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C_{21} steroidal glycosides from the roots of *Cynanchum paniculatum*

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ABSTRACT

As a part of our continuing research for bioactive constituents from *Cynanchum* plants, four new C_{21} steroidal glycosides, cynapanoside D-G (**1**–**4**), together with six known compounds (**5**–**10**) were isolated from the roots of *Cynanchum paniculatum* (Bge.) Kitag. Their structures were elucidated on the basis of 1D- and 2D-NMR spectroscopic data as well as HR-ESI-MS analysis. Compound **8** exhibited potent inhibitory activities against HL-60, HT-29, PC-3 and MCF-7 cell lines with IC_{50} values of 8.3, 7.5, 34.3 and 19.4 μM, respectively and compounds **1**–**4** and **9** displayed moderate cytotoxicity against the four cell lines. The in vitro antioxidant activities of compounds **1**–**4**, **8** and **9** were assayed by DPPH radical scavenging activity. Antibacterial and antifungal activities of compounds **1**–**4**, **8** and **9** were also tested.

1. Introduction

C_{21} steroidal and their glycosides have established themselves as an important class of biologically active compounds. They possess a wide range of pharmacological activities including antitumor, antifungal and cytotoxic activities [1–5]. *Cynanchum paniculatum*, is a perennial herb belonging to the genus *Cynanchum* in the family Asclepiadaceae, which is chiefly distributed in China, Japan and Korea. Its radix and rhizome have been used for rheumatic arthralgia, epigastric pain and distension, toothache, lumbago, traumatic injuries, urticaria and eczema in traditional Chinese medicine [6–8]. It has been reported to be rich in C_{21} steroidal and their glycosides [9], whose chemical structures are classified into normal four-ring C_{21} steroid type and aberrant 13,14:14,15-diseco-pregnane-type [10,11]. As our current interest in the biologically active and structurally unique natural products, the EtOH extract of *C. paniculatum* was investigated, and four new steroidal glycosides, cynapanoside D (**1**), cynapanoside E (**2**), cynapanoside F (**3**) and cynapanoside G (**4**), together with six known ones (**5**–**10**) were isolated.

2. Experimental

2.1. General experiment procedure

Optical rotations were determined using a WZZ-2A (Shanghai base solid Instrument Co., Ltd., Shanghai, China). UV spectra were recorded on a Shimadzu-2201 (Kyoto, Japan). The IR spectrum was obtained from a Bruker IFS-55 spectrophotometer (Karlsruhe, Germany) using KBr pellet. HR-ESI-MS data were measured on a Micro-mass Autospec-Untima TOF mass spectrophotometer (Waters, USA). NMR spectra were run on a Bruker AVANCE-400/-600 spectrometer (Karlsruhe, Germany). Analytical HPLC was carried out on a Shimadzu LC-10AT (Kyoto, Japan) liquid chromatography and preparative HPLC separation was performed on a YMC-Pack ODS-A column(10 × 250 mm, 5 μm; YMC-Pack, Japan), equipped with a Shimadzu LC-8A pump (Kyoto, Japan) and a Shimadzu SPD-10A UV–vis detector (Kyoto, Japan). Sugars analytical HPLC was carried out on a Jasco PU-4180 pump (Kyoto, Japan) and a OR-4090 detector (Kyoto, Japan). HPLC was performed with an Asahipak NH2P-50 4E column (4.6 mm × 250 mm, 5 μm, Japan).

2.2. Plant material

The dried roots (10 kg) of *C. panticulatum* 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. 20120913).

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2.3. Extraction and isolation

The dried roots (10 kg) of *C. panticulatum* were extracted three times with 95% EtOH (each 2 h) and the combined solution evaporated to dryness by a vacuum rotary evaporator to afford a syrup (1300 g). The crude extract was successively partitioned with petroleum ether, ethyl acetate and *n*-butanol to yield three layers of extracts. The ethyl acetate extract (170 g) was fractionated by silica gel column chromatography eluting with CH_2Cl_2-MeOH (100:0–0:100, v/v) to obtain twelve fractions (Fr. A-L) based on TLC analyses. Fr. B (18.3 g) was separated into subfractions (Fr. B1-B9) by silica gel column using PE-EtOAc (100:0–0:100, v/v) as the eluent. Fr. B4 (476 mg) was purified via Sephadex LH-20 eluting with CH_2Cl_2-MeOH (1:1, v/v) to afford four fractions (Fr. B4-1-Fr. B4-4). Compound **10** (6.8 mg) was recrystallized from Fr. B4-2. Fr. D (6.8 g) was applied to a silica gel column to give eight fractions (Fr. D1-D8). Fr. D2 (860 mg) was subjected to Sephadex LH-20 eluting with MeOH to afford five fractions (Fr. D2-1-D2-5). Fr. D2-3 (218 mg) was purified by semi-preparative HPLC eluting with 70% MeOH-H_2O (v = 4 mL/min) to yield compound **2** (26 mg, t_R 40.0 min) and compound **8** (45.3 mg, t_R 70.2 min). Fr. D4 (1.4 g) was further separated over a silica gel column using a solvent system of CH_2Cl_2-MeOH (100:0–0:100, v/v) to give five subfractions (Fr. D4-1-D4-5). Fr. D4-2 (863 mg) was subjected to Sephadex LH-20 eluting with MeOH to afford five fractions (Fr. D4-2-1-D4-2-5). Fr. D4-2-2 (215 mg) was purified by preparative reversed-phase HPLC using 68% MeOH-H_2O (v = 4 mL/min) and then purified by preparative TLC using a solvent system of cyclohexane-acetone (3:2, v/v) to obtain compound **9** (26.5 mg). Fr. E (17.2 g) was separated into subfractions (Fr. E1-E7) by silica gel column

Table 1
^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectroscopic data in C_5D_5N for compounds **1–4.**

