A New Eudesmane Derivative and a New Fatty Acid Ester from *Sambucus williamsii*

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1,4,13-Trihydroxy-eudesm-11(12)-ene, a new eudesmane derivative (3), (9*E*)-8,11,12-trihydroxyoctadecenoic acid methyl ester, a new fatty acid ester (2) and tianshic acid (1) were obtained from the stems of *Sambucus williamsii*. Their structures were elucidated by physiochemical properties and spectroscopic analysis. Both compounds 1 and 2 showed stimulating effects on alkaline phosphatase activity of the osteoblastic UMR106 cell about 1.5 fold at 30 μmol/l while they had no effects on cell proliferation.

Key words eudesmane; fatty acid; *Sambucus williamsii*; UMR106 cell; alkaline phosphatase activity

Sambucus williamsii HANCE, a tree widely distributed in China, has been used for prevention and treatment of bone diseases for thousands of years.[1)] Previous phytochemical investigation indicated that lignans were active constituents of the 60% ethanol extract of the stems of *Sambucus williamsii* which could stimulate either the proliferation or alkaline phosphatase activity of UMR106 cell (results submitted). The rat osteosarcoma UMR106 cell line, with many osteoblast-like phenotypes, has been used as an *in vitro* system to study the molecular mechanism of hormones and growth-factors influencing osteoporosis, such as estrogen, insulin, insulin-like growth factor-I.[2—4)] Stimulating effects on proliferation and differentiation of UMR106 cell may indicate positive action on osteoblast. Further isolation of this plant extract, while the stimulation of UMR106 cell proliferation was still used as *in vitro* marker, afforded tianshic acid (**1**), its methyl ester (**2**) and a new sesquiterpene (**3**) (Fig. 1). Compounds **1** and **2** had shown stimulating effects on alkaline phosphatase (ALP) activity of UMR106 cell.

Results and Discussion

Compound **1** was identified as tianshic acid ((9*E*)-8,11,12-trihydroxyoctadecenoic acid) by comparison with reported NMR data.[5)] Compound **2** was obtained as amorphous powder. The HR-ESI-MS gave a $[M+Na]^+$ peak at *m*/*z* 367.2465, calculated 367.2460 for the molecular formula of $C_{19}H_{36}O_5$. The IR spectrum indicated the presence of hydroxyl group (3356 cm^{-1}), double carbon–carbon bond (2341 cm^{-1}) and ester carbonyl group (1732 cm^{-1}). The ^{1}H- and ^{13}C-NMR data of compound **2** were strikingly similar with those of compound **1**, which also suggested an oxygenated carbonyl group, a *trans* vinylic group, 3 oxygenated methine groups, 11 saturated methylene groups and a methyl group. The major difference was that there was one more methoxyl group appeared in the NMR spectra of compound **2**, δ_H 3.64 (3H, s) and δ_C 52.0. This methoxyl group showed correlation with the carbonyl carbon at δ 176.2 in the HMBC spectrum which suggested compound **2** was methyl ester of compound **1**. Tianshic acid was first isolated from *Aesculus wilsonii*,[6)] Sang S. M. *et al.* had reported isolation of its monoglyceride from *Allium fistulosum* L. To the best of our knowledge, it was the fourth report of isolation of tianshic acid and the second report of isolation of its esters.[5)]

