Two New C_{21} Steroidal Glycosides from *Marsdenia tenacissima* (Roxb.) Wight *et* Arn

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Two new C_{21} steroidal glycosides, tenacissoside L (1), tenacissoside M (2), were isolated from the stems of *Marsdenia tenacissima* (Roxb.) Wight *et* Arn. Their structures were elucidated, respectively, by means of chemical and spectral data, including ESI-MS, HR-ESI-MS, 1D-NMR and 2D-NMR.

Key words Asclepiadaceae; *Marsdenia tenacissima*; C_{21} steroidal glycoside; tenacissoside L; tenacissoside M; anti-cancer

Marsdenia tenacissima (Roxb.) Wight *et* Arn, which grows mainly in Southwest China, has been widely employed to treat inflammation, asthma and cancer.[1)] Previous studies have revealed the structural determination of 14 C_{21} steroidal glycosides,[2—5)] but so far little research on the bioactive compounds has been carried out. Only one article mentioned that their isolates expressed cytotoxicity to the KB cell line.[7)] Our continuing investigations seeking new bioactive compounds from the stems of *Marsdenia tenacissima* (Roxb.) Wight *et* Arn have now led to the isolation of two other new C_{21} steroidal glycosides, tenacissoside L (**1**) and tenacissoside M (**2**). Through MTT experiments, we concluded that **1** and **2** inhibit the proliferation of some tumor cell lines *in vitro*, such as C-26 Colon Carcinoma cell line and Hepal-6 Mouse Hepatic Carcinoma cell line. As summarized in Table 2, the ID_{50} varies since the two compounds demonstrate different sensitivity to the different cell lines. Structural differences of the two compounds also results in different inhibition to the same cell line, thus the differing IC_{50}. With regard to the study of substance basis, this result, in some sense, illustrates why *Marsdenia tenacissima* is adopted as a key traditional Chinese medicine against cancer in folk therapy. The present report mainly describes the isolation and structural elucidation of these two new C_{21} steroidal glycosides.

Results and Discussion

Tenacissoside L (**1**) was obtained as an amorphous powder, Lieberman–Burchard and Keller–Kiliani reactions were positive,[6)] suggesting that it is a steroidal glycoside containing 2-deoxy-hexose. A molecular formula of $C_{42}H_{72}O_{16}$ was established based on a $[M+Na]^+$ peak at HR-ESI-MS *m/z* 855.4713 and ^1H and ^{13}C-NMR spectra (Table 1). Three methyl groups δ_H 1.33 (3H, s, C_{18}-CH_3), δ_C 10.6; δ_H 1.01 (3H, s, C_{19}-CH_3), δ_C 12.7 and δ_H 1.17 (3H, d, J=6.4 Hz, C_{21}-CH_3), δ_C 16.7, indicated its C_{21} steroidal skeleton.[7)] Furthermore, on acid hydrolysis, **3** was separated on silica gel. The ^{13}C-NMR spectrum of **3** (Table 1) was the same as dihydrosarcostin reported before.[8)] Thus, the aglycone moiety was determined to be dihydrosarcostin.

On acid hydrolysis, β-D-cymaropyranose and β-D-thevetopyranose were detected by TLC analysis with authentic samples. Also, the ^1H and ^{13}C-NMR spectra showed three anomeric proton and carbon signals, δ_H 4.86 (1H, dd, J=9.6, 1.6 Hz), δ_C 95.6; δ_H 4.76 (1H, dd, J=9.6, 1.6 Hz), δ_C 99.6 and δ_H 4.31 (1H, d, J=7.6 Hz), δ_C 104.4, along with three 6-methyl groups, δ_H 1.23, 1.28, 1.31 (each 3H, s), δ_C 18.3, 18.4, 17.8 and three methoxyls signals δ_H 3.43, 3.44, 3.66 (each 3H, s), δ_C 58.0, 58.0, 60.7. The ^1H and ^{13}C-NMR spectra are the same as those in previous studies.[9,10)] So, the sugar moieties were comprised of two moles of β-D-cymaropyranoses and one of β-D-thevetopyranose. In addition, all the glycosidic linkages were suggested to be in β-form since the coupling constants of three anomeric proton signals were all in the range of 7—10 Hz.

