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ISSN: 1478-6419 (Print) 1478-6427 (Online) Journal homepage: http://www.tandfonline.com/loi/gnpl20

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To cite this article: Jia-chuan Liu, Li-li Yu, Shao-fei Chen, Xiao-jie Lu, Dan Zhao, Hai-feng Wang, Gang Chen & Yue-hu Pei (2017): Two new 14, 15-secopregnane-type steroidal glycosides from the roots of Cynanchum limprichtii, Natural Product Research, DOI: 10.1080/14786419.2017.1353506

To link to this article: http://dx.doi.org/10.1080/14786419.2017.1353506

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Published online: 16 Jul 2017.

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NATURAL PRODUCT RESEARCH, 2017
https://doi.org/10.1080/14786419.2017.1353506

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Two new 14, 15-secopregnane-type steroidal glycosides from the roots of *Cynanchum limprichtii*

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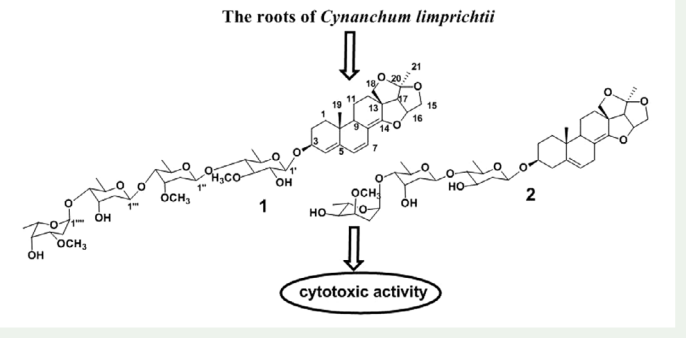
ABSTRACT

Two new steroidal glycosides **1** and **2**, along with three known ones (**3**–**5**), were isolated from the 95% ethanol extract of the roots of *Cynanchum limprichtii* Schltr. The structure of the new compounds was elucidated as 3-O-*α*-L-diginopyranosyl-(1→4)-*β*-D-digitoxopyranosyl-(1→4)-*β*-D-cymaropyranosyl-(1→4)-*β*-D-thevetopyranosyl-14, 16:15, 20:18, 20-triepoxy-14, 15-secopregn-4, 6, 8 (14)-triene (**1**) and 3-O-*α*-L-cymaropyranosyl-(1→4)-*β*-D-digitoxopyranosyl- (1→4)-*β*-D-3-demethyl-2-deoxythevetopyranosyl-14, 16: 15, 20: 8, 20-triepoxy-14, 15- secopregn-5, 8 (14)-diene (**2**) on the basis of spectroscopic analysis together with acidic hydrolysis. All compounds showed cytotoxic activity against the human cancer cell line HL60, with IC_{50} values of 55.36, 65.41, 17.88, 17.68 and 33.5 μM, respectively. While, only compound **3** showed cytotoxicity against the Caco-2 cell line, with an IC_{50} value of 67.47 μM.



ARTICLE HISTORY
Received 16 February 2017
Accepted 13 June 2017

KEYWORDS
Cynanchum limprichtii; steroidal glycoside; cytotoxicity

1. Introduction

Cynanchum limprichtii Schltr. is a perennial herb belong to the genus *Cynanchum* in the family *Asclepiadaceae*, which is chiefly distributed in Kangding, Sichuan Province of China

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Supplemental data for this article can be accessed at https://doi.org/10.1080/14786419.2017.1353506.

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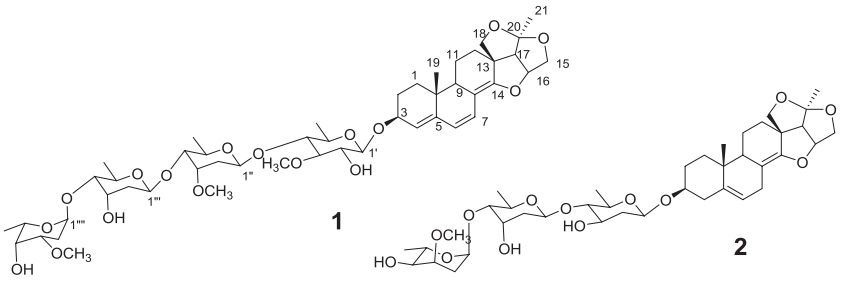

Figure 1. The structures of compounds **1** and **2**.

