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Novel cytotoxic steroidal saponins from the roots of *Liriope muscari* (Decne.) L.H. Bailey†

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Ten new steroidal saponins (**1–10**) and three known steroidal saponins (**11–13**) were isolated from a 70% EtOH extract of the roots of *Liriope muscari* (Decne.) L. H. Bailey. Their structures were determined by analyses of infrared, nuclear magnetic resonance and mass spectroscopic data. These compounds exhibited different levels of cytotoxic activity against MDA-MB-435, 95D, HepG2, HeLa, MCF-7 and A549 cell lines in an *in vitro* bioassay. The structure–activity relationship of these related compounds was also investigated.

Introduction

Liriope muscari (Decne.) L. H. Bailey, a commonly used herb of the Liliaceae family, has been used in China for a long time.[1] The tuber of *L. muscari* exhibits clear therapeutic effects on cough, insomnia, acute and chronic inflammation and cardiovascular diseases.[2–6] Previous phytochemical studies have indicated that saccharides and steroidal compounds are the predominant components of *L. muscari* tubers. The steroidal compounds are considered to be the main active ingredients responsible for the therapeutic effects of *L. muscari* because many of these steroidal saponins display a variety of biological properties, such as anti-inflammatory, hypoglycemic and cardiovascular activities.[7–14] The steroidal constituents, which contain a spiro structure in their parent nucleus, are widely distributed in plants from Dioscoreaceae, Amaryllidaceae, Liliaceae and Agavaceae.[15] Pharmacodynamic studies of the steroidal constituents have shown that they exhibit diverse biological properties, including anticancer, anti-inflammatory, antimicrobial, insecticidal and molluscicidal activities.[16,17] Recently, the antitumorigenic properties of steroidal saponins have attracted attention for the development of antitumor therapies. Significant antitumor activities of some steroidal saponins from *L. muscari* have been demonstrated *in vitro* and *in vivo*.[7,18–27] For example, DT-13, a mixture of 25 (*R*/*S*) steroidal saponins isolated from *L. muscari*, significantly suppresses adhesion and invasion of cancer cells, and has been considered a candidate drug for prevention of cancer metastasis.[18–29]

Although some phytochemical investigations of *L. muscari* have been reported,[7,30–35] the steroidal components of the herb are still not fully elucidated. To promote a better understanding of the steroidal components of *L. muscari* and enable further screening for potentially useful bioactive ingredients, a 70% EtOH extract of *L. muscari* was studied. As a result, 10 novel steroidal saponins (**1–10**) were identified, and three known steroidal saponins (**11–13**) were also obtained. The structures of these compounds were elucidated based on infrared (IR), nuclear magnetic resonance (NMR) and mass spectroscopic data. The cytotoxic activities of the compounds were evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The isolation, structural characterization and cytotoxic activities of the compounds are described in detail in this article. Furthermore, the structure–activity relationship of these related compounds is also discussed to better interpret the activities of these steroidal compounds.

Results and discussion

Compound **1** was obtained as a white amorphous powder and gave a positive Liebermann–Burchard reaction. Its molecular formula was determined as $C_{44}H_{70}O_{18}$ according to HRESI-QTOF-MS data (*m*/*z* 909.4456 $[M + Na]^+$, calcd 909.4454). The ^1H and ^{13}C spectra of **1** (Tables 1–4) displayed the following characteristic signals: two tertiary methyl groups (δ_H 1.34, s; δ_H 0.88, s), two secondary methyl groups (δ_H 1.14, d, $J = 7.0$ Hz; δ_H 1.08, d, $J = 7.0$ Hz), one quaternary carbon (δ_C 109.7) and an olefinic group (δ_C 139.4 and 124.7; δ_H 5.52, brs, 1H). Moreover, the ^1H-NMR spectrum showed three anomeric proton signals [δ_H 5.04 (1H, d, $J = 7.7$ Hz), 5.52 (1H, d, $J = 7.7$ Hz) and 5.29 (1H, d, $J = 7.7$ Hz)], giving heteronuclear single quantum correlation (HSQC) correlations with three anomeric carbon signals at δ_C 100.1, 104.7 and 105.3, respectively. These observations together with the characteristic absorptions of a 25(*S*) spiroketal

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Table 1 ^{13}C NMR data for aglycone moieties of compounds **1–10**[a] (δ in ppm, pyridine-d_5)

Position	1	2	3	4	5	6	7	8	9	10
1	82.6	82.5	82.6	82.4	84.5	82.8	82.5	82.6	84.3	83.0
2	37.2	36.9	36.7	36.5	37.7	37.3	37.0	36.7	37.5	37.4
3	68.1	67.9	68.1	67.9	68.0	68.4	68.1	68.1	67.8	68.3
4	43.7	43.5	43.5	43.4	43.4	43.7	43.4	43.5	43.4	43.6
5	139.4	139.2	139.4	139.2	139.2	140.0	139.6	139.4	140.0	139.8
6	124.7	124.7	124.6	124.4	124.8	124.3	124.1	124.6	124.6	124.4
7	31.9	31.7	31.8	31.7	31.8	32.1	31.8	31.8	31.5	32.0
8	32.9	32.8	32.9	32.7	33.0	33.0	32.7	32.9	33.0	33.2
9	50.3	50.2	50.5	50.1	50.2	50.3	50.0	50.2	50.0	50.4
10	42.7	42.6	42.8	42.6	42.6	43.0	42.7	42.8	42.4	42.9
11	23.9	23.8	23.7	23.4	24.1	23.6	23.4	23.7	23.9	23.7
12	40.3	40.2	40.2	40.0	40.3	40.5	40.2	40.1	40.0	40.3
13	40.2	40.1	40.0	39.9	40.2	40.3	40.0	40.1	40.0	40.4
14	56.8	56.7	56.6	56.4	56.8	57.0	56.8	56.6	56.7	57.0
15	32.3	32.2	32.2	32.0	32.3	32.4	32.2	32.2	32.0	32.4
16	81.2	81.0	81.1	80.8	81.0	81.3	80.9	81.3	81.1	81.5
17	62.8	62.8	62.7	62.7	62.9	63.0	62.8	62.8	62.7	63.1
18	16.7	16.6	16.6	16.4	16.8	16.8	16.5	16.5	16.6	16.7
19	15.0	14.9	14.9	14.7	15.0	15.1	14.9	14.9	14.8	15.1
20	42.5	41.9	42.3	41.7	41.9	42.6	41.8	41.7	41.6	41.9
21	14.8	14.8	14.7	14.7	14.9	14.9	14.8	14.8	14.6	14.9
22	109.7	109.2	109.7	109.0	109.2	109.8	109.0	109.4	109.3	109.5
23	26.4	31.6	26.3	31.5	31.7	26.4	31.6	33.1	32.8	33.0
24	26.2	29.1	26.1	28.9	29.2	26.3	29.0	28.8	28.6	29.0
25	27.6	30.5	27.4	30.3	30.5	27.6	30.4	144.3	144.2	144.6
26	65.0	66.7	64.9	66.5	66.7	65.1	66.6	64.9	64.6	65.0
27	16.3	17.2	16.2	17.0	17.2	16.3	17.1	108.6	108.4	108.6

[a] NMR data were measured at 500 MHz for ^{1}H and at 125 MHz for ^{13}C in pyridine-d_5. Assignments are based on TCOSY, HSQC, and HMBC experiments.

unit at 987, 920, 896, 848, and 920 > 896 cm^{-1} in the IR spectrum and chemical shifts at δ_C 26.4 (C-23), 26.2 (C-24), 27.6 (C-25), and 65.0 (C-26) in the ^{13}C-NMR spectrum suggested that **1** was a (25*S*)-spirostanol derivative containing three sugar units.[36]

