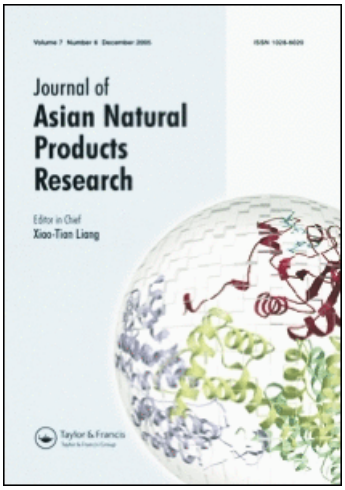

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[a] School of Chemical Biology and Pharmaceutical Sciences, Capital Medical University, Beijing, China [b] School of Pharmaceutical Sciences, Shanxi Medical University, Taiyuan, China [c] Department of Natural Product Chemistry, Shenyang Pharmaceutical University, Shenyang, China [d] College of Chemistry & Enviromental Science, Hebei University, Baoding, China [e] Life Science Division, Graduate School at Shenzhen, Tsinghua University, Shenzhen, China

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Two new steroidal compounds from starfish *Asterias amurensis* Lutken

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[a]*School of Chemical Biology and Pharmaceutical Sciences, Capital Medical University, Beijing 100069, China;* [b]*Life Science Division, Graduate School at Shenzhen, Tsinghua University, Shenzhen 518055, China;* [c]*School of Pharmaceutical Sciences, Shanxi Medical University, Taiyuan 030001, China;* [d]*Department of Natural Product Chemistry, Shenyang Pharmaceutical University, Shenyang 110016, China;* [e]*Key lab for Research and Development of Traditional Chinese Medicine and Natural Products, Research Institute of Tsinghua University in Shenzhen, Shenzhen 518057, China;* [f]*College of Chemistry & Enviromental Science, Hebei University, Baoding 071002, China*

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Two new sulfated steroidal compounds (**1** and **2**), along with three known steroidal saponins (**3**, **4**, and **5**) were isolated from the starfish *Asterias amurensis* Lutken. The structures of new compounds were elucidated as 3β-*O*-sulfated-cholest-5-ene-7α-ol (**1**) and (*E*) 25-*O*-β-D-xylopyranosyl-26, 27-dinor-24(*S*)-methyl-22-ene-15α-*O*-sulfated-5α-cholest-3β,6α-ol (**2**) by extensive NMR experiments and chemical evidence. Their effects on UMR106 cell proliferation were screened by MTT method. The results indicated that compounds **2** and **2a** (0.01–100 μM) significantly promoted the osteoblastic proliferation. The initial structure–activity relationship analysis suggests that the sugar moiety is the necessary group for the activity.

Keywords: starfish; *Asteria amurensis*; steroid; UMR106 cell proliferation

1. Introduction

Steroidal glycosides are the predominant metabolites of starfish and are responsible for their general toxicity.[1,2] According to chemical structures, they have been divided into three main groups: asterosaponins, cyclic steroidal glycosides, and glycosides of polyhydroxylated steroids. The members of the first group are usually sulfated steroidal penta- or hexaglycosides based on a $\geq^{9(11)}$-3β,6α-dioxysteroidal aglycon with a sulfate group attached at C-3 and the oligosaccharide moiety at C-6.[3] These saponins have been reported to exhibit various biological activities, including cytotoxic, hemolytic, antibacterial, antiviral, and antifungal effects.[4] For a long time, starfish *Asterias amurensis* Lutken has been used as a traditional Chinese medicine for the treatment of stomach diseases. Previously, only chemical studies revealed an antitumor acidic polysaccharide (NRP-1) from the starfish *A. amurensis*.[5] Our chemical investigation on the same resulted in the isolation of a new sulfated sterol, a new steroidal glycosides, along with three known starfish saponins. In this paper, we describe the isolation and structure elucidation of the new compounds, and their bioactivities on UMR106 cell proliferation (Figure 1), (Table 3).

