Fitoterapia 97 (2014) 71–77

Contents lists available at ScienceDirect

Fitoterapia

journal homepage: www.elsevier.com/locate/fitote

Cardiac glycosides from the bark of *Antiaris toxicaria*

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ARTICLE INFO

Article history:
Received 14 April 2014
Accepted in revised form 20 May 2014
Available online 28 May 2014

Keywords:
Antiaris toxicaria
Chemical constituents
Cardiac glycoside
Cytotoxicity
Expression of Nur77

ABSTRACT

Five new cardiac glycosides (**1**–**5**, namely antiaroside Y-ZC) together with 19 known compounds were obtained from the bark of *Antiaris toxicaria*. Their chemical structures were determined by IR, HR-ESI-MS, 1D and 2D NMR (HSQC, ^{1}H–^{1}H COSY, HMBC, ROESY). The absolute configuration of sugar unit was defined by acid hydrolysis and appropriate derivatization. Compound **1** was rare 5β-H-10β-H-19-nor-cardenolide, which might derive from decarboxylative derivative of 19-COOH cardenolide. The inhibitory effects of cardiac glycosides **1**–**11** on the viability of NIH-H460 lung cancer cells and their induction of Nur77 expression were evaluated and preliminary structure–activity relationship (SAR) was also discussed.

1. Introduction

Cardiac glycosides are clinically used in the treatment of congestive heart failure and as anti-arrhythmic agents due to their strong inhibitory activity toward the ubiquitous cell surface enzyme Na^+/K^+-ATPase. Recently, analysis of epidemiological data along with results arising from in vitro and in vivo studies demonstrate that cardiac glycosides exhibit potent antiproliferative and apoptotic effects on cancer cells through complex signal transduction mechanisms, and the first cardiac glycoside-based substances are now undergoing clinical trials for cancer treatment [1–9]. The exact mechanisms underlying these effects of cardiac glycosides are not yet fully elucidated.

In our previous search for anticancer agents from plants, about 40 cardiac glycosides were isolated from the latex and stem of *Antiaris toxicaria* by bioassay and chemical guided fractionation [10,11]. Some cardiac glycosides were observed to effectively induce apoptosis and Nur77 protein expression in human NIH-H460 lung cancer cells. In addition, the cardiac glycosides found to induce Nur77 expression were also examined for their modulation of the subcellular localization of Nur77 protein. Our studies revealed that both induction of Nur77 expression and its subsequent translocation from the nucleus to the cytoplasm are critical events in apoptosis induction by cardiac glycosides in cancer cells [10–12]. Herein, we reported the isolation and structural elucidation of 5 new (**1**–**5**) (see Fig. 1.) and 19 known cardiac glycosides from the bark of *A. toxicaria*. In addition, the inhibitory effects of cardiac glycosides **1**–**11** on the viability of NIH-H460 lung cancer cells and their induction of Nur77 expression were evaluated and preliminary structure–activity relationship (SAR) was also discussed.

2. Experimental

2.1. General experimental procedures

Optical rotations were obtained on a P-1020 digital polarimeter (Jasco Corporation). IR spectra were recorded on a JASCO FTIR-480 plus spectrometer. NMR spectra were

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http://dx.doi.org/10.1016/j.fitote.2014.05.013
0367-326X/