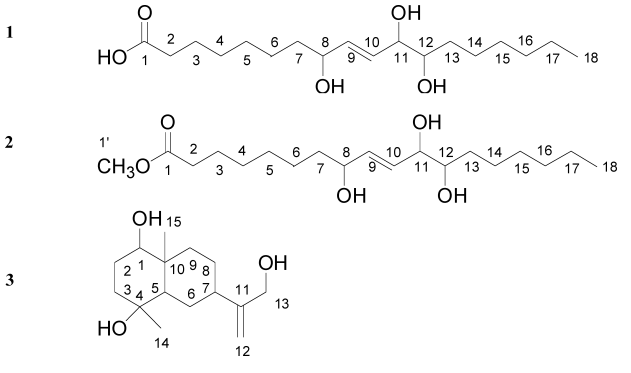

Fig. 1. Structures of Compounds Isolated from *Sambucus williamsii*

Compound **3** was obtained as yellowish oil, $[\alpha]_D^{26}$ −30.2° (c=0.1, MeOH). The HR-ESI-MS gave a $[M+Na]^+$ peak at *m*/*z* 277.1802, calculated 277.1780 for the molecular formula of $C_{15}H_{26}O_3$. The ^{1}H- and ^{13}C-NMR spectra indicated that compound **3** was a eudesmane derivative. The presence of δ 155.5 (s), δ 107.9 (t) and δ 65.2 (t) signals in the ^{13}C-NMR spectrum suggested an 11,12-ene-13-hydroxy substructure,[7)] the corresponding protons appearing at δ 5.02 (1H, s) and δ 4.89 (1H, s), δ 4.06 (2H, s). The appearance of carbon signal at δ 80.3 (C-1), δ 72.3 (C-4) indicated that these 2 carbons were oxygenated. So the structure of compound **3** was elucidated as 1,4,13-trihydroxyeudesm-11(12)-ene. The relative configuration of compound **3** could be established by NOESY spectrum. The methyl proton (δ 0.89, 3H, s, H-15) showed correlations with H-2 (δ 1.63, 2H, m), H-14 (δ 1.08, 3H, s), H-6b (δ 1.25, 1H, m), H-8b (δ 1.41, 1H, m) and H-9a (δ 1.94, 1H, m) in the NOESY spectrum; H-1 (δ 3.23, 1H, m) showed correlations with H-2 (δ 1.63, 2H, m), H-3b (δ 1.52, 1H, m), H-5 (δ 1.28, 1H, m) and H-9b (δ 1.15, 1H, m) in the NOESY spectrum; H-7 (δ 1.99, 1H, m) showed NOESY correlations with H-9b (δ 1.15, 1H, m) and H-5 (δ 1.28, 1H, m). So the methyl groups attached to C-4 and C-10 were on the same side of the planar, and H-1, H-5 and H-7 were on the opposite side. Thus the relative configuration of compound **3** was elucidated as 1β,4α,13-trihydroxy-eudesm-

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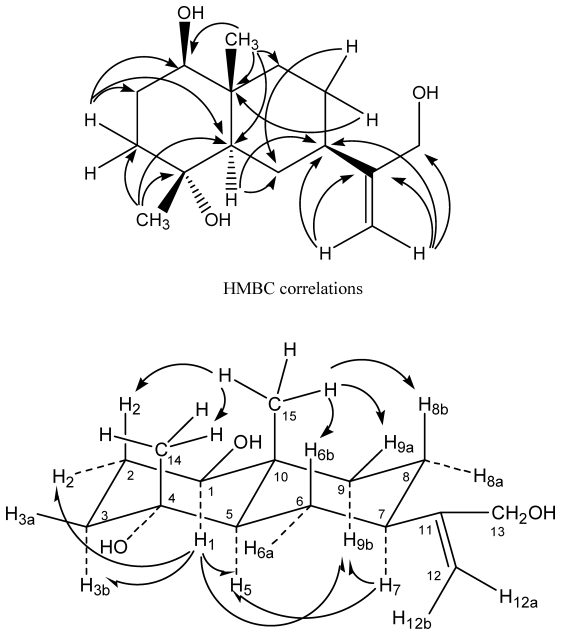

Fig. 2. The Principle HMBC and NOESY Correlations of Compound **3**

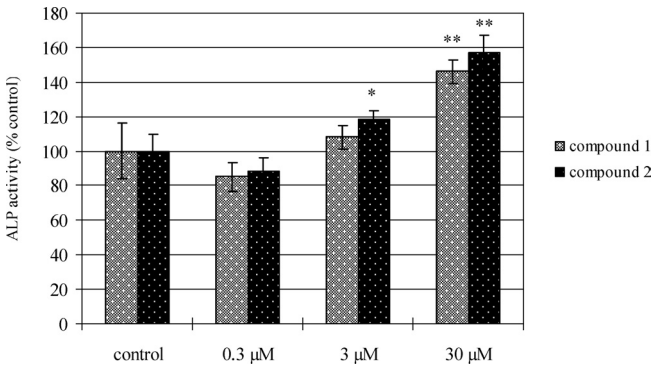

Fig. 3. Effects of Compounds **1** and **2** on UMR106 Cell ALP Activity

Data were expressed as mean±S.D. (n=3). The stimulating rate was expressed as percentage of control (no test sample added in). Statistical significance was determined by Student's t-test. $*p<0.05$, $**p<0.01$.

11(12)-ene. The principle HMBC and NOESY correlations of compound **3** were displayed in Fig. 2.

