Biotransformation of Oleaside A by *Cunninghamella echinulata*

by **Min Lu**[a])[b]), **Qing-Xin Liu**[b]), **Zhu-Zhen Han**[b]), **Xian-Qing Hu**[b]), **Wei-Dong Zhang**[b])[c]), **Ping Wan***[a]), and **Hui-Liang Li***[b])

[a]) Life and Environment Science College, Shanghai Normal University, Shanghai, 200234, P. R. China
[b]) Department of Phytochemistry, School of Pharmacy, Second Military Medical University, No. 325, Guohe Rd., Shanghai 200433, P. R. China
(phone: +86-21-81871245; fax: +86-21-81871245; e-mail: faranli@hotmail.com)
[c]) School of Pharmacy, Shanghai Jiao Tong University, Shanghai 200240, P. R. China

Biotransformation of oleaside A (**1**) by *Cunninghamella echinulata* (ACCC 30369) was carried out to afford two products, (6*R*)-6-hydroxyoleaside A (**2**) and (7*S*)-7-hydroxyoleaside A (**3**). The structures of **2** and **3** were elucidated by extensive NMR analyses and further confirmed by single-crystal X-ray diffraction analysis. We also report herein the X-ray diffraction structure of oleaside A (**1**) for the first time. Compounds **1**–**3** were evaluated for their cytotoxic activities against the A549, HCT116, HepG2, and HL-60 human cancer cell lines.

Introduction. – Microbial transformation is an effective tool for the structural modification of natural bioactive compounds. Its application in asymmetric synthesis is increasing due to its versatility and simplicity [1]. A variety of transformations on natural products such as oxidation, reduction, hydrolysis, isomerization, epimerization, rearrangement, D-homoannulation, *Michael* addition, and reverse aldol reaction have already been performed [2]. *Cunninghamella echinulata*, a filamentous fungus, is recognized for its potential for steroid hydroxylation and has been noted for its ability to mimic mammalian hepatic metabolism with other substrates [3][4].

Oleaside A (**1**) is one of the most important cardiac glycosides derived from the *Nerium oleander* L. Recently, the anticancer effects of cardiac glycosides have been reported [5–7]. In continuation of our biotransformation studies on the natural anticancer products [8][9], we now report the characterization of two metabolites of oleaside A (**1**) in cell suspension of *C. echinulata* (ACCC 30369). These metabolites were identified as (6*R*)-6-hydroxyoleaside A (**2**) and (7*S*)-7-hydroxyoleaside A (**3**) by spectroscopic methods. We also report here the X-ray diffraction structure of these three compounds. Compounds **1**–**3** (see *Fig. 1*) were evaluated for their cytotoxic activities against the A549, HCT116, HepG2, and HL-60 human cancer cell lines.

Results and Discussion. – Compound **2** was obtained as colorless needles. The HR-ESI-MS (positive-ion mode) provided the molecular formula $C_{30}H_{44}O_8$ (*m*/*z* 555.2945 $[M+Na]^+$), requiring nine degrees of unsaturation. In the ^{1}H-NMR spectrum (*Table 1*), three Me signals at δ(H) 0.88 (*s*, Me(19)), 0.96 (*s*, Me(18)), and 1.25 (*d*, $J = 6.5$, Me(6′)) were observed. The ^{13}C-NMR and DEPT spectroscopic data displayed (*Table 1*) 30 C-atom signals, including those of one MeO group, three Me, ten CH_2, and

