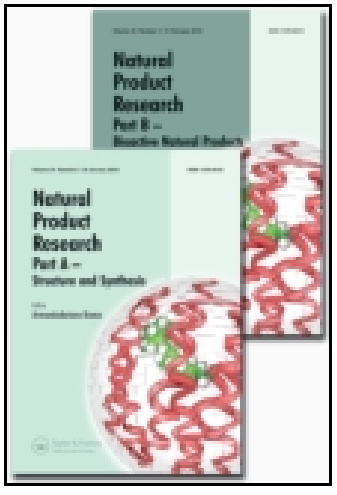

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Five furostanol saponins from fruits of Tribulus terrestris and their cytotoxic activities

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Five furostanol saponins from fruits of *Tribulus terrestris* and their cytotoxic activities

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Two new furostanol saponins, terrestroside A, 3-*O*-{β-D-xylopyranosyl-(1 → 3)-[β-D-xylopyranosyl(1 → 2)]-β-D-glucopyranosyl(1 → 4)-[α-L-rhamnopyranosyl (1 → 2)]-β-D-galactopyranosy}-26-*O*-β-D-glucopyranosyl-L-5a-furost-20(22)-en-(25R)-3β,26-diol (**1**) and terrestroside B, 3-*O*-{β-D-xylopyran-osyl(1 → 3)-[β-D-xylopyranosyl(1 → 2)]-β-D-glucopyranosyl(1 → 4)-[α-L-rhamnopyranosyl-(1 → 2)]-β-D-galactopyranosy}-26-*O*-β-D-glucopyranosyl-5a-furostan-12-one-(25*R*)-*22*-methoxy-3β,26-diol (**2**), together with three known compounds, chloromaloside E (**3**), terrestrinin B (**4**) and terrestroneoside A (**5**) were isolated from the dry fruits of *Tribulus terrestris*. Furthermore, the inhibitory effects of the compounds on tumour cells were evaluated, and compounds **1**–**5** showed potential anti-tumour activity.

Keywords: *Tribulus terrestris*; fruits; furostanol saponins

1. Introduction

Tribulus terrestris L. is an annual creeping herb used in traditional Chinese medicine, and the fruit of this plant, which is known as 'Ci Ji Li', has long been used for the treatment of eye trouble, oedema, abdominal distention, emission and morbid leucorrhoeas (Jiang, 1977). The steroidal saponins in the fruits of *Tribulus terrestris* include spirostanol saponins and furostanol saponins. This article deals with the isolation and structure elucidation of five furostanol saponins. Terrestrosides A (**1**) and B (**2**) were new compounds.

The crude fractions of *T. terrestris* were subjected to normal-phase and reversed-phase silica gel column chromatography and repeated HPLC to afford compounds **1**–**5** (Figure 1). They can be easily deduced to be furostanol saponins by the colour reaction with Herlich's spray reagent on TLC (Kiyosawa et al., 1968). After acid hydrolysis, compounds **1**–**5** yielded glucose, xylose, rhamnose and galactose as sugar residues in a 2 : 2 : 1 : 1 ratio. They were analysed by GC-MS using standard aldononitrile peracetates as authentic samples.

