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Identification of new qingyangshengenin and caudatin glycosides from the roots of *Cynanchum otophyllum*

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ABSTRACT

HPLC analysis of the roots of *Cynanchum otophyllum* Scheind (Asclepiadaceae) led to the isolation of six new pregnane glycosides, specifically otophyllosides N-P (**2**–**4**) and otophyllosides Q-S (**7**–**9**), in addition to the identification of three known C-21 steroidal glycosides, otophylloside A (**1**), otophylloside B (**5**) and caudatin 3-*O*-β-D-glucopyranosyl-(1→4)-β-D-oleandropyranosyl-(1→4)-β-D-cymaropyranosyl-(1→4)-β-D-cymaropyranoside (**6**). The structure of each glycoside was determined by detailed spectroscopic analysis and chemical methods. All compounds contain qingyangshengenin or caudatin aglycones and a straight sugar chain consisting of 4-7 hexosyl moieties with the mode of 1→4 linkage. The optically isomeric monosaccharides, D- and L-cymarose, coexisted in both otophyllosides R (**8**) and S (**9**).

1. Introduction

Cynanchum otophyllum Schneid (Chinese name Qingyangshen) has been used for the treatment of some nervous system and mental disorders, such as epilepsy, depression and Menier's syndrome. The C-21 steroidal glycosides are thought to be the bioactive principles [1–3]. Previously, we reported the isolation and structure elucidation of six pregnane glycosides from the roots [4]. In further studies, *Cynanchum otophyllum* was subjected to preparative HPLC and nine C-21 steroidal glycosides were purified, including six new otophyllosides N-S (**2**–**4** and **7**–**9**). Their structures were determined by detailed spectroscopic analysis and chemical methods. This paper describes the isolation and structural elucidation of the new compounds.

2. Experimental

2.1. General methods

Melting points were measured on an XRC-I micromelting point apparatus and were uncorrected. Optical rotations were obtained using a JASCO P-1020 automatic digital polarimeter. IR spectra were determined using a Bruker Tensor 27 spectrometer in KBr pellets. NMR spectra were performed in C_5D_5N and were recorded on a Bruker DRX-500 instrument with TMS as the internal standard. MS data were detected on a VG Auto Spec-3000 spectrometer. Preparative HPLC was performed on Waters Delta 600-2487 Dual λ Absorbance Detector (Manual-injection, Analytical 7725i, Semi-prep 3725i-119). Silica gel HF_{254} prepared for TLC and silica gel (200–300 mesh) for column chromatography (CC) were obtained from Qingdao Marine Chemical Company (Qingdao, China). A preparative reverse phase C_{18} column (Agilent ZORBAX StableBond C-18 ⌀ 21.2 mm × 250 mm, 7 μm) and an analytical reversed phase C_{18} column (Agilent ZORBAX StableBond C-18 ⌀ 4.6 mm × 250 mm, 5 μm) were employed.

2.2. Plant material

The roots of *C. otophyllum* Schneid were collected in Eryuan County, in the northwest of Yunnan province of China, and identified by Prof. C.R. Yang (Kunming Institute of Botany, Chinese Academy of Sciences). An example specimen is available at the herbarium of Kunming Institute of Botany.

2.3. Extraction and isolation

The components of the air-dried roots of *C. otophyllum* (10 kg) were extracted by three consecutive treatments of 90% EtOH at room temperature. After removal of the organic solvent by vacuum, the residue was suspended in water and partitioned with $CHCl_3$ to yield a $CHCl_3$ fraction (155 g). As described in our previous paper [4], part of the $CHCl_3$ fraction (150 g) was subjected to a silica gel CC and eluted with $CHCl_3$–MeOH (10:1.5) to give five fractions (Frs.

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Table 1
^{13}C NMR data for the aglycone moieties of compounds **2–4** and **7–9** (δ in ppm, in C_5D_5N, 100 MHz).

No.	2	3	4	7	8	9	No.	2	3	4	7	8	9
1	39.3	39.3	39.3	39.3	39.2	39.2	15	33.9	33.9	33.9	33.9	33.8	33.8
2	29.9	29.9	29.9	29.9	29.9	29.9	16	33.2	33.2	33.2	33.0	33.0	33.0
3	77.6	77.6	77.7	77.7	77.6	77.7	17	92.5	92.5	92.5	92.4	92.4	92.4
4	38.9	38.9	38.9	39.0	39.0	39.0	18	10.9	10.9	10.9	10.8	10.8	10.8
5	139.4	139.4	139.4	139.3	139.3	139.3	19	18.2	18.2	18.2	18.2	18.2	18.2
6	119.2	119.2	119.2	119.3	119.2	119.2	20	209.9	209.9	209.8	209.5	209.5	209.5
7	34.8	34.8	34.8	34.8	34.8	34.8	21	27.9	27.9	27.8	27.6	27.6	27.6
8	74.3	74.3	74.4	74.3	74.3	74.3	1′	165.4	165.4	165.4	166.0	166.0	166.0
9	44.5	44.5	44.5	44.6	44.6	44.6	2′	122.0	122.0	122.0	114.2	114.2	114.2
10	37.4	37.4	37.4	37.4	37.4	37.4	3′	132.5	132.5	132.4	165.5	165.5	165.5
11	25.2	25.2	25.2	25.1	25.1	25.1	4′	116.2	116.3	116.2	38.2	38.2	38.2
12	73.4	73.4	73.4	72.6	72.6	72.6	5′	163.7	163.7	163.6	20.9	20.9	20.9
13	58.4	58.4	58.4	58.0	58.0	58.0	6′	116.2	116.3	116.2	21.0	20.9	20.9
14	89.6	89.6	89.6	89.5	89.5	89.5	7′	132.5	132.5	132.5	16.5	16.5	16.5