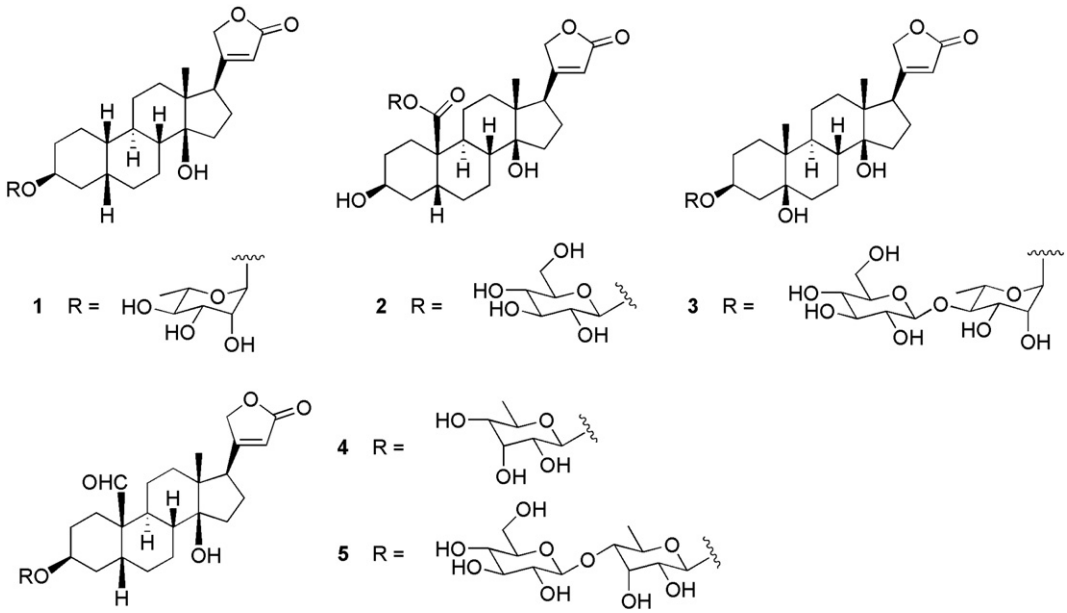

Fig. 1. Chemical structures of compounds **1**–**5.**

measured on Bruker AV 300 and 400. The chemical shifts were given in ppm relative to chemical shifts of solvent resonances (pyridine-d_5: 7.58 and 135.9 ppm). HR-ESI-MS spectra were obtained on a Micromass Q-TOF mass spectrometer. Analysis HPLC was performed on a SHIMADZU LC-20AB Liquid Chromatograph with SPD-M20A Detector using a cosmosil C18 column (4.6 × 250 mm, 5 μm). Preparative HPLC was performed on a SHIMADZU LC-6AD Liquid Chromatograph with SPD-20A Detector using an ODS column [YMC-Pack ODS-A (10.0 × 250 mm, 5 μm, 220 and 254 nm)]. Open column chromatography (CC) was performed using silica gel (200–300 mesh, Qingdao Haiyang Chemical Group Corp, Qingdao), ODS (50 μm, YMC), and Sephadex LH-20 (Pharmacia). TLC analysis was performed on pre-coated silica gel GF254 plates (Qingdao Haiyang Chemical Group Corp, Qingdao).

2.2. Plant material

The bark of *A. toxicaria* was collected from Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Yunnan province, P.R. China in March 2011. The plant was authenticated by Professor Yu Chen of Kunming Institute of Botany, Chinese Academy of Sciences. A voucher specimen (ANTO201103) was deposited in the Institute of Traditional Chinese Medicine & Natural Products, Jinan University.

2.3. Extraction and isolation

The bark of *A. toxicaria* (3.25 kg) was extracted by 95% (v/v) EtOH-H_2O (4 × 30 L) under reflux condition for 2 h every time. The combined ethanol extracts were concentrated under vacuum to obtain the crude extract (135.0 g). The crude extract was subjected to open silica gel CC (ϕ 3.3 × 53 cm) using a $CHCl_3$-MeOH gradient to give 12 fractions (C1–C12). Fraction C7 (3.53 g) was subjected to open silica gel CC (ϕ 3.3 × 25 cm) using a $CHCl_3$-MeOH gradient to give 6 subfractions (C7-1–C7-6). Subfraction C7-5 (1.06 g) was chromatographed over ODS (ϕ 3.3 × 25 cm) MPLC using MeOH-H_2O gradient to give 12 subfractions (C7-5-1–C7-5-12). Subfraction C7-5-6 (65.3 mg) was subjected to preparative RP-HPLC (52% MeOH-H_2O, a flow rate of 3.0 mL/min) to afford compound **6** (t_R = 13.5 min, 32.7 mg). Subfraction C7-5-7 (25.5 mg) was applied to preparative RP-HPLC (58% MeOH-H_2O, a flow rate of 3.4 mL/min) to obtain compound **4** (t_R = 10.0 min, 10.1 mg). Subfraction C7-5-9 (26.6 mg) was applied to preparative RP-HPLC (58% MeOH-H_2O, a flow rate of 3.2 mL/min) to obtain compound **1** (t_R = 15.5 min, 5.0 mg). Fraction C8 (3.33 g) was subjected to ODS (ϕ 3.5 × 13 cm) MPLC eluting with MeOH-H_2O gradient to give 5 subfractions (C8-1–C8-5). Subfraction C8-5 (172.0 mg) was applied to preparative RP-HPLC (35% MeOH-H_2O, a flow rate of 3.0 mL/min) to obtain compound **12** (t_R = 17.0 min, 25.7 mg). Fraction C9 (4.18 g) was chromatographed over ODS (ϕ 3.5 × 13 cm) MPLC eluted with MeOH-H_2O (48%) to give 7 subfractions (C9-1–C9-7). Subfraction C9-4 (339.1 mg) was applied to preparative RP-HPLC (45% MeOH-H_2O, a flow rate of 3.0 mL/min) to obtain compounds **13** (t_R = 16.2 min, 73.5 mg) and **14** (t_R = 20.1 min, 53.9 mg). Subfraction C9-6 (468.3 mg) was subjected to RP-HPLC (50% MeOH-H_2O, a flow rate of 3.0 mL/min) to yield compound **7** (t_R = 35.0 min, 3.0 mg). Fraction C11 was subjected to ODS (ϕ 3.3 × 25 cm) MPLC eluted with MeOH-H_2O gradient to give 8 subfractions (C11-1–C11-8). Subfraction C11-3 (779.0 mg) was subjected to ODS (ϕ 3.3 × 25 cm) MPLC eluted with MeOH-H_2O gradient to give 6 subfractions (C11-3-1–C11-3-6). Subfraction C11-3-4 (132.1 mg) was applied to preparative RP-HPLC (20% MeOH-H_2O, a flow rate of 3.2 mL/min) to obtain compound **15** (t_R = 19.7 min, 16.7 mg). Subfraction C11-3-4-3 (26.3 mg) was applied for semipreparative