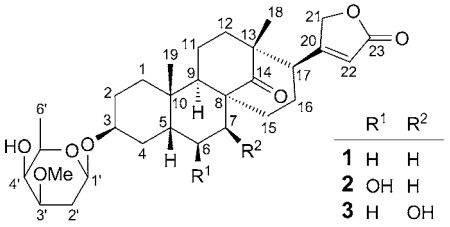

Fig. 1. *Structures of compounds* **1**–**3**

ten CH groups, and six quaternary C-atoms. The ^{13}C-NMR spectrum also indicated the presence of two C=O groups (δ(C) 226.7 (C(14)) and 175.5 (C(23))) and one C=C bond (δ(C) 172.4 (C(20)), 116.6 (C(22))). From the detailed analysis of 1D- and 2D-NMR spectra of compound **2**, a five-membered unsaturated lactone ring was infered by the characteristic ^{13}C signals at δ(C) 172.4 (C(20)), 116.6 (C(22)), 175.5 (C(23)), and 74.3 (C(21)) in ^{13}C-NMR spectrum. The ^{1}H,^{1}H-COSY and HMQC-TOCSY spectrum of **2** indicated four fragments: **a**: CH_2(1)–CH_2(2)–CH(3)–CH_2(4)–CH(5)–CH(6)–CH_2(7); **b**: CH(9)–CH_2(11)–CH_2(12); **c**: CH_2(15)–CH_2(16)–CH(17); **d**: CH(1′)–CH_2(2′)–CH(3′)–CH(4′)–CH(5′)–Me(6′) according to the H-atom spin systems (*Fig. 2*). By combination with HMBCs from Me(19) to C(1), C(5), C(9), and C(10), correlations from H–C(7) to C(8), C(9), and C(14); from Me(18) to C(12), C(13), C(14), and C(17); from H–C(15) to C(8) and C(14), and from H–C(21) and H–C(22) to C(17), the fragments **a**, **b**, and **c**, together with the above mentioned lactone ring moiety, enabled the construction of the oleagenin aglycone sketch. The remaining part of **2**, the fragment **d** and one MeO group, were in accorddence with diginoside sugar moiety, which was confirmed by the HMBCs of the signal of H–C(1′) to those of C(2) and C(3), of signal of Me(6′) to those of C(5′) and C(4′), and signal of MeO to that of C(3′). The key HMBCs H–C(1′)/C(3) and H–C(3)/C(1′) evidenced, without doubt, that diginoside sugar moiety was linked to the aglycone at C(3). Thus, compound **2** could be identified as a hydroxylated analog of oleaside A. Similarly, the position of the OH in aglycone was determined as C(6) from the correlations δ(H) 3.60–3.62 (*m*, H–C(6))/δ(H) 1.72–1.75 (*m*, H–C(5)) and δ(H) 3.60–3.62 (*m*, H–C(6))/δ(H) 2.15–2.22 (*m*, 1 H of CH_2(7)) in ^{1}H,^{1}H-COSY, and correlations δ(H) 3.60–3.62 (*m*, H–C(6))/C(5) (δ(C) 44.0) and C(7) (δ(C) 36.2) in HMBC spectra. Collectively, these data (*Fig. 2*) allowed assignment of the planar structure of **2**.

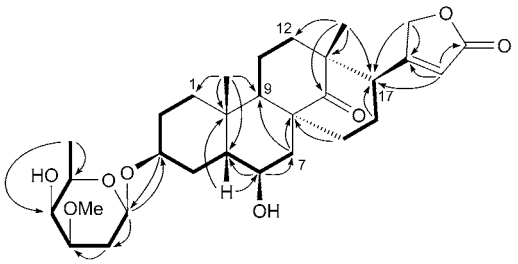

Fig. 2. *Selected key* ^{1}*H,*^{1}*H-COSY* (—)*, and HMB* (H→C) *correlations of* **2**

Table 1. *^{1}H-NMR Data of* **1**–**3**. At 500 MHz in CD_3OD; δ in ppm, J in Hz.

