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Lignans from the stems of *Sambucus williamsii* and their effects on osteoblastic UMR106 cells

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Three new lignans, sambucunol A (**8**) ((+)-*erythro*-1-(4-hydroxy-3-methoxyphenyl)-2-[4-(4-hydroxy-3-methoxycinnamoyloxypropanyl)-2-hydroxyphenoxy]-1, 3-propanediol), sambucunol B (**9**) ((+)-*threo*-1-(4-hydroxyl-3-methoxyphenyl)-2-[4-(4-hydroxy-3-methoxy-cinnamoyloxy propanyl)-2-hydroxy-phenoxy]-1, 3-propanediol) and buddlenol G (**10**) (2-{4-[2, 3-dihydro-3-hydroxymethyl-7-hydroxy-5-(4-hydroxy-3-methoxycinnamoyloxypropanyl)-2-benzofuranyl]-2,6-dimethoxyphenoxy}-1-(4- hydroxy-3-methoxyphenyl) -1, 3-propanediol), along with seven known ones, including (−)-syringaresinol (**1**), (−)-pinoresinol (**2**), 1, 2-bis(4-hydroxy-3-methoxy phenyl)-1, 3-propanediol (**3**), (−)-*erythro*-1-(4-hydroxy-3-methoxyphenyl)-2-[4-(3-hydroxy propanyl)-2-methoxyphenoxy]-1, 3-propanediol (**4**), (−)-*threo*-1-(4-hydroxy-3-methoxyphenyl)-2-[4-(3-hydroxypropanyl)-2-methoxy phenoxy]-1, 3-propanediol (**5**), (−)-lariciresinol (**6**) and (−)-dihydrodehydrodiconiferyl alcohol (**7**), were isolated from the 60% ethanol extract of stems of *Sambucus williamsii* Hance by chromatographic methods. Their structures were established by spectral analysis. The effects of isolated compounds on the osteoblast-like UMR106 cell proliferation and ALP activities were determined. Compounds **2**, **7** and **10** showed stimulating effects both on UMR106 cell proliferation and ALP activity. Compounds **1**, **3**, **6** and **8** stimulated UMR106 cell proliferation, while compounds **4** and **5** induced ALP activity in UMR106 cell.

Keywords: *Sambucus williamsii*; *Caprifoliaceae*; Lignans; UMR106 cell; Proliferation; ALP activity

1. Introduction

Sambucus williamsii Hance is a tree widely distributed in China. It has been used for treatment of bone diseases since ancient times [1]. Clinical studies also showed that Traditional Chinese Medicine compositions containing *Sambucus williamsii* Hance were effective on bone fractures [2,3]. However, chemical investigation on this species has been seldom reported previously. Only several triterpenoids and steroids have been isolated until now [4].

UMR106 cell is a rat osteocarcoma cell line with osteoblast-like phenotype [5,6] and is widely used as *in vitro* model to study the mechanisms of action of different hormones and

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compounds related to osteoporosis [7–10]. The present study deals with isolation of active compounds from the 60% ethanolic extract of stems of *Sambucus williamsii* Hance using stimulation of UMR106 cell proliferation as *in vitro* marker. Ten lignans (compounds **1–10**) were obtained from the active fractions of the extract and their structures were elucidated by spectral analysis. The effects of these compounds on UMR106 cell proliferation and differentiation were determined.

2. Results and discussion

2.1 Bioactivity-guided isolation procedure and tructure elucidation of isolated lignans

Recent study in our laboratory indicated that the 60% ethanolic extract of stems of *Sambucus williamsii* Hance showed anti-osteoporotic effects on ovariectomized rats (data not shown). It also stimulated the proliferation of UMR106 cell *in vitro*. So, isolation procedure was carried out to define active components in this plant while stimulation of UMR106 cell proliferation was used as *in vitro* marker. Ten lignans (compounds **1–10**) were obtained from active fractions of the extract, and their structures were displayed in figure 1.

