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Spirostanol saponins from *Ypsilandra parviflora* induce platelet aggregation

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ABSTRACT

Phytochemical investigation on the whole plants of *Ypsilandra parviflora* led to the isolation of seven new spirostanol saponins, named ypsiparosides A–G, together with 14 known saponins. Their structures were unambiguously established based on extensive spectroscopic evidence and chemical methods. The induced rabbit platelet aggregation activities of the isolates were tested. Compounds **4**, **15**, and **17** showed maximal platelet aggregation rates ranging from 43 to 55% at a concentration of 300 μg/mL. Further experiments exhibited that compounds **4**, **15**, and **17** possessed EC_{50} values of 642.9, 95.3, and 300.8 μg/mL, respectively.

1. Introduction

Plants of genus *Ypsilandra* (Liliaceae) containing five species are mainly distributed in Myanmar and southwest China, of which four are present in China [1]. They have been used in traditional Chinese medicine (TCM) for the treatments of scrofula, urination, edema, uterine bleeding, and traumatic hemorrhage [2]. Previous studies on this genus have led to the isolation of a series of steroidal saponins responsible for diverse bioactivities, such as cytotoxic, antifungal, hemostatic, and anti-HIV effects [3–8].

Y. parviflora, an erect herb, widely grows in mountain slopes and streams at the altitude between 1000 and 1400 m in Guizhou, Hunan, Guangxi, and Guangdong provinces of China [1]. However, the phytochemicals and the biological activities of *Y. parviflora* have not been reported so far. Our bioassay showed that the 70% EtOH extract of *Y. parviflora* showed 67.5% maximal rabbit platelet aggregation rate at a concentration of 1.5 mg/mL. In order to clarify it's bioactive constituents, seven new spirostanol saponins, ypsiparosides A–G (**1**–**7**) (Fig. 1), and 14 known analogues (**8**–**21**) were isolated from the 70% EtOH extract of the whole plants of title species. The known saponins were identified as ypsilandroside C (**8**) [4], ypsilandroside D (**9**) [4], pennogenin-3-*O*-*α*-L-rhamnopyranosyl-(1→4)-*α*-L-rhamnopyranosyl-(1→4)-*β*-D-glucopyranoside (**10**) [9], paris saponin VII (**11**) [10], polyphylloside III [11] [10], taccasuboside B (**13**) [12], isoypsilandroside B (**14**) [3], paris saponin II (**15**) [13], ypsilandroside G (**16**) [4], ypsilandroside M (**17**) [14], ypsilandroside K (**18**) [5], parispseudoside A (**19**) [15], proto-Pb (**20**) [16], and pseudoproto Pb (**21**) [17] by comparison of their spectroscopic data with those reported in the literature. The current paper reports the isolation, structural elucidation, and the induced platelet aggregation activities of these isolates.

2. Experimental

2.1. General experimental procedures

Optical rotations were measured on a JASCO P-1020 digital polarimeter. UV spectra were measured using a Shimadzu UV-2401 PC spectrophotometer. IR spectra were obtained on Bruker Tensor-27 infrared spectrophotometer with KBr pellets. ESI-MS spectra were recorded on a Bruker HTC/Esquire spectrometer, HRESIMS spectra were recorded on an API Qstar Pulsar instrument. NMR experiments were performed on Bruker AM-400, DRX-500, and Avance III 600 instruments with TMS as the an internal standard. Chemical shifts (δ) are expressed in ppm with reference to the solvent signals. Column

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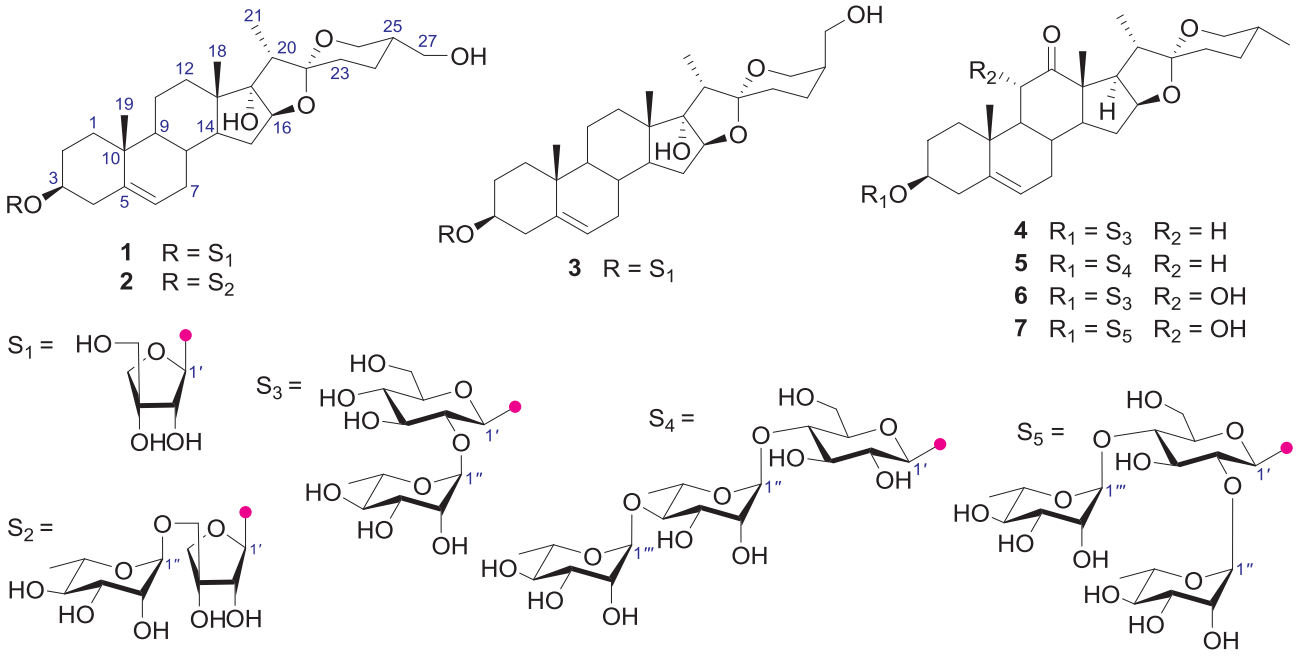