H-Atom	**1**	**2**	**3**
CH_2(1)	1.41–1.46 (*m*), 1.51–1.56 (*m*)	1.40–1.42 (*m*), 1.52–1.55 (*m*)	1.42–1.44 (*m*), 1.52–1.55 (*m*)
CH_2(2)	1.07–1.11 (*m*), 1.12–1.16 (*m*)	1.08–1.10 (*m*), 1.14–1.17 (*m*)	1.07–1.11 (*m*), 1.12–1.16 (*m*)
H–C(3)	4.06 (br. *s*)	4.00 (br. *s*)	3.99 (br. *s*)
CH_2(4)	1.08–1.12 (*m*), 1.89–1.95 (*m*)	1.10–1.15 (*m*), 1.89–1.92 (*m*)	1.10–1.14 (*m*), 1.89–1.92 (*m*)
H–C(5)	1.62–1.64 (*m*)	1.72–1.75 (*m*)	1.78 (*d*, J = 5.1)
CH_2(6) or H–C(6)	1.65–1.68 (*m*), 1.87–1.91 (*m*)	3.60–3.62 (*m*)	1.48–1.52 (*m*), 2.33–2.41 (*m*)
H–C(7) or CH_2(7)	1.56–1.60 (*m*), 1.76–1.79 (*m*)	1.44–1.47 (*m*), 2.15–2.22 (*m*)	3.23 (*dd*, J = 12.8, 4.7)
H–C(9)	2.47 (*d*, J = 8.3)	2.52 (*m*)	2.46–2.50 (*m*)
CH_2(11)	1.78–1.86 (*m*), 2.35–2.45 (*m*)	1.74–1.78 (*m*), 2.32–2.38 (*m*)	1.73–1.81 (*m*), 2.42–2.45 (*m*)
CH_2(12)	2.05–2.15 (*dd*, J = 15.0, 6.3)	2.03–2.15 (*m*)	2.02–2.21 (*m*)
CH_2(15)	1.71–1.80 (*m*), 1.98–2.09 (*m*)	1.73–1.78 (*m*), 2.06–2.10 (*m*)	1.71–1.76 (*m*), 2.02–2.09 (*m*)
CH_2(16)	1.68–1.76 (*m*), 2.80–2.90 (*m*)	1.66–1.70 (*m*), 2.82–2.88 (*m*)	1.67–1.69 (*m*), 2.80–2.84 (*m*)
H–C(17)	3.09 (*d*, J = 7.2)	3.15 (*d*, J = 7.1)	3.12 (*d*, J = 5.1)
Me(18)	0.92 (*s*)	0.96 (*s*)	0.90 (*s*)
Me(19)	0.77 (*s*)	0.88 (*s*)	0.81 (*s*)
CH_2(21)	4.56 (*dd*, J = 17.6, 1.8), 4.68 (*dd*, J = 17.6, 1.8)	4.67 (*dd*, J = 17.3, 1.8), 4.73 (*dd*, J = 17.3, 1.8)	4.68 (*dd*, J = 18.2, 1.9), 4.74 (*dd*, J = 18.2, 1.9)
H–C(22)	5.68 (*t*, J = 1.6)	5.60–5.62 (*m*)	5.77 (*t*, J = 1.6)
H–C(1′)	4.44 (*dd*, J = 9.8, 2.1)	4.48 (*dd*, J = 9.7, 2.1)	4.47 (*dd*, J = 9.8, 2.1)
CH_2(2′)	1.46–1.50 (*m*)	1.44–1.48 (*m*)	0.91 (*d*, J = 7.0)
H–C(3′)	3.34 (*ddd*, J= 12.0, 5.0, 3.0)	3.30–3.35 (*m*)	3.30–3.35 (*m*)
H–C(4′)	3.68 (br. *s*)	3.64 (*d*, J = 2.1)	3.64 (*d*, J = 2.8)
H–C(5′)	3.42 (*dd*, J = 6.5, 1.0)	3.42 (*dd*, J = 6.5, 1.0)	3.42 (*dd*, J = 6.5, 1.0)
Me(6′)	1.33 (*d*, J = 6.5)	1.25 (*d*, J = 6.5)	1.26 (*d*, J = 6.5)
MeO	3.39 (*s*)	3.36 (*s*)	3.36 (*s*)

Table 2. *^{13}C-NMR Data of* **1**–**3**. At 125 MHz in CD_3OD; δ in ppm.

Position	**1**	**2**	**3**	Position	**1**	**2**	**3**
1	31.4	32.9	32.0	16	26.9	27.0	27.1
2	24.1	26.5	27.1	17	53.1	52.7	53.1
3	72.2	72.4	73.2	18	23.3	23.5	23.2
4	29.0	31.6	31.4	19	26.3	28.1	26.6
5	36.8	44.0	38.8	20	170.5	172.4	172.8
6	29.9	72.9	33.9	21	72.8	74.3	74.5
7	26.8	36.2	73.1	22	116.5	116.6	116.9
8	48.8	50.9	54.3	23	173.6	175.5	175.7
9	46.0	47.3	48.8	1′	97.6	98.6	98.8
10	37.3	37.3	38.0	2′	32.0	32.6	32.7
11	21.3	21.8	22.0	3′	78.0	78.9	78.9
12	42.5	43.0	43.3	4′	67.2	67.5	67.4
13	47.3	49.0	49.1	5′	70.3	71.3	71.3
14	220.9	226.7	224.5	6′	16.8	17.0	17.0
15	44.1	43.6	39.1	3′-MeO	55.7	55.9	55.9

The relative configurations of **2** was deduced from the analysis of its NOESY spectrum. In the aglycone segment, Me(19) and H–C(5) were on the same side evidenced by the cross-peak Me(19)/H–C(5), while H–C(6) was on the opposite side, since no significant correlations H–C(6)/H–C(5) or H–C(6)/Me(19) were observed. To confirm the planar structure of **2** and determine the absolute configuration of the important stereogenic center C(6), we attempted to crystallize **2** for single-crystal X-ray diffraction analysis. Fortunately, we were successful in crystallizing **2** in MeOH to yield a single needle crystal. Therefore, based on the crystal structure, and its *Flack* and *Hooft* parameters alone, only the relative configuration can be established. But considering the known configuration of the diginoside sugar moiety, the absolute configuration of **2** was determined to be (3*S*,5*R*,6*R*,8*S*,9*R*,10*R*,13*R*,17*R*) (*Fig. 3*), **2** was named (6*R*)-6-hydroxyoleaside A.