1–5). Further purification was focused on Fr. 1, 2 and 5, mainly by preparative HPLC. Fr. 1 (18.8 g) was separated by silica gel CC with $CHCl_3$–MeOH mixture (95:5, 9:1) to give three fractions (Frs. A–C). Fr. A was chromatographed on silica gel (petrol ether:acetone 2:1, 3:2) to yield **1** (23 mg) and **2** (10 mg). Fr. B was purified by preparative HPLC with MeOH:H_2O (82:18, v/v) to give **5** (8 mg). Fr. C was performed on preparative HPLC and was eluted with MeOH:H_2O (78:22, v/v) to yield **3** (29 mg). Fr. 2 (21.7 g) was subjected to silica gel ($CHCl_3$:MeOH 93:7, 9:1, 85:15) to give two fractions (Frs. I and II). Fr. I was subjected to silica gel (petrol ether:acetone 2:3, 1:2) to yield **8** (12 mg) and **9** (9 mg). Fr. II was purified by preparative HPLC with MeOH:H_2O (77:23, v/v) to give **6** (19 mg). Compounds **4** (18 mg) and **7** (14 mg) were obtained from the portions of Fr. 5 (20.0 g) by preparative HPLC purification through elution with MeOH:H_2O (82:18, v/v) and (65:35, v/v), respectively.

2.3.1. Otophylloside N (**2**)

White amorphous powder, mp 163–165 °C, $[\alpha]_D^{18}$ + 7.7° (*c* = 0.20, MeOH), IR (KBr) ν_{max} 3446, 2971, 2932, 1713, 1610, 1594, 1516, 1452, 1382, 1369, 1308, 1275, 1164, 1093, 1004, 912, 866, 852, 772 cm^{-1}. FAB-MS (negative ion mode) *m/z* 1061 $[M-1]^-$, HRESI-MS (negative ion mode) *m/z* 1061.5284 $[M(C_{55}H_{82}O_{20})-H]^-$ (calcd. 1061.5321). ^{1}H and ^{13}C NMR: see Tables 1–3.

2.3.2. Otophylloside O (**3**)

White amorphous powder, mp 157–160 °C, $[\alpha]_D^{18}$ + 20.3° (*c* = 0.19, MeOH), IR (KBr) ν_{max} 3455, 2972, 2934, 1713, 1610, 1594, 1516, 1451, 1382, 1368, 1309, 1275, 1164, 1096, 1059, 1004, 987, 913, 865, 853, 772 cm^{-1}. FAB-MS (negative ion mode) *m/z* 1076 $[M]^-$, 931 $[M-1-144]^-$. HRESI-MS (negative ion mode) *m/z* 1111.5239 $[M(C_{56}H_{84}O_{20})+Cl]^-$ (calcd. 1111.5244). ^{1}H and ^{13}C NMR: see Tables 1–3.

2.3.3. Otophylloside P (**4**)

White amorphous powder, mp 200–202 °C, $[\alpha]_D^{17}$ + 13.2° (*c* = 0.19, MeOH), IR (KBr) ν_{max} 3439, 2932, 1711, 1634, 1610, 1551, 1515, 1449, 1383, 1368, 1308, 1275, 1164, 1097, 1060, 1004, 912, 854, 773 cm^{-1}. FAB-MS (negative ion mode) *m/z* 1255 $[M-1]^-$, HRESI-MS (negative ion mode) *m/z* 1255.5745 $[M(C_{61}H_{92}O_{27})-H]^-$ (calcd. 1255.5747). ^{1}H and ^{13}C NMR: see Tables 1–3.

2.3.4. Otophylloside Q (**7**)

White amorphous powder, mp 202–204 °C, $[\alpha]_D^{18}$ − 0.0° (*c* = 0.22, MeOH), IR (KBr) ν_{max} 3442, 2969, 2933, 1714, 1643, 1550, 1452, 1381, 1369, 1317, 1276, 1224, 1195, 1164, 1098, 1059, 1004, 912, 865, 824 cm^{-1}. FAB-MS (negative ion mode) *m/z* 1390 $[M]^-$, 1262 $[M-1-127]^-$, 1228 $[M-162]^-$. HRESI-MS (negative ion mode) *m/z* 1425.6831 $[M(C_{68}H_{110}O_{29})+Cl]^-$ (calcd. 1425.6821). ^{1}H and ^{13}C NMR: see Tables 1, 2 and 4 .

2.3.5. Otophylloside R (**8**)

White amorphous powder, mp 158–160 °C, $[\alpha]_D^{19}$ − 27.4° (*c* = 0.20, MeOH), IR (KBr) ν_{max} 3457, 2971, 2934, 1714, 1645, 1549, 1452, 1381, 1369, 1317, 1275, 1223, 1195, 1164, 1102, 1059, 1003, 913, 863, 823 cm^{-1}. FAB-MS (negative ion mode) *m/z* 1515 $[M-1]^-$, 1389 $[M-127]^-$. HRESI-MS (negative ion mode) *m/z* 1551.7863 $[M(C_{76}H_{124}O_{30})+Cl]^-$ (calcd. 1551.7865). ^{1}H and ^{13}C NMR: see Tables 1, 2 and 4.

2.3.6. Otophylloside S (**9**)

White amorphous powder, mp 155–158 °C, $[\alpha]_D^{19}$ − 41.7° (*c* = 0.16, MeOH), IR (KBr) ν_{max} 3453, 2971, 2934, 1715, 1644, 1549, 1452, 1382, 1369, 1317, 1276, 1224, 1195, 1165, 1102, 1058, 1003, 935, 913, 863, 821 cm^{-1}. FAB-MS (negative ion mode) *m/z* 1515 $[M-1]^-$, 1388 $[M-1-127]^-$. HRESI-MS (negative ion mode) *m/z* 1551.7868 $[M(C_{76}H_{124}O_{30})+Cl]^-$ (calcd. 1551.7865). ^{1}H and ^{13}C NMR: see Tables 1, 2 and 4.