*Corresponding authors. Email: liuhongwei60@yahoo.com.cn; yaoxinsheng@vip.tom.com

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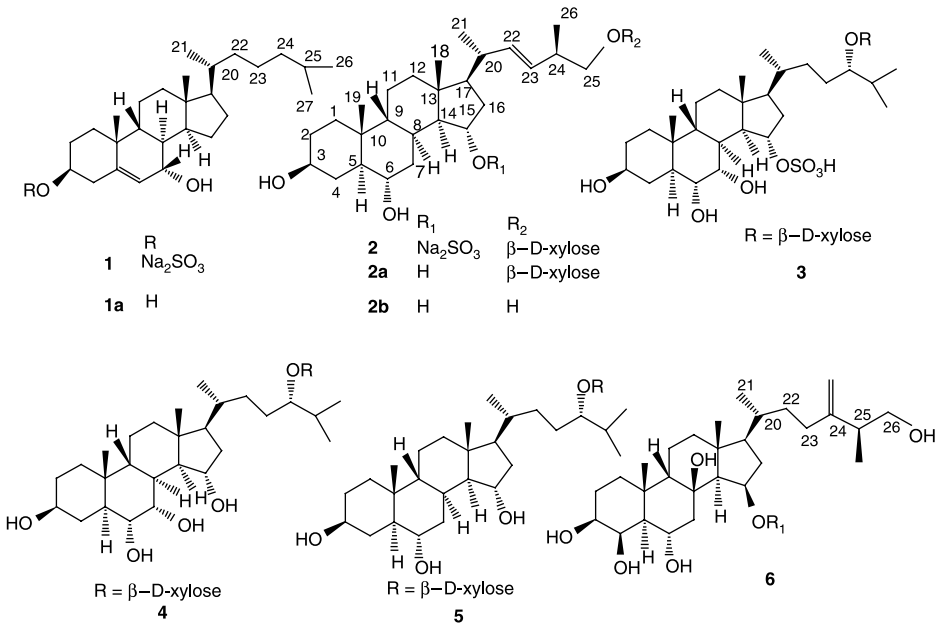

Figure 1. Structures of compounds **1**–**6**.

2. Results and discussion

Compound **1** was obtained as white crystalline needles. The pseudomolecular ion at *m/z* 527.2816 $[M + Na]^+$ in HR TOFMS spectrum and $[M - Na]^-$ at *m/z* 481 in ESI-MS, as well as the ^{13}C and ^1H NMR spectral data for **1** indicated the molecular formula as $C_{27}H_{45}O_5SNa$. The fragment ion peak at *m/z* 407 $[M + Na - NaHSO_4]^+$ in the ESI-MS/MS indicated the presence of a sulfate group in the structure, which was also confirmed by the IR spectrum with the absorption bands at 1211 and 1222 cm^{-1}. The ^1H, ^{13}C NMR, and DEPT spectra displayed resonances due to five methyl groups [δ_H 0.72 (3H, s), 0.89 (6H, d, 6.8 Hz), 0.92 (3H, s), and 1.00 (3H, d, 6.4 Hz); δ_C (12.1, 22.9, 22.7, 18.9, 19.1), one olefinic proton (δ_H 5.58; δ_C 141.3, 128.9), and one multiplet (δ_H 4.73; δ_C 77.6) that was ascribed to a methine proton linked to a carbon bearing a sulfate group, and suggested that the aglycone of **1** has a cholest-5-ene-3,7-dihydroxysteroidal structure skeleton. The correlations of H-18/C-1, 5, 9, 10, H-19/C-12, 13, 14, 17, H-21/C-17, 20, 22, and H-26, 27/C-24, 25 observed in its HMBC spectrum confirmed the suggested structure, and the signal assignments were achieved in combination with ^1H–^1H COSY and HMQC experiments. The key ^1H–^1H COSY and HMBC correlations are presented in Figure 2. The α-configuration of the hydroxyl group at C-7 was confirmed by NOE correlations between H-7 and H-19. To further confirm the structure, compound **1** was converted into the desulfation product **1a** by treatment with a dioxane–pyridine mixture (1:1). Compound **1a** was identified as cholest-5-ene-3β,7α-diol by TLC comparison with the authentic sample and NMR spectral analysis. Based on the above results, the structure of **1** was determined to be 3β-*O*-sulfated-cholest-5-ene-7α-ol.

