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# Neuroprotective polyhydroxypregnane glycosides from *Cynanchum otophyllum*

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ABSTRACT

Five new polyhydroxypregnane glycosides, namely cynanotosides A–E (**1**–**5**), together with two known analogues, deacetylmetaplexigenin (**6**) and cynotophylloside H (**7**), were isolated from the roots of *Cynanchum otophyllum*. Their structures were established by spectroscopic methods and acid hydrolysis. The neuroprotective effects of compounds **1**–**7** against glutamate-, hydrogen peroxide-, and homocysteic acid (HCA)-induced cell death were tested by MTT assay in a hippocampal neuronal cell line HT22. Compounds **1**, **2**, and **7** exhibited protective activity against HCA-induced cell death in a dose-dependent manner ranging from 1 to 30 μM, which may explain the Traditional Chinese Medicine (TCM) use of this plant for the treatment of epilepsy.



## 1. Introduction

*Cynanchum otophyllum* Schneid (Asclepiadaceae), a perennial weed widely distributed in south-west China, is known as "Qingyangshen" in Traditional Chinese Medicine (TCM) for its treatments of epilepsy, rheumatic pain, kidney weakness, and muscle injuries [1]. Previous chemical investigations of this species have resulted in the isolation of a number of pregnane glucosides with the structural variations usually occurring on the substitutions at C-3 and C-12 of the pregnane core [2–5]. Recently, pregnane glycosides have attracted considerable attention for their broad range of bioactivities, such as anti-epileptic activity [6], multidrug-resistance modulating activity [7], immunological activity [8], and antiviral properties [9]. In our continuing search for structurally and biologically interesting metabolites from medical plant resources [10–12], five new pregnane glycosides together with two known steroids (Fig. 1) have been isolated from the roots of *C. otophyllum*. Their structures were established by spectroscopic analyses combined with chemical methods, and three compounds showed neuroprotective effects on homocysteic acid (HCA)-induced cell death screening in the hippocampal neuronal cell line HT22. We report herein the isolation, structural elucidation, and neuroprotective activity of these compounds.

## 2. Experimental

### 2.1. General methods

Optical rotation was recorded on a Perkin-Elmer 341 polarimeter. IR spectra were recorded on a FT-IR Tensor37 spectrometer. NMR spectra were recorded on a Bruker AM-400 and Bruker AM-500 spectrometers at 25 °C. ESIMS and HRESIMS were recorded on a Finnigan LC Q$^{DECA}$ instrument. Silica gel (300–400 mesh, Qingdao Haiyang Chemical Co. Ltd.), $C_{18}$ reverse-phase silica gel (12 nm, S-50 μm, YMC Co. Ltd), Sephadex LH-20 gel (Amersham Biosciences), and Mitsubishi Chemical Industries (MCI) gel (CHP20P, 75–150 μm, Mitsubishi Chemical Industries Ltd.) were used for column chromatography. All solvents used were of analytical grade (Guangzhou Chemical Reagents Company, Ltd.).

### 2.2. Plant material

The roots and stems of *C. otophyllum* (2 kg) were collected in October 2011 from Yunnan province, PR China, and were identified by Prof You-Kai Xu of Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences. A voucher specimen (accession number: QYS201110) has been deposited at the School of Pharmaceutical Sciences, Sun Yat-sen University.

### 2.3. Extraction and isolation

The air-dried powder of the roots and stems of *C. otophyllum* (2.0 kg) was extracted with 95% EtOH (3 × 10 L) at room temp to

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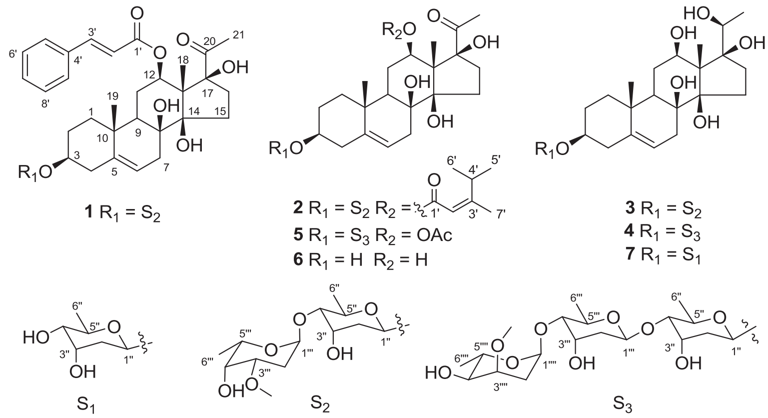