	1		**2**		**3**		**4**	
No.	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C
1	0.91 (m), 1.79[a]	36.2	0.88 (m), 1.80[a]	36.3	0.88 (m), 1.79[a]	36.3	0.99 (m), 1.69 (m)	37.1
2	1.67[a], 2.06[a]	29.8	1.54[a], 2.09[a]	30.3	1.52[a], 2.11[a]	30.1	1.79[a], 2.17 (m)	30.0
3	3.74 (m)	77.3	3.71 (m)	77.2	3.70 (m)	77.2	3.95 (m)	78.5
4	2.27[a], 2.53[a]	38.8	2.28[a], 2.52[a]	38.8	2.26[a], 2.52[a]	38.8	2.64 (m), 2.79[a]	38.8
5	–	140.4	–	140.2	–	140.2	–	139.4
6	5.38 (m)	120.2	5.37 (m)	120.3	5.37 (m)	120.3	5.40 (m)	122.2
7	1.36[a], 1.99[a]	29.7	1.65[a], 2.03[a]	29.8	1.65[a], 2.03[a]	29.9	1.86[a], 2.51 (m)	27.6
8	2.47 (m)	40.4	2.51[a]	40.8	2.50[a]	40.5	1.81[a]	36.8
9	1.20[a]	53.0	1.25[a]	52.7	1.22[a]	52.7	1.10 (m)	46.0
10	—	38.4	—	38.5	—	38.5	—	37.2
11	1.33[a], 2.55[a]	23.7	1.83[a], 2.49[a]	20.6	1.82[a], 2.50[a]	20.7	1.28[a], 1.35[a]	20.8
12	2.11[a], 2.60[a]	28.2	2.14[a], 2.64[a]	28.3	2.13[a], 2.63	28.3	1.18[a], 1.36[a]	38.5
13	–	118.3	–	118.7	–	118.8	–	49.1
14	–	175.2	–	175.3	–	175.4	–	84.7
15	3.92 (dd, 9.3, 9.0) 4.22 (dd, 8.5, 7.3)	67.5	3.97 (t, 9.3) 4.29 (dd, 9.3, 7.5)	66.9	3.97 (t, 9.3) 4.29 (dd, 9.3, 7.5)	67.0	1.79[a], 1.99[a]	34.3
16	5.41 (m)	75.3	5.99 (t, 8.0)	81.8,	5.97 (dd, 8.5, 7.5)	81.8	1.85[a], 2.00[a]	24.2
17	3.53[a]	55.9	–	92.2	–	92.2	2.80[a]	62.8
18	6.45 (s)	143.6	6.63 (s)	144.4	6.65 (s)	144.4	1.05 (s)	15.2
19	0.82 (s)	17.6	0.85 (s)	17.7	0.83 (s)	17.8	0.93 (s)	19.3
20	–	114.1	–	119.5	–	119.5	–	216.5
21	1.51 (s)	24.5	1.73 (s)	20.4	1.73 (s)	20.5	2.17 (s)	32.1
	β-D-Ole		*β*-D-Ole		*β*-D-Ole		*β*-D-Glc	
1′	4.77 (br.d, 9.8)	97.9	4.75 (dd, 9.8, 1.8)	97.9	4.77 (br.d, 9.8)	97.9	5.05 (d, 7.5)	101.1
2′	1.78[a], 2.40[a]	37.7	1.74[a], 2.40[a]	37.7	1.77[a], 2.40[a]	37.8	4.10[a]	84.5
3′	3.55[a]	78.9	3.52[a]	78.9	3.55[a]	78.9	4.21[a]	77.6
4′	3.55[a]	82.9	3.52[a]	82.7	3.51[a]	83.0	4.20[a]	71.2
5′	3.53[a]	71.5	3.53[a]	71.4	3.52[a]	71.5	3.87[a]	78.0
6′	1.44 (s)	18.6	1.42 (s)	18.5	1.41 (s)	18.6	4.30 (m), 4.48 (dd, 11.9, 2.4)	62.3
3′-OMe	3.53 (s)	57.2	3.40 (s)	57.1	3.53 (s)	57.3	–	–
	β-D-Dig		*β*-D-Cym		*β*-D-Dig			
							β-D-Glc	
1″	5.52 (brd, 9.8)	98.5	5.23 (dd, 9.8, 1.8)	98.1	5.51 (brd, 9.8)	98.5	5.26 (d, 7.5)	106.4
2″	1.97[a], 2.41[a]	39.5	1.73[a], 2.06[a]	36.7	1.96[a], 2.39[a]	39.8	4.11[a]	76.8
3″	4.42 (m)	68.5	3.82 (m)	77.6	4.37 (m)	68.8	4.21[a]	77.8
4″	3.57[a]	73.8	3.40[a]	82.0	3.50[a]	82.2	4.28[a]	71.3
5″	4.26 (dd, 8.3, 7.0)	70.3	4.13[a]	69.2	4.57 (br.s)	67.6	3.85[a]	78.8
6″	1.55 (s)	18.8	1.32 (s)	18.3	1.41 (s)	18.4	4.40 (m), 4.54 (dd, 11.9, 2.4)	62.5
3″-OMe	—	—	3.50	58.1	—	—	—	—
			α-L-Cym		*α*-L-Ole			
1‴			4.98 (d, 3.3)	98.7	5.21 (d, 3.0)	100.0		
2‴			1.75[a], 2.39[a]	31.8	1.69[a], 2.44[a]	35.5		
3‴			3.67 (m)	76.1	3.79[a]	78.6		
4‴			3.56[a]	73.1	3.50[a]	76.6		
5‴			4.50 (m)	66.1	4.37[a]	69.3		
6‴			1.50 (s)	18.4	1.48 (s)	18.2		
3‴-OMe			3.34 (s)	56.3	3.30 (s)	56.8		

δ in ppm, *J* values are in parentheses and reported in Hz. The assignments were based on NOESY, HSQC and HMBC experiments. Ole = oleandropyranose, Dig = digitoxopyranose, Cym = cymaropyranose, Glc = glucopyranose.