Compounds **1–7** were identified, by spectral analysis and comparison with published spectral data, as (−)-syringaresinol [11,12], (−)-pinoresinol [13,14], 1, 2-bis(4-hydroxy-3-methoxyphenyl)-1, 3-propanediol [15,16], (−)-*erythro*-1-(4-hydroxy-3-methoxyphenyl)-2-[4-(3-hydroxypropanyl)-2-methoxyphenoxy]-1, 3-propanediol [17], (−)-*threo*-1-(4-hydroxy-3-methoxyphenyl)-2-[4-(3-hydroxy propanyl)-2-methoxyphenoxy]-1, 3-propanediol [17], (−)-lariciresinol [18], (−)-dihydrodehydro diconiferyl alcohol [19] respectively.

Compound **8** was obtained as yellowish oil, $[\alpha]_D^{26} = +4.9$ (*c* 0.1, MeOH). The HRESIMS established the molecular formula as $C_{29}H_{32}O_{10}$ (*m/z* 563.1642 $[M + Na]^+$). The 1D NMR spectra were similar with those of compound **4** except for the presence of a cinnamoyloxy group indicating it was a sesquilignan. Compared with **4**, the resonance of H-9′ was down *field* shifted from δ3.54 to 4.13. And the correlation of H-9′ with the carbonyl carbon (δ169.4, C-9″) could be observed in the HMBC spectrum. The relative small coupling constant $J_{H7-H8} = 4.4$, indicated **8** has *erythro* configuration [20–22]. So the structure of compound **8** was assigned as (+)-*erythro*-1-(4-hydroxy-3-methoxyphenyl)-2-[4-(4-hydroxy-3-methoxycinnamoyloxypropany)-2-hydroxyphenoxy]-1, 3-propanediol (sambucunol A). The NMR spectra of compound **9** were similar with those of **8**. And the elucidation of $^1H-^1H$ COSY, HMQC and HMBC correlations of **9** led to the same planar structure with **8**. The major difference was that the coupling constant of $J_{H7-H8} = 6.1$, which indicated a *threo* diastereoisomer of compound **8** [20–22], (+)-*threo*-1-(4-hydroxy-3-methoxyphenyl)-2-[4-(4-hydroxy-3-methoxy-cinnamoyloxypropanyl)-2-hy droxyphenoxy}-1, 3-propanediol. The ^1H and ^{13}C NMR data of compounds **8** and **9** were displayed in table 1.

Compound **10** was isolated as yellowish oil, $[\alpha]_D^{23} = -25.2$ (*c* 0.1, MeOH). The HRESIMS established the molecular formula as $C_{40}H_{44}O_{14}$ (*m/z* 771.2683 $[M + Na]^+$). The presence of a dihydrobenzofuran ring in the structure of **10** was suggested by the signals at δ5.52 (1H, *d*, $J = 5.6$, H-7), 3.43 (1H, *m*, H-8) in the ^1H NMR spectrum and at δ88.3 (C-7), 56.0 (C-8) in the ^{13}C NMR spectrum. This suggestion was also supported by the correlations between H-7 and C-4′ at δ146.6, H-8 and C-5′ at δ129.5 in the HMBC spectrum. The small coupling constant $J_{H7-H8} = 5.6$ Hz indicated these protons had a *cis* configuration [19]. The presence of an α, β-unsaturated carbonyl ester group was clearly observed in the ^1H spectrum (δ7.54, 1H, *d*,