In the HMBC spectrum, the sugar sequence was determined to be thv (S_3)-cym (S_2)-cym (S_1)-aglycone due to ^1H–^{13}C long-range correlations, as follows, C_3 (δ_H 3.81)→S_1-C_1 (δ_C 95.6), S_1-C_1 (δ_H 4.86)→C_3 (δ_C 77.4); S_1-C_4 (δ_H 3.22)→S_2-C_1 (δ_C 99.6), S_2-C_1 (δ_H 4.76)→S_1-C_4 (δ_C 82.6) and S_2-C_4 (δ_H 3.27)→S_3-C_1 (δ_C 104.4), S_3-C_1 (δ_H 4.31)→S_2-C_4 (δ_C 82.5). The sugar chain was located at C_3 according to glycosylation shifts,[2,11)] δ_{C2} (−3.1 ppm), δ_{C3} (+6.5 ppm) and δ_{C4} (−5.0 ppm) compared with dihydrosarcostin.

Based on the above evidence, the structure of **1** has been established as dihydrosarcostin-3-*O*-β-D-thevetopyranosyl-(1→4)-β-D-cymaropyranosyl-(1→4)-β-D-cymaropyranoside (Fig. 1).

Tenacissoside M (**2**) was obtained as an amorphous powder. Positive Lieberman–Burchard and Keller–Kiliani tests indicated that it was a steroidal glycoside containing 2-deoxy-hexose. The formula $C_{44}H_{74}O_{17}$ was determined by a $[M+Na]^+$ peak at HR-ESI-MS *m/z* 897.4818 and NMR data (Table 1). The ^{13}C-NMR spectrum of **2** showed a resemblance to **1** except that **2** had additional acetyl group signals, δ_H 2.03 (3H, s, $C_{2'}$-CH_3), $\delta_{C2'}$ 21.3 and $\delta_{C1'}$ 169.8. ESI-MS-MS provided further evidence of the presence of an acetyl group due to a $[M-C_2H_3O_2]^+$ peak at *m/z* 837.4. The acetyl group as a substituent was assigned to the 20-hydroxyl group, as judged from the 3-bond correlation between C_{20} (δ_H 5.16) and $C_{1'}$ (δ_C 169.8). Moreover, an acetylation shift was observed at δ_{C20} (+2.4 ppm), and the ^1H-signal of C_{20} shifted down-field to δ_H 5.16. The chiral center C_{20} must be in the *S*-configuration, as judged from the NOES between H_{12} (δ_H 3.37) and H_{20} (δ_H 5.16), H_{20} (δ_H 5.16) and H_{16} ($\delta_{H\alpha}$ 1.72), and H_{21} (δ_H 1.23) and H_{16} ($\delta_{H\beta}$ 1.87). The remaining ^1H and ^{13}C-NMR spectra were the same as those of **1**. Consequently, the aglycone moiety was elucidated as 20-(*S*)-*O*-

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Table 1. NMR Spectral Data of **1** and **2** (in $CDCl_3$)
Aglycone moiety