On the basis of the HSQC and heteronuclear multiple-bond correlation (HMBC) correlations, the aglycone moiety of compound **1** was identified as (25*S*)-ruscogenin.[37] The analyses of chemical shifts and coupling constants (J = 7.5–8.0 Hz) obtained from extensive 1D and 2D NMR experiments allowed the identification of two β-glucopyranosyl units and one β-xylopyranosyl unit in **1**. The sugar residues were further confirmed by co-thin-layer chromatography (co-TLC) with standard sugars after hydrolysis of **1**, and the D-configurations were confirmed by gas chromatography (GC) of their corresponding trimethylsilylthiazolidine derivatives. This procedure was also applied to the other new compounds (**2–10**). The sequence of the sugar chain and the glycosidic position of **1** were determined by HMBC, HSQC and total correlation spectroscopy (TOCSY) experiments. The HMBC correlations (Fig. 1) of the anomeric proton signal at δ_H 5.52 (d, J = 7.7 Hz, Glu H-1″) to δ_C 81.6 (Glu C-2′), from δ_H 5.29 (d, J = 7.7 Hz, Xyl H-1‴) to δ_C 86.0 (Glu C-3′) and from δ_H 5.04 (d, J = 8.0 Hz, Glu H-1′) to δ_C 82.6 (Agly C-1) proved that the sequence of the sugar chain was Glu-(1→2)-[Xyl-(1→3)]-Glu and that the glycosidic site was at C-1. Other key HMBC, HSQC and TOCSY correlations are shown in the ESI data.† The structure of compound **1** was therefore assigned as (25*S*)-ruscogenin-1-*O*-β-D-glucopyranosyl-(1→2)-[β-D-xylop yranosyl-(1→3)]-β-D-glucopyranoside.

The HRESI-QTOF-MS spectrum of compound **2** (*m*/*z* 909.4454 [M + Na]$^+$, calcd 909.4454) supported a molecular formula of $C_{44}H_{70}O_{18}$, the same as compound **1**. A detailed analysis of the NMR data of **2** (Tables 1–4) compared to those for **1** showed that **2** contained the same sugar chain at C-1 as compound **1**. The major difference was that the chemical shifts at δ_C 31.58 (C-23), 29.13 (C-24), 30.47 (C-25) and 66.73 (C-26) in the ^{13}C-NMR spectrum had lower field resonances than those of **1**, indicating that the configuration at C-25 was *R*. This result was further confirmed by the characteristic absorptions of a 25(*R*) spiroketal unit at 982, 921, 902, 870 cm^{-1}, and 902 > 921 cm^{-1} in the IR spectrum. The aglycone moiety of **2** was identified as (25*R*)-ruscogenin by comparison of spectroscopic data to those reported in the literature.[37] Compound **2** was therefor assigned as (25*R*)-ruscogenin-1-*O*-β-D-glucopyranosyl-(1→2)-[β-D-xylopyranosyl-(1→3)]-β-D-glucopyranoside. Key HMBC, HSQC and TOCSY correlations are shown in Fig. 1 and the ESI data.†

The molecular formula of compound **3** was determined as $C_{43}H_{68}O_{17}$ from the ion peak [M + Na]$^+$ at *m*/*z* 879.4344 (calcd 879.4349) in the HRESI-QTOF-MS. The IR absorption bands at 3390 and 1072 cm^{-1} indicated the presence of hydroxyl and C–O groups, respectively. Comparison of the NMR data of **3** obtained from 1D and 2D NMR spectra (Tables 1–4) to those of **1** showed that they contained the same aglycone moiety ((25*S*)-ruscogenin), but differed slightly in the sugar moiety. The major difference

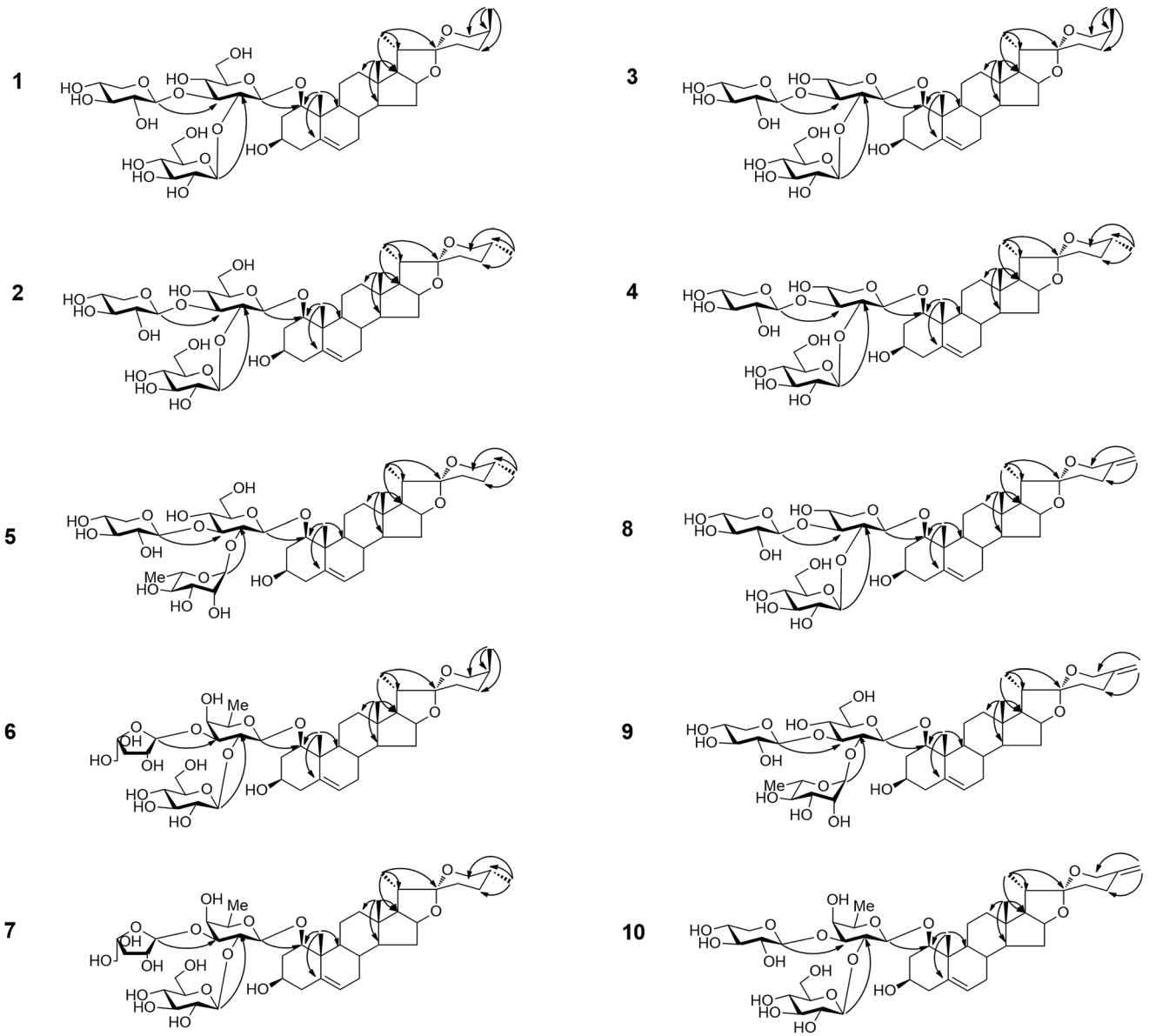

Fig. 1 Key HMBC correlations ofcompounds **1–10**.

was that the β-glucopyranoside unit was replaced by a β-xylopyranosyl unit in **3**. The HMBC correlations (Fig. 1) between the anomeric proton signal at δ_H 5.43 (d, $J = 7.7$, Glu H-1″) to δ_C 79.2 (Xyl C-2′), from δ_H 5.27 (d, $J = 7.7$ Hz, Xyl H-1‴) to δ_C 81.6 (Xyl C-3′) and from δ_H 4.95 (d, $J = 7.0$ Hz, Xyl H-1′) to δ_C 82.6 (Agly C-1) proved that the sequence of the sugar chain was Glu-(1→2)-[Xyl-(1→3)]-Xyl and that the glycosidic site was at C-1. Thus, compound **3** was (25*S*)-ruscogenin-1-*O*-β-D-glucopyranosyl-(1→2)-[β-D-xylopyranosyl-(1→3)]-β-D-xylopyrano side.