[a] Overlapped with other signals.

using CH_2Cl_2-MeOH (100:0–0:100, v/v) as the eluent. Fr. E3 (3 g) was subjected to Sephadex LH-20 eluting with MeOH to afford five fractions (Fr. E3-1-E3-5). Fr. E3-2 (1.1 g) was chromatographed on a silica gel column and eluted with CH_2Cl_2-MeOH (100:0–0:100, v/v) to yield four fractions (Fr. E3-2-1-E3-2-4). Fr. E3-2-2 (323 mg) was purified by semi-preparative HPLC eluting with 68% MeOH-H_2O (v = 4 mL/min) to yield compound **1** (29 mg t_R 111 min). Fr. E4 (1.1 g) was purified via silica gel column eluting with CH_2Cl_2-MeOH (100:0–0:100, v/v) to afford ten fractions (Fr. E4-1-Fr. E4-10). Fr. E4-8 (780 mg) was purified via Sephadex LH-20 eluting with MeOH to afford four fractions (Fr. E4-8-1-Fr. E4-8-4). Fr. E4-8-2 (260 mg) was purified by semi-preparative HPLC eluting with 61% MeOH-H_2O (v = 4 mL/min) to yield compound **3** (32.3 mg, t_R 15.0 min), compound **5** (8.1 mg, t_R 21.3 min), compound **6** (7.6 mg, t_R 40.5 min) and compound **7** (6.4 mg, t_R 31.3 min), respectively. The *n*-butanol fraction (128 g) was subjected to a silica gel column eluted with CH_2Cl_2-MeOH (100:0–0:100, v/v) to obtain thirteen fractions (Fr. A-M). Compound **4** (25.3 mg) was recrystallized from fraction E.

Cynapanoside D (**1**): light yellow amorphous gum, $[\alpha]_D^{20}$ +43.5 (*c* 0.069, MeOH); UV (MeOH) λ_{max}: 216 nm; IR (KBr) ν_{max} cm^{-1}: 3423, 2927, 2852, 1774, 1651, 1449, 1384, 1308, 1161, 1125, 1100, 1065; ^1H NMR and ^{13}C NMR (C_5D_5N) data see Table 1; HR-ESI-MS *m/z*: 657.3174 $[M + Na]^+$ (calcd for $C_{34}H_{50}NaO_{11}$, 657.3251).

Cynapanoside E (**2**): light yellow amorphous gum, $[\alpha]_D^{20}$ −36.9 (c 0.244, MeOH); UV (MeOH) λ_{max}: 215 nm; IR (KBr) ν_{max} cm^{-1}: 3437, 2933, 1726, 1655, 1450, 1383, 1303, 1274, 1107, 1061; ^1H NMR and ^{13}C NMR (C_5D_5N) data see Table 1; HR-ESI-MS *m/z*: 6831.4009 $[M + Na]^+$ (Calcd for $C_{42}H_{64}NaO_{15}$ 831.4143).

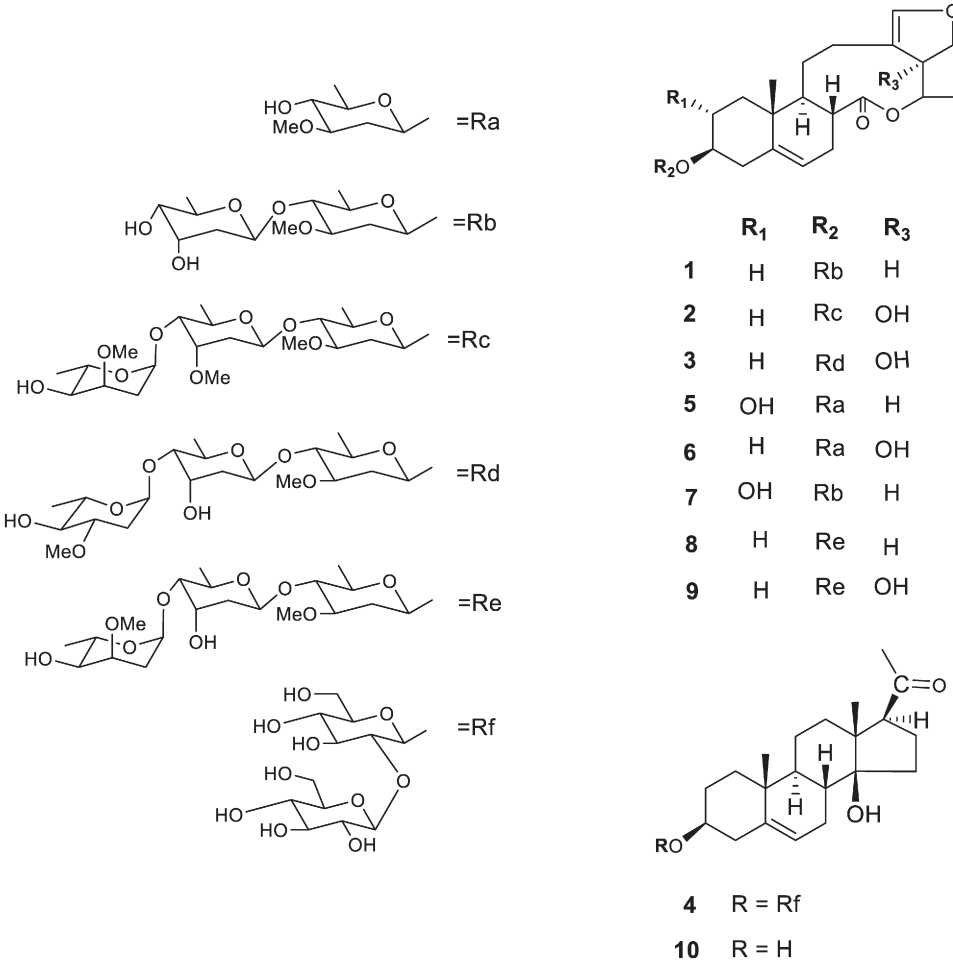
Cynapanoside F (**3**): light yellow amorphous gum, $[\alpha]_D^{20}$ −24.3 (c 0.226, MeOH); UV (MeOH) λ_{max}: 216 nm; IR (KBr) ν_{max} cm^{-1}: 3419, 2935, 1724, 1656, 1453, 1383, 1382, 1304, 1274, 1157, 1058, 987; ^1H NMR and ^{13}C NMR (C_5D_5N) data see Table 1; HR-ESI-MS *m/z*: 817.4011 $[M + Na]^+$ (Calcd for $C_{41}H_{62}NaO_{15}$, 817.3996).