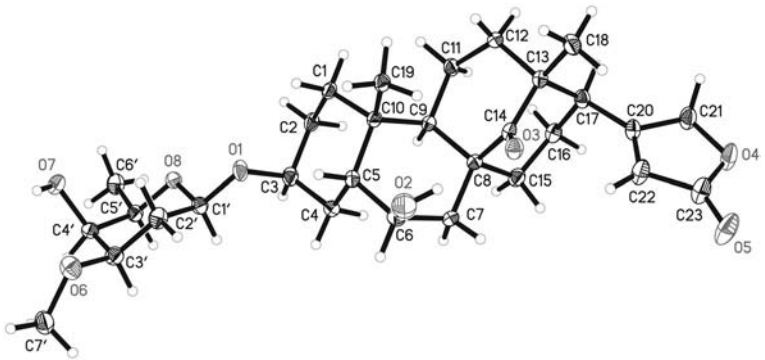

Fig. 3. *Molecular three-dimensional ellipsoid diagram of* **2**

Compound **3** was obtained as colorless needles. Its molecular formula was established as $C_{30}H_{44}O_8$ based on the HR-ESI-MS. The ^{1}H- and ^{13}C-NMR data were consistent with the presence of one MeO group, three Me, ten CH_2, and ten CH groups, one carboxylic C-atom (δ(C) 175.7 (C(23))), one ketone C-atom (δ(C) 224.5 (C(14))), and one C=C bond (δ(C) 172.8 (C(20)) and 116.9 (C(22))). The NMR data of **3** were almost identical to those of (6*R*)-6-hydroxyoleaside A (**2**). Therefore, **3** was considered as an oxidized analog of oleaside A (**1**) too. The OH group was at C(7) due to the HMBCs between H-atom signal at δ(H) 3.21–3.25 (*dd*, J = 12.8, 4.7, H–C(7)), and those of C(8) (δ(C) 54.3) and C(6) (δ(C) 33.9). The relative configuration of **3** was deduced from the NOESY data. H–C(7) and H–C(9) were on the same side as evidenced by the cross-peak δ(H) 3.23 (*dd*, J = 12.8, 4.7, H–C(7))/δ(H) 2.46–2.50 (*m*, H–C(9)). The absolute configuration of **3** was finally confirmed by X-ray diffraction as (3*S*,5*R*,7*S*,8*R*,9*R*,10*S*,13*S*,17*R*) (*Fig. 4*), and **3** was named (7*S*)-7-hydroxyoleaside A.

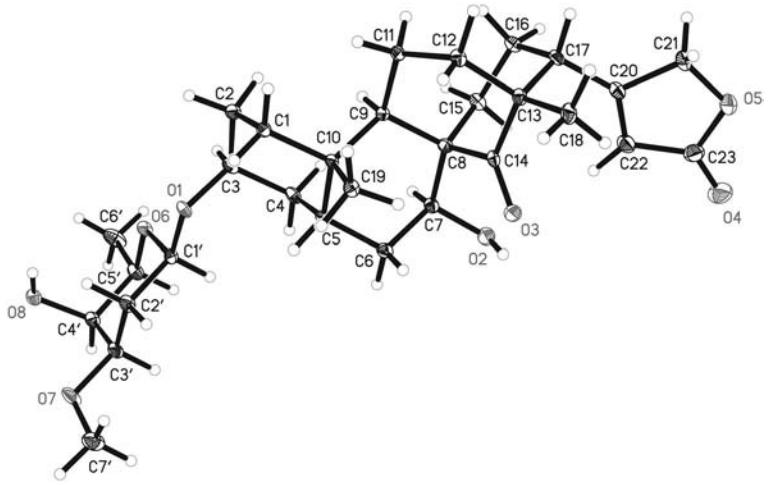

Fig. 4. *Molecular three-dimensional ellipsoid diagram of* **3**

Compounds **1**–**3** were evaluated *in vitro* for their cytoxicities against four human cancer cell lines, A549, HCT116, HepG2, and HL-60, using MTT (= 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) assay with doxororubicin (IC_{50}: 0.368, 0.0426, 0.519, and 0.0629 μg/ml, resp.) as a positive control. Compound **3** exhibited potent inhibitory activity against A549 and HL-60 cell lines, with IC_{50} values of 44.8 and 72.9 μg/ml, respectively. Compounds **1** and **2** showed no activity.