The HRESI-QTOF-MS spectrum of compound **4** (*m*/*z* 879.4344 $[M + Na]^+$ calcd 879.4349) supported a molecular formula of $C_{43}H_{68}O_{17}$, the same as compound **3**. The NMR data of **4** (Tables 1–4) obtained from 1D and 2D NMR spectra were similar to those of **3**. The chemical shifts at δ_C 31.50 (C-23), 28.94 (C-24), 30.27 (C-25) and 66.53 (C-26) in the ^{13}C-NMR spectrum had lower field resonances than those of **3**. Together with the characteristic absorptions of a 25(*R*) spiroketal unit at 982, 921, 902, 870 cm^{-1}, and 902 > 921 cm^{-1} in the IR spectrum, these results indicated that the configuration at C-25 was *R*. The aglycone moiety of compound **4** was identified as (25*R*)-ruscogenin by comparison of the NMR data of **4** to those of compound **1**. The sequence of the sugar chain and the glycosidic position of **4**, determined d by HMBC and TOCSY experiments, showed that **4** contained the same sugar chain at C-1 as compound **3**. The structure of compound **4** was therefore (25*R*)-ruscogenin-1-*O*-β-D-glucopyranosyl-(1→2)-[β-D-xylopyranosyl-(1→3)]-β-D-xy lopyranoside. Key HMBC, HSQC and TOCSY correlations are shown in Fig. 1 and the ESI data.†

The molecular formula of compound **5** was established unequivocally as $C_{44}H_{70}O_{17}$ from the HRESI-QTOF-MS spectrum (*m*/*z* 871.4702 $[M + H]^+$, calcd 871.4686). The IR spectrum indicated the presence of a hydroxyl and a 25(*R*) spiroketal unit. The

Table 2 ^{13}C NMR data for sugar moieties of compounds **1–10**[a] (δ in ppm, pyridine-d_5)

Position	1	2	3	4	5	6	7	8	9	10
1-Glc-1′	100.1	99.9			100.1				99.9	
2′	81.6	81.5			76.1				75.9	
3′	86.0	85.7			88.5				88.2	
4′	70.1	70.0			70.1				69.8	
5′	77.8	77.6			77.6				77.4	
6′	63.2	63.0			63.1				62.9	
1-Xyl-1′			100.2	100.0				100.2		
2′			79.2	79.0				79.2		
3′			81.6	81.4				81.5		
4′			68.9	68.8				68.9		
5′			66.4	66.2				66.3		
1-Fuc-1′						99.9	99.6			100.5
2′						77.9	77.6			78.1
3′						82.6	82.2			83.2
4′						72.1	71.8			72.3
5′						70.8	70.5			70.7
6′						17.1	16.8			17.1
2″-Glc-1″	104.7	104.6	105.1	104.9		104.9	104.5	105.1		104.9
2″	76.6	76.4	76.2	76.0		76.3	76.0	76.2		76.5
3″	77.9	77.7	77.9	77.7		77.9	77.6	77.9		78.1
4″	71.9	71.7	71.7	71.5		72.1	71.7	71.7		72.1
5″	78.6	78.4	78.2	78.1		78.2	77.8	78.2		78.3
6″	63.0	62.8	62.9	62.8		63.4	63.0	62.9		63.4
2″-Rha-1″					101.5				101.3	
2″					72.3				72.1	
3″					72.4				72.1	
4″					74.1				73.9	
5″					69.5				69.2	
6″					19.2				19.0	
3‴-Ara (*f*)-1‴						111.2	110.8			
2‴						83.0	82.7			
3‴						78.0	77.7			
4‴						86.6	86.2			
5‴						62.9	62.5			
3‴-Xyl-1‴	105.3	105.1	105.9	105.7	105.1			105.9	104.9	106.5
2‴	75.0	74.9	74.8	74.6	74.6			74.8	74.4	75.1
3‴	78.8	78.6	78.1	78.0	78.2			78.2	78.0	78.6
4‴	70.8	70.7	70.9	70.7	70.5			70.9	70.3	71.1
5‴	67.4	67.2	67.1	66.9	67.1			67.0	66.9	67.3

[a] NMR data were measured at 500 MHz for ^{1}H and at 125 MHz for ^{13}C in pyridine-d_5. Assignments are based on TCOSY, HSQC, and HMBC experiments.

aglycone moiety of **5** was established as 25(*R*)-ruscogenin by comparison of the NMR data of **5** (Tables 1–4) to those of **2**, and the glycosidic site was identified as C-1. A detailed analysis of 1D and 2D NMR data of **5** compared to those reported for parisverticoside A,[38] and parisyunnanoside G,[39] showed that they shared the same sugar moiety. The sequence of the sugar chain was established as Rha-(1→2)-[Xyl-(1→3)]-Glu by HMBC, HSQC and TOCSY correlations. Thus, compound **5** was (25*R*)-ruscogenin-1-*O*-α-L-rhamnopyranosy l-(1→2)-[β-D-xylopyranosyl-(1→3)]-β-D-glucopyranoside.

Compound **6** has a molecular formula of $C_{44}H_{70}O_{17}$, as indicated by the HRESI-QTOF-MS spectrum (*m*/*z* 893.4461 $[M + Na]^+$, calcd 893.4505). The aglycone moiety of **6** was established as (25*S*)-ruscogenin by comparison of the NMR data of **6** (Tables 1–4) to those of **3**. The ^{1}H-NMR spectrum showed three anomeric proton signals [δ_H 5.36 (1H, d, J = 7.7 Hz), 6.10 (1H, d, J = 2.4 Hz) and 4.79 (1H, d, J = 7.5 Hz)], giving HSQC correlations with three anomeric carbon signals at δ_C 104.5, 110.8 and 99.6, respectively. The HMBC correlations (Fig. 1) of the anomeric proton signal in **6** at δ_H 5.36 (d, J = 7.7, Glu H-1″) to δ_C 77.6 (Fuc C-2′), from δ_H 6.10 (d, J = 2.4 Hz, Ara (*f*)H-1‴) to δ_C 82.2 (Fuc C-3′) and from δ_H 4.79 (d, J = 7.5 Hz, Fuc H-1′) to δ_C 82.5 (Agly C-1) proved that the sequence of the sugar chain was Glu-(1→2)-[Ara (*f*)-(1→3)]-Fuc and that the glycosidic site was at C-1. HSQC and TOCSY correlations are shown in the ESI data.† The structure of **6** was therefore established as (25*S*)-ruscogenin-1-*O*-β-D-glucopyranosyl-(1→2)-[α-L-arabinofuranosyl-(1→3)]-β-D-fucopyranoside.

