A New Nortriterpene from the Root of *Celastrus hypoleucus*

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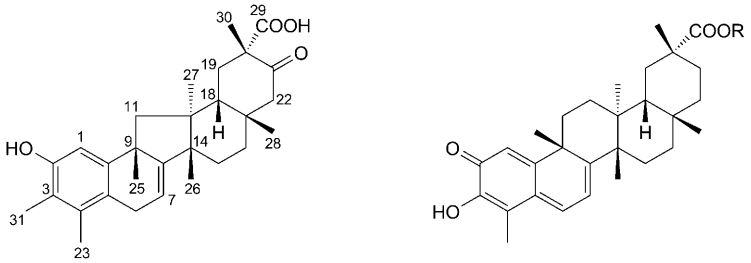
A new nortriterpene, 2-hydroxy-3-methyl-21-oxo-12,24-dinor-*D*:*B*-friedooleana-1,3,5(10),7-tetraen-29-oic acid (**1**), was isolated from the root of *Celastrus hypoleucus*, together with the two known compounds, celastorol (**2**) and pristimerine (**3**). Their structures were elucidated on the basis of spectroscopic analyses. Compounds **1**–**3** exhibited *in vitro* significant antioxidant (against lipid peroxidation; by the TBARS method) and antitumor activities (against cancer cell lines P-388, A-549, HL-60, and BEL-7402).

Introduction. – *Celastrus hypoleucus* (OLIV.) WARB. is a species of the Celastraceae family and commonly named as Nan She Teng (Chinese trade name). It is widely distributed in China and has been used for the treatment of inflammation and detumescence. As part of our continuing investigation of bioactive components from the Celastraceae family [1–7], many new antioxidant and antitumor-active metabolites have been isolated and characterized from *C. hypoleucus* [1][6][7]. In this article, we report a new nortriterpene, 2-hydroxy-3-methyl-21-oxo-12,24-dinor-*D*:*B*-friedooleana-1,3,5(10),7-tetraen-29-oic acid[1]) (**1**), and two known triterpenes, *i.e.*, celastorol (**2**) [8] and pristimerine (**3**) [9], from the root of *C. hypoleucus* (*Fig. 1*). In addition, their antioxidant and antitumor activities were investigated.



1[1])

2 R = H
3 R = Me

Fig. 1. *Compounds* **1**–**3** *isolated from* C. hypoleucus

[1]) Trivial atom numbering, for the systematic name, see *Exper. Part.*

Results and Discussion. – 1. *Chemistry.* The acetone-soluble fraction derived from the crude extract of dried and powdered roots from *C. hypoleucus* was purified by a combination of column chromatography (silica gel) and prep. TLC, as well as by crystallization to yield the three compounds **1**–**3**. Their structures were elucidated by 1D- and 2D-NMR spectra and comparison with literature data.

Compound **1** was obtained as a red lacquer and had the molecular formula $C_{29}H_{38}O_4$, as derived from the positive-ion-mode HR-ESI-MS (*m/z* 451.2834 ($[M+H]^+$)) and the ^{13}C-NMR (DEPT) spectrum (*Table*). The IR spectrum of **1** revealed the presence of OH (3400 cm^{-1}) and C=O groups (1719 cm^{-1}), and of C=C bonds (1653 cm^{-1}). The UV absorption of **1** at 212 and 282 nm indicated the presence of a substituted benzene ring. The ^{1}H-NMR spectrum (*Table*) showed characteristic signals of a *D*:*B*-friedooleana-1,3,5(10),7-tetraene at δ(H) 6.73 (*s*), 3.86 (*d*, *J* = 6.4), and 5.77 (*d*, *J* = 6.4) corresponding to the three protons H–C(1), H_a–C(6), and H–C(7), respectively, five angular Me groups at δ(H) 1.43 (Me(25)), 0.63 (Me(26)), 1.16 (Me(27)), 1.05 (Me(28)), and 1.25 (Me(30)), and two Me groups on an aromatic ring at δ(H) 2.10 (Me(31)) and 2.16 (Me(23)) [10]. In addtion, the ^{1}H-NMR spectrum showed signals of a *d* at δ(H) 2.65 (*J* = 13.2 Hz, 1 H) and a *d* at δ(H) 2.40 (*J* = 13.2 Hz, 1 H) attributed to two geminal H-atoms (CH_2(22)) [10]. The locations of the seven Me groups and two C=O groups, C(29) (δ(C) 183.1) and C(21) (δ(C) 209.4), were deduced from a HMBC experiments (*Fig. 2*). In the ^{1}H,^{1}H-COSY plot, correlations indicated the presence of three spin systems, namely CH_2–CH, (C(6) to C(7)), CH_2–CH_2 (C(15) to C(16)), and CH–CH_2 (C(18) to C(19)). The relative configuration of **1** was derived by a NOESY experiment (*Fig. 2*) in combination with biogenetic considerations [10].