Cynapanoside G (**4**): white needle crystal (MeOH), $[\alpha]_D^{20}$ +26.7 (c 0.071, MeOH); UV (MeOH) λ_{max}: 227, 255 nm; IR (KBr) ν_{max} cm^{-1}: 3444, 2938, 2920, 1675, 1641, 1452, 1384, 1368, 1170, 1075, 1020; ^1H NMR and ^{13}C NMR (C_5D_5N) data see Table 1; HR-ESI-MS *m/z*: 679.3312 $[M + Na]^+$ (Calcd for $C_{33}H_{52}NaO_{13}$, 679.3306).

2.4. Acid hydrolysis of compounds *1–4*

Each solution of **1**, **2** and **3** (6 mg) in MeOH (3 mL) were treated separately with 0.1 N H_2SO_4 (3 mL) at 50 °C for 30 min. Then the mixture was diluted with 6 mL of water and concentrated to 6 mL. The solution was kept at 60 °C for another 30 min, then neutralized with aqueous saturated $Ba(OH)_2$ and the precipitates were filtered off. The filtrate was concentrated and analyzed by TLC with three solvent systems: solvent A, $CHCl_3$-CH_3OH (9:1); solvent B, CH_2Cl_2-C_2H_5OH (9:1); and solvent C, PE-acetone (3:2). The R_f values of cymarose, oleandrose, digitoxose in the order of 0.51, 0.45 and 0.27 with solvent A; 0.58, 0.49, and 0.32 with solvent B; and 0.41, 0.36 and 0.28 with solvent C, respectively [10,12].

A solution of 10 mg of **4** in dioxane (5 mL) was refluxed with 10% HCl (5 mL) at 80 °C for 3 h. After the removal of dioxane, the solution was diluted with H_2O (20 mL) and extracted with CH_2Cl_2 (25 mL × 3), neutralized with 1 N NaOH and then concentrated. Glucose was identified by TLC comparison with authentic sample, respectively.



	R_1	R_2	R_3
1	H	Rb	H
2	H	Rc	OH
3	H	Rd	OH
5	OH	Ra	H
6	H	Ra	OH
7	OH	Rb	H
8	H	Re	H
9	H	Re	OH

4	R = Rf
10	R = H

Fig. 1. The structures of compounds **1**–**10**.

*2.5. Determination of absolute configurations of glucose moieties in **1–4***

The absolute configurations of the glucoses and deoxysugars obtained from water layer of the hydrolysis solution of the compounds by HPLC under the following conditions: column, Asahipak NH2P-50 4E column (4.6 mm × 250 mm, 5 μm, Shodex); flow rate, 0.8 mL/min; solvent, 75% MeCN-H_2O; detection, OR (Jasco OR-4090) detector. Identification of D-oleandrose, L-oleandrose, D-cymarose, L-cymarose, D-digitoxose and D-glucose in each sugar fraction was carried out by a comparison of the retention times and polarities with those of authentic samples (the source of the standards of D-oleandrose, L-oleandrose, D-cymarose, L-cymarose, D-digitoxose were obtained by acid hydrolysis of the known compounds with the same method of those new compounds). D-oleandrose (t_R 15.1 min, positive polarity), L-oleandrose (t_R 12.1 min, negative polarity), D-cymarose (t_R 18.6 min, positive polarity), L-cymarose (t_R 15.8 min, negative polarity), D-digitoxose (t_R 20.7 min, positive polarity) and D-glucose (t_R 12.5 min, positive polarity).

2.6. Biological activity assays

2.6.1. Cytotoxicity assay

A MTT assay was used to determine the cytotoxicity of each compound against four cultured human cancer cell lines. The cell lines used were HL-60 (human leukemic promyelocytic cell), A549 (non-small cell lung adenocarcinoma), PC-3 (prostate cancer cell) and MCF-7 (breast cancer cell). All the cell lines (American Type Culture Collection) was maintained in RPMI-1640 medium (Gibco) with 10% fetal