Compound **2** possesses the molecular formula $C_{31}H_{51}O_{11}SNa$ determined by HRT-OFMS measurement at *m/z* 677.3004 $[M + Na]^+$. The fragment ion peak at *m/z*

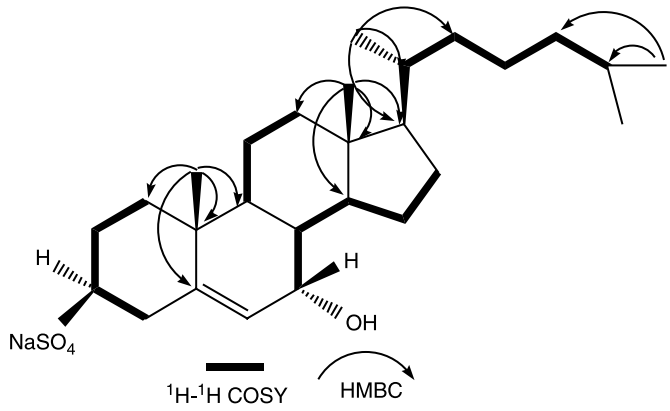

Figure 2. Key $^{1}H-^{1}H$ COSY and HMBC correlations of **1**.

557 $[M + Na-NaHSO_4]^+$ in ESI-MS/MS indicated the presence of a sulfate group in the structure, which was confirmed by the IR spectrum with the absorption bands at 1211 and 1242 cm^{-1}. The IR absorption band at 3379 cm^{-1} suggested the presence of hydroxyl groups. In the ^{1}H NMR spectrum, four methyl groups at δ_H 0.64 (3H, s), 0.82 (3H, s), 0.93 (3H, d, 6.8 Hz), and 0.97 (3H, d, 6.4 Hz), one anomeric proton at δ_H 4.68 (1H, d, 7.2 Hz), two olefinic protons at δ_H 5.25 (dd, 8.0, 14.8 Hz) and 5.35 (dd, 8.4, 14.8 Hz), and several oxygenated proton signals at δ_H (3.5–5.0) were observed. The ^{13}C NMR spectrum of **2** as well as the HMQC experiment showed the presence of 4 methyl groups, 9 methylenes (two oxygenated), 14 methines (seven oxygenated), 2 quaternary carbons, and 2 olefinic carbons (Table 2). The resonances due to the anomeric proton and carbon and other four oxygenated carbons suggested the presence of one pentoside moiety. The analysis of $^{1}H-^{1}H$ COSY spectrum led to the structure fragments depicted with the bold bonds in Figure 3. These structure fragments were connected by HMBC correlations to give a gross structure of **2**. The key HMBC correlations are summarized in Figure 3. The correlation from the anomeric proton to C-25 revealed the location of the sugar moiety. To further confirm the structure of **2**, it was desulfated by treatment with a mixture of dioxane–pyridine (1:1), and **2a** was obtained. The structure of **2a** was completely assigned by ^{1}H, ^{13}C, DEPT, $^{1}H-^{1}H$ COSY, HMQC, HMBC, and NOESY experiments. The relative configurations at C-3, C-6, and C-15 were deduced as 3β, 6α, and 15α on the basis of NOE correlations of H-3/H-5, H-5/H-9, H-6/H-19, H-7β/H-9, H-7α/H-15, H-14/H-17, and H-15/H-18 observed in the NOESY spectrum of **2a** (Figure 4). The sugar connected to C-25 was also determined as xylopyranose by acid hydrolysis and by a comparison of the ^{13}C NMR spectral data with those reported in the literature.[6,7] The β-configuration of this sugar unit was deduced by the coupling constant 7.2 Hz of the anomeric proton. Acid hydrolysis of **2a** gave D-xylose (TLC, GC, optical rotation) and alycon **2b**. The structure of **2b** was established as 26,27-dinor-24-methyl-5α-cholest-22-ene-3β,6α,15α,25-tetrol by NMR spectral data.