Table. *^{1}H- and ^{13}C-NMR Data of Compound* **1**[1]) (400 MHz, $CDCl_3$). δ in ppm, *J* in Hz.

	δ(C)[a])	δ(H)		δ(C)[a])	δ(H)
H–C(1)	109.3	6.73 (*s*)	C(17)	30.9	–
C(2)	140.3	–	H–C(18)	44.3	1.78 (*t*, *J* = 8.0)
C(3)	121.5	–	CH_2(19)	30.5	2.02 (*d*, *J* = 12.6), 2.46 (*d*, *J* = 12.6)
C(4)	120.0	–	C(20)	40.2	–
C(5)	127.9	–	C(21)	209.4	–
CH_2(6)	28.9	1.51 (*d*, *J* = 6.4), 3.86 (br. *s*)	CH_2(22)	51.8	2.40 (*d*, *J* = 13.2), 2.65 (*d*, *J* = 13.2)
H–C(7)	121.6	5.77 (*d*, *J* = 6.4)	Me(23)	11.1	2.16 (*s*)
C(8)	150.2	–	Me(25)	36.7	1.43 (*s*)
C(9)	37.1	–	Me(26)	18.8	0.63 (*s*)
C(10)	142.2	–	Me(27)	22.3	1.16 (*s*)
CH_2(11)	35.6	1.65 (*d*, *J* = 12.6), 2.02 (*d*, *J* = 12.6)	Me(28)	31.5	1.05 (*s*)
C(13)	37.7	–	C(29)	183.1	–
C(14)	43.8	–	Me(30)	32.8	1.25 (*s*)
CH_2(15)	29.7	1.51–1.78 (*m*)	Me(31)	11.1	2.10 (*s*)
CH_2(16)	36.7	1.51–1.78 (*m*)			

[a]) Assignments are supported by the results of DEPT, ^{1}H,^{1}H-COSY, NOESY, HMQC, and HMBC experiments.

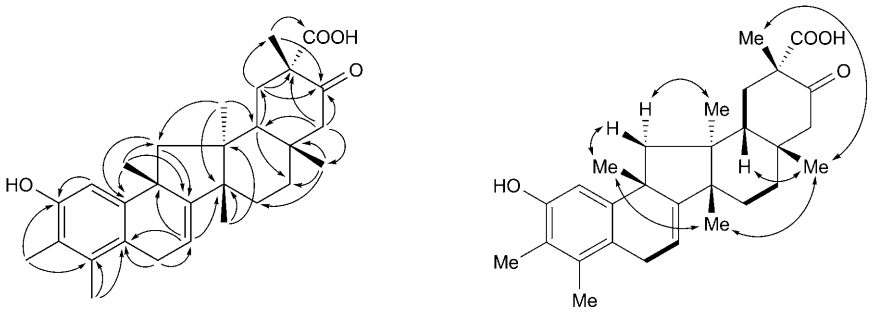


Fig. 2. *Selected HMBCs* (H → C), *Key NOESYs* (H ↔ H) *and* $^1H,^1H$*-COSYs* (H — H) *of compound* **1**