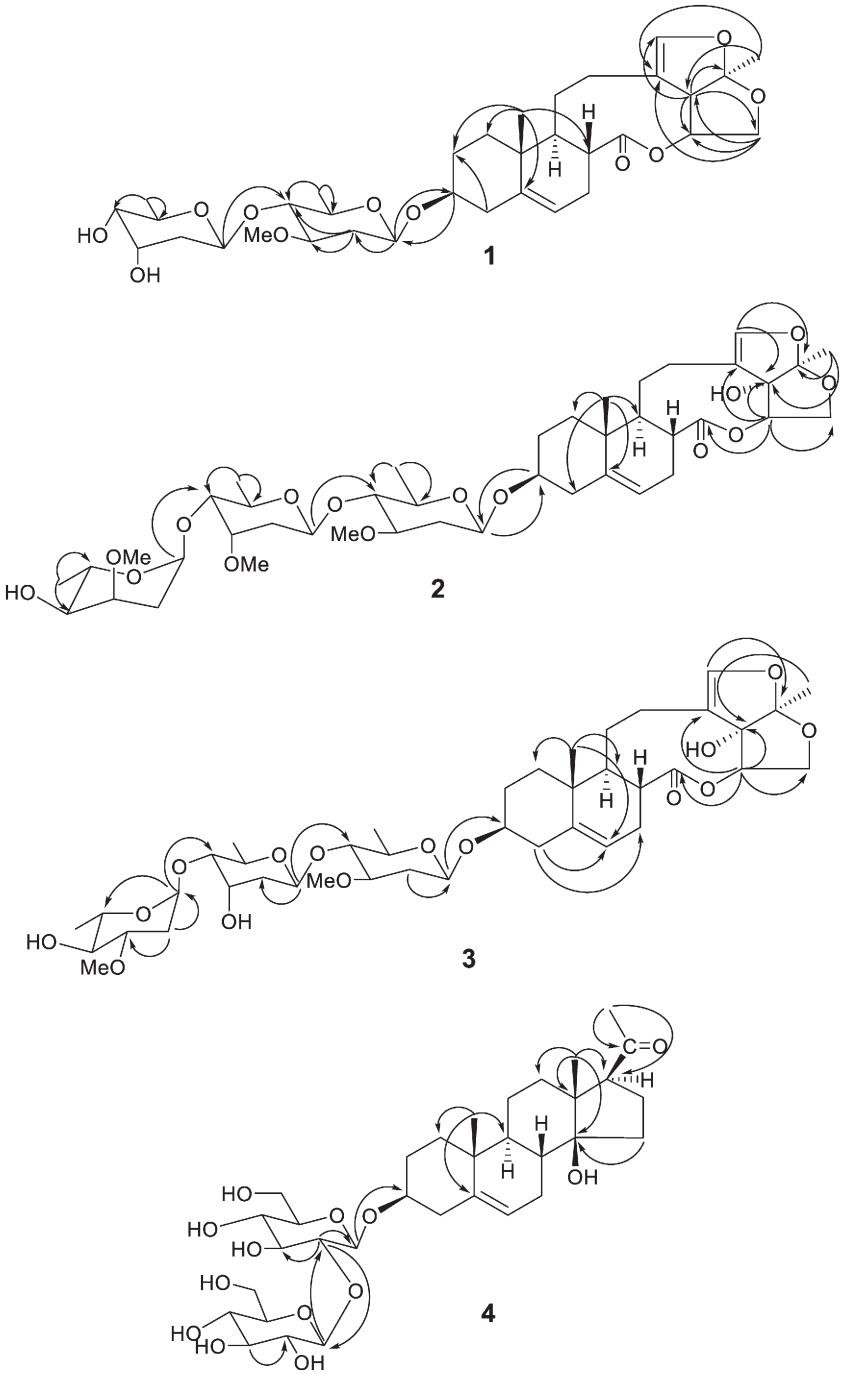


Fig. 2. Key HMBC correlations of compounds **1–4**.