Experimental Part

General. Column chromatography (CC): reversed-phase (RP) silica gel *RP-18* (40–63 μm, *Merck*, Germany), silica gel (SiO_2, 200–300 mesh; *Yantai*, P. R. China), and *Sephadex LH-20* (*Pharmacia Co. Ltd.*). TLC: Silica-gel plates, visualization by spraying with 10% H_2SO_4 in EtOH, followed by heating. Optical rotations: *PelkinElmer 341* polarimer. NMR Spectra: *Bruker AVANCE^{II}* 500 NMR (at 500 (^{1}H)

and 125 MHz (^{13}C); δ in ppm with Me_4Si as internal standard, J in Hz. MS: *Varian MAT-212* mass spectrometer (for ESI) and *Q-Tof micro* mass spectrometer (for HR-ESI); in m/z.

Plant Material. The fresh leaves of *Nerium oleander* L. were collected in School of Pharmacy, Second Military Medical University, Shanghai, P. R. China, in July 2012, and authenticated as *N. oleander* L. by Prof. *Han-Ming Zhang*, School of Pharmacy, Second Military Medical University, P. R. China. A voucher specimen (No. 20120612-2) was deposited with the Herbarium of the School of Pharmacy, Second Military Medical University, Shanghai, P. R. China.

Organisms. The stock culture of *C. echinulata* (ACCC 30369) was maintained on a potato dextrose agar slant. Ten *Erlenmeyer* flasks (1,000 ml), each containing 300 ml of liquid medium consisting of 0.1% peptone, 0.1% yeast extract, 0.1% beef extract, and 0.5% glucose, were inoculated with freshly obtained *C. echinulata* cultured from the agar slant on a rotary shaker at 180 rpm. After cultivation at 28° for 72 h, the *oleaside A* (**1**) soln. (25 mg of **1** dissolved in 250 µl of EtOH) was added to each flask, and the incubation was continued for 4 d.

Microbial Metabolism of Oleaside A (**1**). After 4 d of incubation, the incubation mixtures were pooled and filtered. The filtrate was passed through a *MCI* gel *CHP20P* column, and washed with H_2O to remove sugars, then successively extracted with 40% aq. MeOH, 80% aq. MeOH, and MeOH to give the *Fractions A*, *B*, and *C*, resp. Each *Fr.* was evaporated to dryness in vacuum and analyzed by TLC ($CHCl_3$/MeOH 20 : 1). *Fr. B* was dissolved in 10% aq. MeOH and subjected to CC (*RP* SiO_2 (*ODS*); H_2O/MeOH 70 : 30 to 40 : 60) to give **2** (4.6 mg) and **3** (5.8 mg).

X-Ray Diffraction Analysis. Data collection was performed with a *Bruker APEX2 CCD* and graphite monochromated CuK_α radiation ($\lambda = 1.54178$ Å) at 140 K. *Cell refinement and data reduction: Bruker* SAINT. Program used to solve and refine the structure, SHELXS-2013 and SHELXL-2013, resp., refinement on F^2 with full-matrix least-squares calculations. All non-H-atoms were refined with anisotropic parameters, and all H-atoms were positioned by geometrical calculation and refined by ride-on method with relative isotropic parameters.

Oleaside A (=*4-[(3S,4a*R,*6a*R,*9*R,*10*R,*12a*R,*12b*S*)-Tetradecahydro-10,12b-dimethyl-13-oxo-3-{[(2*R,*4*R,*5S,6*R*)-tetrahydro-5-hydroxy-4-methoxy-6-methyl-2*H*-pyran-2-yl]oxy}-1*H*-6a,10-methanocycloocta[*a*]naphthalen-9-yl]furan-2(5*H*)-one*; **1**). Colorless plate. $C_{30}H_{44}O_7$. ESI-MS: 539 ($[M+Na]^+$). $[\alpha]_D^{23} = +24.2$ ($c = 0.12$, MeOH). Compound **1** was isolated from the fresh leaves of *Nerium oleander* L. with purity of 99.0% by HPLC analysis in our laboratory. The chemical structure was elucidated by detailed NMR analysis, and its data was compared with those in the literature [10]. Herein, the X-ray analysis of **1** (*Fig. 5*) was performed to establish its absolute configuration of (+)-oleaside A.

