Medicine, Academy for Military Medical Science). The other chemicals used in this study were of analytical grade.

3.2. Plant Materials

The stems of *G. sylvestre* were obtained from the Exhibition Center of Guangxi University of Chinese Medicine in August, 2010 and identified as *G. sylvestre* by Professor Wenhui Tan at Guangxi

University of Chinese Medicine. A voucher specimen was preserved at the herbarium of Institute of Pharmacology and Toxicology with the reference number Gs201008002.

3.3. Extraction and Isolation

The dried stems (20 kg) of *G. sylvestre* were extracted with 50% ethanol three times (120 L × 1 h each). Concentration of the combined extracts under reduced pressure afforded 2 kg dry mass. The concentrate was suspended in water and extracted with *n*-butanol to give an *n*-butanol soluble extract (750 g) which was adsorbed on a macroporous resin column. The column was washed at first with water, and then eluted with 15%, 30%, 50%, 70% and 95% ethanol successively. The eluate (120 g) of 50% ethanol was fractionated on a silica gel column, eluting sequentially with chloroform/methanol (20:1-1:5, v/v) to give six fractions (A_1-A_6). Fraction A_4 (6 g) was dissolved in water and adsorbed with a MCI resin using aqueous methanol (0-45%) as elute, giving fractions $A_{4\text{-}1}$-$A_{4\text{-}5}$. Separation of $A_{4\text{-}3}$ (2 g) by preparative HPLC with an ODS-A column (20 × 250 mm), eluting with MeOH/H_2O (80:20) gave compound **1** (900 mg) and **2** (100 mg). HPLC separation of $A_{4\text{-}4}$ (500 mg) using MeOH/H_2O (60:40) as eluent afforded compound **3** (60 mg) and **4** (80 mg). Compound **5** (70 mg) was isolated from the hydrolysate after enzymatic hydrolysis of compound **1** (200 mg). Compounds **2** and **5** were further purified by preparative HPLC eluting with MeOH/H_2O (75:25) to give 92 mg and 63 mg purified products for each compound. Compounds **3** and **4** were processed similarly by preparative HPLC using MeOH/H_2O (55:45) as eluent, and each afforded 48 mg and 70 mg purified products, respectively.

3.4. Isolated Compounds

Compound **1**: colorless powder. $[\alpha]_D^{20}$ +80.8 (c 1.09, MeOH). HRESIMS *m/z*: 1422.6627 $[M+NH_4]^+$ (1422.6694 calcd for $C_{70}H_{104}O_{29}N$). UV (MeOH) λ_{max} (log ε) 205.2 (1.84), 223.2 (1.87), 278.6 (1.81) nm. IR (film) cm^{-1}: 3447, 1718, 1642, 1289, 1034. ^{1}H-NMR and ^{13}C-NMR: See Tables 1–4.

Compound **2**: colorless powder. $[\alpha]_D^{20}$ +71.0 (c 1.44, MeOH). HRESIMS *m/z:* 1383.6593 $[M+H]^+$ (1383.6585 calcd for $C_{68}H_{103}O_{29}$). UV (MeOH) λ_{max} (log ε) 217.2 (1.84), 278.6 (1.77) nm. IR (film) cm^{-1}: 3431, 2513, 2161, 1057. ^{1}H-NMR and ^{13}C-NMR: See Tables 1–4.

Compound **3**: colorless powder. $[\alpha]_D^{20}$ +35.8 (c 1.30, MeOH). HRESIMS *m/z*: 1323.5978 $[M+Na]^+$ (1323.5986 calcd for $C_{63}H_{96}O_{28}Na$). UV (MeOH) λ_{max} (log ε) 206.9 (1.81), 216.3 (1.82), 277.8 (1.84) nm. IR (film) cm^{-1}: 3425, 3932, 2164, 1709, 1167, 1066. ^{1}H-NMR and ^{13}C-NMR: See Tables 1–4.

Compound **4**: colorless powder. $[\alpha]_D^{20}$ +90.8 (c 1.05, MeOH). HRESIMS *m/z*: 1411.6318 $[M+Na]^+$ (1411.6299 calcd for $C_{70}H_{100}O_{28}Na$). UV (MeOH) λ_{max} (log ε) 206.1 (1.86), 223.2 (1.90), 279.5 (1.86) nm. IR (film) cm^{-1}: 3446, 2937, 2518, 2161, 1709, 1167, 1105. ^{1}H-NMR and ^{13}C-NMR: See Tables 1–4.

Compound **5**: colorless powder. $[\alpha]_D^{20}$ +76.8 (c 1.09, MeOH). HRESIMS *m/z*: 1260.6208 $[M+NH_4]^+$ (1260.6166 calcd for $C_{64}H_{94}O_{24}N$). UV (MeOH) λ_{max} (log ε) 205.2 (1.84), 223.2 (1.87), 278.6 (1.81) nm. ^{1}H-NMR and ^{13}C-NMR: See Tables 1–4.

3.5. Enzymatic Hydrolysis of Compound **1**

Compound **1** (200 mg) was dissolved in 40mL water and then diluted with 50 mL acetic acid-sodium acetate buffer (pH = 4.97). After addition of 8 mL β-glucosidase (*Aspergilus niger*, 1 mg/mL), the solution was mixed well and incubated at 50 °C in a water bath for 24 h. The hydrolysate was loaded on a reversed-phase silica gel colunm and successively washed with water and methanol. The methanol eluate was purified by preparative HPLC to give compound **5** (70 mg).

3.6. α-Glucosidase Inhibitory Effect

The α-glucosidase (*S. cerevisia*) inhibitory effect of compounds **1**–**4** were determined using the method adopted previously by Hou *et al.* [45] The tested compounds (8 μL) were premixed with α-glucosidase (0.90 unite/mL, 20 μL) on a 96 wells microplate and diluted with 112 μL phosphate buffer (pH = 6.8). The reaction mixtures were incubated at 37 °C for 15 min and then 20 μL *p*NPG (2.45 mmol/mL) was added to start the reaction. After incubation for another 15 min at 37 °C, the reaction was stopped by adding 80 μL Na_2CO_3 (0.199 mmol/mL). The activity was determined by measuring the release of *p*-nitrophenol at 405 nm with a microplate reader. Acarbose was used as the positive control and DMSO (dimethyl sulfoxide) as the negative control. Inhibitory rates were calculated as follows:

$$\text{Inhibition ratio (\%)} = [(OD_{\text{negative control}} - OD_{\text{test}})/OD_{\text{negative control}}] \times 100\%$$

4. Conclusions

In this study, four new polyoxygenated pregnane glycosides carrying a complex pentasaccharide moiety were obtained from the 50% ethanol extract of the stem of *G. sylvestre*. Their structures were elucidated by intensive spectroscopic analysis with the help of enzymatic hydrolysis of the sugar chain. α-Glycosidase inhibitory activity of these compounds has been investigaed, but the observed data were statistically not significant.

Supplementary Materials

Supplementary materials can be accessed at: http://www.mdpi.com/1420-3049/20/02/3050/s1.

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Author Contributions

R.X. designed the study, did the isolation, identification, and drafted the manuscript. Y.Y. contributed in the interpretation of the spectra and also part of the preparation of the manuscript. Y.Z., F.X.R. and J.L.X. made available the laboratory, including equipment and consumables. N.J.Y. supervised the phytochemical work. Y.M.Z. supervised the phytochemical work and prepared the final manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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Sample Availability: Samples of the compounds **1**–**5** are available from the authors.