(Qin & Li 2011). Many herbs of genus *Cynanchum* have been used as antitussive and expectorants in China for a long time (Pharmacopoeia of the People's Republic of China 2010). Chemical investigation showed that they contained essential oils (Yu et al. 2015), flavonoids (Yildiz et al. 2017) and steroidals. The main native organic compounds are characteristic C_{21} steroidal and their glycosides, which exhibited antitumour, antifungal and cytotoxic activities (Day et al. 2001; Chen et al. 2010; Peng et al. 2008). As our current interest in the biologically active and structurally unique natural products from the genus *Cynanchum*, the EtOH extract of *C. limprichtii* Schltr. was investigated, and two new steroidal glycosides, 3-O-*α*-L-diginopyranosyl-(1→4)-*β*-D-digitoxopyranosyl-(1→4)-*β*-D-cymaropyranosyl-(1→4)-*β*-D-thevetopyranosyl-14, 16:15, 20:18, 20-triepoxy-14, 15-secopregn-4, 6, 8(14)-triene (**1**) and 3-O-*α*-L-cymaropyranosyl-(1→4)-*β*-D-digitoxopyranosyl -(1→4)-*β*-D-3-demethyl-2-deoxythevetopyranosyl-14, 16:15, 20:18, 20-triepoxy-14, 15-secopregn-5, 8 (14)-diene (**2**) (Figure 1) together with three known ones, stauntoside A (**3**) (Zhu et al. 1999), tylophoside A (**4**) (Abe et al. 1999) and glaucogenin C-3-O-*β*-D-digitoxopyranosyl-(1→4)-*β*-D-canaropyranoside (**5**) (Yin et al. 2016) were isolated. Their structures were established on the basis of spectroscopic analyses. Additionally the cytotoxicity of compounds **1**–**5** was evaluated against selected human cancer cell lines, including HL-60 (human leukaemic promyelocytic cell) and Caco-2 (human Caco-2 colon cancer cell).

2. Results and discussion

Compound **1** was obtained as a white amorphous powder, with α_D^{20} + 41.0 (c 0.2, MeOH). Its molecular formula was determined as $C_{48}H_{72}O_{17}$ by its pseudo-molecular ion peak at *m/z* 943.4666 $[M + Na]^+$ in the positive HR-ESI-MS experiment, corresponding to thirteen degrees of unsaturation. The IR spectrum of it showed strong absorption bands at 3451 and 2935 cm^{-1}, indicating the presence of hydroxyl and methyl groups, respectively. The ^1H NMR spectrum of **1** revealed the diagnostic signals of steroidal glycoside, with an aglycone of 14, 15-secopregnane-type skeleton being exhibited by two tertiary methyls resonated at δ_H 0.81 (3H, s, H-19) and 1.59 (3H, s, H-21), two methine protons at δ_H 3.95 (1H, m, H-3) and 4.82 (1H, m, H-16) and two methylene groups at δ_H 3.82 (1H, dd, J = 10.8, 4.5 Hz, H_α-15) and 4.28 (1H, d, J = 10.8 Hz, H_β-15), and at δ_H 4.02 (1H, d, J = 8.8 Hz, H_α-18) and 4.07 (1H, d, J = 8.8 Hz, H_β-18), three characteristic olefinic proton signals at δ_H 6.65 (1H, d, J = 9.7 Hz, H-7), 5.90 (1H, d, J = 9.7 Hz, H-6), and 5.80 (1H, s, H-4). Comparison of the ^1H- and ^{13}C NMR data (Table 1S) with those of stauntosaponin A (Shibano et al. 2012) revealed that **1** contained the same aglycone with stauntosaponin A, which was confirmed by analyses of the 2D NMR spectra (Figures