**Fig. 1.** The structures of compounds **1**–**7** isolated from *C. otophyllum*.

give 120 g of crude extract, which was suspended in $H_2O$ (1 L) and successively partitioned with petroleum ether (PE, 3 × 1 L), EtOAc (3 × 1 L), and *n*-BuOH (3 × 1 L) The EtOAc extract (22 g) was subjected to MCI gel column chromatography (CC) eluted with a $MeOH/H_2O$ gradient (3:7 → 10:0) to afford three fractions (I–III). Fraction I (1.2 g) was purified by silica gel CC ($CHCl_3$/MeOH, 10:1 → 1:1) then a $C_{18}$ reverse-phase CC ($MeOH/H_2O$ 6:4 → 10:0) to give **4** (8 mg) and **5** (6 mg). Fraction II (0.8 g) was subjected to a silica gel CC (CHCl/MeOH, 20:1 → 1:1) then a Sephadex LH-20 column (EtOH) afford **3** (12 mg). Fraction III was separated on a $C_{18}$ reverse-phase CC ($MeOH/H_2O$ 6:4 → 10:0) to give two fractions (Fr. IIIa and Fr. IIIb). Fr. IIIa was subjected to a silica gel CC (CHCl/MeOH, 30:1 → 5:1) to afford **1** (14 mg) and **2** (7 mg). Fr. IIIb was subjected to a Sephadex LH-20 column (EtOH) then a silica gel CC ($CHCl_3$/MeOH, 20:1) to give **6** (22 mg) and **7** (31 mg).

### 2.3.1. Cynanotoside A (*1*)

White, amorphous powder; $[\alpha]^{25}_D$ + 4.4 (*c* 0.09, $CHCl_3$); IR (KBr) $\nu_{max}$ 3440, 1709, 1638, 1457, 1375, 1169, 1087, 991 $cm^{-1}$; $^1H$ and $^{13}C$ NMR see Tables 1 and 2; HRESIMS *m*/*z* 807.3970 $[M + Na]^+$ (calcd. for $C_{43}H_{60}O_{13}Na$, 807.3932).

### 2.3.2. Cynanotoside B (*2*)

White, amorphous powder; $[\alpha]^{25}_D$ − 20.6 (*c* 0.19, $CHCl_3$); IR (KBr) $\nu_{max}$ 3456, 1710, 1642, 1383, 1224, 1168, 1088, 1017, 988 $cm^{-1}$; $^1H$ and $^{13}C$ NMR see Tables 1 and 2; HRESIMS *m*/*z* 787.4268 $[M + Na]^+$ (calcd. for $C_{41}H_{64}O_{13}Na$, 787.4245).

### 2.3.3. Cynanotoside C (*3*)

White, amorphous powder; $[\alpha]^{20}_D$ − 24.0 (*c* 0.05, $CHCl_3$); IR (KBr) $\nu_{max}$ 3421, 1637, 1447, 1373, 1167, 1068, 1017, 993, 754 $cm^{-1}$; $^1H$ and $^{13}C$ NMR see Tables 1 and 2. HRESIMS *m*/*z* 679.3685 $[M + Na]^+$ (calcd. for $C_{34}H_{56}O_{12}Na$, 679.3669).

### 2.3.4. Cynanotoside D (*4*)

White, amorphous powder; $[\alpha]^{20}_D$ − 25.0 (*c* 0.36, $CHCl_3$); IR (KBr) $\nu_{max}$ 3441, 1640, 1375, 1161, 1122, 1064, 996, $cm^{-1}$; $^1H$ and $^{13}C$ NMR see Tables 1 and 2. HRESIMS *m*/*z* 809.4300 $[M + Na]^+$ (calcd. for $C_{40}H_{66}O_{15}Na$, 809.4299).

### 2.3.5. Cynanotoside E (*5*)

White, amorphous powder; $[\alpha]^{20}_D$ − 43.4 (*c* 0.35, $CHCl_3$); IR (KBr) $\nu_{max}$ 3452, 1641, 1411, 1017, $cm^{-1}$; $^1H$ and $^{13}C$ NMR see Tables 1 and 2. HRESIMS *m*/*z* 849.4240 $[M + Na]^+$ (calcd. for $C_{42}H_{66}O_{16}Na$, 849.4249).

### 2.3.6. Deacetylmetaplexigenin (*6*)

White, amorphous powder; $[\alpha]^{20}_D$ + 40.0 (*c* 0.34, MeOH); The optical rotation of 6 was reported for the first time in the current study. The $^1H$ and $^{13}C$ NMR data ($CD_3OD$) agreed well with the literature values [13].