The observed NOESY correlations Me(25)/H_a–C(11), H_b–C(11)/Me(27), Me(25)/Me(26), Me(26)/Me(28), Me(28)/Me(30), and Me(28)/H–C(18) indicated their *cis*-orientation. In contrast, no NOESY correlation was observed between H–C(18) and Me(27), indicating their *trans*-orientation. Therefore, the structure of **1** was elucidated as 2-hydroxy-3-methyl-21-oxo-12,24-dinor-*D* : *B*-friedooleana-1,3,5(10),7-tetraen-29-oic acid.

2. *Bioactivity.* Antioxidant activities of compounds **1**–**3** were evaluated against lipid peroxidation of male Wistar rat heart, liver, and kidneys liposomes by the TBARS (thiobarbituric acid reactive substance) method described in [11]. The results are summarized in *Fig. 3* and compared with the activity of the reference antioxidant HTEMPO (=4-hydroxy-2,2,6,6-tetramethylpiperidin-1-yl-oxy radical), establishing that compounds **1**–**3** can be considered as promising antioxidant candidates. Thus, the present studies suggest that nortriterpenequinone methides are efficient antioxidants and can reduce DNA damage caused by hydroxy-radical oxidation.

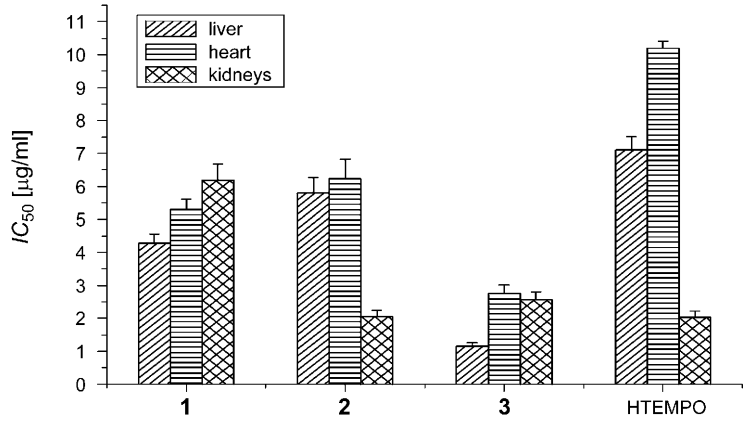


Fig. 3. IC_{50} (μg/ml) *Values of compounds* **1**–**3** *on lipid peroxidation.* The results are presented as the mean ± s.d. ($n = 3$).

Antitumor activities of compounds **1**–**3** were evaluated *in vitro* by using a variety of cultured cancer cell lines (P-388 and HL-60 (by the MTT (thiazolyl blue tetrazolium

bromide) method [12], and A-549 and BEL-7402 by the SRB (sulforhodamine B) method [13]). The results are summarized in *Fig. 4*. Etoposide was included as a standard in these measurements. Thus, compounds **1**–**3** showed potent cytotoxicity against a number of cultured tumor cell lines, with IC_{50} values of less than 50.00 μg/ml.