Compound **7** has the same molecular formula as compound **6** ($C_{44}H_{70}O_{17}$) from the HRESI-QTOF-MS spectrum (*m*/*z* 893.4461 $[M + Na]^+$, calcd 893.4505), and similar NMR data (Tables 1–4). The only difference in the chemical shifts at δ_C C-23, C-24, C-25 and C-26 of **7** was the lower field resonances compared to those of **6** in the ^{13}C-NMR spectrum, indicating that **6** and **7** were a pair of C-25 epimers. The aglycone moiety of compound **7** was

Table 3 ^{1}H NMR data for aglycone moieties of compounds **1–10**[a] (δ in ppm, pyridine-d_5, J in Hz)

Position	1	2	3	4	5	6	7	8	9	10
1	3.96m	3.98m	3.88dd (11.5, 4.1)	3.89dd (11.5, 4.2)	3.90m	3.90dd (10.9, 4.2)	3.90dd (11.0, 4.0)	3.89dd (11.8, 4.3)	3.90m	3.89dd (11.6, 4.3)
2	2.32q-like (11.8)	2.37q-like (12.0)	2.35q-like (11.5)	2.33q-like (11.5)	2.44q-like (12.0)	2.23q-like (11.4)	2.25q-like (12.0)	2.35q-like (11.8)	2.46q-like (12.0)	2.33q-like (11.6)
	2.76m	2.80m	2.79m	2.74m	2.70m	2.68m	2.68br d (12.0)	2.79m	2.71m	2.73m
3	3.74m	3.77m	3.85m	3.84m	3.80m	3.89m	3.89m	3.86m	3.80m	3.87m
4	2.56dd (12.6, 5.2)	2.59dd (11.8, 5.0)	2.59dd (12.5, 5.2)	2.58dd (11.5, 4.2)	2.57dd (11.6, 4.5)	2.60dd (12.5, 5.5)	2.60dd (12.5, 5.5)	2.59m	2.58dd (12.8, 4.3)	2.60dd (12.8, 5.3)
	2.68br d (12.6)	2.71br d (11.8)	2.70br d (12.5)	2.69br d (11.5)	2.71br d (11.6)	2.69m	2.69m	2.70br d (12.1)	2.71m	2.70br d (12.8)
6	5.52br s	5.54br s	5.56d (5.6)	5.57d (5.6)	5.57d (5.7)	5.57d (5.5)	5.56d (5.5)	5.57d (5.6)	5.58d (5.7)	5.57d (5.7)
7	1.68m	1.67m	1.54m	1.58m	1.52m	1.48m	1.50m	1.55m	1.51m	1.56m
	1.85m	1.80m	1.88m	1.85m	1.89m	1.87m	1.90m	1.88m	1.89m	1.93m
8	1.54m	1.54m	1.57m	1.55m	1.61m	1.57m	1.61m	1.57m	1.77m	1.78m
9	1.45m	1.46m	1.41m	1.42m	1.67m	1.39m	1.41m	1.41m	1.67m	1.45m
11	1.56m	1.56m	1.56m	1.54m	1.67m	1.60m	1.62m	1.56m	1.66m	1.56m
	2.80br d (10.3)	2.80br d (10.3)	2.87m	2.84m	2.90m	2.84m	2.85m	2.87m	2.90m	2.87m
12	1.37m	1.37m	1.24m	1.24m	1.53m	1.28m	1.28m	1.23m	1.50m	1.33m
	1.69m	1.67m	1.58m	1.59m	1.72m	1.67m	1.68m	1.57m	1.68m	1.67m
14	1.07m	1.08m	1.08m	1.10m	1.22m	1.11m	1.13m	1.09m	1.20m	1.17m
15	1.42m	1.44m	1.55m	1.45m	1.50m	1.44m	1.50m	1.56m	1.50m	1.47m
	1.97m	1.98m	2.00ddd (12.4, 7.3, 5.4)	2.03ddd (12.3, 7.4, 5.5)	1.99m	2.01m	2.04m	2.00ddd (12.5, 7.5, 5.6)	1.99m	2.01m
16	4.45m	4.48q-like (8.2)	4.50m	4.53m	4.46m	4.50m	4.54m	4.51m	4.44m	4.54q-like (7.3)
17	1.76m	1.80m	1.73m	1.75m	1.84m	1.77m	1.82m	1.74m	1.83m	1.83m
18	0.88s	0.88s	0.85s	0.87s	0.93s	0.87s	0.89s	0.85s	0.92s	0.89s
19	1.34s	1.33s	1.38s	1.38s	1.44s	1.37s	1.37s	1.38s	1.44s	1.38s
20	1.90m	1.97m	1.87m	1.93m	1.99m	1.89m	1.94m	1.93t-like (6.7)	1.97m	1.96t-like (6.8)
21	1.14d (6.9)	1.15d (6.9)	1.11d (6.9)	1.12d (7.0)	1.13d (6.9)	1.12d (6.9)	1.10d (6.9)	1.07d (6.9)	1.08d (6.9)	1.07d (7.0)
23	1.44m	1.44m	1.41m	1.52m	1.69m	1.43m	1.43m	1.56m	1.58m	1.60m
	1.89m	1.87m	1.89m	1.90m	1.92m	1.90m	1.90m	1.76m	1.77m	1.78m
24	1.36m	1.58m	1.36m	1.55m	1.58m	1.35m	1.57m	2.26m	2.24m	2.27m
	2.13m	1.69m	2.14m	1.66m	1.68m	2.14m	1.66m	2.72m	2.72m	2.72m
25	1.58m	1.59m	1.58m	1.57m	1.58m	1.57m	1.57m	—	—	—
26	3.37br d (10.8)	3.51t-like (10.5)	3.35d (10.9)	3.51d (10.9)	3.49t (10.4)	3.37br d (10.9)	3.50d (10.3)	4.03d (12.4)	4.45d (12.0)	4.03d (12.2)
	4.05m	3.60dd (10.5, 3.5)	4.05m	3.58m	3.59m	4.07dd (10.9, 2.7)	3.58m	4.46br d (12.4)	4.04d (12.0)	4.47br d (12.2)
27	1.08d (7.1)	0.70d (5.1)	1.07d (7.1)	0.70d (5.2)	0.70d (5.0)	1.09d (7.1)	0.69d (4.9)	4.81br s	4.81br s	4.82br s
								4.78br s	4.79br s	4.79br s

[a] NMR data were measured at 500 MHz for ^{1}H and at 125 MHz for ^{13}C in pyridine-d_5. Assignments are based on TCOSY, HSQC, and HMBC experiments.

Table 4 ^{1}H NMR data for sugar moieties of compounds **1–10**[a] (δ in ppm, pyridine-d_5, J in Hz)