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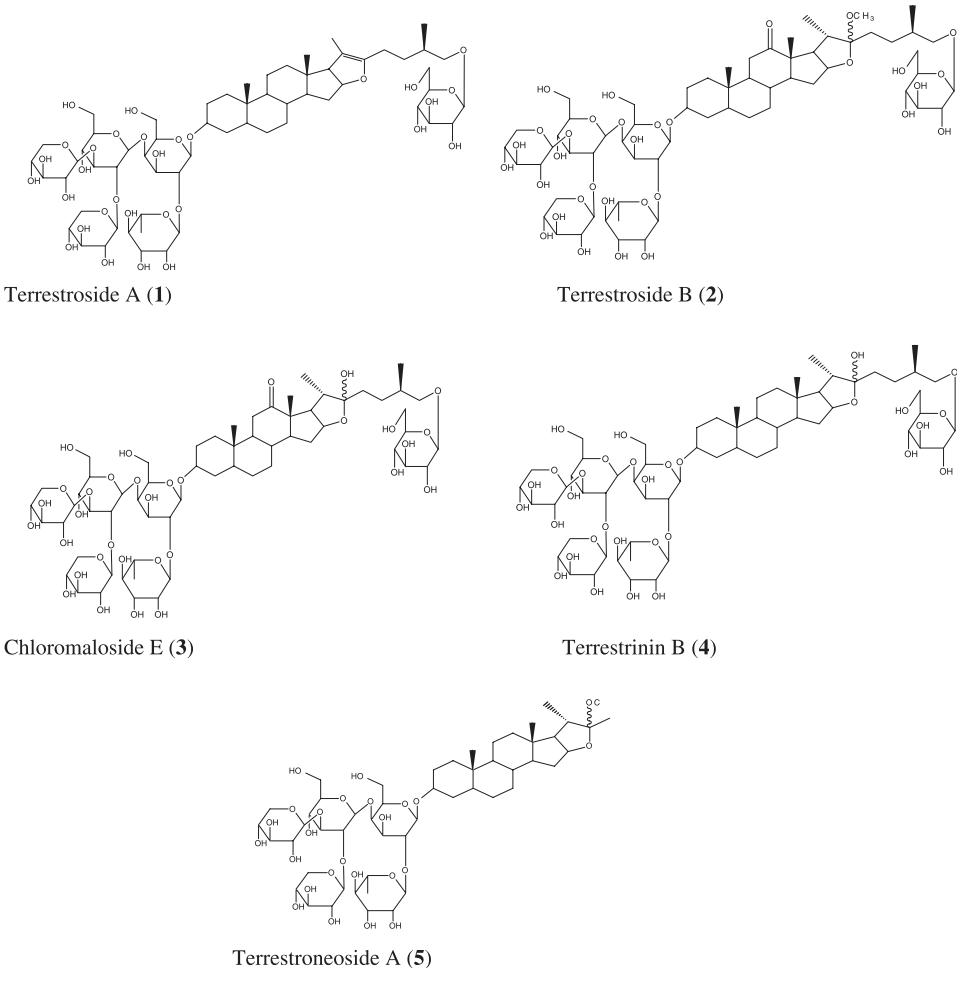

Figure 1. Five compounds isolated from the extract of *T. terrestris*, in which terrestrosides A (**1**) and B (**2**) are new compounds.

2. Results and discussion

Terrestroside A (**1**) was obtained as a white amorphous power with negative optical rotation ($[\alpha]_D^{25}$ −38.2°) (H_2O, *c* 0.68), and was deduced to be a furostanol saponin by the red colour produced with Ehrlich's reagent. In the ESI-MS of **1**, its negative and positive molecular ion peaks were observed at *m/z* 1311.4 $[M-H]^-$ and 1335.3 $[M+Na]^+$. The molecular formula $C_{61}H_{100}O_{30}$ was deduced from HR-TOF-MS and ^{13}C-NMR (Table 1) data analysis.

The ^{1}H-NMR (pyridine-d_5) spectrum of compound **1** displayed six signals of anomeric protons at δ_H 4.84 (1H, d, $J=8.1$ Hz), 4.82 (1H, d, $J=7.8$ Hz), 4.98 (1H, d, $J=7.5$ Hz), 5.22 (1H, d, $J=7.8$ Hz), 5.41 (1H, d, $J=7.7$ Hz), 6.16 (1H, brs), which were characteristic of saponins with six sugar units. Detailed analysis of the ^{13}C-NMR spectrum revealed that 34 signals were assignable to the sugar moiety, 27 to the aglycone, and two signals in the olefinic carbon region at δ_C (103.6, 152.3) could be assigned to C-20 and C-22, respectively.

Table 1. ^{13}C-NMR data for the compounds **1**–**5** (in pyridine-d_5, 100 MHz).