### 2.3.7. Cynotophylloside H (*7*)

White, amorphous powder; $[\alpha]^{20}_D$ + 36.1 (*c* 0.05, MeOH), lit [14] $[\alpha]^{20}_D$ + 30.0 (*c* 2.2, MeOH); the $^1H$ and $^{13}C$ NMR data agreed well with the literature values [14].

### 2.4. Acid hydrolysis of compounds *1*–*5* and comparison with standard sugars

To a solution of each compound (2 mg) in MeOH (1 mL), 0.2 M $H_2SO_4$ (1 mL) was added. The solution was kept at 60 °C for 2 h and then diluted with $H_2O$ (2 mL). The solution was neutralized with satd. aq. $Ba(OH)_2$ and concentrated under vacuum. The residue (the mixture of aglycone and sugars) was subjected to CC (Sephadex LH-20, MeOH) to give fractions of sugars and aglycone. Constituents of each sugar fraction were identified by co-TLC with authentic sugars: cymarose [$R_f$ *ca.* 0.50 in $CHCl_3$/MeOH (8:1)], diginose [$R_f$ *ca.* 0.46 in $CHCl_3$/MeOH (8:1)], and digitoxose ($R_f$ *ca.* 0.40 in $CHCl_3$/MeOH (8:1)]. One of the glycosides **13** (5 mg) was hydrolyzed by the above method to afford digitoxose and diginose. The positive optical rotation of digitoxose $[\alpha]^{20}_D$ = +46.0 (*c* = 0.1, $H_2O$) was indicative of a D-configuration ($[\alpha]^{20}_D$ = +48.4), while the negative optical rotation of diginose $[\alpha]^{20}_D$ = −56.2 (*c* = 0.1, $H_2O$) suggested a L-configuration ($[\alpha]^{20}_D$ = −60.6) [3]. By the same method, digitoxose obtained from **4** and **5** was determined to be the D-isomer, while the cymarose was determined to be the L-form ($[\alpha]^{20}_D$ = −48.2).

### 2.5. Neuroprotective activity assays

HT22 murine hippocampal neuronal cells were maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and incubated at 37 °C under 5% $CO_2$. To study the protective effect of compounds on neuronal death induced by inducers, glutamate, $H_2O_2$ and homocystenic acid (HCA), we seeded cells in 96-well plates (10,000 cells/well) and used 6 wells for each treatment group. HT22 cells were pretreated with compounds at different concentrations for 30 min before exposure to inducers unless stated otherwise. The control group was treated with 0.1% (v/v) DMSO as vehicle control. After 24 h, the cell viability was determined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously

**Table 1**
1H NMR data for compounds **15** in $CDCl_3$ (*J* in Hz, $\delta$ in ppm).

| Position | **1**[a] | **2**[a] | **3**[b] | **4**[b] | **5**[b] |
|---|---|---|---|---|---|
| 1 | 1.10, m | 1.10, m | 1.07, m | 1.06, dt (13.4, 3.0) | 1.09, m |
| | 1.92, m | 1.92, m | 1.86, m | 1.86, m | 1.88, m |
| 2 | 1.63, m | 1.64, m | 1.93, m | 1.67, m | 1.94, m |
| | 1.92, m | 1.93, m | 1.68, m | 1.93, m | 1.62, m |
| 3 | 3.56, m | 3.57, m | 3.56, m | 3.56, m | 3.57, m |
| 4 | 2.32, m | 2.32, m | 2.30, m | 2.30, m | 2.46, m |
| | 2.41, m | 2.40, m | 2.40, dd (13.0, 3.5) | 2.41, dd (13.2, 4.0) | 2.32, m |
| 6 | 5.37, brs | 5.36, brs | 5.37, brs | 5.37, brs | 5.35, brs |
| 7 | 2.18, m | 2.19, m | 2.10, m | 2.10, m | 2.18, m |
| | 2.24, m | 2.19, m | 2.15, m | 2.14, m | 2.18, m |
| 9 | 1.57, dd (11.5, 5.5) | 1.53, m | 1.43, dd (13.5, 2.0) | 1.43, dd (13.2, 2.7) | 1.52, dd (11.1, 6.0) |
| 11 | 1.89, m | 1.85, m | 1.65, m | 1.64, m | 1.82, m |
| | 1.92, m | 1.82, m | 2.02, m | 2.03, m | 1.76, m |
| 12 | 4.70, dd (10.0, 6.0) | 4.56, t (7.5) | 3.57, m | 3.57, m | 4.51, dd (9.1, 6.6) |
| 15 | 2.00, m | 1.97, m | 1.74, m | 1.74, m | 1.93, m |
| | | | 1.86, m | 1.86, m | |
| 16 | 2.88, m | 2.87, m | 1.73, m | 1.76, m | 2.86, m |
| | 1.90, m | 1.86, m | 1.85, m | 1.84, m | 1.82, m |
| 18 | 1.48, s | 1.42, s | 1.36, s | 1.35, s | 1.42, s |
| 19 | 1.14, s | 1.13, s | 1.18, s | 1.18, s | 1.12, s |
| 20 | – | – | 4.06, q (6.5) | 4.05, m | – |
| 21 | 2.20, s | 2.20, s | 1.16, d (6.5) | 1.18, d (5.4) | 2.25, s |
| 2′ | 6.30, d (15.8) | 5.52, brs | | | 1.95, s |
| 3′ | 7.62, d (15.8) | – | | | |
| 4′ | – | 2.37, m | | | |
| 5′ | 7.51, m | 1.06, d (7.0) | | | |
| 6′ | 7.38, m | 1.06, d (7.0) | | | |
| 7′ | 7.39, m | 2.17, s | | | |
| 8′ | 7.38, m | | | | |
| 9′ | 7.51, m | | | | |
| | D-Digit | D-Digit | D-Digit | D-Digit | D-Digit |
| 1″ | 4.94, brd (9.5) | 4.94, brd (9.0) | 4.94, brd (9.0) | 4.93, dd (8.6, 1.5) | 4.92, brd (8.4) |
| 2″ | 1.86, m | 1.86, m | 2.05, m | 2.11, m | 2.10, m |
| | 2.12, m | 2.13, m | 1.77, m | 1.72, m | 1.72, m |
| 3″ | 4.13, d (2.5) | 4.12, d (3.0) | 4.12, d (2.0) | 4.24, m | 4.23, m |
| 4″ | 3.30, dd (10.0, 2.0) | 3.30, dd (10.1, 3.0) | 3.30, dd (9.0, 2.0) | 3.21, m | 3.21, m |
| 5″ | 3.80, m | 3.78, m | 3.78, m | 3.82, m | 3.80, m |
| 6″ | 1.25, d (6.0) | 1.25, d (6.0) | 1.24, d (6.5) | 1.24, d (5.6) | 1.24, d (7.0) |
| | D-Dig | D-Dig | D-Dig | D-Digit | D-Digit |
| 1‴ | 5.07, t (2.0) | 5.06, t (2.0) | 5.06, t (2.0) | 4.89, brd (9.6) | 4.89, brd (8.4) |
| 2‴ | 1.88, m | 1.88, m | 1.90, m | 2.15, m | 2.13, m |
| | 1.97, m | 1.96, m | 1.85, m | 1.75, m | 1.75, m |
| 3‴ | 3.60, m | 3.61, m | 3.58, m | 4.07, m | 4.07, m |
| 4‴ | 3.80, m | 3.80, m | 3.80, brs | 3.24, m | 3.24, m |
| 5‴ | 3.88, q (6.5) | 3.88, q (6.0) | 3.87, m | 3.78, m | 3.79, m |
| 6‴ | 1.30, d (6.5) | 1.30, d (6.5) | 1.30, d (6.5) | 1.22, d (5.8) | 1.22, d (6.4) |
| OMe | 3.39, s | 3.40, s | 3.39, s | – | – |
| | | | | L-cym | L-cym |
| 1⁗ | | | | 4.91, brd (3.0) | 4.90, brd (3.0) |
| 2⁗ | | | | 2.31, m | 2.31, m |
| | | | | 1.80, m | 1.80, m |
| 3⁗ | | | | 3.63, m | 3.63, m |
| 4⁗ | | | | 3.27, m | 3.26, m |
| 5⁗ | | | | 3.85, m | 3.85, m |
| 6⁗ | | | | 1.26, d (6.3) | 1.26, d (6.1) |
| OMe | | | | 3.42, s | 3.42, s |

[a] Measured in $CDCl_3$ at 500 MHz.
[b] Measured in $CDCl_3$ at 400 MHz. Digit = digitoxopyranosyl. Dig = diginopyranosyl. cym = cymaropyranosyl.

described [15]. Optical density was measured using a microplate reader (Bio-Tek, USA) at 570 nm and all data were represented as percent of control.