Fig. 1. Chemical structures of compounds **1–7**.

chromatography (CC) was performed on silica gel (100–200, 200–300, and 300–400 mesh, Qingdao Marine Chemical Co, China), RP-18 (40–63 μm, Merck), and Sephadex LH-20 (GE Healthcare, Sweden). Semi-preparative HPLC was run on Agilent 1100 liquid chromatograph with diode array detector (DAD), Zorbax-SB-C18 column (5 μm; 25 cm × 9.4 mm i.d.). TLC was performed on $HSGF_{254}$ (0.2 mm, Qingdao Marine Chemical Co, China) or RP-18 F_{254} (0.25 mm, Merck). Fractions were monitored by TLC and spots were visualized by heating silica gel plates sprayed with 10% H_2SO_4 in EtOH.

2.2. Plant material

The whole plants of *Y. parviflora* were collected in August 2012, from Leishan County, Guizhou Province, China, and identified by Dr. Rong Li of Kunming Institute of Botany, CAS. A voucher specimen (No. HY0020) was deposited at State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, CAS.

2.3. Extraction and isolation

Dried and powdered plant materials of *Y. parviflora* (4.5 kg) were extracted three times with 70% EtOH (20 L × 3) under reflux for a total of 6 h (3 × 2 h) and the combined extract was concentrated under reduced pressure. The crude extract was partitioned between *n*-BuOH and water to afford *n*-BuOH soluble portion (666 g). Then, the concentrated *n*-BuOH-soluble portion was subjected to CC (silica gel, 200-300 mesh; gradient $CHCl_3$-MeOH 20:1→0:1, v/v) to afford six fractions: A–F (60 g, 40.5 g, 40 g, 82 g, 210 g, and 105 g, respectively). Fr. B was passed through an MCI gel column and eluted with EtOH-H_2O gradient (40:60→90:10, v/v) followed by silica gel CC ($CHCl_3$-MeOH, 25:1→1:1, v/v) to give **13** (8 mg). Fr. D was purified by RP-18 gel (MeOH-H_2O, 40:60→90:10, v/v), repeated silica gel CC ($CHCl_3$-MeOH, 15:1→1:1, v/v), and finally purified by semi-preparative HPLC (MeCN-H_2O, 40:60 v/v; flow rate: 3.0 mL/min) to give **1** (11 mg), **2** (20 mg), **3** (12 mg), **4** (20 mg), **6** (15 mg), **7** (10 mg), and **10** (10 mg). Fr. E was fractionated by a silica gel column ($CHCl_3$–MeOH, 10:1→1:1, v/v) to give two fractions: Fr. 5-1 (80 g) and Fr. 5-2 (70 g). Fr. 5-1 was separated by an RP-18 column (MeOH–H_2O, 45:55→100:0, v/v) and further purified by semi-preparative HPLC (MeCN-H_2O, 40:60 v/v; flow rate: 3.0 mL/min) to afford **5** (8 mg), **6** (30 mg), and **9** (181 mg). Fr. 5-2 was subjected to repeated silica gel CC ($CHCl_3$-MeOH, 10:1→1:1, v/v) and separated by semi-preparative HPLC (MeCN-H_2O, 40:60 v/v; flow rate: 3.0 mL/min) and afforded **11** (15 mg), **15** (20 mg), **16** (23 mg), and **17** (13 mg). Fr. F was subjected to MCI gel column and eluted with aqueous EtOH (70% v/v), repeated silica gel CC ($CHCl_3$-MeOH, 8:1→1:1, v/v), and finally purified by semi-preparative HPLC (MeCN-H_2O, 30:70 v/v; flow rate: 3.0 mL/min) to yield **8** (33 mg), **12** (18 mg), **14** (20 mg), **18** (29 mg), **19** (50 mg), **20** (40 mg), and **21** (92 mg).

2.3.1. Ypsiparoside A (1)

White amorphous powder; $[\alpha]_D^{21}$ −163.7 (*c* 0.6, MeOH); IR (KBr) ν_{max} 3424, 2932, 1631, 1453, 1089, 1042, 910 cm^{-1}; ^1H NMR data see Table 1; ^{13}C NMR data see Table 2; positive ESIMS: *m/z* 601 $[M+Na]^+$; HRESIMS: *m/z* 601.3352 $[M+Na]^+$ (calcd for $C_{32}H_{50}O_9Na$, 601.3353).