2.3.7. Acid hydrolysis of **2–4** and **7–9**

Compounds **2–4** and **7–9** (each 5 mg) were subjected to acid hydrolysis as described in a previous study [4]. The aglycones, as well as monosaccharides, were detected by TLC analysis combined with comparison to known samples. Qingyangshengenin was identified as the aglycone of compounds **2–4**, and caudatin was revealed to be the aglycones of compounds **7–9**. Regarding the monosaccharide component of each compound, cymarose and oleandrose were detected in all of the six new glycosides (**2–4** and **7–9**); glucose was detected in **4** and **7–9**; digitoxose was detected only in **2**.

2.3.8. Determination of glucose moiety configuration

Neutralized hydrolysates of **4** and **7–9** were treated as described in a previous study [4], and the GC analysis of the corresponding trimethylsilylated L-cysteine adducts confirmed a D-configuration of the glucose molecules.

3. Results and discussion

Nine pregnane glycosides (**1–9**) were further isolated from the $CHCl_3$ extracts of the roots of *C. otophyllum* through open column chromatography and preparative HPLC. Six of these were otophyllosides N-S (**2–4** and **7–9**) and had not been previously isolated. Acid hydrolysis indicated that compounds **2–4** and **7–9** were qingyangshengenin and caudatin glycosides, respectively, which were confirmed by the comparison of their spectra data with qingyangshengenin and caudatin [4]. Their structural characteristics were determined by 1D and 2D spectroscopic analysis as well as chemical methods. Concerning the three known compounds, **1** and **5** were identified as otophyllosides A and B, respectively, which are the active ingredients for the

Table 2
^{13}C NMR data for the sugar moieties of compounds **2**–**4** and **7**–**9** (δ in ppm, in C_5D_5N, 100 MHz).

No.	**2** D-digit	**3** D-cym	**4** D-cym	**7** D-cym	**8** D-cym	**9** D-cym
1″	96.4	96.4	96.4	96.4	95.6	95.5
2″	39.1	37.2	37.3	37.2	36.8	36.7
3″	67.6	78.1	78.1	78.0	77.8	77.6
4″	83.4	83.4	83.5	83.4	83.1	83.1
5″	68.6	69.1	69.1	69.1	68.9	68.9
6″	18.7	18.7	18.5	18.5	18.5	18.6
OMe		58.9	58.9	58.7	58.8	58.9

No.	**2** D-cym	**3** D-cym	**4** D-cym	**7** D-cym	**8** D-ole	**9** D-ole
1‴	99.8	100.5	100.5	100.5	101.8	101.9
2‴	36.7	37.0	37.0	37.0	37.2	37.2
3‴	77.7	77.8	77.8	77.8	79.0	79.0
4‴	83.2	83.2	83.2	83.2	81.6	81.6
5‴	69.1	69.0	68.9	68.9	72.1	72.1
6‴	18.5	18.6	18.6	18.7	18.5	18.6
OMe	58.9	59.0	59.0	59.0	56.4	56.4

No.	**2** D-ole	**3** D-ole	**4** D-ole	**7** D-ole	**8** L-cym	**9** L-cym
1⁗	102.0	102.0	101.9	102.0	97.4	97.4
2⁗	37.7	37.7	37.5	37.8	32.4	32.3
3⁗	78.9	78.9	79.5	78.8	73.5	73.6
4⁗	82.7	82.8	83.4	82.6	78.1	78.0
5⁗	71.8	71.8	72.0	71.8	64.7	64.8
6⁗	18.7	18.7	18.8	18.7	18.4	18.4
OMe	57.4	57.4	57.3	57.5	57.2	57.3

No.	**2** D-cym	**3** D-cym	**4** D-glc	**7** D-cym	**8** D-cym	**9** D-cym
1⁗′	98.6	98.6	104.3	98.3	96.4	96.4
2⁗′	35.9	35.9	75.3	36.8	37.2	37.2
3⁗′	79.0	79.0	77.0	78.1	78.1	78.0
4⁗′	74.2	74.2	81.7	83.3	83.4	83.4
5⁗′	71.3	71.2	76.4	69.6	69.0	69.1
6⁗′	19.1	19.1	62.5	18.7	18.6	18.6
OMe	58.1	58.1		58.9	58.9	59.0

No.	**2**	**3**	**4** D-glc	**7** D-glc	**8** D-cym	**9** D-cym
1⁗″			105.1	106.3	100.5	100.5
2⁗″			74.9	74.9	37.0	37.0
3⁗″			78.5	76.6	77.8	77.8
4⁗″			71.6	81.4	83.3	82.5
5⁗″			78.3	76.5	69.0	69.1
6⁗″			62.5	62.4	18.6	18.6
OMe					59.0	58.4

No.	**2**	**3**	**4**	**7** D-glc	**8** D-ole	**9** L-cym
1⁗‴				105.0	101.9	99.0
2⁗‴				74.8	37.5	32.3
3⁗‴				78.6	79.3	73.3
4⁗‴				71.5	83.3	78.9
5⁗‴				78.3	72.1	65.1
6⁗‴				62.4	18.9	18.5
OMe					57.3	56.9

No.	**2**	**3**	**4**	**7**	**8** D-glc	**9** D-glc
1⁗⁗					104.5	102.3
2⁗⁗					75.8	75.3
3⁗⁗					78.7	78.8
4⁗⁗					71.9	71.8
5⁗⁗					78.2	78.5
6⁗⁗					63.1	63.0

D-Digit: β-D-digitoxopyranosyl; D-cym: β-D-cymaropyranosyl; L-cym: α-L-cymaropyranosyl; D-ole: β-D-oleandropyranosyl; D-glc: β-D-glucopyranosyl.