Position	1	2	3	4	5	Position	1	2	3	4	5
						3-o-Glc					
1	37.2	36.6	36.6	37.2	37.2	1″	100.1	100.1	100.1	100.1	100.1
2	29.9	29.9	29.9	29.9	29.9	2″	76.5	76.6	76.6	76.6	76.6
3	76.9	76.5	76.5	77.0	77.0	3″	76.6	76.6	76.6	76.6	76.6
4	34.4	34.1	34.1	34.3	34.3	4″	81.3	81.2	81.2	81.3	81.3
5	44.6	44.3	44.4	44.6	44.6	5″	75.0	75.0	75.0	75.0	75.0
6	28.9	28.6	28.6	29.0	28.6	6″	60.4	60.3	60.4	60.4	60.4
7	32.5	31.6	31.6	32.4	32.4	Rha					
8	35.0	34.2	34.3	35.2	35.2	1‴	101.9	101.9	101.9	101.9	101.9
9	54.4	55.5	55.5	54.4	54.4	2‴	72.4	72.4	72.4	72.4	72.4
10	35.9	36.3	36.3	35.9	35.9	3‴	72.6	72.7	72.6	72.6	72.6
11	21.4	37.9	37.9	21.2	21.1	4‴	73.9	73.9	73.9	73.9	73.9
12	39.8	212.7	212.9	40.2	39.9	5‴	69.3	69.3	69.3	69.3	69.3
13	43.7	55.6	55.7	41.1	41.0	6‴	18.4	18.4	18.4	18.4	18.4
14	54.7	55.7	55.8	56.4	56.3	Glc					
15	34.4	31.3	31.6	32.4	32.0	1⁗	105.3	105.3	105.3	105.3	105.3
16	84.5	79.8	79.6	81.1	81.3	2⁗	81.4	81.4	81.4	81.4	81.4
17	64.6	55.5	55.0	63.9	64.3	3⁗	87.6	87.5	87.6	87.6	87.6
18	14.3	15.9	16.1	16.7	16.4	4⁗	70.4	70.3	70.3	70.4	70.3
19	12.4	11.7	11.8	12.4	12.3	5⁗	77.7	77.7	77.7	77.7	77.7
20	103.6	41.0	41.2	40.6	40.4	6⁗	62.8	62.8	62.8	62.8	62.8
21	11.7	14.9	15.2	16.4	16.2	Xyl					
22	152.3	112.6	110.7	110.6	112.6	1⁗′	105.0	104.9	105.0	105.0	105.0
23	23.6	30.7	37.0	37.2	32.4	2⁗′	75.0	75.0	75.0	75.0	75.0
24	31.4	28.1	28.3	28.3	28.1	3⁗′	78.7	78.7	78.7	78.7	78.7
25	33.4	34.1	34.2	34.2	34.1	4⁗′	70.7	70.6	70.7	70.7	70.7
26	74.9	75.1	75.2	75.2	75.1	5⁗′	67.3	67.3	67.3	67.3	67.3
27	17.3	17.0	17.4	17.4	17.1	Xyl					
26-o-Glc						1⁗″	105.7	105.7	105.7	105.7	105.7
1′	104.8	104.9	104.9	104.9	104.9	2⁗″	75.7	75.8	75.7	75.7	75.8
2′	75.1	75.1	75.1	75.1	75.1	3⁗″	79.0	79.0	79.0	79.0	79.0
3′	78.6	78.6	78.5	78.5	78.6	4⁗″	70.8	70.8	70.8	70.8	70.8
4′	71.7	71.7	71.7	71.6	71.7	5⁗″	67.6	67.6	67.6	67.6	67.6
5′	78.4	78.5	78.4	78.4	78.5						
6′	62.8	62.8	62.8	62.9	62.8						

These assignments can be confirmed through long-range correlations in the heteronuclear multiple bond correlation (HMBC) experiment. Namely, long-range correlations were observed between the following protons and carbons: the methyl protons at δ_H 0.69 (3H, s, 18-CH_3) showed long-range correlations with the carbons at δ_C 43.7 (C-13), 54.7 (C-14), 39.8 (C-12) and 64.6 (C-17); the methyl protons at δ_H 0.84 (3H, s, 19-CH_3) showed long-range correlations with the carbons at δ_C 35.9 (C-10), 37.2 (C-1), 44.6 (C-5) and 54.4 (C-9); the methyl protons at δ_H 1.62 (3H, s, 21-CH_3) showed long-range correlations with the carbons at δ_C 64.6 (C-17), 103.6 (C-20) and 152.3 (C-22); the methyl protons at δ1.01 (3H, d, $J = 6.7$ Hz, 27-CH_3) showed long-range correlations with the carbons at δ_C 31.4 (C-24), 33.4 (C-25) and 74.9 (C-26); the methyl protons at δ_H 1.69 (3H, d, $J = 6.1$ Hz) were assigned to 6-CH_3 of the α-L-rhamnose (Figure 2). Thus, its aglycone moiety was deduced to be a 5a-furost-20(22)-en-3β-2,6-diol structure.