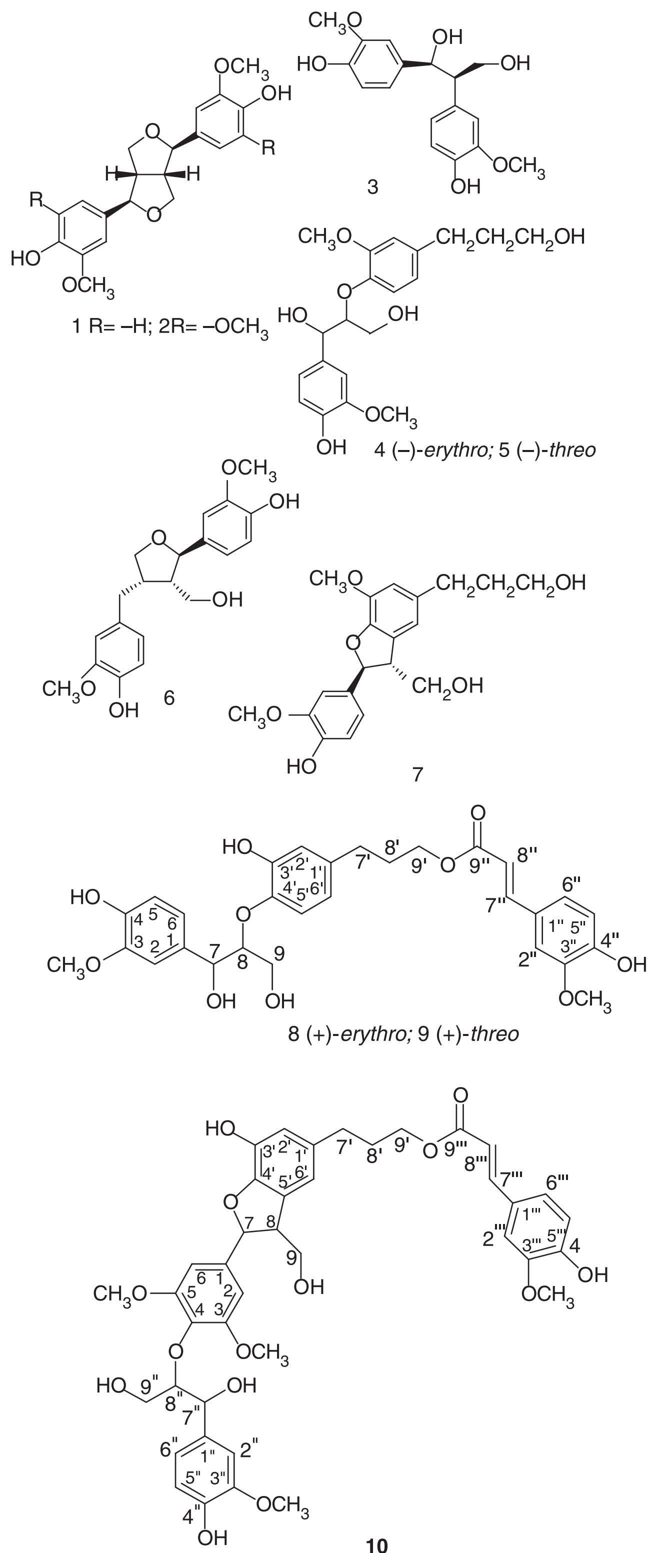

Figure 1. Stuctrues of lignans from *Sambucus williamsii* Hance.

Table 1. ^{1}H and ^{13}C NMR data of compounds **8** and **9** *.

	8		**9**	
Position	^{1}H	^{13}C	^{1}H	^{13}C
1		134.0		133.8
2	6.99 brs	111.7	6.99 brs	111.7
3		148.8		148.9
4		147.1		147.3
5	6.74 d, 8.1	115.9	6.74 d, 8.1	116.0
6	6.83 o	120.8	6.83 o	120.8
7	4.87 d, 4.4	74.1	4.88 d, 6.1	74.2
8	4.15 m	87.7	4.11 m	87.8
9	3.83 o; 3.73 o	62.0	3.70 o; 3.44 dd, 11.9, 5.2	61.8
OCH_3	3.79 s	56.4	3.80 s	56.4
1′		137.8		138.0
2′	6.67 d, 1.8	117.3	6.67 d, 1.8	117.3
3′		149.6		149.6
4′		146.2		145.9
5′	6.92 d, 8.2	119.6	6.92 d, 8.2	119.6
6′	6.52 dd, 8.2, 1.8	120.6	6.56 dd, 8.2, 1.8	120.6
7′	2.60 o	32.6	2.60 o	32.6
8′	1.95 o	31.6	1.95 o	31.6
9′	4.13 brt, 6.6	64.8	4.13 brt, 6.6	64.8
1″		127.7		127.7
2″	7.17 brs	111.7	7.17 brs	111.7
3″		150.8		150.8
4″		149.4		149.4
5″	6.80 d, 8.2	116.5	6.80 d, 8.2	116.5
6″	7.06 dd, 8.2, 1.6	124.2	7.06 dd, 8.2, 1.6	124.2
7″	7.58 d, 15.9	146.8	7.58 d, 15.9	146.8
8″	6.35 d, 15.9	115.5	6.35 d, 15.9	115.5
9″		169.4		169.4
OCH_3	3.87 s	56.5	3.87 s	56.5

* Measured in CD_3OD at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR, with assignment confirmed by $^{1}H-^{1}H$ COSY, HMQC and HMBC.