2.3.2. Ypsiparoside B (2)

White amorphous powder; $[\alpha]_D^{21}$ −122.7 (*c* 0.6, MeOH); IR (KBr) ν_{max} 3426, 2932, 1640, 1453, 1383, 1135, 1052, 979 cm^{-1}; ^1H NMR data see Table 1; ^{13}C NMR data see Table 2; positive ESIMS: *m/z* 747 $[M+Na]^+$; HRESIMS: *m/z* 747.3923 $[M+Na]^+$ (calcd for $C_{38}H_{60}O_{13}Na$, 747.3932).

2.3.3. Ypsiparoside C (3)

White amorphous powder; $[\alpha]_D^{21}$ −141.2 (*c* 0.7, MeOH); IR (KBr) ν_{max} 3425, 2934, 2902, 1631, 1455, 1379, 1221, 1062, 919 cm^{-1}; ^1H NMR data see Table 1; ^{13}C NMR data see Table 2; positive ESIMS: *m/z* 601 $[M+Na]^+$; HRESIMS: *m/z* 601.3345 $[M+Na]^+$ (calcd for $C_{32}H_{50}O_9Na$, 601.3353).

2.3.4. Ypsiparoside D (4)

White amorphous powder; $[\alpha]_D^{21}$ −80.4 (*c* 0.7, MeOH); IR (KBr) ν_{max} 3428, 2930, 2909, 1707, 1637, 1454, 1381, 1048, 981, 919, 899, 868 cm^{-1} (intensity: 899 > 919 cm^{-1}); ^1H NMR data see Table 1; ^{13}C NMR data see Table 2; positive ESIMS: *m/z* 759 $[M+Na]^+$; HRESIMS: *m/z* 759.3929 $[M+Na]^+$ (calcd for $C_{39}H_{60}O_{13}Na$, 759.3932).

2.3.5. Ypsiparoside E (5)

White amorphous powder; $[\alpha]_D^{21}$ −62.5 (*c* 0.7, MeOH); IR (KBr) ν_{max} 3426, 2927, 2853, 1707, 1634, 1455, 1384, 1043, 981, 921, 900, 866 cm^{-1} (intensity: 900 > 921 cm^{-1}); ^1H NMR data see Table 1; ^{13}C

Table 1
^{1}H NMR spectroscopic data of saponins **1–7** in C_5D_5N (δ in ppm, J in Hz).[a]