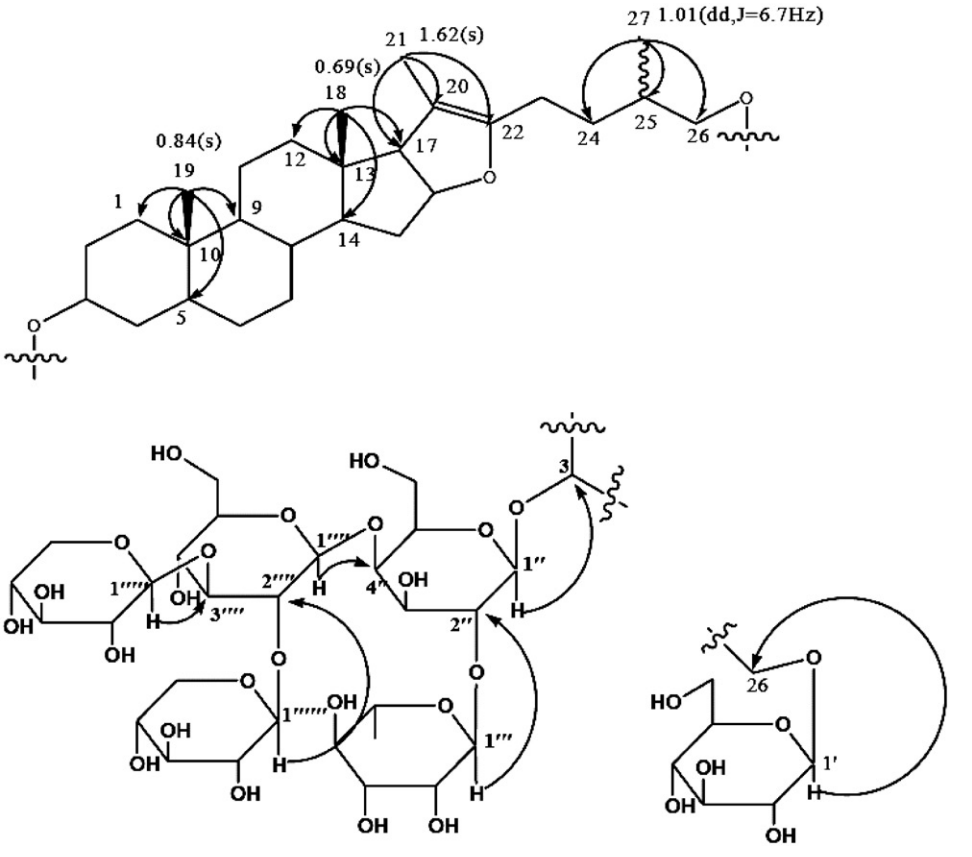

Figure 2. Key HMBC correlations of terrestroside A (**1**).

The C-25 configuration of the compound **1** was '*R*', which was determined by comparison of the ^{13}C-NMR of 26-*O*-*β*-D-glucopyranosyl(25*R*)-5a-furost-20(22)-en12-one-3*β*,26-diol-3-*O*-*β*-D-galactopyranosyl(1-2)-*β*-D-glucopyranosyl(1-4)-*β*-D-galacto-pyranoside (Tu, Ma, & Li, 2002; Wang & Ohtani, 1997). After acid hydrolysis, glucose, xylose, rhamnose and galactose as sugar residues in a 2 : 2 : 1 : 1 ratio was identified by GC-MS analysis with standard aldononitrile peracetates as authentic samples.

Complete assignments of each glycosidic proton system were achieved by analysis of 2D NMR experiments (COSY, TOCSY, HMQC, HMBC and NOESY). Evaluation of spin–spin couplings and chemical shifts allowed the identification of two *β*-D-glucopyranosyl units, two *β*-D-xylopyranosyl units, one *β*-D-galactopyranosy unit and one *α*-L-rhamnopyranosyl unit. The linkage of sugar units was established by HMBC experiment cross peaks between *β*-D-Glc (H-1′)/C_{26}–O; *β*-D-Gal (H-1″)/C_3–O; *α*-L-Rha (H-1‴)/*β*-D-Gal (C-2″); *β*-D-Glc (H-1⁗)/*β*-D-Gal (C-4″); *β*-D-Xyl (H-1⁗′)/*β*-D-Glc (C-2⁗); and *β*-D-Xyl (H-1⁗″)/*β*-D-Glc (C-3⁗) (Figure 1). Comparison of spectroscopic data with that of the structure offered references (Q. Yang & C. Yang, 2000) indicated that they have the same sugar sequence. Thus, the structure of compound **1** was established to be 3-*O*-{*β*-D-xylopyranosyl(1 → 3)-[*β*-D-xylopyranosyl(1 → 2)]-*β*-D-glucopyranosyl(1 → 4)-[*α*-L-rhamnopyranosyl(1 → 2)]-*β*-D-galactopyranosy}-26-*O*-*β*-D-glucopyranosyl-5a-furost-20(22)-en-(25*R*)-3*β*,26-diol.