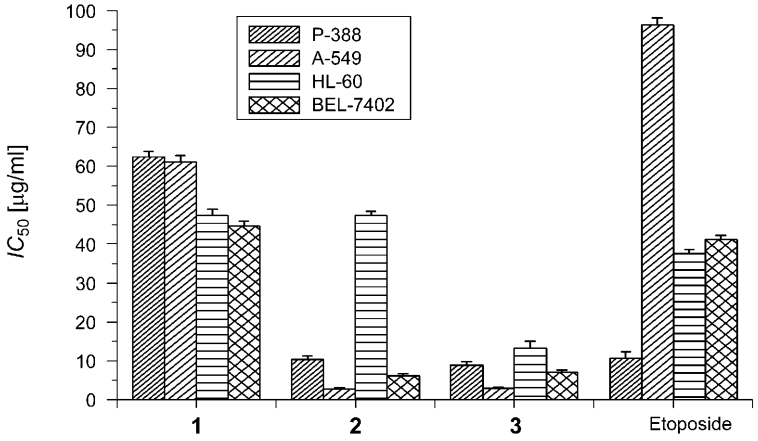


Fig. 4. IC_{50} (μg/ml) *Values of compounds* **1**–**3** *on cancer cell.* The results are presented as the mean ± s.d. ($n = 3$).

In conclusion, the nortriterpenes isolated from the root of *C. hypoleucus* exhibited strong antioxidant and antitumor activities indicating that this plant may represent a valuable source of natural antioxidant and antitumor derivatives.

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Experimental Part

General. TLC: precoated SiO_2 *60* F_{254} plates (*Merck*); detection by UV light or by heating after spraying with 5% H_2SO_4 in EtOH. Column chromatography (CC): silica gel (SiO_2; 200 ± 300 mesh; Qingdao, P. R. China). Optical rotation: *Perkin-Elmer-341* polarimeter. IR: *Nicolet-Avatar-360* FT-IR spectrometer; KBr pellets; $\tilde{\nu}$ in cm^{-1}. UV: *Shimadzu-UV-260* spectrometer; λ_{max} (log ε) in nm. ^{1}H- and ^{13}C-NMR and DEPT spectra: *Bruker-Avance-400* spectrometer; in $CDCl_3$ solns. at 400 and 100 MHz, resp.; δ in ppm rel. to the residual solvent signals (δ(H) 7.24, δ(C) 77.0), *J* in Hz; 2D-NMR experiments included ^{1}H,^{1}H-COSY, HMQC, NOESY, and HMBC. EI-, FAB-, and HR-ESI-MS: *HP-5988* and *Apex™-II-Bruker-4.7-TAS* spectrometer; in *m*/*z* (rel. %).

Plant Material. The roots of *C. hypoleucus* were collected in Xiaolong Mountain, Gansu Province of China, in October 2000, and identified by Prof. *J. Z. Sun*, Lanzhou University, P. R. China. A voucher specimen (No. 2001001) was deposited with the Department of Biology, Lanzhou University, P. R. China.

Extraction and Isolation. The dried, powdered roots of *C. hypoleucus* (1.2 kg) were extracted with acetone (3 × 10 l) by percolation at r.t. to give a residue (100 g) after evaporation. This residue was separated by CC (SiO_2 (800 g; 200–300 mesh), gradient petroleum ether (PE; 60–90°)/acetone 20:1, 16:1, 12:1, 8:1, 4:1, and 0:1 (3500 ml each)): *Fractions 1–20. Fr. 2* (2.0 g) was recrystallized from PE

(300 ml) to give **3** (1000.0 mg; R_f 0.45 (PE/acetone 5:1). *Fr. 7* (3.0 g) was purified by TLC (20 g, 20 × 20 cm): **1** (15.3 mg; R_f 0.45 (PE/acetone 3:1)). *Fr. 8* (2.0 g) was subjected to CC (SiO_2 (30.0 g; 30 × 3 cm), PE/acetone 15:1, 12:1, 9:1, 6:1, 3:1, and 0:1 (300 ml each)): pure **2** (50.2 mg; R_f 0.26 (PE/acetone 4:1)).