Table 3
^{1}H NMR spectral data of compounds **2**–**4** (δ in ppm, J in Hz, in C_5D_5N, 500 MHz).

No.	**2**	**3**[a]	**4**
3	3.87 m	3.85 m	3.85 m
6	5.26 br s	5.29 br s[b]	5.29 br s[b]
12	5.33 dd, 11.5, 4.0	5.33 dd, 11.9, 3.8	5.33 dd, 11.8, 3.5
18	2.09 s	2.09 s	2.09 s
19	1.30 s	1.30 s	1.30 s
21	2.41 s	2.42 s	2.41 s
3′	8.30 d, 8.6	8.30 d, 8.4	8.30 d, 8.6
4′	7.23 d, 8.6	7.23 d, 8.4	7.22 d, 8.6
6′	7.23 d, 8.6	7.23 d, 8.4	7.22 d, 8.6
7′	8.30 d, 8.6	8.30 d, 8.4	8.30 d, 8.6

No.	**2** D-digit	**3**[a] D-cym	**4** D-cym
1″	5.48 br d, 9.2	5.28 br d[b]	5.28 br d[b]
2″	2.07 m, 2.40 m	1.90 m, 2.32 m	1.91 m, 2.32 m
3″	4.64 m	4.08 m	4.08 m
4″	3.52 m	3.52 m	3.66 m
5″	4.30 m	4.21 m	4.22 m
6″	1.43 d, 6.4	1.39 d, 6.1	1.35 d, 6.2
OMe		3.62 s	3.60 s
D-cym-1‴	5.17 br d, 9.8	5.12 br d, 9.9	5.10 br d[c]
2‴	1.78 m, 2.29 m	1.81 m, 2.30 m	1.79 m, 2.31 m
3‴	4.01 m	4.01 m	4.23 m
4‴	3.41 m	3.45 m	3.41 m
5‴	4.18 m	4.17 m	4.16 m
6‴	1.32 d, 6.1	1.39 d, 6.1	1.37 d, 6.2
OMe	3.56 s	3.57 s	3.55 s
D-ole-1⁗′	4.68 br d, 9.7	4.69 br d, 9.5	4.66 br d, 9.3
2⁗′	1.73 m, 2.45 m	1.73 m, 2.48 m	1.73 m, 2.48 m
3⁗′	3.55 m	3.56 m	3.58 m
4⁗′	3.51 m	3.50 m	3.51 m
5⁗′	3.52 m	3.54 m	3.63 m
6⁗′	1.43 d, 6.4	1.44 d, 5.2	1.69 d, 5.6
OMe	3.51 s	3.52 s	3.47 s

No.	**2** D-cym	**3**[a] D-cym	**4** D-glc
1⁗″	5.25 br d, 9.3	5.26 br d[b]	5.06 d[c]
2⁗″	1.77 m, 2.38 m	1.78 m, 2.39 m	4.00 m
3⁗″	3.77 m	3.78 m	4.26 m
4⁗″	3.54 m	3.54 m	4.30 m
5⁗″	4.14 m	4.13 m	3.91 m
6⁗″	1.54 d, 6.3	1.54 d, 6.0	4.48 m, 4.52 m
OMe	3.46 s	3.46 s	
D-glc-1⁗″′			5.18 d, 7.4
2⁗″′			4.10 m
3⁗″′			4.21 m
4⁗″′			4.20 m
5⁗″′			4.02 m
6⁗″′			4.30 m, 4.54 m

D-digit: β-D-digitoxopyranosyl; D-cym: β-D-cymaropyranosyl; L-cym: α-L-cymaropyranosyl; D-ole: β-D-oleandropyranosyl; D-glc: β-D-glucopyranosyl.

[a] Obtained in 400 MHz.
[b] Partial overlap with each other.
[c] Overlap with H_2O signal.

treatment of epilepsy [1]. Compound **6** was identified as caudatin 3-*O*-β-D-glucopyranosyl-(1→4)-β-D-oleandropyranosyl-(1→4)-β-D-cymaropyranosyl-(1→4)-β-D-cymaropyranoside, which was first reported as the product of wallicoside hydrolyzed with β-glucosidase [5], and was then later isolated as a natural product from *Cynanchum caudatum* [6] (Scheme 1).

3.1. Otophylloside N (**2**)

Compound **2** was obtained as a white amorphous powder with $[\alpha]_D^{18} + 7.7^\circ$ (c = 0.20, MeOH). Its molecular formula, $C_{55}H_{82}O_{20}$, was determined by negative HRESI-MS (m/z 1061.5284 $[M-H]^-$, calcd. 1061.5321) and ^{13}C NMR (DEPT) data (Tables 1 and 2). IR spectrum showed the absorption bands for hydroxyl groups

Table 4
^{1}H NMR spectral data of compounds **7**–**9** (δ in ppm, J in Hz, in C_5D_5N, 500 MHz).