Table 2. Results of inhibitory effects of compounds **1**–**5** on tumour cell bioassay (MTT based) *in vitro*.

	IC_{50} (μM)			
Samples	NCI-H46	SF-268	MCF-7	HepG2
1	9.26	7.53	6.18	*
2	4.31	3.47	3.98	6.66
3	3.11	3.03	2.75	*
4	5.68	6.14	4.77	3.59
5	7.21	5.81	8.31	*

Note: *The compounds showed no effects.

Terrestroside B (**2**) was obtained as a colourless crystal with negative optical rotation ($[\alpha]_D^{25}$ −25.2°) (H_2O, *c* 0.56), and was deduced to be a furostanol saponin by the red colour produced with Ehrlich's reagent. In the ESI-MS of **2**, its negative and positive molecular ion peaks were observed at *m/z* 1357.6 $[M-H]^-$ and 1381.6 $[M+Na]^+$. The molecular formula $C_{62}H_{102}O_{32}$ was deduced from HR-TOF-MS and ^{13}C-NMR (Table 1) data analysis.

Comparing spectroscopic data with that of compound **1**, it was found that they share the same sugar sequence and a similar aglycone. However, there were no signals in the olefinic carbon region. In the ^{13}C-NMR spectrum of the compound **2**, a remarkable carbonyl signal at δ_C 212.7 could be assigned to C-12, which was determined by HMBC experiment.

Using the techniques of 2D NMR (COSY, TOCSY, HMQC, HMBC and NOESY) and comparing with compound **1**, the structure of compound **2** was established to be 3-*O*-{β-D-xylopyranosyl (1 → 3)-[β-D-xylopyranosyl (1 → 2)]-β-D-glucopyranosyl (1 → 4)-[α-L-rhamnopyranosyl (1 → 2)]-β-D-galactopyranosy}-26-*O*-β-D-glucopyranosyl-5a-furost-12-one-(25R)-*22*-methoxy-3β, 26-diol.

Compounds **3** (Q. Yang & C. Yang, 2000), **4** (Bedir, Khan, & Walker, 2002; Huang, Tan, Jiang, & Zhu, 2003) and **5** (Sun, Gao, Tu, Guo, & Zhang, 2002; Xu et al., 2000) were known compounds, whose structures were elucidated by comparison of their spectral data with those in the literature.

2.1. *Anti-tumour activity*

The inhibitory effects of compounds **1**–**5** were examined on NCI-H460, SF-268, MCF-7 and HepG2 tumour cells. Five triterpenoid saponins (compounds **1**–**5**) all showed potent anti-tumour activity, and the IC_{50} values for these compounds are shown in Table 2.

3. Experimental

The following instruments were used to obtain physical data: a YANACO digital micromelting point apparatus, uncorrected; NMR (ppm, *J* in Hz): a Bruker AVANCE-400 NMR; ESI-MS: a Bruker Esquire 2000; HPLC: [Detector: an RID-10A SHIMADZU refractive index detector; Pump: an LC-8A SHIMADZU liquid chromatograph; Column: a SHIM-PACK PREP-ODS column (20 × 250 mm)]. MPLC: an EYELA CERAMIC [pump VSP-3050; Fraction collector: DC-1200]. CC: an RP-18 (Merck). Resin: D101. Optical rotation: a Jasco P-1020.

3.1. *Plant material*

The fruit of *T. terrestris* L. was from the Ji-Lin province of China and identified by a company in Nanjing. The voucher of this plant material is on file in our laboratory.