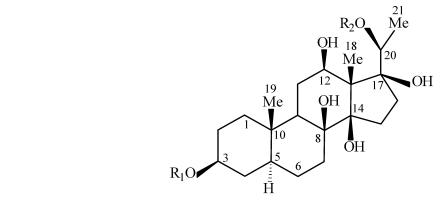
No.	Tenacissoside L (**1**)		Tenacissoside M (**2**)		HMBC ($^1H \rightarrow ^{13}C$)	(**3**)
	δ_H (J=Hz)	δ_C	δ_H (J=Hz)	δ_C		δ_C
1	1.57 m (β)	38.1 t	1.58 m (β)	37.9 t	C5, C9	38.4
	1.85 hidden (α)		1.87 hidden (α)			
2	1.54 m (α)	28.9 t	1.54 m (α)	28.7 t	C3, C4	32.0
	1.84 hidden (β)		1.88 hidden (β)			
3	3.81 (br)	77.4 d	3.81 (br)	76.9 d	S1-C1	70.9
4	1.25 hidden (α)	33.9 t	1.25 hidden (α)	33.8 t	C3, C10	38.9
	1.60 m (β)		1.62 m (β)			
5	1.10 (dd, J=13.0, 2.6)	45.3 d	1.08 (dd, J=12.8, 2.6)	45.1 d	C10, C19	41.5
6	1.22 hidden (α)	24.6 t	1.24 hidden (α)	24.4 t	C8, C10	26.8
	1.63 hidden (β)		1.67 hidden (β)			
7	1.34 m (β)	27.5 t	1.34 m (β)	27.9 t	C6, C8, C14	26.3
	1.80 m (α)		1.79 m (α)			
8	—	75.5 s	—	75.7 s	—	76.2
9	1.18 hidden	46.8 d	1.14 m	46.7 d	C10, C12, C14, C19	47.3
10	—	36.2 s	—	36.0 s	—	36.5
11	1.64 m (α)	34.3 t	1.67 m (α)	33.9 t	C8, C9, C13, C14	34.2
	1.84 m (β)		1.87 μ (β)			
12	3.37 (t, J=2.8)	71.6 d	3.37 (t, J=2.8)	70.8 d	C13, C14	72.2
13	—	58.2 s	—	58.1 s	—	59.0
14	—	88.1 s	—	87.7 s	—	88.6
15	1.73 m (β)	33.2 t	1.71 m (β)	33.7 t	C8, C14, C16, C17	33.8
	1.84 hidden (α)		1.87 m (α)			
16	1.73 m (α)	32.5 t	1.72 m (α)	32.0 t	C13, C15, C17	33.2
	1.84 m (β)		1.87 m (β)			
17	—	88.2 s	—	88.7 s	—	88.2
18	1.33 (3H, s)	10.6 q	1.31 (3H, s)	9.26 q	C12, C13, C14, C17, C20	11.0
19	1.01 (3H, s)	12.7 q	0.96 (3H, s)	12.5 q	C1, C5, C9, C10	13.0
20	4.05 (q, J=6.4)	72.4 d	5.16 (q, J=6.4)	74.8 d	C21	71.8
21	1.17 (3H, d, J=6.4)	16.7 q	1.23 (3H, d, J=6.4)	14.8 q	C17, C20	17.1
20-(*S*)-*O*-acetyl						
1′	—	—	—	169.8 s	—	
2′	—	—	2.03 (3H, s)	21.3 q	C1′	