4S, 5S, 6S and 8S). The ^{1}H NMR spectrum showed four anometic protons [δ_H 4.85 (1H, d, J = 7.8 Hz, H-1′), 5.15 (1H, d, J = 8.1 Hz, H-1″), 5.54 (1H, d, J = 8.2 Hz, H-1‴) and 5.21 (1H, d, J = 3.2 Hz, H-1⁗)], and four methyl groups [δ_H 1.49 (3H, d, J = 5.6 Hz, H-6′), 1.31 (3H, d, J = 6.2 Hz, H-6″),1.42 (3H, d, J = 6.2 Hz, H-6‴) and 1.57 (3H, d, J = 6.3 Hz, H-6⁗)], indicating that it contained four 6-deoxysugars. The sugars were determined to be digitoxose, diginose, cymarose and thevetose by comparing their ^{1}H- and ^{13}C NMR data (Table 1S) with those of corresponding deoxysugar units of Stauntoside F and Stauntoside G (Yu et al. 2013). The splitting patterns of anomeric proton signals indicated that **1** had three sugar units with β-linkages and one with α-linkage (Lin et al. 1995). The linkage positions and sequence of these sugars were ascertained by HMBC correlations from δ_H 5.21 (H-1⁗ of α-L-diginopyranose) to δ_C 83.2 (C-4‴), from δ_H 5.54 (H-1‴ of β-D-digitoxopyranose) to δ_C 82.2 (C-4″), from δ_H 5.15 (H-1″ of β-D-cymaropyranose) to δ_C 82.8 (C-4′), and from δ_H 4.82 (H-1′ of β-D-thevetopyranose) to δ_C 74.6 (C-3)(Figures 5S and 7S). Compound **1** was subjected to acid hydrolysis and HPLC analysis as described above in Section 3.4, which gave one D-digitoxose, one L-diginose, one D-cymarose and one *D*-thevetose in the HPLC test. Therefore, **1** was identified to be 3-O-α-L-diginopyranosyl-(1→4)-β-D-digitoxopyranosyl -(1→4)-β-D- cymaropyranosyl -(1→4)-β-D-thevetopyranosyl-14, 16:15, 20:18, 20-triepoxy-14, 15-secopregn-4, 6, 8(14)- triene.

Compound **2** was obtained as a white amorphous powder, with α_D^{20} −55.5 (c 0.2, MeOH). The positive mode HR-ESI-MS showed a pseudo-molecular ion peak at *m/z* 771.3952 $[M + Na]^+$ (Calcd for $C_{40}H_{60}O_{13}Na$, 771.3926), revealing its molecular formula to be $C_{40}H_{60}O_{13}$. The IR spectrum of **2** displayed strong absorption bands at 3437 and 2934 cm^{-1}, indicating the presence of hydroxyl and methyl groups, respectively. ^{1}H NMR spectrum of **2** revealed the diagnostic signals of steroidal glycoside, with a 14, 15-secopregnane-type skeleton aglycone being exhibited typically by two tertiary methylic groups at δ_H 0.77 (3H, s, H-19) and 1.59 (3H, s, H-21), two methine protons at δ_H 3.71 (1H, m, H-3) and 4.77 (1H, dd, J = 7.8, 3.4 Hz, H-16) and two methylene groups at δ_H 3.80 (1H, dd, J = 10.5, 3.4 Hz, H_α-15) and 4.28 (1H, d, J = 10.5 Hz, H_β-15), and at δ_H 4.03 (1H, d, J = 9.7 Hz, H_α-18) and 4.12 (1H, d, J = 9.7 Hz, H_β-18) and with three 6-deoxysugars being shown by three anometic proton signals at δ_H 4.87 (1H, dd, J = 9.8, 1.7 Hz, H-1′), 5.29 (1H, dd, J = 9.7, 1.6 Hz, H-1″), and 5.02 (1H, d, J = 3.2 Hz, H-1‴) and three methyls at δ_H 1.36 (3H, d, J = 6.1 Hz, H-6′), 1.33 (3H, d, J = 6.2 Hz, H-6″) and 1.43 (3H, d, J = 6.4 Hz, H-6‴). In addition, one methoxyl at δ_H 3.38 (3H, s) was also determined in the ^{1}H NMR spectrum, which were compatible with one methylated 6-deoxypyranose. By comparing its NMR data with that of Cynastauoside C (Yu and Zhao 2016), compound **2** had the same aglycone as that of it and the sugar chain was also linked at its C-3 hydroxyl group except for one sugar unit different. The sugar moieties were speculated by comparing the ^{1}H- and ^{13}C NMR spectroscopic data of **2** (Table 1S) with those in the literature (Zhu et al. 1999) and by HPLC analysis (Section 3.4).The linkage positions and sequence of the three sugars were ascertained by HMBC correlations from δ_H 5.02 (H-1‴ of α-cymaropyranose) to δ_C 80.4 (C-4″ of β-digitoxopyranose), from δ_H 5.29 (H-1″ of β-digitoxopyranose) to δ_C 88.6 (C-4′ of β-3-demethyl-2-deoxythevetopyranose) and from δ_H 5.29 (H-1′ of β-3-demethyl-2-deoxythevetopyranose) to δ_C 76.9 (C-3) (Figure 7S and 15S). Thus, the structure of **2** was determined to be 3-O-α-L-cymaropyranosyl- (1→4)-β-D-digitoxopyranosyl- (1→4)-β-D -3-demethyl-2-deoxythevetopyranosyl-14, 16:15, 20:18, 20-triepoxy-14, 15- secopregn-5, 8 (14)-diene.