3.2. *Extraction and isolation*

The dried fruits of *T. terrestris* (1.8 kg) were thrice extracted with 70% EtOH. The solutions were combined and evaporated to dryness to give a crude extract of 450.6 g. The extract was then subjected to D101 resin column chromatography eluted gradiently with H_2O/EtOH solutions to give H_2O fraction (244.7 g), 20% EtOH fraction (56.2 g), 70% EtOH fraction (66.7 g) and 90% EtOH fraction (83.4 g), respectively. The 20% EtOH fraction (56.2 g) was then subjected to silica gel column chromatography (Wako gel C-300, Wako pure Chemical Industry Ltd., 700 g, 12 × 17 cm, eluted with $CHCl_3$: MeOH : H2O (8 : 2 : 0.2; 7 : 3 : 0.5)) to give nine portions. The fractions were combined on the basis of TLC with Ehrlich's spray reagent. The fourth portion was then separated by silica gel column chromatography and eluted with $CHCl_3$: MeOH 100 : 0–0 : 100) into fractions 1–7, and fraction 4 was further purified by HPLC (Senshu pak PEGASIL ODS Al, 10 × 250 mm, MeOH : H2O (70 : 30), flow rate 4 mL min^{-1}; UV detector at 254 nm) to give five furostanol saponins: compound **1** (504 mg), compound **2** (10 mg), compound **3** (15.4 mg), compound **4** (15.1 mg) and compound **5** (20.3 mg).

3.3. *Structure and identification*

Compound **1**: White power, [m.p.] 215–217°C, $[\alpha]_D^{25}$ –38.2°(H_2O, *c* 0.68), [HR-TOF-MS] 1335.6185 $[M + Na + H]^+$. ESI-MS: 1311.4 $[M - H]^-$, 1335.3 $[M + Na]^+$. For ^1H and ^{13}C-NMR (pyridine-d_5) data, see Tables 1 and 3, respectively.

Compound **2**: Colourless crystal, [m.p.] 220–222°C, $[\alpha]_D^{25}$ –25.2° (H_2O, *c* 0.56), [HR-TOF-MS] 1381.6250 $[M + Na]^+$, ESI-MS: 1357.6 $[M-H]^-$, 1381.6 $[M + Na]^+$. For ^1H and ^{13}C-NMR (pyridine-d_5) data, see Tables 1 and 3, respectively.

Compound **3**: White power, [m.p.] 214–216°C, $[\alpha]_D^{25}$ −29.6° (H_2O, *c* 0.28), [HR-TOF-MS] 1367.6095 $[M + Na]^+$, ESI-MS: 1343.6 $[M - H]^-$, 1367.5 $[M + Na]^+$. For ^1H and ^{13}C-NMR (pyridine-d_5) data, see Tables 1 and 3, respectively.

Compound **4**: White power, [m.p.] 230–232°C, $[\alpha]_D^{25}$ –39.3° (H_2O, *c* 0.50), ESI-MS: 1329.6 $[M - H]^-$, 1353.6 $[M + Na]^+$. For ^1H and ^{13}C-NMR (pyridine-d_5) data, see Tables 1 and 3, respectively.

Compound **5**: Colourless crystal, [m.p.] 225−227°C, $[\alpha]_D^{25}$ –32.5° (H_2O, *c* 0.48), ESI-MS: 1343.8 $[M-H]^-$, 1367.7 $[M + Na]^+$. For ^1H and ^{13}C-NMR (pyridine-d_5) data, see Tables 1 and 3, respectively.

3.4. *Acid hydrolysis and GC-MS*

Each glycoside (5 mg) was heated in a sealed tube with 5mL of aqueous 2 M HCl at 100°C for 2 h. The aglycone was extracted with dichloromethane, and the aqueous residue was evaporated under reduced pressure. Then, 1 mL pyridine and 2 mg $NH_2OH \cdot HCl$ were added to the dry residue, and the mixtures were heated at 100°C for 1 h. After the reaction

Table 3. ^{1}H-NMR data for the compounds **1**–**5** (in pyridine-d_5, 400 MHz).