RP-HPLC (11% CH_3CN-H_2O, a flow rate of 3.0 mL/min) to afford compounds **16** (t_R = 22.5 min, 5.2 mg) and **17** (t_R = 25.2 min, 11.7 mg). Subfraction C11-4 (1.036 g) was applied to preparative RP-HPLC (30% MeOH-H_2O, a flow rate of 3.2 mL/min) to obtain compounds **18** (t_R = 10.8 min, 154.5 mg), **19** (t_R = 15.0 min, 27.8 mg), and **20** (t_R = 19.2 min, 42.7 mg). Subfraction C11-6 (763.0 mg) was subjected to ODS (ϕ 3.3 × 25 cm) MPLC eluted with MeOH-H_2O gradient to give 6 subfractions (C11-6-1–C11-6-6). Subfraction C11-6-4 (27.0 mg) was applied to semipreparative RP-HPLC (25% MeOH-H_2O, a flow rate of 3.2 mL/min) to obtain compound **8** (t_R = 26.0 min, 2.1 mg). Subfraction C11-6-5 (60.5 mg) was applied to semipreparative RP-HPLC (30% MeOH-H_2O, a flow rate of 3.0 mL/min) to obtain compounds **21** (t_R = 12.5 min, 1.9 mg) and **22** (t_R = 21.0 min, 9.4 mg). Subfraction C11-6-6 (186.8 mg) was applied to semipreparative RP-HPLC (20% CH_3CN-H_2O, a flow rate of 3.0 mL/min) to obtain compounds **23** (t_R = 12.6 min, 8.0 mg), **9** (t_R =14.5 min, 2.7 mg), **10** (t_R = 19.5 min, 5.5 mg), **2** (t_R =21.0 min, 7.7 mg), **24** (t_R = 23.8 min, 4.2 mg), **5** (t_R =28.6 min, 10.0 mg), **3** (t_R = 33.0 min, 6.8 mg) and **11** (t_R = 34.5 min, 38.0 mg).

*2.3.1. Antiaroside Y (**1**)*

1.5 of dry wt. (mg/kg) colorless syrup; $[\alpha]_D^{26}$ −14.3 (*c* 2.6, MeOH); IR (KBr) ν_{max} 3418, 2928, 1736, 1618, 1066 cm^{-1}; ^1H NMR and ^{13}C NMR (see Table 1); ESI-MS *m/z* 1035 $[2\ M + Na]^+$, 541 $[M + Cl]^-$; HR-ESI-MS: *m/z* 507.2962 $[M + H]^+$ (calcd. for $C_{28}H_{43}O_8$, 507.2958).

*2.3.2. Antiaroside Z (**2**)*

2.4 of dry wt. (mg/kg) colorless powder; $[\alpha]_D^{26}$ −6.2 (*c* 3.1, MeOH); IR (KBr) ν_{max} 3298, 2882, 1648, 1361, 1050 cm^{-1}; ^1H NMR and ^{13}C NMR (see Table 1); ESI-MS *m/z* 589 $[M + Na]^+$, 601 $[M + Cl]^-$; HR-ESI-MS *m/z* 589.2616 $[M + Na]^+$ (calcd. for $C_{29}H_{42}O_{11}Na$, 589.2625).

*2.3.3. Antiaroside ZA (**3**)*

2.1 of dry wt. (mg/kg) colorless syrup; $[\alpha]_D^{26}$ −13.0 (*c* 3.1, MeOH); IR (KBr) ν_{max} 3305, 2934, 1725, 1036 cm^{-1}; ^1H NMR and ^{13}C NMR (see Table 2); ESI-MS *m/z* 721 $[M + Na]^+$, 743 $[M + Cl]^-$; HR-ESI-MS *m/z* 721.3412 $[M + Na]^+$ (calcd. for $C_{35}H_{54}O_{14}Na$, 721.3411).

*2.3.4. Antiaroside ZB (**4**)*

3.1 of dry wt. (mg/kg) colorless syrup; $[\alpha]_D^{26}$ −22.4 (*c* 5.1, MeOH); IR (KBr) ν_{max} 3419, 2973, 2921, 1750, 1373, 1211 cm^{-1}; ^1H NMR and ^{13}C NMR (see Table 2); ESI-MS *m/z* 1091 $[2\ M + Na]^+$, 579 $[M + Cl]^-$; HR-ESI-MS *m/z* 535.2905 $[M + H]^+$ (calcd. for $C_{29}H_{43}O_9$, 535.2907).