The preliminary effects of compounds **1** and **2** on ALP activity of the osteoblastic UMR106 cell were summarized in Fig. 3. Both compounds didn't show stimulating effects on UMR106 cell proliferation (data not shown) in the predetermined concentration range of 3.0 nM—30.0 μM, while they induced the ALP activity about 1.5 fold at 30.0 μM. Alkaline phosphatase was an important enzyme osteoblast secreted during the progress of differentiation. The inducing effects on this enzyme activity may indicate positive effects on osteoblast differentiation.

Experimental

General The IR spectra were recorded on FT/IR-8400 (Shimadzu, Japan) spectrophotometer. The optical rotations were measured on a P-1020 digital polarimeter (JASCO, Japan). The NMR spectra were measured on a Bruker AV-400 (400 MHz for ^{1}H, 100 MHz for ^{13}C) NMR spectrometer. Chemical shifts were expressed in δ (ppm) and coupling constants (J) were reported in Hertz (Hz). The ESI-MS was performed with a Bruker esquire 2000 mass spectrometer. The HR-ESI-MS was performed on Micromass Q-TOF mass spectrometer. The preparative HPLC and analytical HPLC were performed on SHIMADZU Pak with RID detector. Silica gel 60 (Qingdao Haiyang Chemical Co., Ltd., China), Sephadex LH-20 (Advanced Technology Industrial Co., Ltd.) and ODS (40—75 μm, Fuji Silysia Chemical Ltd., Japan) were used as column chromatography stationary phases. TLC was carried out on Silica gel 60 and the spots were visualized by spraying with 10% H_2SO_4 and heating to 105 °C.

Table 1. The ^{1}H-NMR (400 MHz, CD_3OD) and ^{13}C-NMR (100 MHz, CD_3OD) of Compound **2**

	δ (ppm)		δ (ppm), J in Hz
C-1	176.0 (s)		
C-2	34.8 (t)	H-2	2.30, 2H, t, J=7.4
C-3	26.0 (t)	H-3	1.59, 2H, m
C-4	30.1 (t)	H-4	1.32, 2H, o
C-5	30.3 (t)	H-5	1.35, 2H, o
C-6	26.4 (t)	H-6	1.32, 2H, o
C-7	38.3 (t)	H-7	1.51, 2H, o
C-8	73.0 (d)	H-8	4.04, 1H, dd, J=11.5, 6.0
C-9	136.5 (d)	H-9	5.72, 1H, dd, J=15.6, 5.4
C-10	131.1 (d)	H-10	5.65, 1H, dd, J=15.6, 5.4
C-11	76.5 (d)	H-11	3.89, 1H, m
C-12	75.8 (d)	H-12	3.40, 1H, m
C-13	33.6 (t)	H-13	1.32, 2H, o
C-14	26.6 (t)	H-14	1.51, 2H, o
C-15	30.5 (t)	H-15	1.51, 2H, o
C-16	33.1 (t)	H-16	1.32, 2H, o
C-17	23.7 (t)	H-17	1.32, 2H, o
C-18	14.4 (q)	H-18	0.90, 3H, d, J=6.8
C-1′	52.0 (q)	H-1′	3.64, 3H, s

Extraction and Isolation The stems of *Sambucus williamsii* (30 kg) were cut into chops and extracted with 60% ethanol. Five hundred grams of 900 g extract were suspended in water and then were extracted with chloroform, ethyl acetate and *n*-butanol successively. Forty grams of ethyl acetate extract were chromatographied on silica gel column with gradient $CHCl_3$/MeOH elution. The fraction (5.0 g), eluted by 95/5 $CHCl_3$/MeOH, was applied to Sephadex LH-20 chromatography column eluted with 1/1 $CHCl_3$/MeOH, and then applied to ODS chromatography column eluted with 20%, 60% and 80% methanol (in water) to afford sub-fractions 1, 2 and 3. Compound **3** (3 mg) was obtained after the sub-fraction 1 was applied to RP-HPLC eluted with 25% methanol. Compounds **2** (20 mg) and **1** (5 mg) were obtained after the sub-fractions 2, 3 were applied to RP-HPLC eluted with 60%, 75% methanol, respectively.

Compound **2**: Amorphous powder; IR (KBr) cm^{-1}: 3356, 2932, 2851, 2341, 1732, 1438; MS m/z: 367.2 ([M+Na]$^+$); HR-ESI-MS m/z 367.2465 (Calcd for $C_{19}H_{36}O_5Na$: 367.2460); ^{1}H-NMR (400 MHz, CD_3OD) and ^{13}C-NMR (100 MHz, CD_3OD): see Table 1.