All compounds were tested for their cytotoxicity against two selected human cancer cell lines using MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tera-zolium bromide) colorimetric assay. The results showed that all compounds exhibited good inhibitory activities in HL60 cell line with the IC_{50} values 55.36, 65.41, 17.88, 17.68 and 33.5 μM, respectively. Compound **3** showed cytotoxicity against the Caco-2 cell line (IC_{50} = 67.47 μM) and no significant cytotoxicity against the Caco-2 cell line were observed for others (IC_{50} > 80 μM).

3. Experimental

3.1. General experiment procedures

Optical rotations were determined with a WZZ-2A (Shanghai base solid Instrument Co., Ltd., Shanghai, China). The IR spectra were recorded on a Bruker IFS-55 spectrophotometer (Karlsruhe, Germany) using KBr pellet. HR-ESI-MS spectra were measured on a Micro-mass Autospec-Untima TOF mass spectrophotometer (Waters, USA). One- and two-dimensional NMR spectra were obtained on a Bruker AVANCE-400/-600 spectrometer (Karlsruhe, Germany) with tetramethyldilane (TMS) as an internal standard. Sugars analytical HPLC was carried out on a Jasco PU-4180 pump and an OR-4090 detector (Kyoto, Japan). HPLC was performed with an Asahipak NH2P-50 4E column (4.6 mm × 250 mm, 5 μm, Shodex, Japan). Pre-coated silica gel GF_{254} plates for TLC and silica gel (200–300 mesh) for column chromatography (CC) were obtained from Qingdao Marine Chemical Factory. Sephadex LH-20 (GE Healthcare, USA). ODS silica gel CC (50 μm, YMC, Japan). Reversed phase plates (RP-18 F_{254S}, Merck, Germany).

3.2. Plant material

The dried roots (20 kg) of *C. limprichtii* Schltr. were bought from Anhui Economy People Pharmaceutical Co., Ltd. A voucher specimen was identified by Prof. Jincai Lu of Shenyang Pharmaceutical University and has been deposited in the School of Traditional Chinese Materia Medica of Shenyang Pharmaceutical University (No. 2013052).

3.3. Extraction and isolation

The dried roots (20 kg) of *C. limprichtii* Schltr. were extracted with 95% EtOH three times (each 2 h) under reflux condition. After filtration and concentration in vacuum, the residue (3500 g) was suspended in water and extracted with petroleum ether, EtOAc and *n*-BuOH successively.

The EtOAc fraction (300 g) was subjected to silica gel CC eluted with CH_2Cl_2–MeOH (100:0, 100:2, 100:5, 100:10, 100:20, 100:30, 100:50, 0:100, v/v) to afford five subfractions (Frs. A–E) based on TLC analysis.

Fr. B (92 g) was subjected to silica gel CC eluted with petroleum ether-acetone (100:10, 100:15, 100:20, 100:30, 100:40, 100:50, 100:100, v/v) to give nine subfractions (Frs. B1–B9). Fr. B9 (38 g) was further separated by silica gel CC eluted with petroleum ether-acetone (100:15, 100:25, 100:35, 100:50, 100:100, v/v) to give 10 subfractions (Frs. B9A–B9 J). Fr. B9E (12.5 g) was subjected to silica gel CC eluted with CH_2Cl_2–MeOH (100:1, 100:3, 100:5, 100:10, v/v) to obtain six subfractions (Fr. B9E1-B9E6). Fr. B9E3 (3.1 g) was subjected to Sephadex