Position	**1**[b]	**2**[c]	**3**[b]	**4**[c]	**5**[c]	**6**[b]	**7**[b]
1a	1.77 m	1.74 m	1.74 m	1.51 m	1.49 m	2.90 m	2.74 m
1b	1.00 m	0.96	0.97 m	0.86 m	0.87 m	1.27 m	1.20 m
2a	2.13 m	2.05 m	2.16 m	2.09 m	2.01 m	2.22 m	2.00 m
2b	1.74 m	1.69 m	1.50 m	1.83	1.62	1.81 m	1.73 m
3	3.75	3.68 m	3.72 m	3.89 m	3.82 m	3.98	3.82 m
4a	2.54 dd (13.1, 4.4)	2.50 dd (13.1, 4.3)	2.50 dd (13.1, 4.3)	2.83 dd (13.2, 2.4)	2.72 dd (13.3, 2.5)	2.88 m	2.74 m
4b	2.39 t (12.1)	2.35 t (12.2)	2.35 t (12.1)	2.71 t (12.0)	2.40 t (12.2)	2.81 t (12.0)	2.45 t (12.1)
6	5.28 br d (5.0)	5.25 br d (5.0)	5.25 br d (5.0)	5.25 br s	5.27 br s	5.34 br s	5.33 br s
7a	2.21 m	2.17 m	2.19 m	2.11 m	2.09 m	1.84 m	1.80 m
7b	1.82 m	1.87	1.88	1.86 m	1.85 m	1.68 m	1.60
8	1.90 m	1.87	1.89 m	1.83	1.83 m	1.67	1.60
9	0.97 m	0.93 m	0.93 m	1.30 m	1.30 m	1.44 m	1.36 m
11a	1.58 m	1.56 m	1.55 m	2.54 t (13.7)	2.53 t (13.7)	4.72 d (10.2)	4.66 d (10.2)
11b	1.50 m	1.46 m	1.45 m	2.30 t (12.2)	2.29 dd (14.5, 5.7)		
12a	1.90 m	1.78	1.99 m				
12b	1.62 m	1.51 m	1.59				
14	2.10 m	2.06 m	2.04 m	1.43 m	1.42 m	1.40 m	1.40 m
15a	1.82 m	1.87 m	1.88	1.62 m	1.62	1.80 m	1.80 m
15b	1.54 m	1.51 m	1.59	1.45	1.44 m	1.48	1.45
16	4.52 t (6.9)	4.48 t (6.8)	4.44 t (6.9)	4.47 q (7.7)	4.48 br t (8.8)	4.49 br t (7.3)	4.41 dd (9.3, 3.7)
17				2.81 dd (8.5, 7.0)	2.80 t (7.7)	2.91 t (7.7)	2.79 t (7.7)
18	0.99 s	0.96 s	0.94 s	1.11 s	1.10 s	1.12 s	1.05 s
19	0.98 s	0.96 s	0.95 s	1.08 s	0.94 s	1.39 s	1.16 s
20	2.33 br q (7.2)	2.29 br q (7.2)	2.21 br q (7.2)	1.90 quint (6.9)	1.90 quint (7.0)	1.91 d (6.9)	1.82 m
21	1.28 d (7.2)	1.25 d (7.2)	1.19 d (7.2)	1.34 d (6.9)	1.34 d (7.0)	1.30 d (7.0)	1.20 d (6.7)
23a	2.21 m	2.17 m	1.92 m	1.62 m	1.85 m	1.67	1.60 m
23b	1.82 m	1.77 m	1.53 m	1.45	1.69 m	1.48	1.45
24a	1.89 m	1.86 m	2.22 m	1.68 m	1.68 m	1.58	1.50 m
24b	1.81 m	1.78	1.86 m	1.56	1.57 m	1.54 m	1.49
25	2.09 m	2.05 m	1.88	1.56	1.55 m	1.57	1.49
26a	4.10 dd (10.8, 3.9)	4.07 dd (9.8, 3.6)	4.12 dd (11.0, 2.6)	3.58 dd (10.9, 3.3)	3.56 (10.8, 3.2)	3.57 dd (10.7, 2.9)	3.51
26b	3.93 t (11.2)	3.89 t (11.2)	3.93 t (11.1)	3.47 t (10.7)	3.47 t (10.7)	3.48 t (10.7)	3.38 t (10.6)
27a	3.75	3.71 dd (10.1, 5.2)	4.14 m	0.68 d (5.6)	0.67 d (5.7)	0.70 d (5.8)	0.62 d (5.5)
27b	3.66 t (8.5)	3.63 dd (10.5, 7.4)	3.96 m				
Api-1′	5.78 d (3.0)	5.71 d (2.7)	5.75 d (3.0)				
2′	4.77 d (2.8)	4.61 d (2.5)	4.73 d (2.0)				
4′a	4.65 d (9.4)	4.41 d (9.3)	4.62 d (9.4)				
4′b	4.40 d (9.4)	4.29 d (9.3)	4.37 d (9.4)				
5′a	4.25 d (11.2)	4.32 d (10.1)	4.22 d (11.1)				
5′b	4.21 d (11.2)	3.94 d (10.1)	4.18 d (11.1)				
Glc-1′				5.03 d (7.8)	4.93 d (7.9)	5.06 d (7.8)	4.90 d (7.8)
2′				4.28 m	3.99 t (8.5)	4.28 m	4.31 m
3′				4.29	4.22 m	4.29	3.60 m
4′				4.18 t (8.9)	4.47 m	4.20 m	4.28 m
5′				3.90 t (9.4)	3.67 br 9.4)	3.98	4.10 m
6′a				4.51 br d (11.5)	4.21 br d (11.1)	4.48 t (9.4)	4.11
6′b				4.35	4.08 br d (11.1)	4.37 m	3.99 d (6.7)
Rha-1″		5.35 br s		6.39 br s	5.89 br s	6.41 br s	5.68 br s
2″		4.50 dd (3.3, 1.3)		4.82 br d (1.8)	4.33 m	4.83 dd (3.1, 1.3)	4.48 m
3″		4.46 dd (8.8, 3.4)		4.63 dd (9.3, 3.2)	4.39 m	4.67 dd (9.3, 3.3)	4.45 dd (9.1, 2.4)
4″		4.26 t (9.3)		4.36 t (9.3)	4.48 t (9.3)	4.38 m	4.41 m
5″		4.28 m		5.00 dd (9.5, 6.3)	5.07 m	5.02 m	4.82 m
6″		1.62 d (5.7)		1.79 d (6.1)	1.67 d (6.2)	1.79 d (6.2)	1.57 d (6.2)
Rha-1‴					6.33 br s		6.11 br s
2‴					4.60 br d (9.0)		4.78 m
3‴					4.55 dd (9.3, 3.2)		4.40 dd (9.4, 3.8)
4‴					4.62		4.21 m
5‴					4.40 m		4.24 m
6‴					1.60 d (6.1)		1.49 d (5.9)

[a] Overlapped signals are reported without designating multiplicity.
[b] Recorded at 600 MHz.
[c] Recorded at 400 MHz.

NMR data see Table 2; positive ESIMS: m/z 905 $[M+Na]^+$; HRESIMS: m/z 905.4497 $[M+Na]^+$ (calcd for $C_{45}H_{70}O_{17}Na$, 905.4511).

2.3.6. Ypsiparoside F (6)

White amorphous powder; $[\alpha]_D^{21}$ −82.3 (c 0.7, MeOH); IR (KBr) ν_{max} 3432, 2931, 2873, 1709, 1631, 1455, 1383, 1051, 981, 920, 899, 866 cm^{-1} (intensity: 899 > 920 cm^{-1}); ^{1}H NMR data see Table 1; ^{13}C NMR data see Table 2; positive ESIMS: m/z 775 $[M+Na]^+$; HRESIMS: m/z 775.3872 $[M+Na]^+$ (calcd for $C_{39}H_{60}O_{14}Na$, 775.3881).

2.3.7. Ypsiparoside G (7)

White amorphous powder; $[\alpha]_D^{21}$ −86.6 (c 1.2, MeOH); IR (KBr) ν_{max} 3441, 2931, 2874, 1710, 1633, 1454, 1381, 1052, 981, 921, 900, 866 cm^{-1} (intensity: 900 > 921 cm^{-1}); ^{1}H NMR data see Table 1; ^{13}C NMR data see Table 2; positive ESIMS: m/z 921 $[M+Na]^+$; HRESIMS: m/z 921.4452 $[M+Na]^+$ (calcd for $C_{45}H_{70}O_{18}Na$, 921.4460).