The absolute configuration at C-24 can be established by analysis of the (*R*)- and (*S*)-MTPA esters of **2b**. The (*R*) and (*S*)-MTPA esters were generally obtained by treatment with (*R*)-(−)- and (*S*)-(+)-α-methoxy-α-(trifluoromethyl) phenylacetyl chloride. The ^{1}H NMR spectrum of the (*R*)-MTPA ester of **2b** showed the H-25 methylene proton signal as two close multiple at δ 4.22 (overlapped, H-25), while that of the (*S*)-MTPA ester showed the H-25 proton signal as two well-separated doublets of doublets at δ 4.20 (1H,

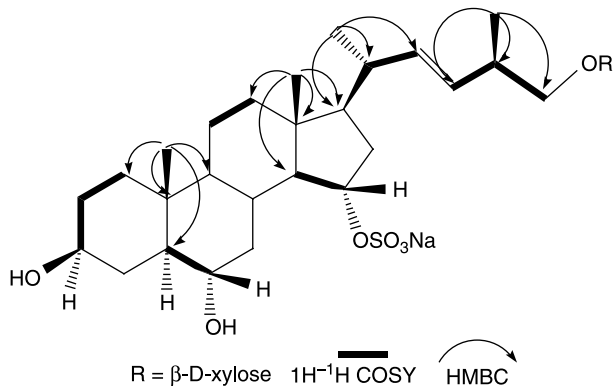

Figure 3. Key $^{1}H-^{1}H$ COSY and HMBC correlations of **2**.

dd, 10.8, 5.6 Hz) and 4.13 (1H, dd, 10.8, 5.6 Hz). The chemical shift difference of H-25 protons in the (*R*)- and (*S*)-MTPA ester of **2b** was consistent with that of H-26 protons of **6** (25*S*) observed in its (*R*)- and (*S*)-MTPA ester.[8] Therefore, a 24*S* configuration was proposed for compound **2**, the same as that for many sterols.[6–8] The structure of **2** was determined as (*E*) 25-*O*-β-D-xylopyranosyl-26,27-dinor-24(*S*)-methyl-22-ene-15α-*O*-sulfated-5α-cholest-3β,6α-ol.

Compounds **3**, **4**, and **5** were identified as known steroidal saponins by a detailed analysis of NMR spectral data and comparing with literature values.[6,7]

The high prevalence of osteoporosis has been regarded as a major public health problem all over the world [9]. In the present work, an *in vitro* osteoblastic model (UMR106 cell line) was used to study the effects of compounds **1**–**5** on osteoblastic proliferation. IGF-1, insulin-like growth factor, was used as a positive control. It is a very important factor for bone formation, and was reported to significantly stimulate osteoblastic proliferation.[10,11] The screening results indicate that compounds **1** and **2b** have no effect on osteoblastic proliferation. Compounds **2** and **2a** (0.01–100 μM) significantly promote osteoblastic proliferation. Moreover, the proliferation rate of **2** is higher than that of **2a** at the same concentrations. Compounds **3** and **5** also promote osteoblastic proliferation at most concentrations. In

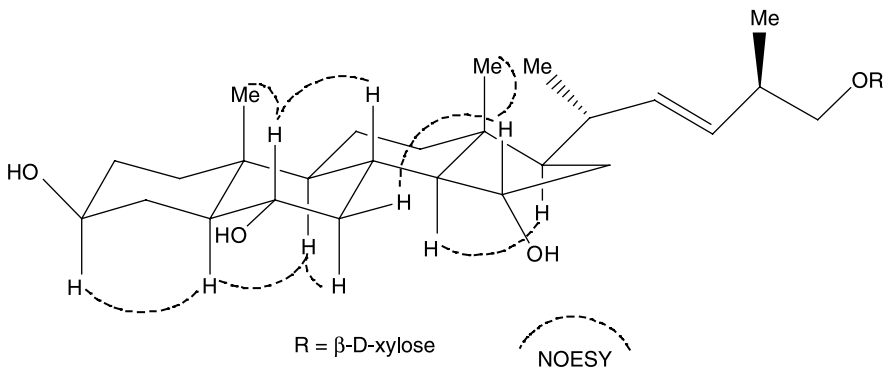

Figure 4. Key NOE correlations of **2a**.

summary, these compounds might have a potential activity against osteoporosis. The initial structure–activity relationship analysis suggests that the sugar moiety of saponins is the necessary group for the activity.