Position	1	2	3	4	5	6	7	8	9	10
1-Glc-1′	5.04d (8.0)	5.05d (7.7)			4.84d (7.7)				4.83d (7.8)	
2′	4.45dd (4.2, 8.0)	4.45m			4.21d (7.7)				4.21d (7.8)	
3′	4.23m	4.23m			4.09d (8.7)				4.08d (9.0)	
4′	3.97m	3.96m			3.88m				3.88m	
5′	3.83m	3.85m			3.81m				3.81m	
6′	4.74dd (11.7, 2.7)	4.77dd (11.7, 2.7)			4.51m				4.51m	
	4.52dd (11.7, 3.5)	4.54dd (11.7, 4.4)			4.24m				4.22m	
1-Xyl-1′			4.95d (7.0)	4.95d (7.0)				4.95d (7.0)		
2′			4.80dd (7.0, 9.0)	4.79dd (7.0, 9.0)				4.81m		
3			4.30m	4.29m				4.29m		
4			4.48m	4.46m				4.48m		
5′			3.71m	3.72m				3.72m		
			4.28m	4.26m				4.28m		
1-Fuc-1′						4.78d (7.9)	4.79d (7.5)			4.85d (7.6)
2′						4.61dd (9.6, 7.5)	4.64dd (9.6, 7.6)			4.24m
3′						4.13dd (9.6, 3.4)	4.14dd (9.6,3.4)			4.20m
4′						4.23m	4.24m			4.24m
5′						3.59m	3.61m			3.74m
6′						1.48d (6.3)	1.48d (6.3)			1.54d (6.2)
2″-Glc-1″	5.52d (7.7)	5.52d (7.7)	5.43d (7.8)	5.42d (7.8)		5.33d (7.8)	5.36d (7.7)	5.44d (7.8)		5.45d (7.9)
2″	4.17m	4.18m	4.15m	4.13m		4.09m	4.06m	4.14m		4.11m
3″	4.33m	4.36m	4.28m	4.27dd (7.5, 3.1)		4.26m	4.23m	4.28m		4.24m
4″	4.31m	4.35m	4.27m	4.26m		4.23m	4.23m	4.27m		4.19m
5″	4.10m	4.10m	3.95m	3.95m		3.86m	3.84m	3.95m		3.93m
6″	4.50m	4.23m	4.46m	4.45m		4.38dd (11.4, 4.6)	4.40br d (11.3)	4.46m		4.42dd (11.5, 4.6)
	4.23m	4.50m	4.61dd (11.6, 2.8)	4.61br d (11.7)		4.50m	4.51m	4.63br d (11.6)		4.60dd (11.5, 2.9)
2″-Rha-1″					6.47br s				6.47br s	
2″					4.82m				4.82m	
3″					4.61dd (8.7, 4.6)				4.62br d (9.5)	
4″					4.35td (9.5, 3.8)				4.34m	
5″					4.88dd (9.5, 6.2)				4.86m	
6″					1.80d (6.2)				1.80d (6.2)	
3‴-Ara (*f*)-1‴						6.10d (2.3)	6.10d (2.4)			
2‴						4.95m	4.97m			
3‴						4.83m	4.83m			
4‴						4.73m	4.77m			
5‴						4.18m	4.21m			
						4.28dd (11.8, 3.5)	4.32br d (11.8)			
3‴-Xyl-1‴	5.29d (7.7)	5.28d (7.7)	5.27d (8.0)	5.26d (8.0)	4.96d (8.0)			5.28d (8.4)	4.96d (7.6)	5.26d (8.2)
2‴	4.02m	4.05m	4.00br d (8.0)	4.00dd (8.0, 3.1)	4.00dd (8.0, 3.5)			3.98dd (8.4, 3.2)	4.00m	3.97br d (8.2)

Table 4 (*Contd.*)

Position	1	2	3	4	5	6	7	8	9	10
3‴	4.04m	4.04m	4.15m	4.15m	4.10m			4.15m	4.10m	4.12m
4‴	4.13m	4.17m	4.16m	4.16m	4.14m			4.16m	4.11m	4.15m
5‴	4.28dd (12.4, 3.9)	4.31dd (11.5, 5.1)	4.31m	4.31dd (11.1, 4.6)	4.27dd (11.5, 5.2)			4.31m	4.27dd (11.4, 5.1)	4.31dd (11.3, 5.2)
	3.73m	3.75m	3.71m	3.74m	3.70dd (11.5, 9.7)			3.71m	3.70t-like (11.4)	3.72m

[a] NMR data were measured at 500 MHz for ^{1}H and at 125 MHz for ^{13}C in pyridine-d_5. Assignments are based on TCOSY, HSQC, and HMBC experiments.

identified as (25*R*)-ruscogenin by comparison of the NMR data to those of **2**. HMBC, HSQC and TOCSY experiments indicated that **7** had the same sequence of the sugar chain and glycosidic position as compound **6**. Key HMBC and HSQC correlations are shown in Fig. 1 and the ESI data.† The structure of **7** was therefore established as (25*R*)-ruscogenin-1-*O*-β-D-glucopyranosyl-(1→2)-[α-L-arabinofuranosyl-(1→3)]-β-D-fucopyranoside.

Compound **8** has the molecular formula $C_{43}H_{66}O_{17}$, as deduced from the HRESI-QTOF-MS data (*m*/*z* 877.4188 [M + Na]$^+$, calcd 877.4192). The ^{1}H- and ^{13}C-NMR data (Tables 1–4) of **8** were similar to those of **3**, with the exception of some differences in the carbon signals due to the presence of an *exo*-olefinic group (δ_H 4.81, 4.78; δ_C 144.3, 108.6) and the disappearance of signals for a secondary methyl (CH_3-27) in **8**. The location of an *exo*-double bond ($\Delta^{25(27)}$) was confirmed by the long-range correlations of H-27 (δ_H 4.81, 4.78) to C-24 (δ_C 28.8), C-25 (δ_C 144.3) and C-26 (δ_C 64.9) in the HMBC spectrum indicating that the aglycone moiety was neoruscogenin.[37] The sugar moiety and glycosidic site of **8** were established to be the same as those of **3** by HMBC and TOCSY experiments. Key long-range correlations are shown in Fig. 1 and the ESI data.† Thus, **8** was characterized as neoruscogenin-1-*O*-β-D-glucopyranosyl-(1→2)-[β-D-xylopyr-anosyl-(1→3)]-β-D-xylopyranoside.

The molecular formula of compound **9** was determined to be $C_{43}H_{68}O_{17}$ from the pseudo-molecular ion peak [M + H]$^+$ at *m*/*z* 869.4529 (calcd 869.4529) in the HRESI-QTOF-MS, differing from that of **5** by 2 Da, and corresponding to an additional double bond. The aglycone moiety of **9** was established as neoruscogenin by comparison of 1D and 2D NMR spectra (Tables 1–4) of compound **9** to those of **8**. A detailed analysis of NMR data of **9** in comparison with those of **5** showed that **9** had the same sugar moiety and glycosidic site as **5**. The sequence of the sugar chain and the glycosidic position of **9** were further confirmed by HMBC, HSQC and TOCSY experiments. Thus, compound **9** was established as neoruscogenin-1-*O*-α-L-rhamnopyranosy-*l*-(1→2)-[β-D-xylop yranosyl-(1→3)]-β-D-glucopyranoside.

The molecular formula of compound **10** was determined by HRESI-QTOF-MS to be $C_{44}H_{68}O_{17}$ (*m*/*z* 891.4321 [M + Na]$^+$, calcd 891.4349). The NMR data of **10** (Tables 1–4) based on HSQC, HMBC and TOCSY were similar to those of **11** {(25*R*)-ruscogenin-1-*O*-β-D-glucopyranosyl-(1→2)-[β-D-xylopyranosyl-(1→3)]-β-D-fucopyranoside, Fig. 2},[30] except for the appearance