Sugar moiety

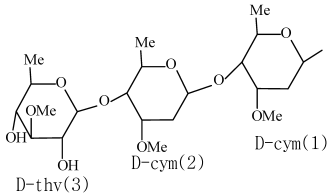
No.	Tenacissoside L (**1**)		Tenacissoside M (**2**)		HMBC ($^1H \rightarrow ^{13}C$)
	δ_H (J=Hz)	δ_C	δ_H (J=Hz)	δ_C	
Cym (1)					
1	4.86 (dd, J=9.6, 1.6)	95.6 d	4.85 (dd, J=9.6, 1.6)	95.4 d	C3, S1-C2
2	1.54 m (β)	35.6 t	1.54 m (β)	35.4 t	S1-C1, S1-C3, S1-C4
	2.07 m (α)		2.06 br (α)		
3	3.50 br	77.1 d	3.61 br	76.9 d	S1-OCH_3
4	3.22 (dd, J=9.6, 2.8)	82.6 d	3.22 (dd, J=9.6, 2.4)	82.5 d	S1-OCH_3, S1-C5, S1-C6, S2-C1
5	3.84 br	68.6 d	3.84 br	68.4 d	S1-C1, S1-C4, S1-C6
6	1.23 (3H, d, J=6.4)	18.3 q	1.22 (3H, d, J=6.4)	18.1 q	S1-C4, S1-C5
3-OCH_3	3.43 (3H, s)	58.0 q	3.43 (3H, s)	57.9 q	S1-C3
Cym (2)					
1	4.76 (dd, J=9.6, 1.6)	99.6 d	4.76 (dd, J=9.6, 1.6)	99.5 d	
2	1.60 m (β)	35.2 t	1.62 m (β)	35.1 t	S2-C1, S2-C3, S2-C4
	2.14 br (α)		2.13 br (α)		
3	3.53 br	76.9 d	3.50 (t, J=8.4)	76.7 d	S2-OCH_3
4	3.27 (dd, J=9.6, 2.8)	82.5 d	3.27 (dd, J=9.6, 2.8)	82.4 d	S2-OCH_3, S2-C5, S2-C6, S3-C1
5	3.91 br	68.5 d	3.92 br	68.3 d	S2-C1, S2-C4, S2-C6
6	1.28 (3H, d, J=6.0)	18.4 q	1.28 (3H, d, J=6.0)	18.2 q	S2-C4, S2-C5
3-OCH_3	3.44 (3H, s)	58.0 q	3.44 (3H, s)	57.8 q	S2-C3
Thv (3)					
1	4.31 (d, J=7.6)	104.4 d	4.30 (d, J=7.6)	104.2 d	
2	3.61 br	74.5 d	3.61 (br)	74.4 d	S3-C3, S3-C4
3	3.11 (t, J=8.8)	85.6 d	3.10 (t, J=8.8)	85.4 d	S3-OCH_3, S3-C2, S3-C4
4	3.19 (t, J=8.8)	74.7 d	3.19 (t, J=8.8)	74.6 d	S3-C3, S3-C5, S3-C6
5	3.37 hidden	71.7 d	3.37 hidden	71.5 d	S3-C1, S3-C3, S3-C6
6	1.31 (3H, d, J=6.0)	17.8 q	1.31 (3H, d, J=6.0)	17.7 q	S3-C4, S3-C5
3-OCH_3	3.66 (3H, s)	60.7 q	3.66 (3H, s)	60.6 q	S3-C3, S3-C4

Table 2. Suppressing Tumor Cells Proliferation Data of **1** and **2**

Compound tested	Cell line tested	IC_{50} ($\mu g \cdot ml^{-1}$)
Tenacissoside L (**1**)	C-26	252.1
	Hepal-6	162.8
Tenacissoside M (**2**)	C-26	166.6
	Hepal-6	105.4



S =



1 R_1 = S R_2 = H

2 R_1 = S R_2 = Ac

3 R_1 = H R_2 = H

Fig. 1. The Structures of Compounds **1**—**3**

acetyl-dihydrosarcostin.

The sugar moieties of **2** were identifed as β-D-cymaropyranoses and β-D-thevetopyranose by characteristic signals in ^{1}H and ^{13}C-NMR spectra: three anomeric proton and carbon signals at δ_H 4.85 (1H, dd, J=9.6, 1.6 Hz), δ_C 95.4; δ_H 4.76 (1H, dd, J=9.6, 1.6 Hz), δ_C 99.5 and δ_H 4.30 (1H, d, J=7.6 Hz), δ_C 104.2, along with three 6-methyl groups (δ_H 1.22, 1.28, 1.31, each 3H, s; δ_C 18.1, 18.2, 17.7) and three methoxyl groups (δ_H 3.43, 3.44, 3.66, each 3H, s; δ_C 57.9, 57.8, 60.6). The NMBC spectra due to the sugar moieties, C_3 (δ_H 3.81)→S_1-C_1 (δ_C 95.4), S_1-C_1 (δ_H 4.85)→C_3 (δ_C 76.9); S_1-C_4 (δ_H 3.22)→S_2-C_1 (δ_C 99.5), S_2-C_1 (δ_H 4.76)→S_1-C_4 (δ_C 82.5) and S_2-C_4 (δ_H 3.27)→$_3$-C_1 (δ_C 104.2), S_3-C_1 (δ_H 4.30)→S_2-C_4 (δ_C 82.4), coincided exactly with those of **1**, so that the sugar moiety of **2** was considered to share the same sequence as **1**. Finally, the sugar chain was presumed to be attached to the 3-hydroxyl group because of glycosylation shifts, C_2 (−3.3), C_3 (+6.0) and C_4 (−5.1) as compared with **3**. Therefore, the structure of **2** was determined to be 20-(*S*)-*O*-acetyl-dihydrosarcostin-3-*O*-β-D-thevetopyranosyl-(1→4)-β-D-cymaropyranosyl-(1→4)-β-D-cymaropyranoside (Fig. 1).