No.	**7**	**8**[a]	**9**[a]
3	3.84 m	3.84 m	3.85 m
6	5.28 br s[b]	5.27 br s[b]	5.28 br s[b]
12	5.04 dd, 12.1, 4.2	5.04[c]	5.04[c]
18	1.98 s	1.98 s	1.98 s
19	1.31 s	1.31 s	1.30 s
21	2.50 s	2.50 s	2.49 s
2′	5.85 s	5.85 s	5.86 s
4′	2.25 m	2.26 m	2.23 m
5′	0.92 d, 7.0	0.92 d, 6.7	0.92 d, 7.3
6′	0.94 d, 7.0	0.93 d, 6.7	0.93 d, 7.3
7′	2.26 s	2.26 s	2.25 s
D-cym-1″	5.26 br d[b]	5.26 br d[b]	5.24 br d[b]
2″	1.90 m, 2.31 m	1.83 m, 2.30 m	1.80 m, 2.29 m
3″	4.07 m	4.00 m	4.00 m
4″	3.52 m	3.43 m	3.42 m
5″	4.21 m	4.16 m	4.15 m
6″	1.37 d, 6.0	1.36 d, 6.2	1.35 d[b]
OMe	3.48 s	3.52 s	3.53 s

No.	**7** D-cym	**8**[a] D-ole	**9**[a] D-ole
1‴	5.11 br d[c]	4.64 br d, 9.8	4.64 br d, 9.8
2‴	1.80 m, 2.30 m	1.62 m, 2.48 m	1.62 m, 2.48 m
3‴	4.00 m	3.39 m	3.40 m
4‴	3.49 m	3.37 m	3.36 m
5‴	4.15 m	3.45 m	3.45 m
6‴	1.38 d, 6.0	1.37 d[b]	1.36 d[b]
OMe	3.55 s	3.29 s	3.27 s

No.	**7** D-ole	**8**[a] L-cym	**9**[a] L-cym
1⁗	4.66 br d, 9.6	5.07 br s	5.07 br s
2⁗	1.71 m, 2.43 m	1.87 m, 2.32 m	1.84 m, 2.33 m
3⁗	3.51 m	3.77 m	3.77 m
4⁗	3.47 m	3.88 dd, 8.5, 2.7	3.88 m
5⁗	3.49 m	4.74 m	4.71 m
6⁗	1.39 d, 5.7	1.56 d, 6.2	1.54 d, 6.4
OMe	3.49 s	3.38 s	3.40 s
D-cym-1⁗′	5.25 br d[b]	5.27 br d[b]	5.27 br d[b]
2⁗′	1.76 m, 2.29 m	1.89 m, 2.31 m	1.89 m, 2.30 m
3⁗′	4.05 m	4.07 m	4.08 m
4⁗′	3.61 m	3.50 m	3.51 m
5⁗′	4.25 m	4.19 m	4.20 m
6⁗′	1.61 d, 6.2	1.37 d[b]	1.36 d[b]
OMe	3.60 s	3.58 s	3.59 s

No.	**7** D-glc	**8**[a] D-cym	**9**[a] D-cym
1⁗′	4.88 d, 7.7	5.10 br d, 9.5	5.11 br d, 9.8
2⁗′	3.98 m	1.78 m, 2.30 m	1.78 m, 2.30 m
3⁗′	4.27 m	3.99 m	3.99 m
4⁗′	4.28 m	3.63 m	3.45 m
5⁗′	3.93 m	4.19 m	4.19 m
6⁗′	4.50 m, 4.52 m	1.38 d[b]	1.37 d[b]
OMe		3.54 s	3.52 s

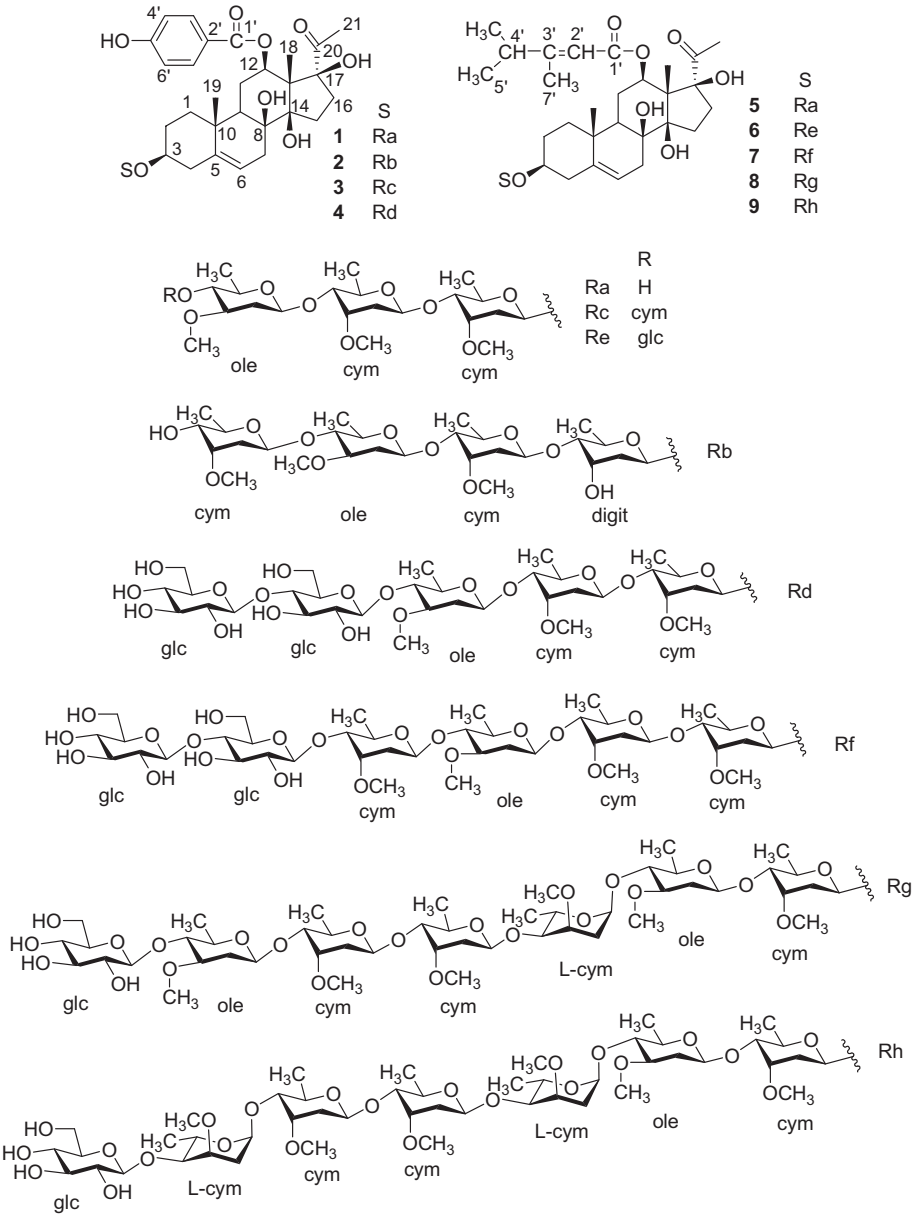
No.	**7** D-glc	**8**[a] D-ole	**9**[a] L-cym
1⁗′′	5.19 d, 7.7	4.67 br d, 10.4	4.94 br s
2⁗′′	4.10 m	1.71 m, 2.47 m	1.77 m, 2.32 m
3⁗′′	4.20 m	3.61 m	3.93 m
4⁗′′	4.20 m	3.70 m	3.97 m
5⁗′′	4.01 m	3.63 m	4.68 m
6⁗′′	4.29 m, 4.52 m	1.69 d, 5.9	1.46 d, 6.4
OMe		3.50 s	3.42 s
D-glc-1⁗′′′		5.11 d, 7.7	5.01 d, 7.8
2⁗′′′		4.00 m	3.99 m
3⁗′′′		4.22 m	3.97 m
4⁗′′′		4.20 m	4.22 m
5⁗′′′		3.95 m	4.25 m
6⁗′′′		4.35 dd, 11.5, 5.3 4.52 br d, 11.5	4.38 dd, 11.2, 4.9 4.57 br d, 11.2