$J = 15.9$, H-7‴; 6.32, 1H, *d*, $J = 15.9$, H-8‴) and ^{13}C spectrum (δ146.8, C-7‴; 115.3, C-8‴; 169.4, C-9‴) of compound **10**. The HMBC correlation indicated that the ester group linked a guaiacyl moiety to a propanol residue, which attached at C-1′ of **10**.

A symmetrical aromatic ring was suggested by the ^{1}H and ^{13}C NMR signals, δ6.74 (2H, *s*, H-2 and H-6), 3.82 (2-OMe, *s*) and δ103.9 (C-2 and C-6), 56.7 (2-OMe), 154.3 (C-3 and C-5). The HMBC correlation between H-7, C-2 and C-6 indicated this symmetric aromatic moiety was attached to C-7 *via* C-1. There was a 1, 2-diaryl glycerol moiety in the structure of compound **10**, δ4.96 (1H, *d*, $J = 6.9$, H-7″), 4.05 (1H, *m*, H-8″), 3.72 and 3.33 (2H, *o*, H-9″); the corresponding carbon signals were observed at δ 74.5 (C-7″), 89.0 (C-8″) and 61.8 (C-9″). The HMBC correlation between H-8″ and C-4 indicated this glycerol moiety was attached to the symmetric aromatic ring at C-4 position. This assignment was also supported by the fact that there was no NOESY interaction between H-8″ with any aromatic protons. The coupling constant $J_{H7''-H8''} = 6.9$ Hz suggested a threo configuration of these 2 protons [20–22].

Thus, the structure of **10** was elucidated as 2-{4-[2, 3-dihydro-3-hydroxymethyl-7-hydroxy-5-(4-hydroxy-3-methoxycinnamoyloxypropanyl)-2-benzofurany l]-2, 6-dimethoxyphenoxy}-1-(4-hydroxy-3-methoxyphenyl)-1, 3-propanediol. The ^{1}H and ^{13}C NMR data of **10** were displayed in table 2. The key HMBC correlations were shown in figure 2.

Table 2. ^{1}H and ^{13}C NMR data of compounds **10** *.

Postition	^{1}H	^{13}C	*Position*	^{1}H	^{13}C
1		136.7	1″		133.4
2	6.74 s	103.9	2″	6.98 brs	111.7
3		154.3	3″		148.7
4		140.1	4″		147.2
5		154.3	5″	6.73 o	115.8
6	6.74 s	103.9	6″	6.84 brd, 7.9	120.9
7	5.52 d, 5.6	88.3	7″	4.96 d, 6.9	74.5
8	3.43 m	56.0	8″	4.05 m	89.0
9	3.74 o; 3.70 o	65.2	9″	3.72 o; 3.33 o	61.8
$OCH_3 \times 2$	3.82 s	56.7	OCH_3	3.81s	56.5
1′		136.3	1‴		127.4
2′	6.60 s	117.2	2‴	7.17 brs	111.7
3′		142.1	3‴		149.6
4′		146.6	4‴		151.2
5′		129.5	5‴	6.78 d, 8.2	116.6
6′	6.60 s	116.7	6‴	7.05 brd, 8.2	124.2
7′	2.63 m	32.9	7‴	7.54 d, 15.9	146.8
8′	1.97 m	31.8	8‴	6.32 d, 15.9	115.3
9′	4.16 t, 6.3	64.9	9‴		169.4
			OCH_3	3.87 s	56.4

* Measured in CD_3OD at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR, with assignment confirmed by $^{1}H-^{1}H$ COSY, HMQC and HMBC.

2.2 *Effects of isolated lignans on UMR106 cell proliferation and ALP activity*

UMR106 is rat clonal cell line with well characterized osteoblast-like phenotypic properties [23] and has been demonstrated to be an excellent *in vitro* cellular system for studies of the physiology of osteoblasts.