*2.3.5. Antiaroside ZC (**5**)*

3.1 of dry wt. (mg/kg) colorless syrup; $[\alpha]_D^{26}$ −17.9 (*c* 4.1, MeOH); IR (KBr) ν_{max} 3421, 2967, 2928, 1733, 1456, 1070 cm^{-1}; ^1H NMR and ^{13}C NMR (see Table 2); ESI-MS *m/z* 719 $[M + Na]^+$, 731 $[M + Cl]^-$; HR-ESI-MS *m/z* 719.3250 $[M + Na]^+$ (calcd. for $C_{35}H_{52}O_{14}Na$, 719.3255).

2.4. Acid hydrolysis and sugar analysis

Acid hydrolysis reactions of compounds **1**–**3** [13]. The cardiac glycosides (0.5 mg each) were hydrolyzed using 2 mL of 2 N HCl for 1 h at 80–90 °C. The resulting mixtures were extracted with EtOAc (2 × 2 mL). The aqueous layers were concentrated and heated with L-cysteine methyl ester in 1 mL pyridine at 60 °C for 1 h. Sugar (D/L) standards were also derivatized using L-cysteine methyl ester in the same manner. Then arylisothiocyanates were added to the reaction mixtures and heated for 1 h at 60 °C. The reaction mixtures were analyzed using C18 HPLC (25% MeOH-H_2O (0.01% HCOOH), a flow rate of 0.8 mL/min) with a UV detector (250 nm). The retention times (min) of the derivatized standards were as follows: D-glucose (21.05), L-glucose (18.34), and L-rhamnose (34.67). By comparing retention times with those of the standards, the rhamnoses in compounds **1** and **3** were determined to be L-configurations; the glucoses in **2**, **3**, and **5** were determined to be D-configurations.

2.5. MTT assay

Cells were counted by using a hemocytometer, equally distributed in 96-well plates (5 × 10^3 cells per well) and treated with cardiac glycosides **1**–**11** and digoxin for 48 h, and cell proliferation was evaluated with an MTT assay procedure as previously described [14,15]. To determine cell viability, the medium was removed and cells were incubated with 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) at a final concentration of 0.5 mg/mL in RPMI 1640 medium containing 10% FBS for 2 h in the dark at 37 °C. Then 100 μL DMSO was added to the wells. Cultures were incubated at room temperature (RT) for 5 min and read at 492 nm.

2.6. Western blotting analysis for Nur77 expression

Equal amounts of the lysates were electrophoresed on an 8% SDS-PAGE gel and transferred onto polyvinylidene difluoride membranes, as reported previously [16,17], which were then blocked with 5% nonfat milk in TBST [50 mmol/L Tris–HCl (pH 7.4), 150 mmol/L NaCl, and 0.1% Tween 20] for 1 h, incubated with various primary antibodies overnight at 4 °C and incubated with secondary antibodies for 1 h. Immunoreactive products were detected by using chemiluminescence with an enhanced chemiluminescence system (ECL, Amersham Biosciences). The dilutions of the primary antibodies were anti-Nur77 (Cell signal, 3960) in 1: 1,000, anti-PARP (BD Biosciences, 556494) in 1: 1,000. The blots were reprobed with anti-β-actin antibody for loading control.

3. Results and discussion

Compounds **1**–**5** gave positive reaction with Keddle reagent, indicating they were cardiac glycosides.

Compound **1** was obtained as colorless syrup. The HR-ESI-MS showed quasimolecular ion at *m/z* 507.2962 $[M + H]^+$ (calcd. for 507.2958), indicating the molecular formula of $C_{28}H_{42}O_8$ and accounting for 8 degrees of unsaturations. The IR spectrum of **1** displayed prominent absorption maxima at 3418, 2928, 1736, 1618, and 1066 cm^{-1}, indicating the

Table 1
^{1}H and ^{13}C NMR data for compounds **1** and **2** (pyridine-d_5, δ in ppm, *J* in Hz).

Position	1		2	
	δ_C[b]	δ_H[a]	δ_C[b]	δ_H[a]
1a/b	27.0	1.84 m, 1.58 m	22.1	2.42 m, 1.56 m
2a/b	26.5	1.83 m, 1.27 m	26.2	2.92 m, 1.44 m
3	73.4	4.20 br s	65.5	4.41 br s
4a/b	30.6	1.60 m	28.3	2.13 m, 1.67 m
5	30.9	2.09 m	32.4	3.24 m
6a/b	32.7	2.09 m, 1.54 m	34.3	2.07 m, 1.67 m
7a/b	22.5	2.14 m, 1.33 m	22.5	2.13 m, 1.43 m
8	42.6	1.31 m	42.1	2.63 m
9	34.6	1.52 m	36.0	1.97 m
10	48.8	1.35 m	50.8	–
11	22.7	1.85 m, 1.68 m	29.1	1.83 m, 1.27 m
12	40.3	1.41 m	40.5	1.46 m
13	50.6	–	50.6	–
14	84.4	–	85.3	–
15	33.7	1.86 m, 1.46 m	33.2	2.14 m, 1.89 m
16	27.8	2.11 m, 1.99 m	27.7	2.11 m, 1.67 m
17	51.9	2.78 m	51.8	2.81 m
18	16.7	1.01 s	16.6	1.20 s
19	–	–	176.2	–
20	176.5	–	176.6	–
21	74.2	5.33 dd (18.1, 1.5) 5.33 dd (18.1, 1.8)	74.1	5.04 dd (18.1, 1.5) 5.05 dd (18.1, 1.8)
22	118.0	6.14 s	118.0	6.13 s
23	174.9	–	175.0	–
1′	100.3	5.43 br s	96.0	6.39 d (8.2)
2′	72.9	4.55 m	74.4	4.14 t (8.2)
3′	73.4	4.55 m	79.3	4.27 m
4′	74.6	4.32 m	71.5	4.30 m
5′	70.6	4.30 m	79.9	4.04 m
6′	19.1	1.69 d (5.6)	62.6	4.47 m, 4.36 m