Position	**1**	**2**	**3**	**4**	**5**
1	0.77, 1.54	0.77, 1.33	0.70, 1.33	0.72, 1.54	0.72, 1.54
2	1.72, 2.00	1.74, 2.00	1.71, 1.95	1.74, 2.00	1.74, 2.00
3	3.90	4.11	4.11	3.90	3.89
4	1.63, 1.87	1.63, 1.89	1.64, 1.89	1.64, 1.89	1.64, 1.89
5	0.89	0.84	0.84	0.90	0.89
6	1.15	1.14	1.14	1.13	1.14
7	0.79, 1.50	0.71, 1.52	0.71, 1.52	0.78, 1.50	0.79, 1.50
8	1.36	1.74	1.74	1.40	1.40
9	0.49	0.86	0.86	0.50	0.49
10	/	/	/	/	/
11	1.22, 1.42	2.23, 2.37	2.25, 2.40	1.22, 1.39	1.17, 1.38
12	1.04, 1.70	/	/	1.68, 1.04	1.00, 1.63
13	/	/	/	/	/
14	0.79	1.33	1.36	1.01	0.98
15	1.32, 1.94	1.53, 2.00	1.53, 2.05	1.39, 2.00	1.32, 1.94
16	4.78	4.37	4.84	4.90	4.43
17	2.42	2.88	2.88	1.93	1.72
18	0.69	1.03	1.11	0.85	0.78
19	0.84	0.85	0.86	0.84	0.84
20	/	2.18	2.18	2.21	2.19
21	1.62	1.41	1.54	1.31	1.18
22	/	/	/	/	/
23	2.21	2.03	2.03	0.78	0.78
24	1.80, 1.42	1.32, 1.77	1.61, 2.03	1.77, 2.01	1.32, 1.77
25	1.93	1.91	1.91	1.91	1.89
26	3.60, 3.92	3.58, 3.91	3.60, 3.91	3.60, 3.91	3.58, 3.91
27	1.01	0.98	0.98	0.97	0.99
26-o-Glc					
1″	4.82	4.83	4.80	4.79	4.84
2″	3.99	3.99	3.99	3.99	3.99
3″	4.20	4.21	4.20	4.20	4.20
4″	4.18	4.21	4.20	4.18	4.18
5″	3.92	3.92	3.92	3.92	3.92
6″	4.35, 4.52	4.35, 4.52	4.35, 4.52	4.35, 4.52	4.35, 4.52
3-o-Glc					
1″	4.84	4.82	4.81	4.83	4.84
2″	4.44	4.44	4.44	4.44	4.44
3″	4.11	4.11	4.11	4.11	4.11
4″	4.46	4.46	4.46	4.46	4.46
5″	3.92	3.92	3.92	3.92	3.92
6″	4.16, 4.66	4.16, 4.66	4.19, 4.68	4.19, 4.68	4.19, 4.68
Rha					
1‴	6.16	6.18	6.17	6.16	6.18
2‴	4.71	4.72	4.73	4.73	4.71
3‴	4.50	4.52	4.51	4.53	4.50
4‴	4.18	4.20	4.20	4.20	4.18
5‴	4.89	4.87	4.87	4.87	4.87
6‴	1.69	1.69	1.69	1.69	1.71
Glc					
1⁗	4.98	4.98	4.98	4.98	4.99
2⁗	4.23	4.23	4.23	4.23	4.23

(*Continued*)

Table 3. Continued.

Position	1	2	3	4	5
3‴	4.04	4.05	4.05	4.05	4.04
4‴	3.77	3.81	3.80	3.80	3.80
5‴	3.80	3.81	3.80	3.80	3.80
6‴	3.99, 4.48	3.99, 4.48	3.99, 4.48	3.99, 4.48	3.99, 4.48
Xyl					
1⁗	5.22	5.23	5.22	5.22	5.23
2⁗	3.92	3.92	3.92	3.92	3.92
3⁗	4.04	4.04	4.04	4.04	4.04
4⁗	4.07	4.07	4.07	4.07	4.07
5⁗	3.64, 4.19	3.64, 4.19	3.64, 4.19	3.64, 4.19	3.64, 4.19
Xyl					
1⁗	5.41	5.42	5.42	5.40	5.42
2⁗	3.39	3.39	3.39	3.39	3.39
3⁗	4.03	4.03	4.03	4.03	4.03
4⁗	4.45	4.45	4.45	4.45	4.45
5⁗	3.46, 4.73	3.46, 4.73	3.46, 4.73	3.46, 4.73	3.46, 4.73

mixtures were cooled, 1.5 mL of Ac_2O was added and the mixtures were heated at 100C for 1 h. The reaction mixtures were evaporated under reduced pressure, and the resulting aldononitrile peracetates were analysed by GC-MS using standard aldononitrile peracetates as authentic samples. Glucose, xylose, rhamnose and galactose were identified for compounds **1** and **2** in a 2 : 2 : 1 : 1 ratio.

3.5. *Inhibition on NCI-H460, SF-268, MCF-7 and HepG2 tumour cell bioassay*

The inhibitory effects of compounds on tumour cells were measured using the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay method (Mosmann, 1983).

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