LH-20 gel CC eluted with MeOH to yield three subfractions (Frs. B9E3A–B9E3C), Fr.B9E3B (2.3 g) was further separated by silica gel CC eluted with petroleum ether-acetone (100:25, 100:50, v/v) to give seven subfractions (Frs. B9E3B1–B9E3B7), and then Fr. B9E3B6 (463 mg) was subjected to ODS silica gel CC eluted with MeOH–H_2O (2:1, 3:1, 4:1, v/v) to yield eight subfractions (Frs. B9E3B6A–B9E3B6H). Compound **4** (22 mg) was purified from Fr. B9E3B6F (121 mg) by silica gel CC eluted with petroleum ether-acetone (100:25, v/v). Fr. B9F (10 g) was subjected to silica gel CC eluted with CH_2Cl_2–MeOH (100:2, 100:3, 100:5, 100:10, v/v) to afford four subfractions (Frs.B9F1–B9F4). Fr. B9F1 (5 g) further produced nine subfractions (Frs. B9F1A–B9F1I) by silica gel CC eluted with petroleum ether-acetone (100:10, 100:20, 100:30, 100:50, v/v), Fr. B9F1E (1 g) was separated by Sephadex LH-20 gel CC eluted with MeOH to yield compound **2** (32 mg). Fr. B9F3 (1.2 g) was separated by Sephadex LH-20 gel CC eluted with MeOH to yield eight subfractions (Frs. B9F3A–B9F3H), Compound **5** (25 mg) was purified from Fr. B9F3C (130 mg) by silica gel CC eluted with petroleum ether-acetone (100:30, v/v). Fr. B9H (12 g) was subjected to silica gel CC eluted with petroleum ether-acetone (100:20, 100:30, 100:40, v/v) to obtain nine subfractions (Frs. B9H1–B9H9). Fr. B9H6 (1.56 g) was further separated by Sephadex LH-20 gel CC eluted with MeOH to yield six subfractions (Frs. B9H6A–B9H6F), and then Fr. B9H6D (301 mg) was subjected to ODS silica gel CC eluted with MeOH–H_2O (80:20, v/v) to give compound **3** (40 mg). Fr. B9H7 (4 g) was subjected to silica gel CC eluted with petroleum ether-EtOAc (1:3, 1:5, 1:7, v/v) to yield six subfractions (Frs. B9H7A–B9H7F). Fr. B9H7E (1.5 g) was separated by Sephadex LH-20 gel CC eluted with MeOH to yield compound **1** (175 mg).

3.3.1. 3-O-α-L-diginopyranosyl-(1→4)-β-D-digitoxopyranosyl -(1→4)-β-D-cymaropyranosyl-(1→4)-β-D-thevetopyranosyl-14, 16:15, 20: 18, 20-triepoxy-14, 15-secopregn-4, 6, 8 (14)-triene (1)