Table 2
^{13}C NMR spectroscopic data of saponins **1–7** in C_5D_5N.

Position	**1**[a]	**2**[b]	**3**[a]	**4**[b]	**5**[b]	**6**[a]	**7**[a]
1	38.0 t	37.6 t	37.4 t	37.0 t	36.8 t	38.9 t	38.6 t
2	30.0 t	30.0 t	30.2 t	29.9 t	29.7 t	30.1 t	29.7 t
3	77.9 d	77.4 d	77.8 d	77.8 d	77.6 d	77.7 d	77.9 d
4	39.7 t	39.2 t	39.1 t	38.8 t	38.9 t	39.2 t	39.1 t
5	141.3 s	140.8 s	140.0 s	140.6 s	140.5 s	141.3 s	141.0 s
6	122.2 d	121.8 d	121.6 d	121.5 d	121.4 d	121.1 d	121.0 d
7	32.6 t	32.4 t	31.9 t	31.6 t	31.6 t	31.8 t	31.2 t
8	32.8 d	32.4 d	32.1 d	30.9 d	30.7 d	31.5 d	31.1 d
9	50.6 d	50.2 d	50.0 d	52.3 d	52.1 d	60.2 d	59.8 d
10	37.6 s	37.1 s	36.9 s	37.7 s	37.4 s	39.5 s	39.1 s
11	21.4 t	21.0 t	20.8 t	37.5 t	37.4 t	73.7 d	73.4 d
12	32.8 t	31.8 t	32.1 t	212.7 s	212.6 s	213.3 s	213.1 s
13	45.7 s	45.2 s	45.0 s	55.0 s	55.0 s	53.8 s	53.6 s
14	53.5 d	53.0 d	52.9 d	56.0 d	56.0 d	55.8 d	55.5 d
15	32.6 t	32.3 t	32.2 t	31.8 t	31.4 t	31.6 t	31.5 t
16	90.5 d	90.0 d	90.0 d	79.7 d	79.5 d	79.7 d	79.5 d
17	90.6 s	90.2 s	89.9 s	54.0 d	54.0 d	54.0 d	53.6 d
18	17.7 q	17.2 q	17.1 q	15.9 q	15.7 q	15.6 q	15.3 q
19	19.9 q	19.5 q	19.3 q	18.8 q	18.6 q	18.9 q	18.5 q
20	45.4 d	44.9 d	45.1 d	42.6 d	42.4 d	43.3 d	42.0 d
21	10.0 q	9.9 q	9.6 q	13.9 q	13.8 q	13.8 q	13.4 q
22	110.8 s	110.3 s	110.2 s	109.3 s	109.2 s	109.2 s	109.1 s
23	32.3 t	31.9 t	27.3 t	31.8 t	31.6 t	31.6 t	31.2 t
24	24.1 t	23.6 t	21.1 t	29.2 t	29.0 t	29.0 t	28.7 t
25	39.5 d	39.1 d	36.0 d	30.6 d	30.4 d	30.4 d	30.0 d
26	64.4 t	64.0 t	60.4 t	66.9 t	66.8 t	66.8 t	66.6 t
27	64.9 t	64.4 t	61.2 t	17.3 q	17.2 q	17.2 q	16.8 q
Api-1′	108.9 d	108.2 d	108.3 d				
2′	78.5 d	78.0 d	77.8 d				
3′	80.7 d	78.9 d	80.1 s				
4′	75.3 d	74.9 d	74.7 d				
5′	65.8 t	70.6 t	65.1 t				
Glc-1′				100.3 d	102.3 d	100.1 d	101.8 d
2′				77.6 d	75.5 d	77.6 d	79.8 d
3′				79.7 d	76.4 d	79.6 d	76.5 d
4′				71.8 d	77.4 d	71.6 d	77.5 d
5′				78.4 d	77.1 d	77.7 d	76.0 d
6′				62.5 t	61.2 t	62.4 t	60.9 t
Rha-1″		102.2 d		102.1 d	102.2 d	102.0 d	101.7 d
2″		72.1 d		72.6 d	73.8 d	72.5 d	72.4 d
3″		72.8 d		72.9 d	70.3 d	72.8 d	72.7 d
4″		74.0 d		74.2 d	80.1 d	74.1 d	79.5 d
5″		70.0 d		69.5 d	68.1 d	69.4 d	67.9 d
6″		18.7 q		18.3 q	18.8 t	18.6 q	18.4 t
Rha-1‴					103.1 d		102.7 d
2‴					72.9 d		72.0 d
3‴					72.7 d		72.2 d
4‴					73.3 d		73.3 d
5‴					70.3 d		69.9 d
6‴					18.4 q		17.9 q

[a] Recorded at 150 MHz.
[b] Recorded at 100 MHz.