2-Hydroxy-3-methyl-21-oxo-12,24-dinor-D:B-*friedooleana-1,3,5(10),7-tetraen-29-oic Acid* (= rel-*(2*R*,4a*R*,6a*R*,12b*R*,13a*R*,13b*S*)-2,3,4,4a,5,6,6a,8,12b,13,13a,13b-dodecahydro-11-hydroxy-2,4a,6a,9,10, 12b,13a-heptamethyl-3-oxo-1*H*-dibenzo[a,i]fluorene-2-carboxylic Acid*; **1**). Red lacquer: $[\alpha]_D^{20} = -3.3$ ($c = 0.65$, $CHCl_3$). IR (KBr): 3400, 2926, 2859, 1719, 1455, 1372, 1265, 1104, 1027, 728. UV (EtOH): 212 (2.11), 282 (0.64). ^{1}H- and ^{13}C-NMR (400 and 100 MHz, resp., $CDCl_3$): *Table*. EI-MS: 450 (M^+). FAB-MS (pos.): 451 ($[M+H]^+$). HR-ESI-MS: 451.2834 ($[M+H]^+$, $C_{29}H_{39}O_4^+$; calc. 451.2848).

Antioxidant-Activity Assay. The heart, liver, and kidneys liposomes from male Wistar rats (*ca.* 4-week old) were obtained after decapitation and homogenated in ice-cold 10 mM *Tris*·HCl buffer (pH 7.4) with a *Teflon*-glass homogenizer. Each homogenate was centrifuged for 10 min at 1000*g*, and the supernatant was used in the test. Lipid peroxidation was stimulated in assays containing 250 µl of rat heart, liver, or kidneys homogenate by the additions of 0.02 mM $FeCl_2$ and 0.25 mM ascorbic acid, and the mixture was incubated for 30 min at 37°. The reaction was stopped by the addition of 35% $HClO_4$ soln. (0.05 ml). After centrifugation for 10 min at 1000*g*, 200 µl of the resulting supernatant was added to 100 µl of an aq. soln. containing 0.5% TBA (= 2-thiobarbituric acid = 2-sulfanylpyridine-4,6-diol) and left at 80° for 1 h. Then, the mixture was cooled to r.t., and its absorbance at 532 nm was measured. The known antioxidant agent HTEMPO was used as a positive control.

Antitumor-Activity Assay. 1. *MTT Assay.* Cells were cultured in *Dulbecco*'s minimum essential medium (DMEM) supplemented with 5% fetal calf serum (FCS), gentamycin sulfate (0.004%), glucose (0.57%), and $NaHCO_3$ (0.12%). Cells were seeded into 96-well flat-bottomed plates at a concentration of $3.0 \cdot 10^5$ cells per ml. After 24 h, the cells were treated with the compounds, which were diluted with culture medium to produce six concentrations. MTT Labeling reagent (50:1) was added and the absorbance (560 nm) read after 72 h. Experiments were carried out three times in triplicate. The known antitumour agent etoposide was used as a positive control for the P-388 and HL-60 cell lines.

2. *SRB Assay.* The cell suspension described in the MTT assay was seeded in 96-well microtiter plates (100 µl per well). After 24 h, the cells were treated with the compounds. Each compound was initially dissolved in a small amount of DMSO and diluted further in medium to produce six concentrations. The compound solns. (100 µl per well of each concentration) was added to the plates in six replicates. By these serial dilutions, the final mixture used for treating the cells contained not more than 0.5% of DMSO, the same as in the solvent-control wells. The final volume in each well was 200 µl. The plates were incubated for a select exposure time of 48 h. Then, 100 µl of ice-cold 40% CCl_3COOH was added to each well, left at 4° for 1 h, and washed five times with dist. H_2O. The CCl_3COOH-fixed cells were stained for 30 min with 50 µl of 0.4% (*w*/*v*) SRB in 1% AcOH. The plates were washed five times with 1% AcOH and air-dried overnight. On the day of reading the plates, bound dye was solubilized with 100 µl of 10 mM *Tris* base. The absorbance of each well was read at 560 nm. Experiments were carried out three times in triplicate. Etoposide was used as a positive control for the A-549 and BEL-7402 cell lines.

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