The assignments of H and C signals are based on HSQC, ^{1}H–^{1}H COSY, and HMBC experiments.
[a] Recorded at 300 MHz (for ^{1}H).
[b] Recorded at 400 MHz (for ^{1}H).

presence of hydroxyl and carbonyl functionalities. The ^{1}H and ^{13}C NMR signals for **1** were assigned using 1D and 2D NMR experiments (see Table 1), which were similar to those of antiaroside I [18]. The ^{1}H NMR spectrum for **1** showed characteristic of butenolide ring protons at δ 6.14 (1H, s, H-22), 5.33 (1H, dd, 18.1, 1.5, H-21a), and 5.04 (1H, dd, 18.1, 1.5, H-21b), two methyl protons at δ 1.01 (3H, s, H-18) and δ 1.69 (3H, d, 5.6, H-6′), and one anomeric proton at δ 5.43 (1H, br s, H-1′). The ^{13}C NMR resonances at δ 100.3 (C-1′), 72.9 (C-2′), 73.4 (C-3′), 74.6 (C-4′), 70.6 (C-5′), and 19.1 (C-6′) revealed the presence of rhamnose moiety in **1**. Analyses of ^{13}C NMR and DEPT 135 signals revealed that **1** possessed four quaternary carbons, having one quaternary carbon less than antiaroside I. The presence of a carbon signal at δ 30.9 (CH, C-5 in **1**) and the absence of a quaternary carbon signal at δ 72.2 (C-5 in antiaroside I) and a downfield shift of C-10 from δ 37.4 to δ 48.8 in **1** suggested that **1** was a 5-deoxy derivative of antiaroside I. The L-configuration of rhamnose unit in **1** was defined via acid hydrolysis and appropriate derivatization of the resulting sugar [13]. Key HMBC correlation between δ 5.43 (1H, br s, H-1′) and δ 73.4 (d, C-3) confirmed that the sugar moiety was located at C-3 position of the aglycone. Thus, compound **1** was identified as 19-nor-digitoxigenin 3β-O-α-L-rhamnopyranoside named antiaroside Y.

Compound **2** was obtained as a colorless powder. The HR-ESI-MS showed quasimolecular ion at *m/z* 589.2616 $[M + Na]^+$ (calcd. for 589.2625), suggesting the molecular formula of $C_{29}H_{42}O_{11}$ and accounting for 9 degrees of unsaturations. The IR spectrum of **2** displayed prominent absorption maxima at 3298, 2882, 1648, 1361, 1050 cm^{-1}, indicating the presence of hydroxyl and carbonyl functionalities. The ^{1}H and ^{13}C NMR signals for **2** were assigned using 1D and 2D NMR experiments (see Table 1) which were similar to those of antiaroside R [11], except for having one oxygen atom less than antiaroside R. The ^{1}H NMR spectrum for **2** showed characteristic of butenolide ring protons at δ 6.13 (1H, s, H-22), 5.33 (1H, dd, 18.1, 1.8, H-21a), and 5.05 (1H, dd, 18.1, 1.8, H-21b), one methyl protons at δ 1.20 (3H, s, H-18), and one anomeric proton at δ 6.39 (1H, d, 8.2, H-1′). The ^{13}C NMR spectrum showed two carbonyl carbon signals at δ 175.0 (s, C-23) and 176.2 (s, C-19), indicating that sugar moiety was attached at C-19 position of aglycone. The ^{13}C NMR resonances at δ 96.0 (C-1′), 74.4 (C-2′), 79.3 (C-3′), 71.5 (C-4′), 79.9 (C-5′), and 62.6 (C-6′) revealed the presence of glucose moiety in **2**. The presence of a carbon signal at δ 32.4 (C-5 in **2**) and the absence of a quaternary carbon signal at δ 75.1 (C-5 in antiaroside R) suggested that **2** was a 5-deoxy derivative of antiaroside R. The large *J* value of H-1′ ($J = 8.2$ Hz) indicated that the anomeric proton in **2** was β-orientated. The D-configuration of glucose unit in **2** was defined via acid hydrolysis and appropriate derivatization of the resulting sugar [13]. Key HMBC correlation between H-1′ (δ 6.39, 1H, d, 8.2)

Table 2
^{1}H and ^{13}C NMR data for compounds **3**–**5** (pyridine-d_5, δ in ppm, J in Hz).