Compound **3**: Yellowish oil; $[\alpha]_D^{26}$ −30.2° (c=0.1, MeOH); HR-ESI-MS m/z 277.1802 (Calcd for $C_{15}H_{26}O_3Na$: 277.1780); ^{1}H-NMR (400 MHz, CD_3OD) and ^{13}C-NMR (100 MHz, CD_3OD): see Table 2.

Cell Culture The UMR106 cell was maintained in DMEM supplemented with 5% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin in humid

The UMR106 cell line was purchased from American Type Culture Collection, No. CRL-1661. DMEM (Dulbecco's Modified Eagle's Medium), FBS (Fetal Bovine Serum) and 0.5% trypsin–5.3 mM EDTA (10×) were purchased from GIBCOBRL. Penicillin, streptomycin (100×) was obtained from Invitrogen Corporation. 24-Well plates and plastic dishes (100×20 mm) for cell culture were obtained from FALCON (U.S.A.). 4-Nitrophenyl phosphate disodium salt hexahydrate was purchased from Fluka. $MgCl_2 \cdot 6H_2O$ was obtained from BIOASIA Ltd., China. Triton X-100 and Tris Base were obtained from AMRESCO. Coomasie reagent for protein assay was purchased from Bio-Rad. All compounds were dissolved in ethanol with concentration of 1 mg/ml as storage, and were diluted in DMEM when used.

Plant Materials *Sambucus williamsii* was collected in Liaoning Province of China and was identified by Prof. Jiang Zerong, Shenyang Pharmaceutical University, Shenyang, China. A voucher specimen (YYXJSW-2003) was kept in Shenzhen Research Center of Traditional Chinese Medicine and Natural Products, Shenzhen, China.

Table 2. The ^{1}H-NMR (400 MHz, CD_3OD) and ^{13}C-NMR (100 MHz, CD_3OD) of Compound **3**

	δ (ppm)		δ (ppm), J in Hz
C-1	80.3 (d)	H-1	3.23, 1H (m)
C-2	29.4 (t)	H-2	1.63, 2H (m)
C-3	41.9 (t)	H-3	1.73, 1H (m); 1.52, 1H (m)
C-4	72.3 (s)		
C-5	54.2 (d)	H-5	1.28, 1H (m)
C-6	27.6 (t)	H-6	1.91, 1H (m); 1.25, 1H (m)
C-7	43.0 (d)	H-7	1.99, 1H (m)
C-8	28.3 (t)	H-8	1.61, 1H (m); 1.41, 1H (m)
C-9	42.1 (t)	H-9	1.94, 1H (m); 1.15, 1H (m)
C-10	40.3 (s)		
C-11	155.5 (s)		
C-12	107.9 (t)	H-12	5.02, 1H (s); 4.89, 1H (s)
C-13	65.2 (t)	H-13	4.06, 2H (s)
C-14	22.6 (q)	H-14	1.08, 3H (s)
C-15	13.8 (q)	H-15	0.89, 3H (s)

5% CO_2 air at 37 °C. The cells were routinely sub-cultured when 80% confluence using 0.05% trypsin–0.53 mM EDTA.

ALP (EC 3.1.3.1) Activity The cells were seeded to 24-well plate at 37500/well in 5% FBS/DMEM and cultured for 3 d. Then followed by treatment with various concentrations of test samples in 1% FBS/DMEM for 24 h, cells were harvested in cell lysis buffer and assayed for ALP activities. For 40 μl cell lysate or 40 μl dd H_2O, 1 ml alkaline buffer containing 0.01 mM *p*-nitrophenyl phosphate, 0.1 mM sodium carbonate and 1 nM magnesium chloride was added. The reaction solution was then incubated for 30 min at 37 °C in darkness. Five hundred microliters 0.1 M NaOH was added to stop the reaction. The absorbance was measured on Thermo Spectronic spectrometer at 405 nm. Another 40 μl cell lysate was used for protein content determination by Bradford method. The inducing effects of ALP activity was quantified as $[(A_{405,\text{test sample}}-A_{405,\text{dd H}_2\text{O}})/30\,\text{min}/\mu\text{g}$ total cellular protein$]/[(A_{405,\text{control}}-A_{405,\text{dd H}_2\text{O}})/30\,\text{min}/\mu\text{g}$ total cellular protein$]$.

Statistics Results were expressed as means±S.D. (n=3). Statistical significance was assessed by Student's t-test. * $p<0.05$, ** $p<0.01$.

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