2.4. Determination of absolute configuration of sugars in 1–7 by HPLC

The absolute configuration of the sugar moieties was determined by the method described in the literature [18]. Compounds **1–7** (2.0 mg, each) were refluxed with 6 M CF_3COOH (1,4-dioxane/H_2O 1:1 v/v, 1.0 mL) on a water bath for 2.0 h at 90 °C. After cooling, the reaction mixture was extracted with $CHCl_3$ (3 × 5 mL). Next, the aqueous layer was evaporated to dryness using a rotary evaporator. The dried residue was dissolved in 1 mL pyridine mixed with L-cysteine methyl ester hydrochloride (1.0 mg) (Aldrich, Japan) and heated at 60 °C for 1.0 h. Then, *o*-tolyl isothiocyanate (5 μL) (Tokyo Chemical Industry Co., Ltd., Japan) was added to the mixture, which was heated at 60 °C for 1.0 h. Similarly, the standard monosaccharides, D-Api (1.0 mg), D-Glc (1.0 mg), and L-Rha (1.0 mg) were subjected to L-cysteine methyl ester hydrochloride (5.0 mg) with pyridine (5.0 mL) respectively. After heating at 60 °C for 1.0 h, *o*-tolyl isothiocyanate (20 μL) was added to each sugar derivative and kept at 60 °C for 1.0 h. Analytical HPLC was performed on a ZORBAX SB-C_{18} column (250 × 4.6 mm i.d., 5 μm, Aglient, U.S.A.) at 35 °C with gradient elution of 20→50% CH_3CN for 30 min at a flow rate of 1.0 mL/min. Peaks were detected by a UV detector at 254 nm. The absolute configurations of the sugar moieties of new compounds were identified as D-Api (t_R = 16.18 min), D-Glc (t_R = 11.25 min), and L-Rha (t_R = 16.36 min), respectively, by comparing the retention time of detected peaks with those of the standard ones.

2.5. Platelet aggregation assays

Turbidometric measurements of platelet aggregation of the samples were performed in a Chronolog Model 700 Aggregometer (Chronolog Corporation, Havertown, PA, USA) according to Born's method [19,20]. Platelet aggregation studies were completed within 3.0 h of preparation of platelet-rich plasma (PRP). Immediately after preparation of PRP, 250 μL was incubated in each test tube at 37 °C for 5.0 min and then 2.5 μL of compounds (300 μg/mL) were individually added. The changes in absorbance as a result of platelet aggregation were recorded. The extent of aggregation was estimated by the percentage of maximum increase in light transmittance, with the buffer representing 100% transmittance. ADP (adenosine diphosphate) was used as a positive control with a 53.3 ± 6.5% maximal platelet aggregation rate at a concentration of 25 μg/mL. 1% DMSO was used as a blank control with a 3.0% maximal platelet aggregation. Data counting and analysis was done on SPSS 16.0, with experimental results expressed as mean ± standard error.

3. Results and discussion

Ypsiparoside A (**1**) was obtained as a white amorphous powder. Its molecular formula was deduced as $C_{32}H_{50}O_9$ by HRESIMS at *m/z* 601.3352 $[M+Na]^+$ (calcd. for $C_{32}H_{50}O_9Na$, 601.3353) and ^{13}C NMR data, indicating eight degrees of unsaturation. The IR absorptions revealed the presence of hydroxy (3424 cm^{-1}) and double bond (1631 cm^{-1}) functionalities. In the ^{1}H NMR spectrum (Table 1) for the aglycone moiety of **1**, two tertiary methyls at δ_H 0.98 and 0.99 (each 3H, s), a secondary methyl at δ_H 1.28 (3H, d, J = 7.2 Hz), an olefinic proton at δ_H 5.28 (1H, br d, J = 5.0 Hz), and an anomeric proton at δ_H 5.78 (1H, d, J = 3.0 Hz), as well as signals for four oxygen-bearing methylene protons [δ_H 4.10 (1H, dd, J = 10.8, 3.9 Hz), 3.93 (1H, t, J = 11.2 Hz), 3.75 (1H, overlapped), and 3.66 (1H, t, J = 8.5 Hz)] and two oxygen-bearing methine protons [δ_H 3.75 (1H, overlapped) and 4.52 (1H, t, J = 6.9 Hz)] were observed. The ^{13}C NMR (Table 2) and HSQC spectra displayed 27 carbon resonances for the aglycone moiety, comprising three methyls, eleven methylenes (two oxygenated ones), eight methines (one olefinic and two oxygenated ones), and five quaternary carbons (one olefinic, one ketal, and one oxygenated).

The planar structure of the aglycone of **1** was established by the inspection of its 1D and 2D NMR data (including ^{1}H–^{1}H COSY, HSQC, and HMBC). The ^{1}H–^{1}H COSY spectrum disclosed that the aglycone of **1** had five partial structures. The connectivity of these fragments and the other functional groups was determined by the HMBC correlations: from δ_H 0.98 (H_3-19) to δ_C 38.0 (t, C-1), 37.6 (s, C-10), 50.6 (d, C-9), and 141.3 (s, C-5); from δ_H 2.54 and 2.39 (H_2-4) to C-5; from δ_H 0.99 (H_3-18) to δ_C 32.8 (t, C-12), 45.7 (s, C-13), 53.5 (d, C-14), and 90.6 (s, C-17); from δ_H 4.52 (H-16) to C-17; from δ_H 1.28 (H_3-21) to C-17 and δ_C 110.8 (s, C-22); from δ_H 4.10 (H-26a), 1.82 (H-23b), and H-16 to C-22. The overall structure of the aglycone of **1** was thus determined as 27-hydroxypennogenin [(25*S*)-spirost-5-en-3*β*,17*α*,27-triol], featuring a hydroxy group at C-17 [4]. The relative stereochemistry of the aglycone of **1** was determined via cross-peaks observed in the ROESY spectrum. The ROESY correlations of δ_H 0.98 (H_3-19*β*) with δ_H 1.77 (H-1a) and of δ_H 1.00 (H-1b) with δ_H 3.75 (H-3) inferred that OH-3 was *β*-oriented. Likewise, the ROESY correlations of δ_H 4.52 (H-16) with 4.10 (H-26a) and of δ_H 2.09 (H-25) with 3.93 (H-26b) revealed that Me-25 had *α*-