Position	3		4		5	
	δ_C[b]	δ_H[a]	δ_C[b]	δ_H[a]	δ_C[b]	δ_H[a]
1a/b	26.5	1.71, m/1.44, m	29.0	1.44, m/1.22, m	28.9	1.47, m/1.22, m
2a/b	26.6	2.10, m/1.83, m	26.0	2.11, m/1.58, m	26.0	2.08, m/1.59, m
3	75.0	4.29, br s	73.5	4.33, (br s) overlap	73.6	4.27, br s
4a/b	35.1	2.16, m/1.70, m	30.1	1.78, m/1.47, m	30.1	1.80, m/1.75, m
5	74.0	–	30.3	2.55, m	30.2	2.59, m
6a/b	35.8	1.88, m/1.49, m	22.4	2.05, m/1.27, m	22.3	2.06, m/1.27, m
7a/b	24.7	2.27, m/1.31, m	22.6	2.08, m/1.60, m	22.6	2.07, m/1.57, m
8	41.3	1.86, m	42.6	2.15, m	42.6	2.15, m
9	39.5	1.63, m	36.4	1.87, m	35.5	1.84, m
10	41.5	–	51.7	–	51.7	–
11	22.4	2.14, m/1.41, m	21.7	2.03, m/1.87, m	21.7	2.08, m/1.87, m
12	40.3	1.46, m	40.2	1.45, m/1.32, m	40.2	1.44, m/1.34, m
13	50.4	–	50.5	–	50.5	–
14	85.2	–	84.7	–	84.8	–
15	33.5	2.08, m/1.89, m	32.8	2.05, m/1.82, m	32.8	2.05, m/1.81, m
16	27.6	2.11, m/1.96, m	27.6	2.08, m/1.96, m	27.6	2.06, m/1.95, m
17	51.7	2.83, m	51.7	2.78, m	51.7	2.78, m
18	16.5	1.05, s	16.5	1.10, s	16.5	1.10, s
19	17.6	1.07, s	207.1	9.57, s	207.0	9.57, s
20	176.3	–	176.3	–	176.3	–
21	74.1	5.33, dd (18.1, 1.2) 5.31, dd (18.0, 1.6)	74.1	5.05, dd (18.1, 1.2) 5.03, dd (18.0, 1.6)	74.1	5.30, dd (18.0, 1.7) 5.03, dd (18.0, 1.7)
22	118.1	6.16, s	118.1	6.13, s	118.1	6.13, s
23	174.9	–	174.9	–	174.9	–
1′	100.2	5.43, br s	101.1	5.33, d (8.0)	100.8	5.29, d (7.8)
2′	72.3	4.51, m	72.9	4.00, dd (7.9, 2.9)	72.4	3.96, dd (8.0, 3.0)
3′	73.1	4.56, m	73.7	4.68, t (2.9)	72.9	4.35, m
4′	85.0	4.41,m	74.8	3.71, dd (9.3, 2.4)	83.9	3.84, dd (9.5, 2.4)
5′	69.2	4.18, m	70.8	4.32, m	69.2	4.47, m
6′	18.8	1.71, d (6.2)	19.2	1.63, d (6.1)	18.6	1.70, d (6.3)
1″	107.2	5.23, d (7.6)			106.7	5.07, d (8.0)
2″	76.8	4.13, t (8.1)			75.6	3.97, m
3″	78.9	4.20, m			78.7	4.25, m
4″	71.8	4.25, br s			72.0	4.23, m
5″	78.9	3.81, m			78.6	3.94, m
6″	63.0	4.44, m/4.39, m			62.9	4.45, m/4.32, m

The assignments of H and C signals are based on HSQC, ^{1}H–^{1}H COSY, and HMBC experiments.
[a] Recorded at 300 MHz (for ^{1}H).
[b] Recorded at 400 MHz (for ^{1}H).

and C-19 (δ 176.2) confirmed that the sugar moiety was located at C-19 position of the aglycone. Thus, compound **2** was named antiaroside Z.