## 3. Results and discussion

Compound **1**, a white amorphous powder, has a molecular formula of $C_{43}H_{60}O_{13}$ as determined by HR-ESI-MS at *m*/*z* 807.3970 $[M + Na]^+$ (calcd. 807.3932). The IR spectrum exhibited the absorption bands for hydroxyl (3440 $cm^{-1}$), ketone (1709 $cm^{-1}$), and benzene (1638 and 1457 $cm^{-1}$) functionalities. Lieberman–Burchard and Keller–Kiliani tests suggested that **1** was a steroidal glycoside. The $^1H$ and $^{13}C$ NMR spectra of **1** showed the characteristic signals from a cinnamoyl group [$\delta_H$ 6.30 (1H, d, $J$ = 15.8 Hz), 7.62 (1H, d, $J$ = 15.8 Hz), 7.51 (2H, m), 7.38 (2H, m), and 7.39 (1H, m); $\delta_C$ 165.8, 117.7, 145.4, 134.3, 128.2 (C $\times$ 2), 128.9 (C $\times$ 2), and 130.4], a pregnane core [$\delta_H$ 1.48 (3H, s), 1.14 (3H, s), and 2.20 (3H, s), together with 21 carbon signals], and two sugar units ($\delta_H$ 4.94 and 5.07; $\delta_C$ 95.8 and 99.7). Aforementioned information indicated compound **1** was a pregnane glycoside comprising a cinnamoyl group and two sugar units. The pregnane core in **1** was identified as deacetylmetaplexigenin (**6**) by comparison of its $^1H$ and $^{13}C$ NMR data with those of **6** [13]. In the $^1H$-NMR spectrum

**Table 2**
$^{13}$C NMR data for compounds **1**–**5** in $CDCl_3$.

| Position | **1**[a] | **2**[a] | **3**[b] | **4**[b] | **5**[b] |
|---|---|---|---|---|---|
| 1 | 38.8 | 38.8 | 39.0 | 38.9 | 38.7 |
| 2 | 28.9 | 28.9 | 29.0 | 29.0 | 28.8 |
| 3 | 78.0 | 78.0 | 77.9 | 77.9 | 77.8 |
| 4 | 38.8 | 38.8 | 38.8 | 38.8 | 38.7 |
| 5 | 140.7 | 140.7 | 139.8 | 139.7 | 141.0 |
| 6 | 117.7 | 117.6 | 118.4 | 118.4 | 117.3 |
| 7 | 34.2 | 34.3 | 34.6 | 34.6 | 34.0 |
| 8 | 74.4 | 74.3 | 73.8 | 73.8 | 74.5 |
| 9 | 43.7 | 43.8 | 43.7 | 43.7 | 43.6 |
| 10 | 37.2 | 37.2 | 37.0 | 37.1 | 37.2 |
| 11 | 24.2 | 24.3 | 28.6 | 28.5 | 24.2 |
| 12 | 72.7 | 71.5 | 70.9 | 70.8 | 72.5 |
| 13 | 58.0 | 57.9 | 57.8 | 57.8 | 57.6 |
| 14 | 88.0 | 88.0 | 87.9 | 87.9 | 88.2 |
| 15 | 33.1 | 33.1 | 33.5 | 33.4 | 32.5 |
| 16 | 32.0 | 31.9 | 32.6 | 32.6 | 32.1 |
| 17 | 91.5 | 91.5 | 88.0 | 88.0 | 91.7 |
| 18 | 9.4 | 9.4 | 10.1 | 10.1 | 9.2 |
| 19 | 18.6 | 18.6 | 18.4 | 18.4 | 18.7 |
| 20 | 209.1 | 208.8 | 72.5 | 72.5 | 209.4 |
| 21 | 27.4 | 27.1 | 16.9 | 17.0 | 27.3 |
| 1′ | 165.8 | 165.9 | | | 170.0 |
| 2′ | 117.7 | 113.0 | | | 20.7 |
| 3′ | 145.4 | 166.8 | | | |
| 4′ | 134.3 | 38.2 | | | |
| 5′ | 128.2 | 20.8 | | | |
| 6′ | 128.9 | 20.9 | | | |
| 7′ | 130.4 | 16.5 | | | |
| 8′ | 128.9 | | | | |
| 9′ | 128.2 | | | | |
| | D-Digit | D-Digit | D-Digit | D-Digit | D-Digit |
| 1″ | 95.8 | 95.8 | 95.7 | 95.7 | 95.8 |
| 2″ | 37.5 | 37.5 | 37.6 | 37.0 | 37.0 |
| 3″ | 67.8 | 67.8 | 67.8 | 66.5 | 66.5 |
| 4″ | 80.9 | 80.9 | 80.9 | 82.6 | 82.6 |
| 5″ | 68.1 | 68.1 | 68.1 | 68.6 | 68.6 |
| 6″ | 18.2 | 18.1 | 18.2 | 18.1 | 18.1 |
| | D-Dig | D-Dig | D-Dig | D-Digit | D-Digit |
| 1‴ | 99.7 | 99.6 | 99.6 | 98.3 | 98.3 |
| 2‴ | 29.6 | 29.6 | 29.6 | 36.7 | 36.7 |
| 3‴ | 74.3 | 74.4 | 74.4 | 67.4 | 67.4 |
| 4‴ | 67.4 | 67.4 | 67.4 | 79.3 | 79.3 |
| 5‴ | 66.7 | 66.7 | 66.7 | 68.0 | 68.0 |
| 6‴ | 16.9 | 16.9 | 17.0 | 18.2 | 18.2 |
| OMe | 55.6 | 55.6 | 55.6 | – | – |
| | | | | L-cym | L-cym |
| 1⁗ | | | | 97.6 | 97.5 |
| 2⁗ | | | | 30.9 | 30.9 |
| 3⁗ | | | | 75.1 | 75.1 |
| 4⁗ | | | | 71.9 | 71.9 |
| 5⁗ | | | | 65. 9 | 65.9 |
| 6⁗ | | | | 17.8 | 17.8 |
| OMe | | | | 56.3 | 56.3 |