configuration. Furthermore, from the ROESY correlations of H_3-18β with δ_H 1.90 (H-8), of δ_H 0.97 (H-9) with δ_H 2.10 (H-14), and of δ_H 4.52 (H-16) with H-14, the relative configurations at the other chiral centers were found similar as those of (25*S*)-spirost-5-en-3β,17α,27-triol.

The remaining five carbon resonances in **1** were ascribed to a pentose sugar. The D-apiofuranosyl in **1** was identified by the result of it's HPLC analysis of its derivative upon comparison with standard sugar, and the β-orientation of anomeric proton in the sugar was confirmed by comparing the chemical shifts of δ_C 108.9 (d, C-1′), 78.5 (d, C-2′), 80.7 (s, C-3′), 75.3 (t, C-4′), and 65.8 (t, C-5′) with those of the corresponding carbons of α- and β-D-apiofuranoside and α- and β-L-apiofuranoside [21,22]. The glycosylation site in **1** was assigned from the HMBC spectrum which showed key correlations between δ_H 5.78 (H-1′) and δ_C 77.9 (C-3). Based on the above evidences, structure **1** was elucidated as 27-hydroxypennogenin-3-*O*-β-D-apiofuranoside.

Ypsiparoside B (**2**) was isolated as a white amorphous powder with the molecular formula of $C_{38}H_{60}O_{13}$, based on the HRESIMS ion at *m/z* 747.3923 $[M+Na]^+$ (calcd. for $C_{38}H_{60}O_{13}Na$, 747.3932) and ^{13}C NMR data. Comparison of its ^1H and ^{13}C NMR spectra (Tables 1 and 2) with those of **1** indicated that they had the same aglycone, except for the presence of six additional signals corresponding to a rhamnose unit in **2**. The α-configuration for the anomeric carbon of L-rhamnopyranosyl moiety was determined by the characteristic signals at δ_C 72.8 (C-3″) and 70.0 (C-5″) [23]. Acid hydrolysis of **2** with 6 M TFA gave α-L-rhamnose as confirmed by HPLC analysis of its derivative. The downfield chemical shift of C-5′ (δ_C 70.6) indicated that the rhamnose moiety was linked to the C-5′ of Apiose unit. The linkages of the sugar residues were further confirmed from HMBC correlations between δ_H 5.71 (H-1′) of Api and δ_C 77.4 (C-3) of the aglycone and between δ_H 5.35 (H-1″) of Rha and C-5′ (δ_C 70.6) of Api. Hence, structure **2** was established to be 27-hydroxypennogenin-3-*O*-α-L-rhamnopyranosyl-(1→5)-β-D-apiofuranoside.

Ypsiparoside B (**3**) had the same molecular formula of $C_{32}H_{50}O_9$ as that of **1** based on its HRESIMS at *m/z* 601.3345 $[M+Na]^+$ and ^{13}C NMR data. Careful comparison of the ^1H and ^{13}C NMR spectroscopic data of **3** with those of **1** suggested that they were similar pennogenin-type saponins with the significant difference observed for the upfield chemical shifts of C-24 (δ_C 24.1→21.1), C-26 (δ_C 64.4→60.4), and C-27 (δ_C 64.9→61.2). The configuration of 25*R* was thus assigned, and this was supported by the ROESY correlations of δ_H 4.44 (H-16) with 4.12 (H-26a) and of δ_H 1.88 (H-25) with H-26a. Thus, structure **3** was established as 27β-hydroxypennogenin-3-*O*-β-D-apiofuranoside.

Ypsiparoside D (**4**) gave a pseudo-molecular ion peak at *m/z* 759.3929 $[M+Na]^+$ in its HRESIMS, corresponding to a molecular formula of $C_{39}H_{60}O_{13}$. The intensity of the absorptions (899 > 919 cm^{-1}) in its IR spectrum implied that the absolute configuration of C-25 was *R* [24]. The ^1H NMR spectrum (Table 1) of **4** showed four typical steroid methyl signals at δ_H 0.68 (d, J = 5.6 Hz, H-27), 1.08 (s, H-19), 1.11 (s, H-18), 1.34 (d, J = 6.9 Hz, H-21), an olefinic proton at δ_H 5.25 (br s, H-5), as well as two anomeric protons at δ_H 5.03 (d, J = 7.8 Hz, H-1′) and 6.39 (br s, H-1″). The ^{13}C NMR spectrum displayed a carbonyl signal at 212.7 (s, C-12), a ketal signal at δ_C 109.3 (s, C-22), a pair of trisubstituted olefinic signals at δ_C 140.6 (s, C-5) and 121.5 (d, C-6), as well as two anomeric signals at δ_C 100.3 (d, C-1′) and 102.1 (d, C-1′). These characteristic signals suggested that **4** was a diosgenin-type saponin with the same aglycone (gentrogenin) as that of ypsilandroside G (**16**). However, compound **4** contained only two sugar units. In the same manner as that for **3**, an α-configuration of the L-rhamnopyranosyl moiety was assigned, whereas a β-configuration to the D-glucopyranosyl unit ($^3J_{1,2}$ > 7.0 Hz) was determined by the coupling constants of the anomeric protons. Based on HPLC analysis of sugar derivatives, the sugar moiety was identified as β-D-glucose and α-L-rhamnose. The linkages of the sugar residues were unambiguously assigned by the HMBC correlations between δ_H 6.39 (H-1″) of Rha and δ_C 77.6 (C-2′) of Glc and between δ_H 5.03 (H-1′) and δ_C 77.8 (C-3) of the aglycone. The other parts of compound **4** were identical to those of gentrogenin based on 2D NMR experiments. Accordingly, structure **4** was identified as gentrogenin 3-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside.