Compound **3** was obtained as colorless syrup. The molecular formula of $C_{35}H_{54}O_{14}$ was determined by the HR-ESI-MS at m/z 721.3412 $[M + Na]^+$ (calcd. for 721.3411), and accounting for 9 degrees of unsaturations. The IR spectrum of **3** displayed prominent absorption maxima at 3305, 2934, 1725, and 1036 cm^{-1}, indicating the presence of hydroxyl and carbonyl functionalities. The ^{1}H and ^{13}C NMR signals for **3** were assigned using 1D and 2D NMR experiments (see Table 2). The ^{1}H NMR spectrum showed resonance characteristic of butenolide ring protons at δ 6.16 (1H, s, H-22), 5.33 (1H, dd, 18.1, 1.2, H-21a), and 5.05 (1H, dd, 18.1, 1.2, H-21b). The ^{1}H NMR spectrum also displayed three methyl protons at δ 1.05 (3H, s, H-18), 1.07 (3H, s, H-19), and 1.71 (3H, d, 6.2, H-6′), two anomeric protons at δ 5.43 (1H, br s, H-1′) and 5.23 (1H, d, 7.6, H-1″). Analyses of ^{1}H–^{1}H COSY, HSQC, and HMBC spectra indicated that the aglycone for **3** was periplogenin [18]. The sugar units for **3** were identified as glucose and rhamnose units according to their ^{1}H and ^{13}C NMR resonances. The L-configuration of rhamnose unit and D-configuration of glucose unit were determined via acid hydrolysis and appropriate derivatization of the resulting sugar [13]. The β-orientation of anomeric proton for glucose unit was defined by large coupling value of H-1″ (J = 7.6 Hz). The HMBC correlation between δ 5.23 (1H, d, 7.6, H-1″) and δ 85.0 (d, C-4′) suggested that glucose unit was linked to C-4 of rhamnose unit; HMBC correlation between 5.43 (1H, br s, H-1′) and δ 75.0 (d, C-3) revealed that the sugar chain was attached to C-3 position of aglycone. Thus, compound **3** was identified as periplogenin 3β-O-β-D-glucopyranosyl (1–4)-α-L-rhamnopyranoside named antiaroside ZA.

Compound **4** was obtained as colorless syrup. The HR-ESI-MS showed quasimolecular ion at m/z 535.2905 $[M + H]^+$ (calcd. for 535.2907), suggesting the molecular formula of $C_{29}H_{42}O_9$ and accounting for 9 degrees of unsaturations. The IR spectrum of **4** displayed prominent absorption maxima at 3419, 2973, 2921, 1750, 1373, and 1211 cm^{-1}, indicating the presence of hydroxyl and carbonyl functionalities. The ^{1}H and ^{13}C NMR signals for **4** were assigned using 1D and 2D NMR experiments (see Table 2). The ^{1}H NMR spectrum for **4** showed characteristic

Table 3
The antiproliferative effects of compounds **1**–**11** on NIH-H460 cancer cells.[a]

Compound	IC_{50} (nM)	Compound	IC_{50} (nM)
1	978.2	**7**	140.2
2	>10 μM	**8**	253.2
3	1212	**9**	229.3
4	158.4	**10**	50.03
5	34.18	**11**	26.82
6	27.03	Digoxin	712.2

[a] NIH-H460 cells were incubated for 48 h with compounds **1**–**11** and cell viability was then evaluated by the MTT assay. Digoxin was used as positive control.

of butenolide ring protons at δ 6.13 (1H, s, H-22), 5.31 (1H, dd, 18.0, 1.6, H-21a), and 5.03 (1H, dd, 18.0, 1.6, H-21b), one methyl protons at δ 1.10 (3H, s, H-18), and one anomeric proton at δ 5.33 (1H, d, 8.0, H-1′). The ^{13}C NMR resonances at δ 101.1 (C-1′), 72.9 (C-2′), 73.7 (C-3′), 74.8 (C-4′), 70.8 (C-5′), and 19.2 (C-6′) revealed the presence of 6-deoxy-D-allose moiety in **4**. The aglycone for **4** was identified as cannogenin by comparison of ^{13}C NMR resonance with that of malayoside (**6**), which was confirmed by analyses of ^{1}H–^{1}H COSY, HSQC, and HMBC spectra [10,19,20]. Key HMBC correlation between δ 5.33 (1H, d, 8.0, H-1′) and δ 73.5 (d, C-3) confirmed that the sugar moiety was located at C-3 position of the aglycone. The large J value of H-1′ (J = 8.0 Hz) indicated that the anomeric proton for 6-deoxy-D-allose moiety in **4** was β-orientated. Hence, compound **4** was established for cannogenin 3β-O-6-deoxy-β-D-allopyranoside named antiaroside ZB.