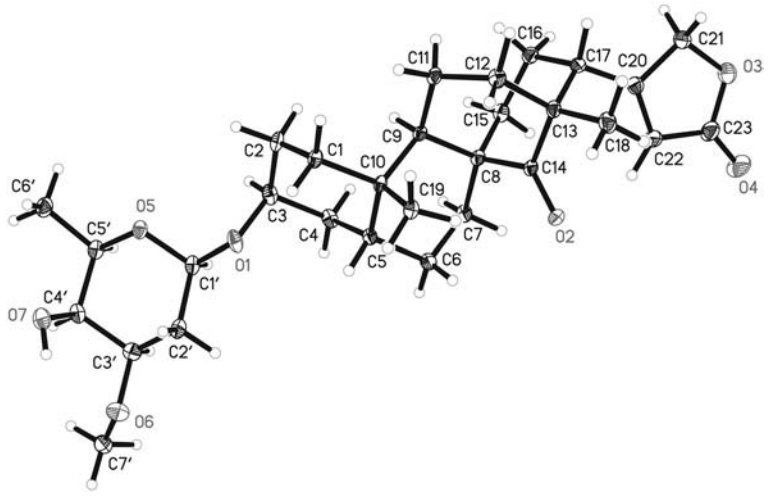

Fig. 5. *Molecular three-dimensional ellipsoid diagram of* **1**

X-Ray Diffraction Analysis of **1**[1]). Single crystal for analysis was obtained from MeOH. *Crystallgraphic data:* $C_{30}H_{44}O_7$; M_r 516.65; crystal size, $0.25 \times 0.10 \times 0.02$ mm, colorless plate; space group, $P2_12_12_1$; $a = 7.3821$ (2) Å, $b = 12.1963$ (4) Å, $c = 30.2148$ (9) Å, $\alpha = 90°$, $\beta = 90°$, $\gamma = 90°$; $V = 2720.37$ (14) Å^3; $Z = 4$; $D_x = 1.261$ g cm^{-3}; $F_{000} = 1120$; μ(CuK_α) 0.71 mm^{-1}. 20882 reflections (4923 unique, $R_{int} = 0.064$) were measured in the range θ from 2.9° to 69.1° and limiting indices $8 \geq h \geq -8$, $14 \geq k \geq -14$, $35 \geq l \geq -31$. The final phase converged to $R_1 = 0.040$ ($wR_2 = 0.106$) for 4503 observed reflections ($I > 2\sigma(I)$) and 339 refined parameters, and Goodness-of-fit on F^2 was 1.036. The *Flack* parameter was $x = 0.00$ (12) [11]. Further analysis of the absolute configuration was performed using likelihood methods [12] with PLATON [13]. A total of 1949 *Bijvoet* pairs were included in the calculations. The resulting value of the *Hooft* parameter was $y = -0.03(13)$. These results indicated that the absolute configuration has been correctly assigned.

*(6*R*)-6-Hydroxyoleaside A (=(9*S*)-9-(2,5-Dihydro-5-oxo-furan-3-yl)tetradecahydro-5-hydroxy-10,12b-dimethyl-13-oxo-1*H*-6a,10-methanocycloocta[*a*]naphthalen-3-yl 2,6-Dideoxy-3-*O*-methylhexopyranoside*; *4-[(3*S*,4a*R*,5*R*,6a*S*,9*R*,10*R*,12a*R*,12b*R*)-Tetradecahydro-5-hydroxy-10,12b-dimethyl-13-oxo-3-{[(2*R*,4*R*,5*S*,6*R*)-tetrahydro-5-hydroxy-4-methoxy-6-methyl-2*H*-pyran-2-yl]oxy}-1*H*-6a,10-methanocycloocta[*a*]naphthalen-9-yl]furan-2(5*H*)-one*; **2**). Colorless needles. $[\alpha]_D^{23} = +13.5$ (*c* 0.01, CH_3OH). ^{1}H- and ^{13}C-NMR: see *Tables 1* and *2*, resp. 2D-NMR (HMBC; 500 MHz, $CDCl_3$): H–C(6)/C(5), C(7), H–C(7)/C(6), C(8). ESI-MS: 555 ($[M + Na]^+$). HR-ESI-MS: 555.2945 ($[M + Na]^+$, $C_{30}H_{44}NaO_8^+$; calc. 555.2934).