D-digit: β-D-digitoxopyranosyl; D-cym: β-D-cymaropyranosyl; L-cym: α-L-cymaropyranosyl; D-ole: β-D-oleandropyranosyl; D-glc: β-D-glucopyranosyl.
[a] Obtained in 400 MHz.
[b] Partial overlap with each other.
[c] Overlap with H_2O signal.

(3446 cm^{-1}), carbonyl groups (1713 cm^{-1}), and benzene rings (1610 and 1594 cm^{-1}). The ^{1}H NMR spectrum of **2** revealed the presence of three singlet methyl groups [δ_H 1.30, 2.09, 2.41 (each 3H, s, Me-19, 18, 21)], one olefinic proton [δ_H 5.26 (brs, H-6)] and four aromatic protons on a *para*-substituted benzene ring [δ_H 7.23 (2H, d, J = 8.6 Hz, H-4′,6′), and 8.30 (2H, d, J = 8.6 Hz, H-3′,7′)] in its aglycone moiety. Its negative FAB-MS exhibited a base peak at m/z 137, which could represent the fragmented ion peak of a hydroxybenzoyl ester group. The above features, together with the TLC comparison with known samples after acid hydrolysis, suggested that the aglycone was qingyangshengenin [4]. Proton signals of the sugar moiety could be assigned to four secondary methyl groups [δ_H 1.32 (3H, d, J = 6.1 Hz), 1.43 (2 × 3H, d, J = 6.4 Hz), and 1.54 (3H, d, J = 6.3 Hz)], three methoxyl groups [δ_H 3.46, 3.51, and 3.56] and four anomeric protons [δ_H 4.68 (brd, J = 9.7 Hz), 5.17 (brd, J = 9.8 Hz), 5.25 (brd, J = 9.3 Hz), and 5.48 (brd, J = 9.2 Hz)]. The multiples suggested that the sugar chain consisted of four 2,6-dideoxy-sugar units with β-linkage. The ^{13}C NMR shifts of each sugar unit were assigned unambiguously by HSQC, HMBC and HSQC-TOCSY analyses (Table 2), and we identified the presence of one digitoxopyranosyl, one oleandropyranosyl and two cymaropyranosyl units. Comparison with published spectroscopic data, the absolute configuration of the deoxysugars was consistent with D-series [1,7,8]. The sequence of the four sugar units located at C-3 of the aglycone was elucidated by HMBC spectrum, in which distinct correlations from δ_H 5.48 (β-D-digitoxopyranosyl H-1″) to δ_C 77.6 (C-3); from δ_H 5.17 (inner β-D-cymaropyranosyl H-1‴) to δ_C 83.4 (β-D-digitoxo-pyranosyl C-4″); from δ_H 4.68 (β-D-oleandropyranosyl H-1⁗) to δ_C 83.2 (inner β-D-cymaropyranosyl C-4‴); from δ_H 5.25 (terminal β-D-cymaropyranosyl H-1⁗′) to δ_C 82.7 (β-D-oleandropyranosyl C-4⁗) were observed. Thus, the structure of otophylloside N (**2**) was established as qingyangshengenin-3-*O*-β-D-cymaropyranosyl-(1→4)-β-D-oleandropyranosyl-(1→4)-β-D-cymaropyranosyl-(1→4)-β-D-digitoxopyra-noside.