White amorphous powder, α_D^{20} + 41.0 (c 0.2, MeOH); IR(KBr) ν_{max} 3451, 2973, 2935, 1677, 1634, 1453, 1382, 1308, 1258, 1166, 1064, 1023, 938, 869, 721 and 637 cm^{-1}. HR-ESI-MS *m/z*: 943.4668 $[M + Na]^+$ (calcd for $C_{48}H_{72}O_{17}Na$, 943.4662). ^1H NMR (400 MHz, C_5D_5N)*δ*: 1.34 (m, H_α-1), 1.54 (m, H_β-1), 2.24 (m, H_α-2), 1.94 (m, H_β-2), 4.56 (brt, 7.8, H-3), 5.80 (brs, H-4), 5.90 (d, 9.7, H-6), 6.65 (d, 9.7, H-7), 2.13 (dd, 11.2, 5.8, H-9), 1.62 (o, H_β-11), 1.21 (dd, 13.7, 11.2, H_α-11), 1.99 (m, H_β-12), 1.45 (m, H_α-12), 4.28 (d, 10.8, H_β-15), 3.82 (dd, 4.3, 10.8, H_α-15), 4.82 (dd, 7.8, 4.3, H-16), 2.80 (d, 7.8, H-17), 4.02 (d, 8.8, H_β-18), 4.07 (d, 8.8, H_α-18), 0.81 (s, H-19), 1.59 (s, H-21), *β*-D-the: 4.85 (d, 7.8, H-1′), 3.95 (m, H-2′), 3.73 (m, H-3′), 3.74 (m, H-4′), 3.70 (m, H-5′), 1.49 (d, 5.6, H-6′), 3.96 (s, 3′-OCH_3), *β*-D-cym: 5.15 (d, 8.1, H-1″), 2.41 (m, H_β-2″), 1.68 (m, H_α-2″), 3.94 (m, H-3″), 3.40 (m, H-4″), 4.22 (m, H-5″), 1.31 (d, 6.2, H-6″), 3.54 (s, 3″-OCH_3), *β*-D-digt: 5.54 (d, 8.2, H-1‴), 2.46 (m, H_β-2‴), 2.04 (m, H_α-2‴), 4.66 (m, H-3‴), 3.50 (dd, 9.9, 2.6, H-4‴), 4.32 (m, H-5‴), 1.42 (d, 6.2, H-6‴), *α*-L-dign: 5.21 (d, 3.2, H-1⁗), 2.41 (m, H_β-2⁗), 2.11 (m, H_α-2⁗), 3.86 (m, H-3⁗), 4.08 (m, H-4⁗), 4.31 (m, H-5⁗), 1.57 (d, 6.3, H-6⁗), 3.32 (s, 3⁗-OCH_3). ^{13}C NMR(100 MHz, C_5D_5N) *δ*: 33.5 (C-1), 27.2 (C-2), 75.4 (C-3), 125.1 (C-4), 144.5 (C-5), 125.7 (C-6), 122.6 (C-7), 108.2 (C-8), 44.2 (C-9), 35.5 (C-10), 20.4 (C-11), 30.8 (C-12), 54.8 (C-13), 155.2 (C-14), 72.1 (C-15), 86.1 (C-16), 62.0 (C-17), 77.3 (C-18), 17.6 (C-19), 118.4 (C-20), 22.6 (C-21), *β*-D-the: 103.3 (C-1′), 74.6 (C-2′), 85.8 (C-3′), 82.8 (C-4′), 71.7 (C-5′), 18.7 (C-6′), 60.5 (C-3′-OCH_3), *β*-D-cym: 99.6 (C-1″), 34.9 (C-2″), 77.4 (C-3″), 82.2 (C-4″), 69.3 (C-5″), 18.6 (C-6″), 57.3 (C-3″-OCH_3), *β*-D-digt: 98.9 (C-1‴), 39.1 (C-2‴), 67.68 (C-3‴), 83.2 (C-4‴), 68.8 (C-5‴), 18.5 (C-6‴), *α*-L-dign: 101.2 (C-1⁗), 30.9 (C-2⁗), 75.8 (C-3⁗), 67.5 (C-4⁗⁗), 67.72 (C-5⁗), 17.74 (C-6⁗), 55.0 (C-3⁗ -OCH_3).

3.3.2. 3-O-α-L-cymaropyranosyl- (1→4)-β-D-digitoxopyranosyl- (1→4)-β-D -3-demethyl-2-deoxythevetopyranosyl-14, 16: 15, 20: 18, 20-triepoxy-14, 15-secopregn-5, 8 (14)-diene (2)