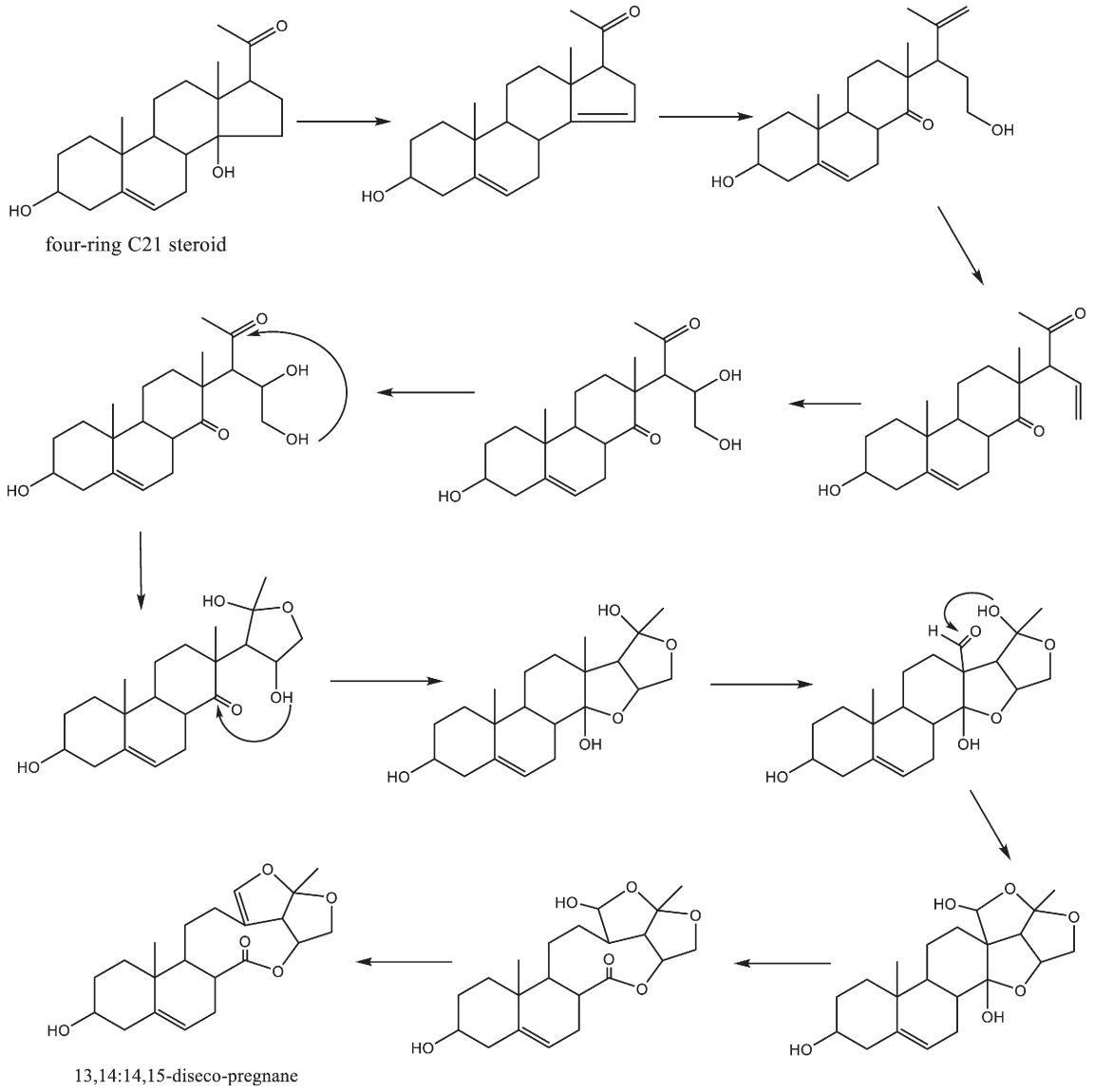


Fig. 3. Proposed the biological cleavage of the ring C/D junction for 13,14:14,15-diseco-pregnane-type steroid.

bovine serum, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 1 mmol/L L-glutamine, and 10% (v/v) heat-inactivated FBS in a humidified atmosphere of 5% CO_2 at 37.0 °C. Logarithmic phase cells were used for experiments. Appropriate dilutions of the test samples were added to the cultures. The growth inhibition was calculated comparing with control cells. 5-Fluorouracil was used as a positive control. The cytotoxicity of 5-Fluorouracil against the HL-60, HT-29, PC-3 and MCF-7 cell lines were estimated by their IC_{50} values, 6.38, 7.90, 7.77, and 17.01 μM, respectively, for these 4 cell lines.

Table 2
Cytotoxicity data of compounds **1**–**5**[a].

Compound	HL-60	HT-29	PC-3	MCF-7
1	13.4	>40	>40	>40
2	28.9	21.9	>40	>40
3	33.2	>40	>40	>40
4	>40	>40	>40	>40
7	>40	>40	>40	>40
8	8.3	7.5	34.3	19.4
9	10.1	25.4	36.6	28.9
5-Fluorouracil	6.38	7.90	7.77	17.0

HL-60, human leukemic promyelocytic cell, HT-29, human colon cancer cell, PC-3, human prostate cancer cell and MCF-7, human breast cancer cell.
[a] Data expressed as IC_{50} values (μM).

2.6.2. 1-Diphenyl-2-picrylhydrazyl free radical (DPPH·) scavenging assay

The DPPH·scavenging activity was assessed according to the method described with minor modifications with lower DPPH concentration (0.2 mM) and different sample to DPPH ratio (1:1). To a 100 μL aliquot of the sample with different concentrations was added 100 μL of DPPH solution (0.2 mM) in a 96-well microplate [13]. The mixture was shaken vigorously and incubated in darkness for 30 min. The absorbance of the reaction solution at 517 nm was recorded using a varioskan flash multimode reader. Ascorbic acid was used as a positive control. The percentage of scavenging DPPH versus concentration was plotted using the following equation: DPPH scavenging activity (%) = [1-(S-SB)/(C-CB)] × 100%, where S, SB, C and CB are the absorption of the sample, the blank sample, the control and the blank control, respectively. All experiments were tested in triplicate.

2.6.3. Antimicrobial bioassay

The MIC values for all compounds were determined by the dilution method. For sample preparation, each of the test compounds was dissolved in DMSO and then diluted with sterile broth to a concentration

of 500 μg/mL. Further dilutions of the compounds in the test medium were prepared at the required quantities of 250, 125, etc. down to 3.9 μg/mL. Chloramphenicol and fluconazole were used as positive controls for bacteria and fungus, respectively. The in vitro antimicrobial activity of the compounds was tested by tube-dilution technique using individually pack-aged, flat bottomed, 96-well microtiter plates (NCCLS. 2000). Bacterial strains were maintained on LB medium for 48 h at 37 °C and fungal strains were on PDA medium for 48 h at 28 °C. The cell density for bacteria was 2–4 × 107 CFU/mL and 2–4 × 105 CFU/mL for fungus. A serial dilution of compounds were performed in the microplates and incubated for 12 h. The last tube with no growth of microorganism was recorded to represent the MIC value expressed in μg/mL.

3. Results and discussion

3.1. Structure elucidation

Cynapanoside D (**1**), was afforded as light yellow amorphous gum, $[\alpha]_D^{20}$ +43.5 (*c* 0.069, MeOH). Its positive HR-ESI-MS showed an ion peak at *m/z* 657.3174 $[M + Na]^+$ (calcd. 657.3251), indicating a molecular formula of $C_{34}H_{50}O_{11}$. The ^{1}H NMR spectrum of **1** revealed the presence of two tertiary methyl groups [δ_H 0.82 (3H, s, H-19) and 1.51 (3H, s, H-21)], two oxygen-substituted methine protons [δ_H 3.74 (1H, m, H-3) and 5.41 (1H, m, H-16)], two oxygen-substituted methylene protons [δ_H 3.92 (1H, dd, J = 9.3, 9.0 Hz, H_α-15) and 4.22 (1H, dd, J = 8.5, 7.3 Hz, H_β-15)], one olefinic proton [δ_H 5.38 (1H, m, H-6)], and one olefinic deshielded proton [δ_H 6.45 (1H, s, H-18)] connected with the trisubstituted double bond. The ^{1}H and ^{13}C NMR data (Table 1) of the aglycone part of **1** were almost identical to those of glaucogenin C [14], except for the glycosidation shifts at C-2 (−2.5 ppm), C-3 (+6.2 ppm) and C-4 (−4.2 ppm). Thus, the aglycone structure of **1** was confirmed to be glaucogenin C by detailed 1D NMR and 2D NMR (HSQC, HMBC and NOESY) spectral analysis, and the sugar chain was linked at its C-3 hydroxyl group. The proton signals showed two anomeric proton signals at [δ_H 4.77 (1H, br.d, J = 9.8 Hz, H-1′) and 5.52 (1H, br.d, J = 9.8 Hz, H-1″)], one methoxy group [δ_H 3.53 (3H, s, C-3′ –OCH_3)], and two methyl groups [δ_H 1.44 (3H, d, J = 6.0 Hz, H-6′) and 1.55 (3H, d, J = 6.0 Hz, H-6″)] of the sugar moiety. The splitting patterns of anomeric proton signals indicated that **1** had two sugar units with β-linkages. The linkage positions and sequence of the two sugars were ascertained by HMBC correlations from δ_H 5.52 (H-1″ of β-digitoxopyranose) to δ_C 82.9 (C-4′ of β-oleandropyranose) and from δ_H 4.77 (H-1′ of β-oleandropyranose) to δ_C 77.3 (C-3). Acid hydrolysis of **1** afforded two sugars (oleandrose and digitoxose). Identification of D-oleandrose and D-digitoxose were performed by HPLC analysis with an optical rotation detector and comparison of R_f values with the literature [15]. In addition, the correlations observed in the NOESY experiment also supported the proposed relative configuration of **1**, in which NOESY correlations of H-19 to H-11β and H-8, of H-16 to H-17 and H-15α, of H-1′ to H-3′ and H-5′, of H-1″ to H-5″ indicated the orientation of the aglycone and two sugars. Thus, the structure of **1** was established as glaucogenin C-3-*O*-β-D-digitoxopyranosyl-(1 → 4)-β-D-oleandropyranoside and named cynapanoside D.