[a] Measured in $CDCl_3$ at 125 MHz.
[b] Measured in $CDCl_3$ at 100 MHz. Digit = digitoxopyranosyl. Dig = diginopyranosyl. cym = cymaropyranosyl.

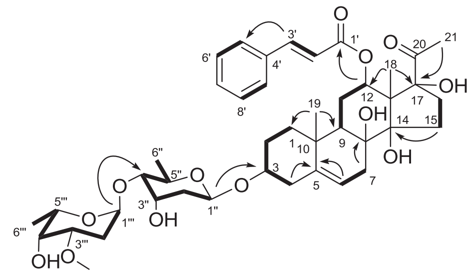

**Fig. 2.** Selected $^1H$–$^1H$ COSY (▬) and HMBC (→) correlations of **1**.

the observation of an anomeric H-atom at $\delta_H$ 4.94 (brd, $J$ = 9.5 Hz), a CH at $\delta_H$ 3.30 (dd, $J$ = 10.0, 2.0 Hz) and a secondary Me at $\delta_H$ 1.25 ($d$ = 6.0 Hz) indicated a $\beta$-digitoxopyranose sugar unit, while the characteristic carbon signals at $\delta_C$ 99.7, 29.6, 74.3, 67.4, 66.7, 16.9, and 55.6 suggested that the other sugar unit was an $\alpha$-diginopyranose [3]. This was further confirmed by TLC comparison of the acidic hydrolyzates of **1** with standard sugar samples. The absolute configurations of $\beta$-digitoxopyranose and $\alpha$-diginopyranose were assigned as D and L, respectively, by comparison of their optical rotation with those of authentic sugars. Detailed 2D analysis fulfilled the connections among cinnamoyl, sugars, and deacetylmetaplexigenin moieties (Fig. 2). HMBC correlation from an oxymethine (4.70, dd, $J$ = 10.0, 6.0 Hz, H-12) to a carbonyl at 165.8 (C-1′) located the cinnamoyl group at C-12. The digitoxopyranose was linked to C-3 by HMBC correlation of H-1″/C-3, which caused the severely downfield shifted carbon signal of C-3 ($\delta_C$ 78.0) with respect to the corresponding signal in **6** ($\delta_C$ 71.6). The sugar sequence was established as $\alpha$-L-diginopyranosyl-(1 → 4)-$\beta$-D-digitoxopyranose by HMBC correlations of H-1‴/C-4″. Thus the structure of **1** was determined as depicted and given a name cynanotoside A.