White amorphous powder, α_D^{20} −55.5 (c 0.2, MeOH); IR(KBr) ν_{max} 3437, 2971, 2934, 1638, 1451, 1382, 1311, 1163, 1090, 1062, 992, 901, 869, 737 and 620 cm^{-1}. HR-ESI-MS *m/z*: 771.3952 $[M + Na]^+$ (Calcd for $C_{40}H_{60}O_{13}Na$, 771.3926). ^1H NMR (400 MHz, C_5D_5 N) δ: 2.02 (m, H-1), 2.32 (m, H_β-2), 1.83 (dt, 14.2, 3.7, H_α-2), 3.71 (m, H-3), 2.56 (m, H_β-4), 2.30 (m, H_α-4), 5.38 (brs, H-6), 1.62 (m, H_β-7), 1.59 (m, H_α-7), 2.12 (m, H-9), 1.29 (m, H_β-11), 1.15 (m, H_α-11), 2.11 (m, H -12), 4.28 (d, 10.5, H_β-15), 3.64 (dd, 10.5, 3.4, H_α-15), 4.77 (dd, 7.8, 3.4, H-16), 2.80 (d, 7.8, H-17), 4.12 (d, 9.7, H_β-18), 4.03 (d, 9.7, H_α-18), 0.77 (s, H-19), 1.59 (s, H-21), β-D-3-demeth: 4.87 (dd, 9.8, 1.7, H-1′), 2.45 (m, H_β-2′), 2.01 (m, H_α-2′), 4.17 (m, H-3′), 3.35 (m, H-4′), 3.56 (m, H-5′), 1.33 (d, 6.2, H-6′); β-D-digt: 5.29 (dd, 9.7, 1.6, H-1″), 2.47 (m, H_β-2″), 1.95 (m, H_α-2″), 4.52 (m, H-3″), 3.45 (dd, 9.4, 3.0, H-4″), 4.18 (m, H-5″), 1.36 (d, 6.1, H-6″); α-L-cym: 5.02 (d, 3.2, H-1‴), 2.36 (m, H_β-2‴), 1.84 (m, H_α-2‴), 3.80 (m, H-3‴), 3.71 (m, H-4‴), 4.48 (m, H-5‴), 1.43 (d, 6.4, H-6‴), 3.38 (s, 3‴-O$\underline{C}H_3$). ^{13}C NMR(100 MHz, C_5D_5 N) δ: 37.9 (C-1), 30.3 (C-2), 76.9 (C-3), 38.9 (C-4), 141.1 (C-5), 120.4 (C-6), 25.8 (C-7), 104.4 (C-8), 44.9 (C-9), 36.4 (C-10), 20.1 (C-11), 31.8 (C-12), 53.7 (C-13), 152.7 (C-14), 72.4 (C-15), 84.3 (C-16), 63.6 (C-17), 76.6 (C-18), 18.7 (C-19), 118.3 (C-20), 22.6 (C-21), β-D-3-demeth: 98.0 (C-1′), 40.2 (C-2′), 69.3 (C-3′), 88.6 (C-4′), 70.9 (C-5′), 18.1 (C-6′); β-D-digt: 99.8 (C-1″), 38.2 (C-2″), 67.5 (C-3″), 80.4 (C-4″), 70.9 (C-5″), 18.07 (C-6″); α-L-cym: 98.5 (C-1‴), 32.1 (C-2‴), 76.4 (C-3‴), 72.5 (C-4‴), 67.4 (C-5‴), 18.3 (C-6‴), 56.7 (C-3‴O$\underline{C}H_3$).

3.4. Acid hydrolysis of compounds 1 *and* 2

Each solution of **1** and **2** (6 mg) in MeOH (5 mL) was added 0.1 N H_2SO_4 (5 mL) and kept at 50 °C for 30 min. The solution was diluted with 10 mL of water. The hydrolysed mixture was kept at 60 °C for another 30 min, then neutralised with aqueous saturated $Ba(OH)_2$ and the precipitates were filtered off. After the removal of MeOH, the solution was diluted with H_2O (10 mL) and extracted with CH_2Cl_2 (25 mL × 3), and the aqueous layer was concentrated. The absolute configurations of the deoxysugars were identified as D-thevetose, D-cymarose, L-cymarose, D-3-demethyl-2-deoxythevetose, D-digitoxose and L-diginose by HPLC analysis (column: Asahipak NH2P-50 4E; carrier: 75% CH_3CN in H_2O (1.0 mL/min); detection: OR detector) of the aqueous solution in comparison with authentic sugars (the source of the standards of sugars were obtained by acid hydrolysis of known compounds with the same method of the new compound). D-cymarose (t_R 8.78 min positive polarity), L-cymarose (t_R 9.15 min, negative polarity), D-digitoxose (t_R 11.7 min, positive polarity), L-diginose (t_R 7.23 min, negative polarity), D-3-demethyl-2-deoxythevetose (t_R 14.9 min, positive polarity) and D-thevetose (t_R 5.99 min, positive polarity).

3.5. Cytotoxic assay

The MTT assay was used to determine the cytotoxicity of each compound against two cultured human cancer cell lines HL-60 and Caco-2. 5-Fluorouracil was used as a positive control. The cytotoxicity of 5-Fluorouracil against the HL-60 and Caco-2 cell lines were estimated by their IC_{50} values 6.38, and 17.01 μM, respectively, for these two cell lines.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the National Natural Science Foundation of China [grant number 31370375].

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