Cynapanoside E (**2**) was obtained as light yellow amorphous gum, $[\alpha]_D^{20}$ −36.9 (*c* 0.24, MeOH). The formula of **2** was determined to be $C_{42}H_{64}O_{15}$ by the $[M + Na]^+$ ion peak at *m/z* 831.4009 (Calcd for $C_{42}H_{64}NaO_{15}$ 831.4143) in HR-ESI-MS. The ^{1}H and ^{13}C NMR data (Table 1) of the aglycone part of **2** were almost identical to those of neocynapanogenin F [8], except for the glycosidation shifts at C-2 (−1.0 ppm), C-3 (+6.7 ppm) and C-4 (−3.1 ppm), so the sugar was determined to be linked to the C-3 hydroxyl group of the aglycone. The ^{1}H NMR spectrum of **2** showed three secondary methyl and three methoxyl methyl signals of deoxysugars. The anomeric configuration of sugars was deduced by *J* values at [δ_H 4.75 (1H, dd, J = 9.8, 1.8 Hz, H-1′), 5.23 (1H, dd, J = 9.8, 1.8 Hz, H-1″) and 4.93 (1H, d, J = 3.3 Hz, H-1‴)] as β-, β-, and α-form, respectively. In the HMBC spectrum, correlations of H-1′ (δ 4.75) with C-3 (δ 77.2), H-1″ (δ 5.23) with C-40 (δ 82.7) and H-1‴ (δ 4.93) with C-40 (δ 82.0) were observed, indicating the sugar moiety of **2** was deduced to be α-cymaropyranosyl-(1 → 4)-β-cymaropyranosyl-(1 → 4)-β-oleandropyranosyl group by comparison with data in the literature as shown in Fig. 2 [16]. Acid hydrolysis of **2** gave oleandrose and cymarose and they were identified by comparison of their spectroscopic data and R_f values with those reported in the literatures [10,15,13,17]. Identification of D-oleandrose, D-cymarose and L-cymarose were also analyzed by HPLC with the detection by using the optical rotation (OR) detectors. The HPLC analysis exhibited a positive peak for the oleandrose, a positive and a negative peaks for the cymaroses, suggesting D-, L-form of the cymaroses and D-form of the oleandrose. In addition, the NMR data of the two cymaroses were identical to those of Sublanceoside G_3 [18], so the outer cymarose was determined as α-L-cymarose, and the inner one was β-D-cymarose. In the NOESY spectrum, correlations of H-19 to H-4 *endo* and H-11 *endo*, indicated that H-19, H-4 *endo* and H-11 *endo* are on the same face of the molecule. Consequently, all the aforementioned evidences characterized **2** as neocynapanogenin F 3-*O*-α-L-cymaropyranosyl-(1 → 4)-β-D-cymaropyranosyl-(1 → 4)-β-D-oleandropyranoside, and was designated cynapanoside E.

Cynapanoside F (**3**), $[\alpha]_D^{20}$ −24.3 (*c* 0.23, MeOH), was yielded as light yellow amorphous gum. Its molecular formula was determined as $C_{41}H_{62}O_{15}$ by the $[M + Na]^+$ ion peak at *m/z* 817.4011 (Calcd for $C_{41}H_{62}NaO_{15}$, 817.3996) in HR-ESI-MS. From its ^{13}C NMR data (Table 1), it was apparent that **3** possessed the same aglycone as that of **2**. The ^{1}H NMR spectrum of **3** showed three anomeric protons signals at [δ_H 4.77 (1H, br.d, J = 9.8 Hz, H-1′), 5.21 (1H, d, J = 3.0 Hz, H-1‴), and 5.51 (1H, br.d, J = 9.8 Hz, H-1″)] in its sugar moiety, indicating the presence of one sugar with α-linkage and two sugars with β-linkages. The ^{1}H and ^{13}C NMR (Table 1) spectra of the deoxysugar units of **3** were similar to Cynanside A [19]. The HMBC experiments of **3** showed correlations from δ_H 5.21 (H-1‴ of α-oleandrose) to δ_C 82.2 (C-4″ of β-digitoxose), 5.51 (H-1″ of β-digitoxose) to δ_C 83.0 (C-4′ of β-oleandrose) and δ_H 4.77 (H-1′ β-oleandrose) to δ_C 77.2 (C-3), which further confirmed the linkage sequence. Acid hydrolysis of **3** yielded oleandrose and digitoxose. The absolute configuration of oleandrose and digitoxose were identified as D-, L-oleandrose and D-digitoxose according to the HPLC analysis. Moreover, the NMR data sugar moiety of **3** were the same as those of a previously reported compound, amplexicosides B [12], which indicated that the inner oleandrose was β-D-oleandrose, and the outer one was α-L-oleandrose. Therefore, the structure of **3** was elucidated as neocynapanogenin F 3-*O*-α-L-oleandropyranosyl-(1 → 4)-β-D-digitoxopyranosyl-(1 → 4)-β-D-oleandropyranoside and named cynapanoside F.