Ypsiparoside E (**5**) exhibited a $[M+Na]^+$ ion peak in the HRESIMS at *m/z* 905.4497 in accordance with the molecular formula of $C_{45}H_{70}O_{17}$. The ^1H and ^{13}C NMR spectroscopic data (Tables 1 and 2) indicated that **5** had the same aglycone as **4**, except for the presence of an additional rhamnose moiety. The NMR signals arising from the sugar chain were in good agreement with those of **10**, indicating the presence of one β-D-glucopyranosyl and two α-L-rhamnopyranosyl units. The connectivities of the sugar units were unambiguously established by the following HMBC correlations: δ_H 6.33 (H-1‴) of Rha with δ_C 80.1 (C-4″) of Rha, δ_H 5.89 (H-1″) of Rha with δ_C 77.4 (C-4′) of Glc, and δ_H 4.93 (H-1′) of Glc with δ_C 77.6 (C-3). Comprehensive 1D and 2D NMR data analysis resulted in the full assignment of its ^1H and ^{13}C NMR signals. Structure **5** was thus deduced as gentrogenin 3-*O*-α-L-rhamnopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranoside.

The molecular formulae of ypsiparosides F (**6**) and G (**7**) were determined as $C_{39}H_{60}O_{14}$ and $C_{45}H_{70}O_{18}$ by HRESIMS at *m/z* 775.3872 $[M+Na]^+$ and 921.4452 $[M+Na]^+$, respectively. The NMR spectra of the aglycones of **6** and **7** closely resembled that of **5** except for the presence of an oxygenated methine (δ_C 73.7 in **6** and 73.4 in **7**) and the absence of a methylene (δ_C 37.4 in **5**). These data suggested that the aglycones of **6** and **7** were the 11-hydroxylated derivatives of **5**, which were further supported by the ^1H–^1H COSY cross peaks of H-9/H-11 and the HMBC correlations from H-11 to C-9 and C-12. The α-configuration of OH-11 in **6** and **7** was assigned by the ROESY correlation of H-11 with H_3-19. The sugar moieties of **6** were determined to be the same as those of **4** while those of **7** were identical to those of compound **5** by their almost identical NMR spectroscopic data. The HMBC correlations between the anomeric protons and their respective carbons established the linkage of the sugars. Thus, **6** and **7** were characterized as 11α-hydroxygentrogenin-3-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside and 11α-hydroxygentrogenin-3-*O*-α-L-rhamnopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside, respectively.

All the compounds except **10**, **21**, and **21** were evaluated for their induced rabbit platelet aggregation activities and the DMSO was used as a blank control. The results showed that saponins **4**, **15**, and **17** at a concentration of 300 μg/mL had maximal induced-platelet aggregation rates (MPAR) of 44, 55 and 43% (Fig. 2), respectively. Further experiments displayed that **15**, **17**, and **4** possessed EC_{50} values of 95.3 ± 21.3, 300.8 ± 16.7 and 642.9 ± 33.4 μg/mL, respectively. These results and our published literature [25,26] demonstrate that chemical constituents with the induced-platelet aggregation of *Y. parviflora* are spirostanol saponins **4**, **10**, **15**, and **17**.

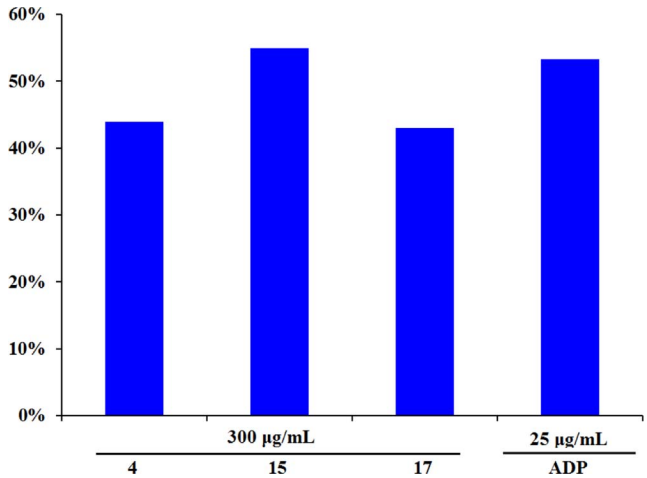

Fig. 2. Platelet aggregation activities of **4**, **15** and **17**.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.steroids.2017.05.004.

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