Compound **2** had a molecular formula $C_{41}H_{64}O_{13}$ as revealed by the HR-ESI-MS at $m/z$ 787.4268 $[M + Na]^+$ (calcd. 787.4245). The NMR spectra of **2** was very similar to those of **1**, except for the presence of an ikemaoyl group [$\delta_H$ 5.52 (1H, brs), 2.37 (1H, m), 1.06 (6H, d, $J$ = 7.0 Hz), 2.17 (3H, s); $\delta_C$ 165.9, 113.0, 166.8, 38.2, 20.8, 20.9, 16.5] in **2** instead of a cinnamoyl group in **1**. HMBC correlation from an oxymethine [$\delta_H$ 4.56 (t, $J$ = 7.5 Hz, H-12)] to the carbonyl at 165.9 (C-1′) located the ikemaoyl group at C-12. Comparison of the optical rotation of the sugars obtained from the acid hydrolysate of **2** with those of authentic sugar samples further confirmed the presence of an $\alpha$-L-diginopyranose and a $\beta$-D-digitoxopyranose in **2**. Thus the structure of **2** was determined as depicted and given the trivial name cynanotoside B.

Compound **3** was assigned the molecular formula $C_{34}H_{56}O_{12}$ on the basis of HR-ESI-MS at $m/z$ 679.3685 $[M + Na]^+$ (calcd. 679.3669). The NMR spectra of **3** bore a resemblance to those of **1**, with the notable differences being the absence of the signals for a cinnamoyl and a ketone groups and the presence of a doublet methyl ($\delta_H$ 1.16, d, $J$ = 6.5 Hz) and an additional oxymethine ($\delta_H$ 4.06, q, $J$ = 6.5 Hz; $\delta_C$ 72.5). This implied that the aglycone of **3** was probably sarcostin, a C-20 reduced derivative of deacetylmetaplexigenin (**6**). Comparison of the 1D NMR data of **3** with those of sarcostin confirmed the presence of sarcostin aglycone [8]. The assignment of $^1H$ and $^{13}C$ NMR signals of **3** was achieved by detailed 2D NMR analysis. The absolute configurations of the sugar units in **3** were confirmed as $\alpha$-L-diginopyranose and a $\beta$-D-digitoxopyranose using the same methods as described in **1** and **2**. Compound **3** was given the trivial name cynanotoside C.

The molecular formula of compound **4** was determined to be $C_{40}H_{66}O_{15}$ by the HR-ESI-MS at $m/z$ 809.4300 $[M + Na]^+$ (calcd. 809.4299). The NMR data of **4** showed the presence of three sugar units and a sarcostin aglycone. Characteristic signals of $\delta_H$ 4.93 (dd, $J$ = 8.6, 1.5 Hz), 4.89 (d, $J$ = 9.6 Hz); $\delta_C$ 95.7, 98.3, 37.0 ($CH_2$), and 36.7 ($CH_2$) in 1D NMR spectra indicated the presence of two $\beta$-digitoxopyranose. The third sugar unit was deduced to be $\alpha$-cymaropyranose by diagnostic signals at $\delta_H$ 4.91 (d, $J$ = 3.0); $\delta_C$ 97.6, and 30.9 ($CH_2$) in 1D NMR spectra [3]. Interpretation of the 2D-NMR data ($^1H$–$^1H$-COSY, HMQC, HMBC, and NOESY) not only confirmed the presence of a three-sugar unit at C-3 but also established the sugar sequence as 3-$O$-$\alpha$-cymaropyranosyl-(1 → 4)-$\beta$-digitoxopyranosyl-(1 → 4)-$\beta$-digitoxopyranoside. Particularly, the HMBC correlations of H-1⁗/C-4‴ and H-1‴/C-4″ suggested the connection of the three sugars via two (1 → 4) linkages. The absolute configurations of the digitoxopyranoses and cymaropyranose were determined as D and L, respectively, by using the same methods described above. The structure of **4** was thus determined as depicted and given the trivial name cynanotoside D.