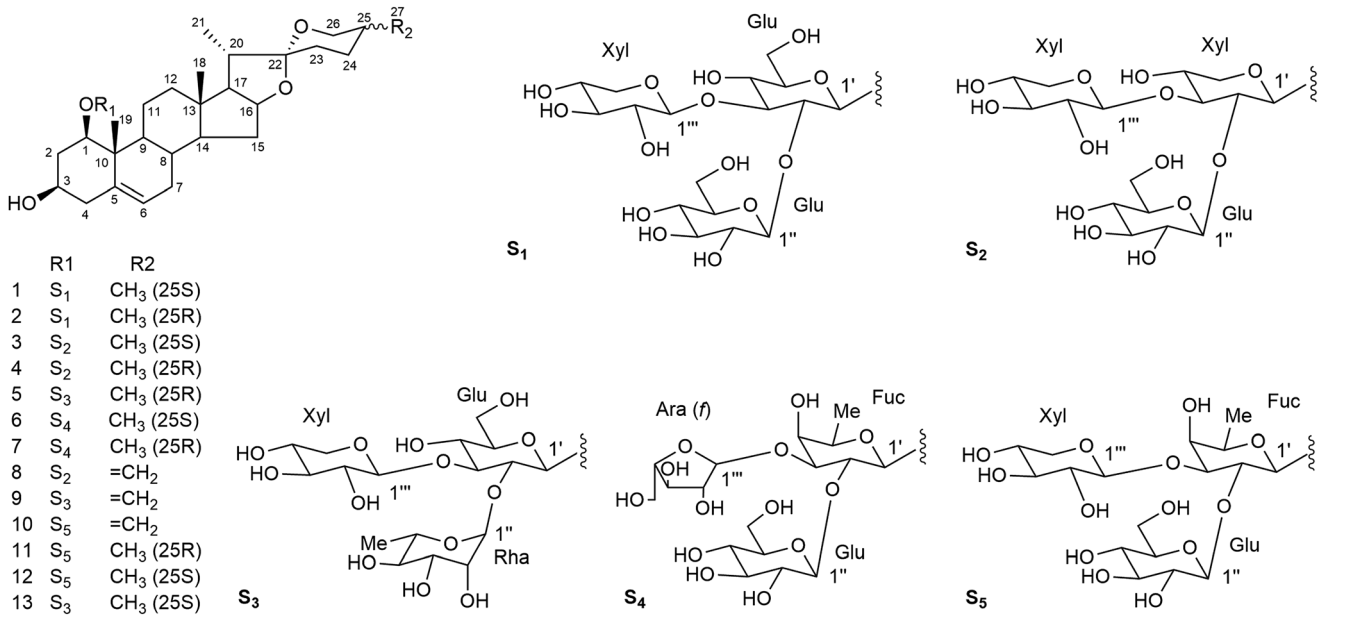

Fig. 2 Chemical structures of compounds **1**–**13**.

of signals for an *exo*-olefinic group (δ_H 4.82, 4.79; δ_C 144.6, 108.6) and the disappearance of the signals for a secondary methyl (CH_3-27) in **10**. The location of an *exo*-double bond ($\Delta^{25(27)}$) was confirmed by the long-range correlations of H-27 (δ_H 4.82, 4.78) to C-24 (δ_C 29.0), C-25 (δ_C 144.6) and C-26 (δ_C 65.0) in the HMBC spectrum. The sequence of the sugar chain and the glycosidic position of **10** determined by HMBC and TOCSY experiments suggested that **10** had the same sugar moiety and glycosidic site as **11**. Therefore, **10** was characterized as neoruscogenin-1-*O*-β-D-glucopyranosyl-(1 → 2)-[β-D-xylopyranosyl-(1 → 3)]-β-D-fucopyranoside.

The three known compounds were identified as (25*R*)-ruscogenin-1-*O*-β-D-glucopyranosyl-(1 → 2)-[β-D-xylopyranosyl-(1 → 3)]-β-D-fucopyranoside (**11**),[30] (25*S*)-ruscogenin-1-*O*-β-D-glucopyranosyl-(1 → 2)-[β-D-xylopyranosyl-(1 → 3)]-β-D-fucopyranoside (**12**),[30] and (25*S*)-ruscogenin-1-*O*-α-L-rhamnopyranosyl-(1 → 2)-[β-D-xylopyranosyl-(1 → 3)]-β-D-glucopyranoside (**13**)[40] by comparison of spectroscopic data with data reported in the literature.

According to the features of the chemical structures shown in Fig. 2, compounds **1–13** can be divided into three groups based on their aglycone moiety, including the (25*S*)-ruscogenin group (compounds **1**, **3**, **6**, **12** and **13**), (25*R*)-ruscogenin group (compounds **2**, **4**, **5**, **7** and **11**), and neoruscogenin group (compounds **8**, **9** and **10**). Compounds **3**, **4** and **8** share the same A–E rings and Glu-(1 → 2)-[Xyl-(1 → 3)]-Xyl glycoside chain at C-1. The only difference between them is that the C-25 configurations of the aglycone moieties are 25*S*, 25*R* and 25, 27 double-bond for **3**, **4** and **8**, respectively. A similar situation prevails for compounds **13**, **5** and **9** and for compounds **12**, **11** and **10**. Compounds **1** and **2**, and compounds **6** and **7** are two pairs of C-25 *S*/*R* epimers.

All isolates were tested for their *in vitro* cytotoxic activity against MDA-MB-435, 95D, HepG2, HeLa, MCF-7 and A549 cell lines. As summarized in Table 5, compounds **11** and **12** exhibited the best cytotoxicity against the MDA-MB-435 cell line among all of the cytotoxicity data, with IC_{50} values of 4.71 and 5.91 μM, respectively. Compound **5** showed no significant cytotoxicity (IC_{50} > 50 μM) against HeLa and MCF-7 cell lines. Compounds **7**, **8** and **11** were also inactive against different cell lines: **7** *vs.* MDA-MB-435, HepG2, MCF-7 and A549 cell lines; **8** *vs.* HepG2, MCF-7 and A549 cell lines; **11** *vs.* HepG2 and MCF-7 cell lines. For all tested cell lines, the configurations at C-25 (25*R*, 25*S* and 25, 27-double bond) in the aglycone can be deduced to be unrelated to the cytotoxicity of compounds containing β-D-glu-(1 → 2)-[β-D-xyl-(1 → 3)]-β-D-xyl or β-D-glu-(1 → 2)-[β-D-xyl-(1 → 3)]-β-D-glu sugar chains at C-1 because of the almost similar IC_{50} values of **1–4** and **8** *vs.* all cell lines. The C-25*R* compounds containing a β-D-glu-(1 → 2)-[β-D-ara-(1 → 3)]-β-D-fuc sugar chain were less active than their epimers (**6** and **7** *vs.* all cell lines). Interestingly, our cytotoxicity data suggest that the C-25 configuration does not influence the cytotoxicity of compounds containing β-D-rha-(1 → 2)-[β-D-xyl-(1 → 3)]-β-D-glu or β-D-glu-(1 → 2)-[β-D-xyl-(1 → 3)]-β-D-fuc sugar chains against the 95D cell line (**5**, **9**, **13**, **10–12** *vs.* 95D). However, these C-25*R* compounds were less active than their epimers against HeLa, MCF-7 and A549 cell lines (**5**, **13**; **11**, **12** *vs.* HeLa, MCF-7 and A549). Additionally, for HeLa and HepG2 cell lines, C-25*S* compounds containing a β-D-glu-(1 → 2)-[β-D-xyl-(1 → 3)]-β-D-fuc sugar chain displayed similar cytotoxicity to those of 25, 27-double bond compounds (**10** and **12** *vs.* HeLa and HepG2). In summary, the configuration at C-25 in the aglycone and the sugar chain may together determine the cytotoxicity of steroidal saponins, and further studies are required to define the underlying chemical and biological mechanisms.

Experimental section

General

Optical rotations were measured using a JASCO P-1020 digital polarimeter (JASCO Corporation, Easton, MD, USA). IR data (KBr disks, in cm^{-1}) were recorded on a Perkin-Elmer FT-IR Spectra 400 (Perkin-Elmer, Norwalk, CT, USA). NMR spectra using pyridine-d_5 as the solvent were recorded using a Bruker Avance 500 NMR spectrometer (Bruker Corporation, Faellanden, Switzerland)

Table 5 Cytotoxic effects of compounds **1–13** on human cancer cells (n = 6)