X-Ray Diffraction Analysis of **2**[1]). Single crystal for analysis was obtained from MeOH. *Crystallgraphic data:* $C_{30}H_{44}O_8$; M_r 532.65; crystal size, $0.20 \times 0.14 \times 0.06$ mm, colorless plates; space group, $P2_12_12_1$; $a = 7.4182$ (2) Å, $b = 12.2709$ (2) Å, $c = 30.1226$ (6) Å, $\alpha = 90°$, $\beta = 90°$, $\gamma = 90°$; $V = 2741.96$ (10)Å^3; $Z = 4$; $D_x = 1.290$ g cm^{-3}; $F_{000} = 1152$; μ(CuK_α) 0.75 mm^{-1}. Absorption correction were applied with multi-scan (max./min. transmission, 0.9562/0.8641). 17436 reflections (4960 unique, $R_{int} = 0.036$) were measured in the range θ from 3.9° to 69.4° and limiting indices $7 \geq h \geq -9$, $14 \geq k \geq -14$, $33 \geq l \geq -36$. The final phase converged to $R_1 = 0.053$ ($wR_2 = 0.152$) for 4960 observed reflections ($I > 2\sigma(I)$) and 349 refined parameters, and Goodness-of-fit on F^2 was 1.008. Absolute structure: *Flack* [11] and *Hooft et al.* [12] [*Hooft* parameter $y = -0.76$ (9) (1987 *Bijvoet* pairs)] *Flack* parameter $x = -0.64$ (14).

*(7*S*)-7-Hydroxyoleaside A (=(9*S*)-9-(2,5-Dihydro-5-oxofuran-3-yl)tetradecahydro-6-hydroxy-10,12b-dimethyl-13-oxo-1*H*-6a,10-methanocycloocta[*a*]naphthalen-3-yl 2,6-Dideoxy-3-*O*-methylhexopyranoside*; *4-[(3*S*,4a*R*,6*S*,6a*R*,9*R*,10*R*,12a*R*,12b*S*)-Tetradecahydro-6-hydroxy-10,12b-dimethyl-13-oxo-3-{[(2*R*,4*R*,5*S*,6*R*)-tetrahydro-5-hydroxy-4-methoxy-6-methyl-2*H*-pyran-2-yl]oxy}-1*H*-6a,10-methanocycloocta[*a*]naphthalen-9-yl]furan-2(5*H*)-one*; **3**). Colorless needles. $[\alpha]_D^{23} = +12.8$ ($c = 0.01$, MeOH). ^{1}H - and ^{13}C-NMR: see *Tables 1* and *2*, resp. 2D-NMR (HMBC; 500 MHz, $CDCl_3$: H–C(6)/C(5), C(7), H–C(7)/C(6), C(8). ESI-MS:555 ($[M + Na]^+$). HR-ESI-MS: 555.2942 ($[M + Na]^+$, $C_{30}H_{44}NaO_8^+$; calc. 555.2934).

X-Ray Diffraction Analysis of **3**[1]). Single crystal for analysis was obtained from MeOH. *Crystallgraphic data:* $C_{31.5}H_{51}O_{10}$; M_r 589.72; crystal size, $0.30 \times 0.28 \times 0.08$ mm, colorless needles; space group, $P2_12_12_1$; $a = 11.9641$ (3) Å, $b = 8.1765$ (2) Å, $c = 31.3782$ (7) Å, $\alpha = 90°$, $\beta = 98.8°$, $\gamma = 90°$; $V = 3033.42$ (13)Å^3; $Z = 4$; $D_x = 1.291$ g cm^{-3}; $F_{000} = 1280$; μ(CuK_α) 0.779 mm^{-1}. Absorption correction were applied with multi-scan (max./min. transmission, 0.753/0.568). 10314 reflections (4977 unique, $R_{int} = 0.048$) were measured in the range θ from 2.9° to 69.3° and limiting indices $13 \geq h \geq -14$, $9 \geq k \geq -9$, $36 \geq l \geq -37$. The final phase converged to $R_1 = 0.044$ ($wR_2 = 0.115$) for 4871 observed reflections ($I > 2\sigma(I)$) and 373 refined parameters, and Goodness-of-fit on F^2 was 1.018. The *Flack* parameter was $x = 0.09$ (8) [11], and the *Hooft* parameter was $y = 0.07$ (8), indicating that the absolute structure has been correctly assigned. The asymmetric unit contains one molecule of **3**, one molecule of MeOH, and half of a H_2O molecule plus another site for a disordered solvent molecule, presumed to be MeOH, but this solvent molecule could not be modeled adequately. Therefore, its contribution to the intensity data was removed by using the SQUEEZE routine of the PLATON program [13]. Omission of the disordered

---

[1]) CCDC-908568, 908567, and 908569 contain the supplementary crystallographic data for **1**–**3**, resp.. These data can be obtained free of charge *via* http://www.ccdc.cam.ac.uk/data_request/cif.

solvent molecules left two cavities of 64 Å^3 per unit cell. The number of electrons contributing to each void in the structure was calculated by the SQUEEZE routine to be *ca*. 17e. Allowing for one MeOH molecule per cavity yields 18e, and this was used in the subsequent calculation of the empirical formula, formula weight, density, linear absorption coefficient, and *F*(000).