IGF-I, the positive control in our *in vitro* studies, is a peptide growth factor derived from live and extrahepatic tissues and stimulates skeletal tissue growth. Leung *et al.* [9] reported that IGF-I (0–10 nM) stimulated proliferation of UMR106 cell in a dose-dependent manner. It increased cell number maximally by 36.9 ± 1.2% above the control level. Alkaline

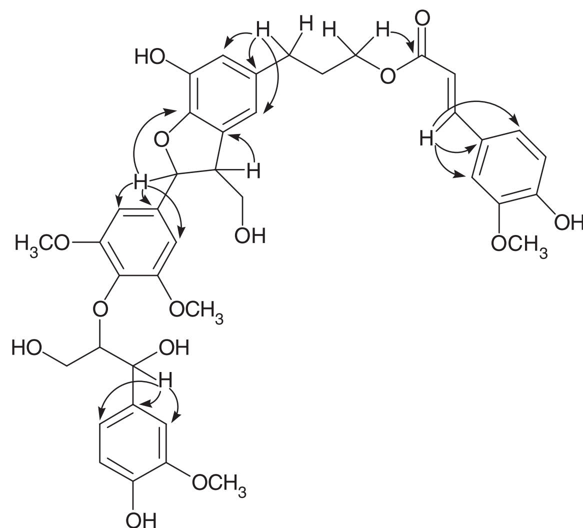

Figure 2. The key HMBC correlations of **10**.

Table 3. Positive effects of isolated lignans on UMR106 cell proliferation and alkaline phosphatase activities.

1) Positive effects of isolated lignans on UMR106 cell proliferation.

Proliferation (% control)	*1.0 nmol/l*	*10 nmol/l*	*0.1 μmol/l*	*1.0 μmol/l*
1	130.1 ± 7.8 **	134.7 ± 1.5 **	135.0 ± 5.4 **	107.6 ± 16.3
2	99.9 ± 10.9	102.7 ± 9.4	113.8 ± 2.8 **	99.6 ± 5.8
3	113.5 ± 2.2 *	142.0 ± 3.2 **	83.7 ± 3.4 **	88.1 ± 2.7 **
6	124.1 ± 8.3 *	114.1 ± 7.9	97.7 ± 5.5	88.7 ± 3.0 *
7	113.3 ± 7.0 *	103.6 ± 10.2	124.8 ± 5.9 **	109.7 ± 9.6
8	149.3 ± 8.9 **	150.7 ± 4.6 **	135.8 ± 13.4 **	114.1 ± 3.9 **
10	119.2 ± 7.6 *	114.7 ± 12. 1	146.8 ± 6.7 **	105.6 ± 8.6

2) Positive effects of isolated lignans on UMR106 cell alkaline phosphatase activities.

Proliferation (% control)	*10.0 nmol/l*	*1.0 μmol/l*	*0.1 μmol/l*	*10.0 μmol/l*
2	80.9 ± 19.3	89.8 ± 6.5	90.8 ± 19.1	171.6 ± 9.1 **
4	95.9 ± 6.5	98.8 ± 4.0	135.5 ± 7.8 **	167.7 ± 6.0 **
5	106.0 ± 14.2	143.1 ± 3.2 **	110.1 ± 4.7	133.5 ± 9.8 **
7	85.8 ± 2.7 **	91.2 ± 2.8 *	103.6 ± 6.8	150.9 ± 2.5 **
10	104.6 ± 4.1%	107.8 ± 4.1	151.5 ± 2.1 **	57.4 ± 5.7 **

Results were expressed as mean ± s.d. from triplicate determinations. Cell proliferation rate and the stimulating rate of ALP activities were presented as the percentage of control value. IGF-I (10μg/L) was used as positive control in cell proliferating experiment, and the stimulating rate was 20% above the control value. The degree of significance was determined by Student's *t* test. * $p < 0.05$ versus control value; ** $p < 0.01$ versus control value.

phosphatase is a membrane-bound glycoprotein produced by many kinds of human cells, including differentiated osteoblast. The enzyme was first isolated and purified from UMR106 cell by Hsu *et al.* [5], and then was used to study the possible mechanism of action of various compounds regarding osteoporosis, such as vitamin B12 [24], vanadium derivatives [25] and estrodiol [7]. The ability of a compound to stimulate UMR106 cell proliferation and ALP activity may indicate its positive effects on bone formation.