3. Experimental

3.1 General experimental procedures

The melting points were determined on a Yanaco MP-S_3 micromelting point apparatus, and are uncorrected. Optical rotations were measured using a P-1020 digital polarimeter (JASCO Corporation). FAB-MS and HR-MS were obtained using a JEOL JMS-DX302 spectrometer. ESI-MS was conducted using a Bruker esquire 2000 mass spectrometer. IR spectra were recorded on a SHIMADZU FT/IR-8400 spectrometer. ^{1}H and ^{13}C NMR, along with 2D NMR spectra were obtained on a Bruker AV-400 (400 MHz for ^{1}H and 100 MHz for ^{13}C) NMR spectrometer, using TMS as an internal standard. GC was done on a HP-5890 SERIES II spectrometer, with a SE30 capillary column (12 m, 0.22 mm i.d.) and hydrogen flame ionization detector (FID, 270°); the column temperature was 170–250°C with a rate of 5°C/min and the carrier gas was N_2 (30 ml/min). TLC was performed on silica gel $60F_{254}$ and the spots were visualized by spraying with 10% H_2SO_4 and heating. LH-20 (Amersham Biosciences) and ODS (Lobar, 40–63 μm, Merck) were used for column chromatography. Preparative HPLC was performed using an ODS column (C-18, 250 × 20 mm, SHIMADZU Pak; detector: RID).

3.2 Plant material

The starfish *A. amurensis* Luken was collected in August 2004 (The North Sea of China, Guangxi), identified by Dr Zhonghua Cai (Graduate School at Shenzhen, Tsinghua University, Shenzhen), and deposited (2005HX001) at Graduate School at Shenzhen, Tsinghua University.

3.3 Extraction and isolation

Ground starfish (5 kg) was subjected to exhaustive extraction with methanol by refluxing (10 l × 3). The extracts were concentrated *in vacuo*. The residue (680 g) was suspended in water (4 l), and partitioned with chloroform and water-saturated *n*-butanol. The butanol-soluble part (350 g) was subjected to silica gel column chromatography (8 × 35 cm) using chloroform–methanol (100:0, 98:2, 97:3, 95:5, 90:10, 80:20, 70:30, 60:40, 0:100) as elution solvent to give 15 fractions (1–15). Fractions 8 and 9 eluted by chloroform–methanol (80:20, 70:30) were found to be rich in steroidal constituents. Fraction 8 (13.3 g) was further chromatographed on an ODS column with methanol–water as the elution solvents to afford 13 subfractions (8-1–13). Subfraction 8-2 was passed through a Sephadex LH-20 column ($CHCl_3$:CH_3OH, 1:1) and finally purified by preparative HPLC (MeOH:H_2O, 55:45) to give **1** (29.0 mg). Subfraction 8-8 was first passed through a Sephadex LH-20 column ($CHCl_3$:CH_3OH, 1:1), and then purified by preparative HPLC (MeOH:H_2O, 78:22) to give compounds **4** (13.9 mg) and **5** (17.0 mg). Fraction 9 was treated in the same way as that of fraction 8. It was first subjected to ODS column separation to give 10 subfractions (9-1–10). Subfraction 9-2 was further purified using Sephadex LH-20 column ($CHCl_3$:CH_3OH, 1:1) and preparative HPLC (MeOH:H_2O, 65:35) to give **2** (24.3 mg) and **3** (29.0 mg).