Experimental

General Procedure Melting points were recorded on a Kofler hot stage apparatus and were uncorrected. Optical rotations were determined on a Perkin-Elmer 341 polarimeter. IR spectrum was obtained in KBr on a Nicolet FT-IR 200SXY spectrophotometer. ^{1}H and ^{13}C-NMR spectra were measured on a Varian Unity INOVA 400/45 NMR spectrometer in $CDCl_3$ with TMS as the internal standard. HR-EI-MS were measured on a Bruker Daltonios BioTOF Q. Column chromatography was carried out on macroporous adsorption resin (D141, Chenguang Chemical Industry Academy), silica gel H (Qindao Sea Chemical Factory), FUJI (ODS-Q_3) gel (50 μm, Mitsubishi Chemical Company). TLC analysis were performed on silica gel and RP-18 F_{254} plates with the following solvent systems, 1: CH_3OH–$CHCl_3$, 2: cyclohexane–acetone and 3: H_2O–CH_3OH. Spots were detected with 10% H_2SO_4 reagent. Abbreviations are used for sugars in the report: cym for β-D-cymaropyranose and thv for β-D-thevetopyranose.

Plant Material The *Marsdenia tenacissima* was collected in Jiulonggou Chongzhou district, Sichuan province, China, in June 2003, and was identified as *Marsdenia tenacissima* by Professor Shu Wang of West China College of Pharmacy, Sichuan University.

Extraction and Isolation Fresh stems of *Marsdenia tenacissima* (15 kg) were dried, cut into small chips and decocted in water. The dark water solution obtained was adsorbed on macroporous adsorption resin (D 141), then 10%, 30%, 50% and 70% EtOH were used sequentially for elution. The elution of 70% EtOH was evaporated under reduced pressure to produce a yellow crude glycoside mixture (10 g), which showed positive Lieberman–Burchard and Keller–Kiliani reactions. The crude glycoside mixture was subjected to silica gel with a solvent of $CHCl_3$–CH_3OH (99 : 1—1 : 1) to afford six parts. Part A (3.0 g) was chromatographed on FUJI (ODS-Q_3) gel with CH_3OH–H_2O (5 : 5—8 : 2) to give fraction I (mainly **1**) and fraction II (mainly **2**). Fraction I and II were rechromatographed, respectively, on silica gel and eluted with cyclohexane–acetone (2 : 1) to yield pure **1** (100 mg) and **2** (110 mg).

Tenacissoside L (**1**): White amorphous powder, mp 158—162 °C; $[\alpha]_D^{20}$ +45.2° (c=0.5, $CHCl_3$). IR (KBr) cm^{-1}: 3440, 2935, 1636, 1086; ^{1}H and ^{13}C-NMR see Table 1; HR-ESI-MS *m/z*: 855.4713 $[M+Na]^+$ Calcd for $C_{42}H_{72}O_{16}$+Na, 855.4698.

Tenacissoside M (**2**): White amorphous powder, mp 154—156 °C; $[\alpha]_D^{20}$ +52.0° (c=0.5, $CHCl_3$). IR (KBr) cm^{-1}: 3447, 2935, 1719, 1637, 1087; ^{1}H and ^{13}C-NMR see Table 1; HR-ESI-MS *m/z*: 897.4818 $[M+Na]^+$ Calcd for $C_{44}H_{74}O_{17}$+Na, 897.4803.

Acid Hydrolysis of Compunds 1 and 2 Each compound (50 mg) was dissolved in 5 ml MeOH and 1 ml 1% H_2SO_4, and heated with stirring at 60 °C for 60 min. After cooling, the reaction mixture was diluted with H_2O and extracted with $CHCl_3$. The $CHCl_3$ phase was evaporated in a vacuum to afford $CHCl_3$ extract. Each $CHCl_3$ extract was chromatographed on silica gel H to give **3**, identified as dihydrosarcostin by ^{13}C-NMR data (see Table 1).

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