The increase in both the proliferation and ALP activity of UMR106 cells occurred with compounds **2**, **7** and **10**. Compounds **1**, **3**, **6**, **8** showed only stimulating effects on UMR106 cell proliferation while compounds **4** and **5** only induced ALP activity of UMR106 cell. The ability to stimulate ALP activities, not cell proliferation, in UMR106 cells appeared to be occurred in dose-dependent manner. Table 3 showed the positive effects of isolated compounds on proliferation and alkaline phosphatase activities of UMR106 cells.

Lignans are phytoestrogen distributed widely in the plant kingdom with abroad biological activities, such as antitumor, antimitotic, antiviral, miscellaneous physiological effects. They also showed effects on nucleic acids and inhibited enzyme activity [26]. However, lignans regulation of osteoblast-like cells has been seldom reported previously [27].

3. Experimental

3.1 General experimental procedures

The UV spectra were obtained with UV2401PC (Shimadzu, Japan) spectrophotometer. The optical rotations were determined 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. The ESIMS was performed with a Bruker esquire 2000 mass spectrometer. The preparative HPLC and analytical HPLC were performed on a SHIMADZU Pak with RI 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.

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 were purchased from GIBCOBRL. Penicillin, streptomycin, glutamine were obtained from Invitrogen Corporation. 24-well plates, 96-well plates and plastic dishes for cell culture were obtained from FALCON (USA). MTT (3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide) was purchased from Amersham Pharmacia Biotech (Little Chalfont, UK). 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 stock, and were diluted in DMEM when used.

3.2 Plant material

Sambucus williamsii Hance was collected in Northeast China and was identified by Prof. Zerong Jiang, Shenyang Pharmaceutical University, Shenyang, China. A voucher specimen (YYXJSW-2003) is kept in Shenzhen Research Centre of Traditional Chinese Medicine and Natural Products, Shenzhen, China.

3.3 Extraction and isolation

The stems of *Sambucus williamsii* Hance (30 kg) were cut into pieces and extracted with 60% ethanol twice. 400 g of 900 g extract were reserved for *in vivo* experiment. The rest of extract was suspended in water and extracted with chloroform, ethyl acetate, *n*-butanol successively. The 30 g of 100 g concentrated chloroform fraction were applied to silica gel 60 column chromatography (60 × 370 mm), eluted with hexane/EtOAc (95:5, 7:3) and $CHCl_3$/MeOH (7:3), 2500 ml each. The lignan-containing fraction (18 g), eluted by $CHCl_3$/MeOH (7:3), was further isolated by silica gel MPLC (50 × 350 mm) with gradient $CHCl_3$/MeOH elution (98:2, 95:5, 9:1, 8:2; 450 ml each). The eluent was combined into 7 sub-fractions according to TLC properties. Sub-fraction 1 (1.2 g, eluted by $CHCl_3$/MeOH (98:2)) was eluted with $CHCl_3$/MeOH (1:1) on Sephadex LH-20 chromatography column (25 × 370 mm). The fraction between 100 ~ 200 ml was concentrated and applied to ODS MPLC (17 × 160 mm) eluted with 200 ml MeOH/H_2O (4:6). Compounds **1** and **2** were obtained after further separation by preparative HPLC (MeOH/H_2O (3:7), 10 ml/min). Sub-fraction 2 (3.5 g, eluted by $CHCl_3$/MeOH (95:5)) was applied to Sephadex LH-20 (25 × 370 mm) chromatography column, eluted with $CHCl_3$/MeOH (1:1). The fraction between 100 ~ 250 ml was concentrated and applied to ODS MPLC (15 × 200 mm) eluted with MeOH/H_2O (2:8, 3:7, 4:6 and 1:1), 300 ml each. The fraction eluted by MeOH/H_2O (2:8) was applied to preparative HPLC and eluted with MeOH/H_2O (1.5:8.5), 10 ml/min, then compounds **3**, **4**, **5** were obtained. The fraction eluted by MeOH/H_2O (3:7) was applied to preparative HPLC, eluted with MeOH/H_2O (2.5:7.5), 10 ml/min, then afforded compounds **6** and **7**. The fraction eluted by MeOH/H_2O (1:1) was applied to preparative HPLC, eluted with MeOH/H_2O (4:6), 10 ml/min, and afforded compounds **8**, **9** and **10**.