	IC_{50} (means ± SD, μM)					
Compounds	MDA-MB-435	95D	HepG2	HeLa	MCF-7	A549
1	15.99 ± 1.03	20.13 ± 1.18	49.68 ± 1.57	39.98 ± 1.20	47.30 ± 1.56	36.35 ± 1.39
2	26.01 ± 0.85	30.00 ± 0.51	40.52 ± 0.96	33.42 ± 1.39	39.12 ± 1.02	36.01 ± 1.31
3	18.07 ± 1.34	25.67 ± 0.41	37.17 ± 1.71	21.58 ± 1.42	45.82 ± 1.44	43.53 ± 1.16
4	17.68 ± 2.50	17.83 ± 0.37	29.48 ± 1.64	22.23 ± 1.43	42.16 ± 1.26	43.20 ± 1.53
5	19.63 ± 0.76	10.82 ± 0.18	15.26 ± 1.29	NA[a]	NA[a]	35.56 ± 1.46
6	16.34 ± 0.60	14.34 ± 0.33	27.10 ± 0.84	14.76 ± 0.52	35.21 ± 2.02	24.69 ± 0.76
7	NA[a]	22.15 ± 1.41	NA[a]	42.56 ± 3.75	NA[a]	NA[a]
8	24.52 ± 0.91	36.12 ± 1.08	NA[a]	24.30 ± 1.55	NA[a]	NA[a]
9	17.54 ± 1.39	11.09 ± 0.15	—[b]	—[b]	—[b]	—[b]
10	9.74 ± 0.62	10.64 ± 0.21	15.48 ± 0.52	11.02 ± 0.42	10.02 ± 0.73	21.25 ± 1.42
11	4.71 ± 0.75	11.62 ± 2.00	NA[a]	26.36 ± 2.01	NA	23.56 ± 2.64
12	5.91 ± 0.27	11.20 ± 0.17	12.76 ± 0.74	8.00 ± 0.45	17.88 ± 0.97	8.226 ± 0.78
13	9.75 ± 0.34	19.58 ± 0.67	15.24 ± 1.53	14.03 ± 0.61	16.30 ± 0.73	13.99 ± 0.64
5-Fluorouracil	116.8 ± 13.93	83.55 ± 10.66	91.9 ± 16.20	251.3 ± 19.93	568.3 ± 54.37	244.8 ± 21.23

[a] No activity (IC_{50} > 50 μM). [b] Not measured due to insufficient amount of compounds.

with tetramethylsilane as the internal standard. ESI-MS, HRESI-TOF-MS, and HRESI-QTOF-MS experiments were performed on an Agilent 1100 Series MSD Trap mass spectrometer (Agilent Technologies, Santa Clara, CA, USA), Agilent 6210 ESITOF spectrometer, and Agilent 6520 ESIQTOF spectrometer, respectively. Silica gel (100–200 mesh and 200–300 mesh; Qingdao Marine Chemical Factory, Qingdao, China), D101 macroporous resin (Tianjin Pesticide Co., Tianjin, China), and YMC RP-C18 (50 μm, YMC, Tokyo, Japan) were used for column chromatography (CC). Preparative high-performance liquid chromatography (HPLC) was carried out using an Agilent 1100 Series HPLC instrument equipped with a diode array detector. Fractions obtained from CC were analyzed by TLC using silica gel GF254 (Qingdao Marine Chemical Factory, Qingdao, China) plates. GC analysis was conducted on an Agilent 6890 gas chromatograph. L-Cysteine methyl ester hydrochloride, trimethylchlorosilane, hexamethyldisilazane, standard D-glucose, D-fucose, L-rhamnose, L-arabinose, and D-xylose were purchased from Sigma-Aldrich Trading Co. Ltd. (Shanghai, China).

Plant material

Dried roots of *L. muscari* were purchased from Fujian Province, People's Republic of China, in October 2014, and authenticated by one of the authors (B. Y, Y.). A voucher specimen (No. 20141010) was deposited at the herbarium of Jiangsu Key Laboratory of TCM Evaluation and Translational Research, China Pharmaceutical University, Nanjing, China.

Extraction and isolation

Dried roots of *L. muscari* (10 kg) were crushed and extracted with 70% EtOH (2 h, 3 × 80 L), and then the extract was concentrated under vacuum to afford a residue. The residue was suspended in 20% EtOH and subjected to D101 macroporous resin CC, eluted successively with EtOH–H_2O (20 : 80, 85 : 15, 95 : 5, v/v) to afford a saponin-rich fraction (85% EtOH eluting fraction, 100 g).

The saponin-rich fraction was subjected to silica gel CC eluted with a gradient of $CHCl_3$–MeOH–H_2O (90 : 10 : 1 to 30 : 70 : 5, v/v) to afford three fractions (Fr. 1–3). Fr. 1 was chromatographed on a silica gel column, eluted with a gradient of $CHCl_3$–MeOH–H_2O (85 : 15 : 1.5 to 70 : 30 : 3, v/v), to give two sub-fractions (Fr. 1a, 1b). Fr. 2 was chromatographed on an ODS column with a gradient of MeOH–H_2O (40 : 60 to 70 : 30, v/v) as the mobile phase to afford two sub-fractions (Fr. 2a, 2b). Fr. 1a was further purified using an Agela Venusil PAH Prep C-18 column, eluted with CH_3CN–H_2O (45 : 55, v/v) to yield compounds **1** (15 mg), **2** (20 mg), **3** (20 mg) and **4** (30 mg). Fr. 1b was further purified using an Agela Venusil PAH Prep C-18 column, eluted with CH_3CN–H_2O (50 : 50, v/v) to yield compounds **5** (50 mg), **6** (10 mg), **7** (30 mg) and **13** (40 mg). Fr. 2a was further purified using an Agela Venusil PAH Prep C-18 column, eluted with CH_3CN–H_2O (45 : 55, v/v) to yield compounds **8** (60 mg), **11** (300 mg) and **12** (200 mg). Compounds **9** (8 mg) and **10** (10 mg) were isolated from Fr. 2b using an Agela Venusil PAH Prep C-18 column, eluted with CH_3CN–H_2O (50 : 50, v/v).

Characterization of new compounds

Compound 1. White amorphous powder; $[\alpha]_{23}^{D}$ −46.89 (c 0.10, MeOH); IR (KBr) ν_{max}: 3424 (OH), 2905 (CH), 987, 920, 896, 848 (intensity 920 > 896, (25*S*)-spiroketal) cm^{-1}; ^{1}H-NMR (pyridine-d_5, 500 MHz) data and ^{13}C-NMR (pyridine-d_5, 300 MHz) data: see Tables 1–4; ESI-MS *m*/*z* 909 $[M + Na]^+$; HRESI-QTOF-MS *m*/*z* 909.4456 $[M + Na]^+$ (calcd for $C_{44}H_{70}O_{18}Na$, 909.4454).

Compound 2. White amorphous powder; $[\alpha]_{23}^{D}$ −56.27 (c 0.10, MeOH); IR (KBr) ν_{max}: 3476 (OH), 2950 (CH), 982, 921, 902, 870 (intensity 902 > 921, (25*R*)-spiroketal) cm^{-1}; ^{1}H-NMR (pyridine-d_5, 500 MHz) data and ^{13}C-NMR (pyridine-d_5, 300 MHz) data: see Tables 1–4; ESI-MS *m*/*z* 909 $[M + Na]^+$; HRESI-QTOF-MS *m*/*z* 909.4454 $[M + Na]^+$ (calcd for $C_{44}H_{70}O_{18}Na$, 909.4454).

Compound 3. White amorphous powder; $[\alpha]_{23}^{D}$ −43.48 (c 0.10, MeOH); IR (KBr) ν_{max}: 3441 (OH), 2905 (CH), 987, 920, 896, 848 (intensity 920 > 896, (25*S*)-spiroketal) cm^{-1}; ^{1}H-NMR (pyridine-d_5, 500 MHz) data and ^{13}C-NMR (pyridine-d_5, 300 MHz) data: see Tables 1–4; ESI-MS *m*/*z* 879 $[M + Na]^+$; HRESI-QTOF-MS *m*/*z* 879.4344 $[M + Na]^+$ (calcd for $C_{43}H_{68}O_{17}Na$, 879.4349).

Compound 4. White amorphous powder; $[\alpha]_{23}^{D}$ −59.73 (c 0.10, MeOH); IR (KBr) ν_{max}: 3449 (OH), 2950 (CH), 982, 921, 902, 870 (intensity 902 > 921, (25*R*)-spiroketal) cm^{-1}; ^{1}H-NMR (pyridine-d_5, 500 MHz) data and ^{13}C-NMR (pyridine-d_5, 300 MHz) data: see Tables 1–4; ESI-MS *m*/*z* 879 $[M + Na]^+$; HRESI-QTOF-MS *m*/*z* 879.4372 $[M + Na]^+$ (calcd for $C_{43}H_{68}O_{17}Na$, 879.4349).