3.3.1 Compound *1*

Colorless crystals; mp 126–128°C. $[\alpha]_D^{25}$ − 11.7 (c = 1.0, MeOH). IR (KBr) ν_{max} 3444, 2954, 2869, 1651, 1469, 1222, 1211, 1068 cm^{-1}. ^{1}H and ^{13}C NMR spectral data, see Table 1. Positive ESI-MS *m/z* 527 $[M + Na]^+$; negative ESI-MS *m/z* 481 $[M - Na]^+$, HR-ESI-MS *m/z* 527.2816 $[M + Na]^+$ (calcd for $C_{27}H_{45}O_5SNa_2$, 527.2783).

Table 1. ^{1}H and ^{13}C NMR spectral data for compounds **1** (pyridine-d_5) and **1a** ($CDCl_3$)[a].

	1		1a
No.	δ_C	δ_H (J = Hz)	δ_C
1	37.1	1.73 (m)	36.9
2	29.9	1.30, 1.78 (m)	31.5
3	77.6	4.73 (m)	73.4
4	39.4	2.53 (t, 8.4 Hz), 2.93 (m)	41.7
5	141.3		143.5
6	128.9	5.58 (br. s)	125.4
7	72.3	4.03 (d, 8.0)	71.4
8	40.9	1.61 (m)	40.9
9	48.8	1.03 (m)	48.8
10	36.6		36.2
11	24.1	1.20, 1.40 (m)	23.8
12	39.8	1.16, 2.00 (m)	39.6
13	43.1		42.9
14	56.9	1.23 (m)	56.0
15	21.3	1.41 (m)	21.0
16	27.0	1.78, 2.27 (m)	26.4
17	56.0	1.12 (m)	55.5
18	12.1	0.72 (s)	11.8
19	18.9	0.92 (s)	18.8
20	36.1	1.43 (m)	36.1
21	19.1	1.00 (d, 6.4)	19.1
22	36.7	1.07 (m)	36.4
23	29.0	1.94, 2.40 (m)	29.6
24	39.6	1.16, 2.00 (m)	39.5
25	28.3	1.56 (m)	28.0
26	22.7	0.89 (d, 6.8)	22.5
27	22.9	0.89 (d, 6.8)	22.7

[a] ^{1}H and ^{13}C NMR signal assignments were made on the basis of ^{1}H–^{1}H COSY, HMQC, HMBC, and NOESY experiments.

3.3.2 Compound **2**

Amorphous powder. $[\alpha]_D^{25}$ + 18.9 (c = 1.0, MeOH). IR (KBr) ν_{max} 3379, 2939, 2869, 1651, 1458, 1242, 1211, 1053 cm^{-1}. ^{1}H and ^{13}C NMR spectral data, see Table 2. Positive EIMS m/z 677 $[M + Na]^+$; negative ESI-MS m/z 631 ($[M - Na]^+$, HR-ESI-MS m/z 677.3004 $[M + Na]^+$ (calcd for $C_{31}H_{51}O_{11}SNa_2$, 677.2980) (Table 3).

3.4 Desulfation of 1 and 2

A solution of **1** (10 mg) in a mixture of dioxane–pyridine (1:1, 2 ml) was heated for 2 h at 100°C. The solution was concentrated *in vacuo* to dryness and the residue was chromatographed on a silica gel column with chloroform–methanol (20:1, 10:1, 9:1, 7:1) as the eluent to give **1a** that was identified by NMR spectral data analysis and TLC comparison with the authentic ($CHCl_3$:CH_3-OH, 20:1; R_f = 0.4). Compound **2** (20 mg) was treated in the same manner, and the residue was finally purified by silica gel column chromatography with chloroform–methanol–water (80:20:1) as the eluent to give **2a** (14 mg).

3.4.1 Compound **1a**

Amorphous powder. $[\alpha]_D^{25}$ − 12.3 (c = 1.0, MeOH). Positive ESI-MS m/z 403 $[M + H]^+$. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 0.70 (3H, s, H-19), 0.87 (3H, d, J = 6.8 Hz, H-26), 0.88 (3H, d, J = 6.8 Hz, H-27), 0.92 (3H, d, J = 6.4 Hz, H-21), 1.07 (3H, s, H-18), 5.29 (1H, m, H-6), 3.85 (1H, m, H-3). ^{13}C NMR spectral data, see Table 1.