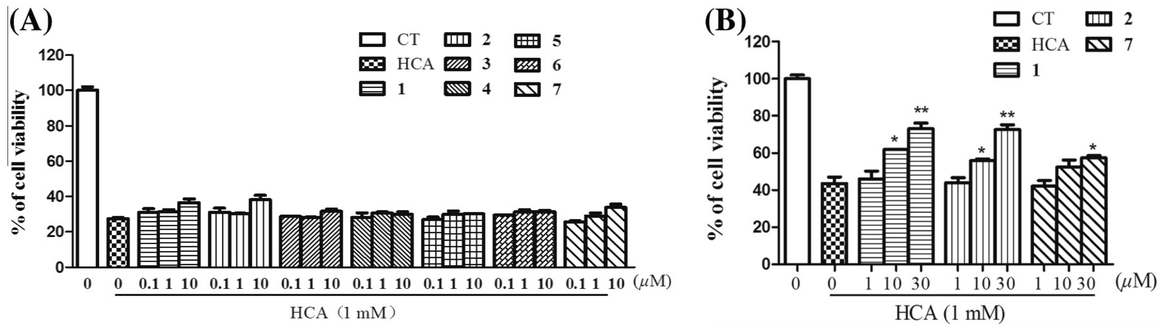


**Fig. 3.** Neuroprotective effects of compounds **1**–**7** against HCA-induced cell death in mice hippocampal HT22 cells. (A) Compounds **1**, **2**, and **7** exerted slightly beneficial effects against HCA-induced cell death at 10 μM. (B) Compounds **1**, **2**, and **7** dose-dependently prevented HCA-induced cell death. $^*p < 0.05$, $^{**}p < 0.01$ vs. control group (CT).

Compound **5** exhibited the molecular formula $C_{42}H_{66}O_{16}$ based on the HR-ESI-MS at *m/z* 849.4240 ($[M + Na]^+$ (calcd. 849.4249). The $^1$H-and $^{13}$C-NMR spectra of **5** (Tables 1 and 2) indicated that it was a triglycoside. The aglycone moiety of **5** shared a high similarity with those of **6** except for the presence of an additional acetyl group [$\delta_H$1.95 (3H, s); $\delta_C$ 20.7 and 170.0]. HMBC correlations from an oxymethine ($\delta_H$ 4.51, H-12) to the carbonyl ($\delta_C$ 170.0) linked the acetyl group to C-12. Three deoxysuger units in **5** were characterized by NMR signals at $\delta_H$ 4.92 (brd, $J$ = 8.4 Hz), 4.89 (brd, $J$ = 8.4 Hz), and 4.90 (brd, $J$ = 3.0 Hz); $\delta_C$ 95.8, 98.3, and 97.5, which were almost identical to those in **4**, indicating that **5** possessed a 3-*O*-α-cymaropyranosyl-(1 → 4)-β-digitoxopyranosyl-(1 → 4)-β-digitoxopyranoside sugar sequence. The sugar moiety was linked to C-3 by HMBC correlation from H-1″ to C-3. The absolute configurations of the digitoxopyranoses and cymaropyranose were determined as D and L, respectively, by using the same methods described above. Detailed 2D analysis allowed the full assignments of 1D NMR data of **5**. Thus **5** was determined as depicted and given the trivial name cynanotoside E.

The known compounds deacetylmetaplexigenin (**6**) [13] and cynotophylloside H (**7**) [14] were identified by comparison of their NMR data with those in literature. A survey of analogous glycosides from the Asclepiadaceae family suggested that all the β-configured 2,6-dideoxysugars have the D-configuration, while the α-configured sugars are L-sugars. In addition, C-2 of a 2-deoxysugar (cymarose, digitoxose, or diginose) that possesses an α-L-configuration usually appears in the $^{13}$C-NMR spectrum at *ca.* 32.0 ppm or less, while that of a β-D-configured 2-deoxysugar normally resonates at a lower field with a chemical shift larger than 34.0 ppm [3].

*C. otophyllum* has been widely used as a treatment for epilepsy in Traditional Chinese Medicine [1]. Epilepsy is a highly prevalent serious brain disorder, and oxidative stress is considered as a contributing factor to the onset and evolution of this disease [16,17]. To investigate the potential chemistry related to the anti-epilepsy usage of this plant, we examined compounds **17** in three oxidative stress models induced by glutamate, $H_2O_2$, and homocysteic acid (HCA), respectively, using MTT assay in a hippocampal neuronal cell line HT22. Compounds **17** failed to reverse the decrease of cell viability caused by glutamate- and $H_2O_2$-induced cell death, while **1**, **2**, and **7** exhibited slightly beneficial effects on HCA-induced cell death at 10 μM (Fig. 3A). To verify the protection effect of **1**, **2**, and **7** on HCA model, we increased the maximum concentration of these compounds to 30 μM in a reset testing, in which **1**, **2**, and **7** showed significant dose-dependent protection to HCA-induced cell death ranging from 1 to 30 μM (Fig. 3B). HCA-induced model leads to the death of neurons by depletion of glutathione, the cells major intracellular antioxidant. Moreover, HCA is also considered to be related to NMDA-independent epilepsy in human being [18] and used to establish epilepsy models in immature rats [19]. Thus, the protective effect of **1**, **2**, and **7** on this model may explain the TCM use of this plant for the treatment of epilepsy. However, the exact mechanisms and detailed connections between this model and epilepsy require further investigation.

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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.2013.06.007.

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