3.3.1 ***Erythro*****-1-(4-hydroxy-3-methoxyphenyl)-2-[4-(4-hydroxy-3-methoxy-cinnamoyl-oxypropanyl)-2-hydroxylphenoxy]-1,3-propanediol (8)**. Yellowish oil; 9.8 mg; $[\alpha]_D^{26}$ + 4.9 (*c* 0.1, MeOH); λ_{max}^{MeOH} (log ε): 230 (3.35), 284 (3.02) nm; ESIMS *m/z* 563.2 $[M + Na]^+$, 539.2 $[M - H]^-$; HREIMS *m/z* 563.1642 $[M + Na]^+$ (calcd for $C_{29}H_{32}O_{10}Na$, 563.1603); ^1H NMR (400 MHz, CD_3OD) and ^{13}C NMR (100 MHz, CD_3OD): see table 1.

3.3.2 ***Threo*****-1-(4-hydroxy-3-methoxyphenyl)-2-[4-(4-hydroxy-3-methoxy-cinnamoyl-oxypropanyl)-2-hydroxyphenoxy]-1,3-propanediol (9)**. Yellowish oil; 8.9 mg; $[\alpha]_D^{26}$ + 5.2 (*c* 0.1, MeOH); λ_{max}^{MeOH} (log ε): 231 (3.35), 284 (3.02) nm; ESIMS *m/z* 563.2 $[M + Na]^+$, 539.2 $[M - H]^-$; HREIMS *m/z* 563.1582 $[M + Na]^+$ (calcd for $C_{29}H_{32}O_{10}Na$, 563.1603); ^1H NMR (400 MHz, CD_3OD) and ^{13}C NMR (100 MHz, CD_3OD): see table 1.

3.3.3 2-{4-[2,3-dihydro-3-hydroxymethyl-7-hydroxy-5-(4-hydroxy-3-methoxycinna-moyloxypropanyl)-2-benzofuranyl]-2,6-dimethoxyphenoxy}-1-(4-hydroxy-3-methoxy-phenyl)-1,3-propanediol (10). Yellowish oil; $[\alpha]_D^{23}$ − 25.2 (*c* 0.1, MeOH); UV λ_{max}^{MeOH} (log ε): 204 (4.02), 286(3.22), 324(3.24) nm; ESIMS *m/z* 771.2 $[M + Na]^+$, 747.2 $[M - H]^-$; HREIMS *m/z* 771.2683 $[M + Na]^+$ (calcd for $C_{40}H_{44}O_{14}Na$, 771.2629); ^1H NMR (400 MHz, CD_3OD) and ^{13}C NMR (100 MHz, CD_3OD): see table 2.

3.4 Cell culture

UMR106 cells were maintained at 37°C in humid air containing 5% CO_2 in DMEM supplemented with 5% FBS (fetal bovine serum), 100 U/ml penicillin, 100 μg/ml streptomycin. The cells were subcultured every 2 days using 0.05% trypsin- 0.53 mM EDTA. When 80% confluence, the cells were plated into 96-well plate or 24-well plate in 5% FBS in DMEM with 7500 cells / well or 37 500 cells / well.

3.5 Cell proliferation

UMR106 cell proliferation was determined by MTT assay just as described previously by Chen *et al.* [28]. Briefly, the cells wee starved of FBS for 24 hours after being seeded in 96-well plates for 1 day. Then, the test samples were added at predetermined concentrations for 24 hours. 10 μL MTT (5 mg/ml) was added to every well at the end of experiment. After the cells were incubated at 37°C in darkness for 4 hours, 100 μl DMSO was added to dissolve the formazan crystals. The absorbance was measured on POLAR star Galaxy at 575 nm, with DMEM as blank.

3.6 ALP (alkaline phosphatase) activities

The cells were cultured in 24-well plates with 5% FBS in DMEM for 3 days, followed by treatment with various concentrations of test samples in 1% FBS in DMEM for 24 hours. 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 mmol p-nitrophenyl phosphate, 0.1 mmol sodium carbonate and 1 nmol magnesium chloride was added. The reaction

solution was then incubated for 30 minutes at 37°C in darkness. 500 μl 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 $(A_{405,\ \mathrm{test\ sample}} - A_{405,\ \mathrm{dd\ H2O}})/(A_{405,\ \mathrm{control}} - A_{405,\ \mathrm{dd\ H2O}})$/30 minute/μg total cellular protein.

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