Cynapanoside G (**4**), $[\alpha]_D^{20}$ +26.7 (*c* 0.071, MeOH), was obtained as white needle crystal (MeOH). It gave the molecular formula as $C_{33}H_{52}O_{13}$ by the $[M + Na]^+$ ion peak at *m/z* 679.3312 (Calcd for $C_{33}H_{52}NaO_{13}$ 679.3306) according to the HR-ESI-MS. The ^{1}H NMR spectrum of **4** revealed the presence of three tertiary methyl groups [δ_H 0.93 (3H, s, H-19), 1.05 (3H, s, H-18) and 2.17 (3H, s, H-21)], one olefinic proton [δ_H 5.40 (1H, m, H-6)] in its aglycone moiety. The ^{1}H NMR and corresponding ^{13}C NMR spectral data (Table 1) were similar to those of the aglycone of Carumbelloside **I** [12]. The ^{1}H NMR spectrum of **4** showed two anomeric proton signals at [δ_H 5.05 (1H, d, J = 7.5 Hz, H-1′), and 5.26 (1H, d, J = 7.5 Hz, H-1″)] in its sugar moiety, indicating the presence of two sugars with β-linkages. Acid hydrolysis of **4** yielded glucopyranose, and identification of D-glucopyranose was performed by HPLC analysis and co-TLC with standard samples. Compared with Carumbelloside **I** [12], the sugar chain was also linked to the C-3 hydroxyl group of aglycone. The difference between the sugar components of **4** and those of Carumbelloside **I** was that the sugar sequence of **4** changed into β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranosyl by the HMBC correlations from δ_H 5.26 (H-1″ of terminal β-D-glucopyranose) to δ_C 84.5 (C-2′ of β-D-glucopyranose). Thus, the structure of **4**

was determined to be 3β, 14β-dihydroxypregn-5-en-20-one 3-*O*-β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranoside (Fig. 1) and named cynapanoside G.

The known compounds were identified as glaucoside A (**5**) [20], neocynapanogenin F 3-*O*-β-D-oleandropyranoside (**6**) [21], glaucogenin A 3-*O*-β-D-digitoxopyranosyl-(1 → 4)-*O*-β-D-oleandropyranoside (**7**) [22], cynatratoside B (**8**) [14], (3β,8β,9α,16α,17α)-14,16β:15,20α:18,20β-triepoxy-16β:17α-dihydroxy-14-oxo-13,14:14,15-disecopregna-5,13(18)-dian-3-yl α-cymaropyranosyl-(1 → 4)-β-digitoxopyransyl-(1 → 4)-β-oleandropyranoside (**9**) [9] and 3β,14-dihydroxy-14β-pren-5-en-20-one (**10**) [10] by comparison of their physico-chemical and spectroscopic properties with previously reported values.

13,14:14,15-Diseco-pregnane-type compounds are novel C_{21} steroidals. Their main cleavage pattern were C/D ring broken and a series of oxidation reactions. The possible mechanism of biological cleavage of the ring C/D junction was proposed. (Fig. 3).

3.2. Biological activities

Compounds **1**–**4** and **7**–**9** were evaluated for their cytotoxicities against HL-60 (human leukemic promyelocytic cell), HT-29 (human colon cancer cell), PC-3 (human prostate cancer cell) and MCF-7 (human breast cancer cell) cell lines using MTT assays in vitro (Table 2). 5-Fluorouracil was used as a positive control and their IC_{50} values were 6.38, 7.90, 7.77 and 17.01 μM, respectively. As shown in Table 2, 13,14:14,15-diseco-pregnane-type glycosides displayed better cytotoxicity than normal four-ring C_{21} steroid type glycosides (IC_{50} value: **1**–**3**, **7**–**9** > **4**). The removal of hydroxyl at the 17 position significantly increased the activity (IC_{50} value: **1** > **7**; **8** > **9**). Compound **8** showed higher inhibitory effect than compound **1**, which may be attributed to the number of the sugars in **8** was more than that of **1**. The IC_{50} values of compound **8** against the above four cell lines displayed 8.3, 7.5, 34.3 and 19.4 μM, respectively, and HL-60 and HT-29 cell lines were more sensitive to **8** than PC-3 and MCF-7 cell lines. The potency of compound **8** against HT-29 cell line was higher than that of 5-fluorouracil.

The antioxidant activities of compounds **1**–**4**, **8** and **9** were evaluated by their ability to scavenge DPPH radical. All compounds were inactive with IC_{50} values above 200 μM in the DPPH radical scavenging assay.

Antibacterial and antifungal activities of compounds **1**–**4**, **8** and **9** were tested. The antimicrobial activities of the compounds **1**–**4**, **8** and **9** were assessed towards *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans*. None of these compounds exhibited antibacterial activities against *Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli*. All compounds showed weak antifungal activities against *Candida albicans* with MIC value of 500 μg/mL. Fluconazole was used as positive control against *Candida albicans* (SC5314) with MIC value of 500 μg/mL.

Conflict of interests

The authors declare that there are no conflicting interests.

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Appendix A. Supplementary data

The NMR and HR-ESI-MS spectra of compounds **1**–**4** were available as supporting information in the Supplementary data. Supplementary data associated with this article can be found in the online version, at doi: http://dx.doi.org/10.1016/j.fitote.2016.07.001

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