MTT Cytotoxicity Assays. The cytotoxic activities were evaluated against four human cancer cell lines, A549, HCT116, HepG2, and HL-60, obtained from the *American Type Culture Collection* (*ATCC*, Rockville, MD). Cells were seeded in 96-well plates at a cell density of 2×10^5 per well and treated for 4 h with various concentrations of compounds **1**–**3**. After 72 h of incubation, MTT (= 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) was added to all wells. Plates were incubated for another 4 h, and cell viability was measured by observing absorbance at 570 nm on a *WellscanMK-2* (Labsystems, Finland). Doxorubicin was used as a positive control [14][15].

This work was supported by program *NCET Foundation*, *NSFC* (81230090), partially supported by *Global Research Network for Medicinal Plants* (GRNMP) and *King Saud University*, *Shanghai Leading Academic Discipline Project* (B906), *FP7- PEOPLE-IRSES-2008* (TCMCANCER Project 230232), *Key Laboratory of Drug Research for Special Environments*, *PLA*, *Shanghai Engineering Research Center for the Preparation of Bioactive Natural Products* (10DZ2251300) and the *Scientific Foundation of Shanghai China* (09DZ1975700, 09DZ1971500,10DZ1971700, 11QA1408200), *National Major Project of China* (2011ZX09307-002-03), and *National Key Technology R&D Program of China* (2012BAI29B06).

REFERENCES

[1] Atta-ur-Rahman, M. I. Choudhary, S. G. Musharraf, *Front. Nat. Prod. Chem*. **2005**, *1*, 133.
[2] W. Charney, H. L. Herzog, 'Microbial Transformations of Steroids', Academic Press, New York, 1967, p. 16.
[3] W. Yang, P. J. Davis, *Drug Metab. Dispos.* **1992**, *20*, 38.
[4] M. Hezari, P. J. Davis, *Drug Metab. Dispos.* **1992**, *20*, 882.
[5] P. Yang, D. G. Menter, C. Cartwright, D. Chan, S. Dixon, M. Suraokar, GT. Mendoza, N. Llansa, R. A. Newman, *Mol. Cancer Ther*. **2009**, *8*, 2319.
[6] T. E. Dudek, D. C. No, E. Seung, V. D. Vrbanac, L. Fadda, P. Bhoumik, C. L. Boutwell, K. A. Power, A. D. Gladden, L. Battis, E. F. Mellors, T. R. Tivey, X. Gao, M. Altfeld, A. D. Luster, A. M. Tager, T. M. Allen, *Sci. Transl. Med*. **2012**, *4*, 143ra99.
[7] Y. Dong, S. Yin, J. Li, C. Jiang, M. Ye, H. Hu, *Apoptosis* **2011**, *16*, 394.
[8] Y.-F. Lü, K.-Y. Chen, H.-L. Li, Y.-H. Pei, R.-H. Liu, W.-D. Zhang, *Helv. Chim. Acta* **2008**, *91*, 819.
[9] K.-Y. Chen, Y.-F. Lü, W.-D. Zhang, J. Tang, R.-H. Liu, H.-L. Li, *Helv. Chim. Acta* **2013**, *96*, 1072.
[10] F. Abe, T. Yamauchi, *Chem. Pharm. Bull*. **1979**, *27*, 1604.
[11] H. D. Flack, *Acta Crystallogr. A* **1983**, *39*, 876.
[12] R. W. W. Hooft, L. H. Straver, A. L. Spek, *J. Appl. Crystallogr.* **2008**, *41*, 96.
[13] A. L. Spek, *Acta Crystallogr. D* **2009**, *65*, 148.
[14] J. Carmichael, W. G. DeGraff, A. F. Gazdar, J. D. Minna, J. B. Mitchell, *Cancer Res.* **1987**, *47*, 936.
[15] T. Mosmann, *J. Immunol. Meth*. **1983**, *65*, 55.

Received April 27, 2013