3.4.2 Compound **2a**

Amorphous powder. $[\alpha]_D^{25}$ + 20.6 (c = 1.0, MeOH). Positive ESI-MS m/z 575 $[M + Na]^+$. ^{1}H and ^{13}C NMR spectral data, see Table 2.

3.5 Acid hydrolysis of 2a

A solution of glycoside **2a** (10 mg) in aqueous 2 N CF_3COOH was heated at 100°C for 2 h in a sealed ample. The reaction mixture was evaporated *in vacuo*. The obtained residue was dissolved in water and extracted with $CHCl_3$ two times. The $CHCl_3$ extracts were isolated on a column with silica gel using chloroform–methanol (20:1, 10:1) as the eluent to give **2b** (8 mg). Xylose was identified in the aqueous layer by TLC on Si gel using BuOH/EtOH/H_2O (4:1:2) as the eluent and by GLC using standard aldononitrile peracetate of xylose as a reference sample (*Rt* 6 min).[12] The D-configuration of

Table 2. ^{1}H and ^{13}C NMR spectral data for compounds **2**, **2a**, and **2b** in pyridine (400 MHz)[a].

	2		**2a**		
No.	δ_C	δ_H ($J = Hz$)	δ_C	δ_H ($J = Hz$)	**2b**
1	38.0	1.00, 1.63 (m)	38.1	1.03, 1.71 (m)	38.7
2	32.3	1.70, 2.05(m)	32.4	1.78, 2.08(m)	31.9
3	70.9	3.88 (m)	71.0	3.92 (m)	71.9
4	33.6	1.63 (m)	33.7	1.73 (m)	33.0
		2.97 (br.d, 12.0)		3.03 (m)	
5	52.3	1.37 (m)	52.7	1.38 (m)	52.8
6	68.9	3.62 (m)	68.9	3.74 (m)	70.2
7	41.5	1.53, 3.15 (m)	43.2	1.22, 3.00 (m)	42.8
8	34.2	1.63 (m)	34.6	1.78 (m)	35.3
9	54.2	0.71 (m)	54.5	0.80 (m)	55.4
10	36.4		36.5		37.3
11	21.2	1.18, 1.44(m)	21.4	1.24, 1.50 (m)	22.2
12	40.2	1.10, 1.84 (m)	40.4	1.23, 1.89 (m)	41.3
13	42.7		43.6		44.6
14	60.7	1.43 (m)	63.7	1.43 (m)	63.9
15	79.7	4.94 (t, 9.2)	72.7	4.13 (t, 9.2)	74.0
16	39.0	2.19, 2.60 (m)	41.9	2.02 (m)	41.9
17	53.7	1.41 (m)	53.7	1.53 (m)	54.8
18	13.5	0.64 (s)	13.7	0.72 (s)	13.9
19	13.7	0.82 (s)	13.8	0.93 (s)	13.9
20	40.2	1.98 (m)	40.0	2.00 (m)	40.9
21	20.8	0.97 (d, 6.4)	20.7	1.02 (d, 6.4)	21.1
22	137.1	5.35 (dd, 8.4, 14.8)	136.7	5.40 (m)	137.1
23	131.4	5.25 (dd, 8.0, 14.8)	130.3	5.40 (m)	131.6
24	38.3	2.56 (m)	37.0	2.60 (m)	40.5
25	75.0	3.54 (t, 9.6)	74.3	3.65 (t, 9.6)	68.4
		3.82 (dd, 4.8, 9.6)		3.87 (dd, 4.8, 9.6)	17.3
26	17.5	0.93 (d, 6.8)	17.6	1.14 (d, 6.4)	
Xyl-1	105.4	4.68 (d, 7.2)	105.1	4.73 (d, 7.2)	
		4.04 (m)	74.7	4.00 (m)	
2	74.3	4.13 (m)	78.4	4.13 (m)	
3	77.7	4.14 (m)	71.1	4.20 (m)	
4	71.1	3.62 (m)	67.1	3.68 (m)	
5	66.9	4.28 (dd, 4.0, 11.6)		4.36 (dd, 5.2, 11.2)	

[a] ^{1}H and ^{13}C NMR signal assignments were made on the basis of ^{1}H–^{1}H COSY, HMQC, HMBC, and NOESY experiments.

xylose was assigned by the observed optical rotation $[\alpha]_D + 9.5$ ($c = 0.5$, H_2O).