3.2. Otophylloside O (**3**)

The molecular formula of compound **3** was determined to be $C_{56}H_{84}O_{20}$ on the basis of negative HRESI-MS (m/z 1111.5239 $[M+Cl]^-$, calcd. 1111.5244). Similar IR absorption frequency, NMR characteristics and acidic hydrolysis indicated that **3** possessed the same aglycone, qingyangshengenin, as compound **2**. The fragment ion peak m/z 931 $[M-1-144]^-$ in negative FAB-MS confirmed a terminal deoxysugar in **3**. TLC results from acid hydrolysis revealed the presence of cymarose and oleandrose. The sugar moiety was elucidated to be a single chain connected to C-3 of the aglycone, and the sequence of the sugar units was identical to those of the 3-*O*-tetraglycosides isolated from *Cynanchum caudatum* identifying cynanchogenin or caudatin as the aglycone [9,10]. The structure of otophylloside O (**3**) was determined to be qingyangshengenin-3-*O*-β-D-cymaropyranosyl-(1→4)-β-D-oleandropyranosyl-(1→4)-β-D-cymaropyranosyl-(1→4)-β-D-cymaropyranoside.



Scheme 1. Qingyangshengenin and caudatin glycosides (**1**–**9**) isolated from *Cynanchum otophyllum*.

3.3. Otophylloside P (**4**)

The molecular formula of compound **4**, $C_{61}H_{92}O_{27}$, was determined by the negative HRESI-MS data (*m*/*z* 1255.5745 $[M-H]^-$, calcd. 1255.5747). It was identified as qingyangshengenin 3-*O*-pentoside based on its ^1H and ^{13}C NMR spectra (Tables 1–3). Acid hydrolysis showed that its sugar moiety consisted of three types of sugar units: cymarose, oleandrose, and glucose. Detailed 2D NMR revealed that the sugar sequence was consistent with that of wallicoside [5]. Thus, the structure of otophylloside P (**4**) was elucidated to be qingyangshengenin-3-*O*-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-β-D-oleandropyranosyl-(1→4)-β-D-cymaropyranosyl-(1→4)-β-D-cymaropyranoside.

3.4. Otophylloside Q (**7**)

Compound **7** was obtained as white amorphous powder with $[\alpha]_D^{18}$ − 0.0° (*c* = 0.22, MeOH). The negative HRESI-MS showed a quasi-molecular ion peak at *m*/*z* (1425.6831 $[M+Cl]^-$, calcd. 1425.6821), in agreement with the molecular formula $C_{68}H_{110}O_{29}$, which was further supported by ^{13}C NMR and DEPT experiments. IR spectrum showed absorption bands for hydroxyl (3442 cm^{-1}) and carbonyl (1714 cm^{-1}) groups as well as a C=C band (1643 cm^{-1}). The ^1H NMR spectrum of **7** exhibited four singlet methyl groups [δ_H 1.31, 1.98, 2.26, 2.50 (each 3H, s, Me-19,18,7′,21)], two doublet methyl groups [δ_H 0.92 and 0.94 (each 3H, d, *J* = 7.0 Hz, Me-5′,6′)], and two olefinic protons [δ_H 5.28 (brs, H-6) and 5.85 (s, H-2′)]. These spectral characteristics suggested that the aglycone was caudatin [4], which could be consistent with the fragment ion peaks at *m*/*z* 1262 $[M-1-127]^-$ resulting from the loss of ikemaoyl ester group in negative FAB-MS spectrum. Acid hydrolysis of **7** led to the detection of caudatin and a sugar mixture of cymarose, oleandrose, and glucose by TLC analysis and comparison to known samples. Its negative FAB-MS exhibited fragment ion peaks at *m*/*z* 1228 $[M-162]^-$, revealing a terminal glucosyl unit in the sugar moiety. The ^1H NMR spectral data of compound **7** contained six anomeric proton signals [δ_H 4.66 (brd, *J* = 9.6 Hz), 4.88 (d, *J* = 7.7 Hz), 5.11 (brd, partially overlapped), 5.19 (d, *J* = 7.7 Hz), 5.25 (brd, par-

tially overlapped), 5.26 (brd, partially overlapped)], four secondary methyl groups [δ_H 1.37 (3H, d, J = 6.0 Hz), 1.38 (3H, d, J = 6.0 Hz), 1.39 (3H, d, J = 5.7 Hz), 1.61 (3H, d, J = 6.2 Hz)], and four methoxyl groups [δ_H 3.48, 3.49, 3.55, and 3.60] in the sugar moiety, indicating the existence of four deoxy sugars and two glucose units. The signals of each sugar unit were assigned by HSQC, HMBC and HSQC-TOCSY analyses (Tables 2 and 4), suggesting that the proportion of cymarose and oleandrose was 3:1; their absolute configuration was determined to be D-series by comparison with the spectroscopic data in the literature [6,9,10]. The sugar sequence of **7** was studied by the same method described above, and it was found to be coincident with otophylloside M [4] except for an excess terminal glucosyl unit. Therefore, the structure of **7** was concluded to be caudatin-3-*O*-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-β-D-cymaropyranosyl-(1→4)-β-D-oleandropyranosyl-(1→4)-β-D-cymaropyranosyl-(1→4)-β-D-cymaropyranoside and was named otophylloside Q.