Compound 5. White amorphous powder; $[\alpha]_{23}^{D}$ −67.28 (c 0.10, MeOH); IR (KBr) ν_{max}: 3416 (OH), 2903 (CH), 984, 920, 900, 865 (intensity 900 > 920, (25*R*)-spiroketal) cm^{-1}; ^{1}H-NMR (pyridine-d_5, 500 MHz) data and ^{13}C-NMR (pyridine-d_5, 300 MHz) data: see Tables 1–4; ESI-MS *m*/*z* 893 $[M + Na]^+$; HRESI-QTOF-MS *m*/*z* 871.4702 $[M + H]^+$ (calcd for $C_{44}H_{70}O_{17}Na$, 871.4686).

Compound 6. White amorphous powder; $[\alpha]_{23}^{D}$ −55.86 (c 0.10, MeOH); IR (KBr) ν_{max}: 3424 (OH), 2905 (CH), 987, 920, 896, 848 (intensity 920 > 896, (25*S*)-spiroketal) cm^{-1}; ^{1}H-NMR (pyridine-d_5, 500 MHz) data and ^{13}C-NMR (pyridine-d_5, 300 MHz) data: see Tables 1–4; ESI-MS *m*/*z* 893 $[M + Na]^+$; HRESI-QTOF-MS *m*/*z* 893.4461 $[M + Na]^+$ (calcd for $C_{44}H_{70}O_{17}Na$, 893.4505).

Compound 7. White amorphous powder; $[\alpha]_{23}^{D}$ −67.59 (c 0.10, MeOH); IR (KBr) ν_{max}: 3385 (OH), 2907 (CH), 982, 920, 900, 864 (intensity 900 > 920, (25*R*)-spiroketal) cm^{-1}; ^{1}H-NMR (pyridine-d_5, 500 MHz) data and ^{13}C-NMR (pyridine-d_5, 300 MHz) data: see Tables 1–4; ESI-MS *m*/*z* 893 $[M + Na]^+$; HRESI-QTOF-MS *m*/*z* 893.4462 $[M + Na]^+$ (calcd for $C_{44}H_{70}O_{17}Na$, 893.4505).

Compound 8. White amorphous powder; $[\alpha]_{23}D$ −65.48 (c 0.10, MeOH); IR (KBr) ν_{max}: 3452 (OH), 2944, 2844, 1064 (CH), 1048, 983, 922, 895, 838 cm^{-1}; ^{1}H-NMR (pyridine-d_5, 500 MHz) data and ^{13}C-NMR (pyridine-d_5, 300 MHz) data: see Tables 1–4; ESI-MS *m*/*z* 877 $[M + Na]^+$; HRESI-QTOF-MS *m*/*z* 877.4189 $[M + Na]^+$ (calcd for $C_{43}H_{66}O_{17}Na$, 877.4192).

Compound 9. White amorphous powder; $[\alpha]_{23}^{D}$ −60.83 (c 0.10, MeOH); IR (KBr) ν_{max}: 3443 (OH), 2937, 2849, 1062 (CH), 1049, 985, 922, 901, 843 cm^{-1}; ^{1}H-NMR (pyridine-d_5, 500 MHz) data and ^{13}C-NMR (pyridine-d_5, 300 MHz) data: see Tables 1–4;

ESI-MS *m/z* 891 $[M + Na]^+$; HRESI-QTOF-MS *m/z* 869.4529 $[M + H]^+$ (calcd for $C_{44}H_{69}O_{17}$, 869.4529).

Compound 10. White amorphous powder; $[\alpha]_{23}^{D}$ −57.71 (c 0.10, MeOH); IR (KBr) ν_{max}: 3441 (OH), 2937, 2849, 1062 (CH), 1049, 985, 922, 901, 844 cm^{-1}; ^{1}H-NMR (pyridine-d_5, 500 MHz) data and ^{13}C-NMR (pyridine-d_5, 300 MHz) data: see Tables 1–4; ESI-MS *m/z* 891 $[M + Na]^+$; HRESI-QTOF-MS *m/z* 891.4320 $[M + Na]^+$ (calcd for $C_{44}H_{68}O_{17}Na$, 891.4349).

Acid hydrolysis of compounds 1–10

Each compound (2 mg) was refluxed with 2 mL of 2 M HCl (dioxane–H_2O, 1 : 1) at 100 °C for 4 h. The solution was diluted with H_2O (2 mL) and extracted with EtOAc (1 mL × 3) after the removal of dioxane. The aqueous layer was evaporated under vacuum, and the residue was diluted with H_2O (50 mL). This procedure was repeated until a neutral residue was obtained (10–15 times), and then it was analyzed by TLC over silica gel (Me_2CO–*n*-BuOH–H_2O, 6 : 3 : 1) together with authentic sugar samples. The remaining residue was dissolved in pyridine (300 μL), and L-cysteine methyl ester hydrochloride (4 mg) was added to it. The mixture was kept at 60 °C in an oil bath for 1.5 h. Then 300 μL of hexamethyldisilazane–trimethylchlorosilane (2 : 1) was added and the mixture was heated at 60 °C in an oil bath for a further 30 min. After centrifugation, the supernatant was analyzed by GC under the following conditions: capillary column, HP-5 (0.32 mm × 30 m × 0.5 μm); flame ionization detection; detector temperature, 280 °C; injection temperature, 250 °C; initial temperature, 200 °C and an initial time of 8 min, 6 °C min^{-1} to 260 °C and then held for 2 min; carrier, N_2; split ratio, 1/50. The monosaccharides present in compounds **1**, **2**, **3**, **4** and **8** were confirmed to be D-glucose and D-xylose by comparing the retention times (T_R) of monosaccharide derivatives with the derivatives prepared similarly from standard sugars. In the same procedure, D-glucose, L-rhamnose and D-xylose were identified from compounds **6** and **7**, D-glucose, L-arabinose and D-fucose for compound **5**, and D-glucose, D-xylose and D-fucose from compound **10**.

Cytotoxicity assay

Cell lines and culture. MDA-MB-435, 95D, HepG2, HeLa, MCF-7 and A549 cells were obtained from the Cell Bank of the Shanghai Institute of Chinese Academy of Sciences and cultured with high glucose DMEM medium or RPMI 1640 medium basic (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Life Technologies Corporation, Carlsbad, CA, USA). All cells were maintained in the logarithmic phase under a humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

MTT assay. Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method as described in the literature.[7] The cells were seeded in 96-well plates at a density of 1×10^4 cells with 100 μL culture medium per well for 12 h. Aliquots of fresh medium (100 μL) containing test compounds (0–100 μM) were added to each well. The medium was removed after an additional 48 h of cultivation in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. An aliquot (100 μL) of fresh medium containing 0.5 mg mL^{-1} MTT was added to the plate, and the cells were cultured for an additional 3 h. The medium solution was then removed, and an aliquot of DMSO (150 μL) added to the plate. The optical density of the formazan solution was analyzed by detection of the absorbance at 570 nm and reference wavelength of 650 nm on a microplate reader.

Conclusions

In this work, ten new steroidal saponins (**1–10**) and three known steroidal saponins (**11–13**) were isolated from the root of *L. muscari*. Their structures were determined using IR, NMR and mass spectroscopic data. These compounds exhibited different cytotoxic activity against six cancer cell lines *in vitro*. Structure–activity analysis of these related compounds revealed that the C-25 configuration of the aglycone moiety and the sugar chain may together determine the cytotoxicity of steroidal saponins. These results provide a basis for evaluating the structure–activity relationships of other steroidal saponins, as well as for developing these compounds as potential anticancer drugs.

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