Compound **5** was obtained as colorless syrup. The HR-ESI-MS showed the quasimolecular ion at m/z 719.3250 $[M + Na]^+$ (calcd. for 719.3255), indicating the molecular formula $C_{35}H_{52}O_{14}$ and accounting for 10 degrees of unsaturations. The IR spectrum of **5** displayed prominent absorption maxima at 3421, 2967, 2928, 1733, 1456, and 1070 cm^{-1}, indicating the presence of hydroxyl and carbonyl functionalities. The ^{1}H and ^{13}C NMR signals for **5** were assigned using 1D and 2D NMR experiments (see Table 2), which were similar to those of **4** except for resonance signals of sugar residues. The ^{1}H NMR spectrum displayed two anomeric protons at δ 5.29 (1H, d, 7.8, H-1′) and 5.07 (1H, d, 8.0, H-1″), indicating that it was a disaccharide glycoside. The ^{13}C NMR resonances at δ 100.8 (C-1′), 72.4 (C-2′), 72.9 (C-3′), 83.9 (C-4′), 69.2 (C-5′), and 18.6 (C-6′) revealed the presence of 6-deoxy-D-allose moiety; δ 106.7 (C-1″), 75.6 (C-2″), 78.7 (C-3″), 72.0 (C-4″), 78.6 (C-5″), and 62.9 (C-6″) revealed the presence of glucose moiety in **5**. The β-orientation of both anomeric protons for 6-deoxy-D-allose and glucose units were defined by large coupling value of H-1′ (J = 7.8 Hz) and H-1″ (J = 8.0 Hz). The D-configuration of glucose unit in **5** was defined via acid hydrolysis and appropriate derivatization of the resulting sugar [13]. The HMBC correlation between δ 5.29 (1H, d, 7.8, H-1′) and δ 73.6 (d, C-3) revealed that the sugar chain was attached to C-3 position of aglycone; HMBC correlation between δ 5.07 (1H, d, 8.0, H-1″) and δ 83.9 (d, C-4′) suggested that glucose unit was linked to C-4 of 6-deoxy-D-allose. Thus, compound **5** was identified as cannogenin 3-O-β-D-glucopyranosyl (1–4)-β-D-allopyranoside named antiaroside ZC.

Nineteen known compounds were identified as malayoside (**6**) [10,20], peripalloside (**7**) [21], toxicarioside O (**8**) [22], convalloside (**9**) [23], periplogenin glucoside (**10**) [24], 3-O-β-D-glucopyranosyl-(1 → 4)-α-L-rhamnopyranosylcannogenin (**11**) [25], antiaroside J (**12**) [11], deglucocheirotoxin (**13**) [26], strophalloside (**14**) [27], antiarosideK (**15**) [11], antiaroside L (**16**) [11], antiaroside M (**17**) [11], β-antiarin (**18**) [28], antialloside (**19**) [26], α-antiarin (**20**) [21], glucostrophanthidin (**21**) [23,27], antiaroside R (**22**) [11], 19-(glucosyloxy)-3β,5,14-trihydroxy-5β-card-20(22)-enolide (**23**) [29], and convallaoxin (**24**) [27] by comparison of their physical and spectroscopic data with those reported previously.

The cytotoxicity of cardiac glycosides **1**–**11** toward human NIH-H460 lung cancer cells were evaluated using MTT assays (see Table 3). Digoxin was used as positive control. Compounds **5**, **6**, **10** and **11** showed significant inhibitory effects on the proliferation of NIH-H460 cells with IC_{50} values of 25–50 nM. Preliminary structure–activity relationship analysis revealed that cardiac glycosides with both an aldehyde functional group at C-10 and β-H substitution at C-5 in the aglycone (cardiac glycosides **5**, **6**, and **11**) exhibited stronger cytotoxic activities. In addition, α-L-rhamnose substitution at C-3 position of the aglycone showed stronger cytotoxicity (cardiac glycosides **4**/**6** and **5**/**11**), which was consistent with previous reports [11,30].

Our previous studies suggested that the cardiac glycosides exerted their apoptotic effect through the Nur77-dependent apoptotic pathway [11]. In order to explore the effects of cardiac glycosides **1**–**11** on expression of Nur77, protein level induction of Nur77 was determined by using Western blotting (see Fig. 2). The result demonstrated that all compounds exhibited strong induction of Nur77 expression at concentrations of 50 nM in 3 h. Interestingly, cardiac glycosides **1**–**3** and digoxin, which exhibited medium cytotoxicity, also showed strong induction of Nur77 expression. This phenomenon suggested that cytotoxicity of

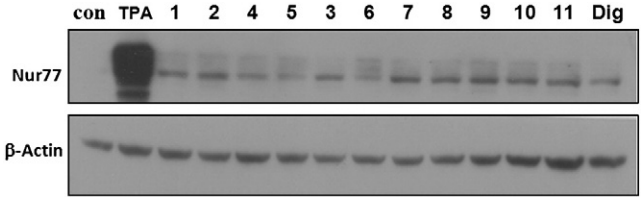

Fig. 2. Induction of Nur77 expression by **1**–**11.**

cardiac glycosides was not proportional to their function of induction of Nur77 expression, which might be interpreted that both induction of Nur77 expression and its subsequent translocation from the nucleus to the cytoplasm are critical events in apoptosis induction by cardiac glycosides in cancer cells.

Acknowledgment

The authors are grateful to Mr. T. Shi, Mr. J. Geng, and Dr. Y. Yu for the HRESIMS and NMR measurements. This work was supported by grants from the National Natural Science Foundation of China (81001373 and 91129302) and the Fund of the State Key Laboratory of Drug Research (SIMM1403KF-15).

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