3.5.1 *Compound **2b***

Amorphous powder. Positive ESI-MS *m/z* 443 $[M + H]^+$. ^{1}H and ^{13}C NMR spectral data, see Table 2.

3.6 *Preparation of MTPA esters*

Compound **2b** was divided into two equal parts and treated with (*R*)-(−)- and (*S*)-(+)-α-methoxy-α-(trifluoromethyl) phenylacetyl chloride (20 μmol) in dry pyridine (40 μl) for 24 h at room temperature to afford (*S*)-MTPA ester and (*R*)-MTPA ester, respectively. The reaction was monitored by TLC (ODS, MeOH) and stopped when the original spot had disappeared. After removal of solvent, the product was purified by reversed-phase HPLC on a YMC-Pack ODS column (250 × 10 mm i.d., 5 μm, 120 Å) and analyzed by ^{1}H NMR spectra.

(*S*)-MTPA ester of **2b**: ^{1}H NMR ($CDCl_3$), δ 4.20 (1H, dd, 10.8, 5.6, H-26), 4.13 (1H, dd,

Table 3. The stimulative effects of compounds **1**–**5** on UMR106 cell proliferation.

Compounds	Increase in cell proliferation (%)[a]					
	50 μM	10 μM	1 μM	0.1 μM	0.01 μM	0.001 μM
1	–	–[b]	–	–	–	
2	52.74%*	64.40%*	76.66%*	67.82%*	63.12%*	20.34*
2a	24.10%	22.18%	20.30%*	23.46%*	31.25%*	15.23*
2b	–	–	–	–	–	
3	–	15.68%	23.69%*	30.56%*	40.78%*	10.68*
4	–	–	–	13.44%	17.36%*	–
5	2.77%	17.28%	28.65%*	20.94%*	23.22%*	–

*$P < 0.05$.
[a] The increase in cell proliferation was determined with reference to a blank experiment. IGF-1 was used as positive control, which showed 11.79% proliferation at 10 ng/mL.
[b] Inactive.

10.8, 5.6, H-26), 0.62 (3H, s, H-18), 0.74 (3H, s, H-19), 0.90 (3H, d, $J = 6.8$ Hz, H-25), 0.95 (3H, d, $J = 6.4$ Hz, H-21).

(*R*)-MTPA ester of **2b**: ^{1}H NMR ($CDCl_3$), δ 4.23 (1H, overlapped, H-26), 4.20 (1H, dd, overlapped, H-26), 0.62 (3H, s, H-18), 0.74 (3H, s, H-19), 0.90 (3H, d, $J = 6.8$ Hz, H-25), 0.95 (3H, d, $J = 6.4$ Hz, H-21).

3.7 Osteoblastic proliferation stimulation assay

UMR106 cells were applied to evaluate the effects of compounds on the proliferation of osteoblasts, which were widely used as a developed osteoblast model in studying the effective mechanism of anti-osteoporosis drugs on osteoblasts.[13,14] UMR106 cells were seeded in a 96-well plate at a density of $1.6 \times 10^4/cm^2$, and incubated for 24 h in minimum essential medium supplemented with 10% fetal calf serum prior to the addition of tested compounds (dissolved in 1% dimethyl sulfoxide, DMSO), and then cultured for 48 h. The cells were then treated with MTT (20 μl, 5 mg/ml) for 4 h prior to the end of the experiment. At the end of this experiment, the supernatant was removed and DMSO (100 μl) was added to dissolve formazan. The absorbance at 570 nm was measured on a microplate spectrophotometer (Bio-rad Model 680, USA) with a reference at 655 nm. The percentage of proliferation rate was evaluated according to the formula: (ODsample − ODcontrol)/ODcontrol × 100%. The positive control, IGF-1, showed an increase of 11.79% in osteoblastic proliferation at a concentration of 10 ng/ml.

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