3.5. Otophylloside R (**8**)

The molecular formula of compound **8** was determined to be $C_{76}H_{124}O_{30}$ on the basis of negative HRESI-MS (m/z 1551.7863 $[M+Cl]^-$, calcd. 1551.7865) and ^{13}C NMR (DEPT) spectrum. The aglycone of **8** was inferred to be caudatin based on a comparison of the NMR features to a known sample [4] and the fragment ion peak at m/z 1389 $[M-127]^-$ in negative FAB-MS. Its ^1H NMR spectrum exhibited seven anomeric proton signals at δ_H 4.64 (brd, J = 9.8 Hz), 4.67 (brd, J = 10.4 Hz), 5.07 (brs), 5.10 (brd, J = 9.5 Hz), 5.11 (d, J = 7.7 Hz), 5.26 (brd, partially overlapped), and 5.27 (brd, partially overlapped), revealing the existence of six β-linkages and one α-linkage. The observation of six secondary methyl and six methoxyl groups (Table 4) suggested the sugar moiety contained six deoxysugar units. In HSQC spectrum, seven anomeric carbon signals at δ_C 101.8, 101.9, 97.4, 100.5, 104.5, 95.6, and 96.4 were attributed to the above anomeric protons, respectively. The other signals were assigned by HSQC, HMBC and HSQC-TOCSY studies, indicating the existence of three D-cymaropyranosyl units, two D-oleandro-pyranosyl units, one L-cymaropyranosyl unit, and one glucopyranosyl unit by comparison with the data in Refs. [5,6,11,12]. These seven sugar units were in a single chain connected to C-3 of the aglycone, and the sequence of the sugars was illustrated by the distinct correlations in HMBC spectrum: from δ_H 5.11 (β-D-glucopyranosyl H-1‴‴‴) to δ_C 83.3 (outer β-D-oleandropyranosyl C-4‴‴), from δ_H 4.67 (outer β-D-oleandro-pyranosyl H-1‴‴) to δ_C 83.3 (outer β-D-cymaropyranosyl C-4‴‴), from δ_H 5.10 (outer β-D-cymaropyranosyl H-1‴‴) to δ_C 83.4 (middle β-D-cymaro-pyranosyl C-4‴), from δ_H 5.27 (middle β-D-cymaropyranosyl H-1‴) to δ_C 78.1 (α-L-cymaropyranosyl C-4‴), from δ_H 5.07 (α-L-cymaropyranosyl H-1‴) to δ_C 81.6 (inner β-D-oleandropyranosyl C-4‴), from δ_H 4.64 (inner β-D-oleandropyranosyl H-1‴) to δ_C 83.1 (inner β-D-cymaro-pyranosyl C-4″), and from δ_H 5.26 (inner β-D-cymaropyranosyl H-1″) to δ_C 77.6 (C-3). Therefore, the structure of otophylloside R (**8**) was determined to be caudatin 3-*O*-β-D-glucopyranosyl-(1→4)-β-D-oleandropyranosyl-(1→4)-β-D-cymaropyranosyl-(1→4)-β-D-cymaropyranosyl-(1→4)-α-L-cymaropyranosyl-(1→4)-β-D-oleandropyranosyl-(1→4)-β-D-cymaropyranoside.

3.6. Otophylloside S (**9**)

Compound **9** has the same molecular formula $C_{76}H_{124}O_{30}$ as **8** based on the negative HRESI-MS data (m/z 1551.7868 $[M+Cl]^-$, calcd. 1551.7865). The IR spectrum and NMR features, together with the TLC results of the acidic hydrolysate, demonstrated that compound **9** was also a caudatin glycoside. Carefully comparing of the ^1H and ^{13}C NMR spectra of **8** and **9** indicated that a set of the characteristic signals of β-D-oleandropyranosyl unit in **8** [δ_H 4.67 (brd, J = 10.4 Hz), 1.69 (d, J = 5.9 Hz), 3.50 (s), and δ_C 101.9, 37.5, 79.3, 83.3, 72.1, 18.9, 57.3] was absent in **9**. Instead, a set of signals arising from an α-L-cymaropyranosyl unit [δ_H 4.94 (brs), 1.46 (d, J = 6.4 Hz), 3.42 (s), and δ_C 99.0, 32.3, 73.3, 78.9, 65.1, 18.5, 56.9] existed in **9**. The sequence of the sugars and the location of the sugar chain were confirmed by HMBC, and the sixth sugar unit was the only difference. Because of the variation from β-D-oleandrosyl in **8** to α-L-cymarosyl in **9**, the C-4 signal of the fifth sugar unit (β-D-cymaropyranosyl) shifted from δ_C 83.3 in **8** to δ_C 82.5 in **9**, and both the anomeric proton and carbon signals of terminal glucopyranosyl unit in **9** moved to lower frequency shifts [δ_H 5.01 (d, J = 7.8 Hz) and δ_C 102.3]. These features were identical to those of wilfosides C1G and G1G, whose sugar chains implied a β-D-glucopyranosyl-(1→4)-α-L-cymaropyranosyl-(1→4)-β-D-cymaropyranosyl fragment [11,12]. Therefore, the structure of **9** was confirmed to be caudatin 3-*O*-β-D-glucopyranosyl-(1→4)-α-L-cymaropyranosyl-(1→4)-β-D-cymaropyranosyl-(1→4)-β-D-cymaropyranosyl-(1→4)-α-L-cymaropyranosyl-(1→4)-β-D-oleandropyranosyl-(1→4)-β-D-cymaropyranoside and was named otophylloside S.

It is noted that most pregnane glycosides isolated from *C. otophyllum* possessed either qingyangshengenin or caudatin as aglycones, which contain a straight sugar chain at C-3 of the aglycone with different composition and various connecting sequences [1,4,13]. In addition, otophyllosides R and S (**8** and **9**) were comprised of caudatin as the aglycone and seven sugar units, with the coexistence of D- and L-cymarosyl optically isomeric monosaccharides. The configurations of the cymarosyl moieties in compounds **8** and **9** were determined by comparing their ^{13}C NMR chemical shifts with the published configurations [11]. The relationship between the ^{13}C NMR data and absolute configuration of cymarosyl moiety was confirmed by a detailed analysis of the purified D- and L-cymarose by acid hydrolysis. It was shown that the orientations of methyl and oxymethyl at C-3 position affect the carbon signals strongly in 2,6-dideoxy sugars. Therefore, the configurations of digitoxosyl and oleandrosyl moieties could also be determined by the ^{13}C NMR data.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.steroids.2011.03.019.

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