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STEROIDAL SAPONINS FROM THE RHIZOMES OF *HOSTA SIEBOLDII* AND THEIR CYTOSTATIC ACTIVITY ON HL-60 CELLS

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Key Word Index—*Hosta sieboldii*; Liliaceae; rhizomes; steroidal saponins; spirostanol saponins; furostanol saponins; cytostatic activity; HL-60 cells.

Abstract—A total of eighteen steroidal saponins were isolated from the rhizomes of *Hosta sieboldii*, one of which appeared to be the first isolation from a plant source and six to be new compounds. The structures of the new saponins were determined by spectral data and a few chemical transformations to be (25*R*)-2α,3β-dihydroxy-5α-spirostan-12-one (manogenin) 3-*O*-{*O*-β-D-glucopyranosyl-(1→2)-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside}, (25*R*)-2α,3β-dihydroxy-5α-spirost-9-en-12-one (9,11-dehydromanogenin) 3-*O*-{*O*-β-D-glucopyranosyl-(1→2)-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside}, 9,11-dehydromanogenin 3-*O*-{*O*-β-D-glucopyranosyl-(1→2)-*O*-[*O*-α-L-rhamnopyranosyl-(1→4)-β-D-xylopyranosyl-(1→3)]-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside}, (25*R*)-2α,3β-dihydroxy-26-β-D-glucopyranosyloxy-22-methoxy-5α-furostan-12-one 3-*O*-{*O*-β-D-glucopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside}, (25*R*)-2α,3β-dihydroxy-26-β-D-glucopyranosyloxy-22-methoxy-5α-furost-9-en-12-one 3-*O*-{*O*-β-D-glucopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside} and (25*R*)-5α-spirostan-2α,3β,12β-triol 3-*O*-{*O*-α-L-rhamnopyranosyl-(1→2)-β-D-galactopyranoside}, respectively. Cytostatic activity of the isolated saponins on leukaemia HL-60 cells was examined.

INTRODUCTION

The genus *Hosta* (Liliaceae), with about 20 species, is distributed in east Asia. Takeda and co-workers made phytochemical examinations on the neutral fractions of the saponified methanolic extract of the domestic *Hosta* plants in Japan, such as *Hosta plantaginea*, *H. sieboldiana*, *H. longipes*, *H. montana* var. *liliflora* and *H. kiyosumiensis* and disclosed the occurrence of several steroidal sapogenins [1, 2]. Although the steroids are expected to be present as glycosides in the plants, there has been no exploration of steroidal saponins in the genus *Hosta*, except for our recent reports concerning the steroidal saponins of *H. longipes* [3, 4] and *H. plantaginea* var. *japonica* [5]. As a part of our contribution to the study of this genus, we have now established phytochemical screening of the rhizomes of *H. sieboldii*. This resulted in the isolation of a total of eighteen steroidal saponins, one of which appeared to be the first isolation from a plant source and six to be new compounds. This paper reports the identification and structural determination of the isolated saponins based on spectroscopic data, including two-dimensional NMR techniques and the result of hydrolysis. Cytostatic activity of the isolated saponins on leukaemia HL-60 cells is also described (Table 2).

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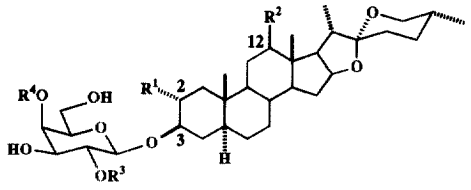
RESULTS AND DISCUSSION

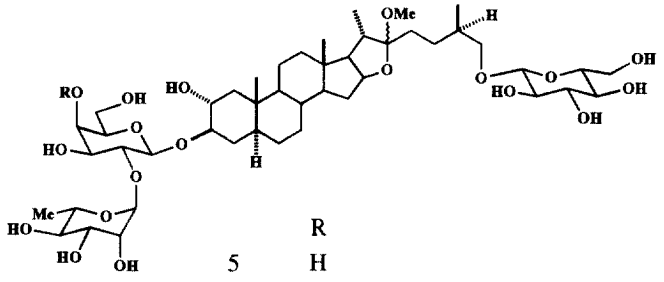
The concentrated 1-butanol-soluble phase of the methanolic extract of *H. sieboldii* rhizomes was repeatedly chromatographed on silica gel, octadecylsilanized (ODS) silica gel and on Diaion HP-20 to furnish eighteen saponins (**1**–**18**). Compounds **1**–**12** were identified as (25*R*)-5α-spirostane-2α,3β-diol (gitogenin) 3-*O*-β-D-galactopyranoside (**1**) [6], gitogenin 3-*O*-{*O*-α-L-rhamnopyranosyl-(1→2)-*O*-β-D-galactopyranoside} (**2**) [4], gitogenin 3-*O*-{*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[β-D-glucopyranosyl-(1→4)]-β-D-galactopyranoside} (**3**) [4], (25*R*)-5α-spirostan-3β-ol (tigogenin) 3-*O*-{*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[β-D-glucopyranosyl-(1→4)]-β-D-galactopyranoside} (**4**) [4], (25*R*)-26-*O*-β-D-glucopyranosyl-22-*O*-methyl-5α-furostan-2α,3β,22ξ,26-

Table 1. ^{13}C NMR spectral data for compounds **1**, **10–18**, **18a** and gitogenin in pyridine-d_5

C	1	10	11	12	13	14	15	16	17	18	18a	gitogenin
1	45.7	45.0	43.4	45.0	45.0	43.5	43.4	45.0	43.4	45.7	46.4	46.5
2	70.6	70.2	70.3	70.1	70.2	70.3	70.3	70.1	70.2	70.6	73.0	73.1
3	85.1	83.9	83.5	83.8	84.4	83.8	83.4	83.8	83.4	85.5	76.7	76.7
4	34.2	33.9	33.8	33.9	34.0	33.8	33.7	33.9	33.7	33.6	37.1	37.2
5	44.6	44.4	42.5	44.3	44.5	42.5	42.4	44.4	42.4	44.7	45.2	45.3
6	28.1	27.8	27.1	27.8	27.8	27.1	27.1	27.8	27.1	28.2	28.4	28.4
7	32.1	31.4	32.5	31.4	31.4	32.5	32.5	31.3	32.4	31.9	31.9	32.4
8	34.6	33.7	36.2	33.6	33.7	36.2	36.1	33.6	36.1	33.8	33.8	34.7
9	54.4	55.3	170.5	55.3	55.4	170.5	170.5	55.3	170.5	53.5	53.7	54.7
10	36.8	37.2	40.5	37.2	37.3	40.5	40.5	37.2	40.5	36.9	37.5	37.6
11	21.4	38.0	120.1	38.0	38.1	120.1	120.1	38.0	120.0	31.8	31.9	21.6
12	40.1	212.4	204.2	212.5	212.4	204.2	204.2	212.5	204.2	79.1	79.2	40.2
13	40.8	55.3	51.4	55.3	55.3	51.4	51.3	55.7	51.7	46.5	46.6	40.9
14	56.3	55.7	52.7	55.7	55.7	52.7	52.6	55.5	52.5	55.0	55.1	56.5
15	32.2	31.5	31.8	31.5	31.5	31.8	31.8	31.5	31.6	32.1	32.2	32.2
16	81.1	79.7	80.2	79.6	79.7	80.2	80.2	79.9	80.4	81.2	81.2	81.2
17	63.0	54.3	54.5	54.2	54.3	54.5	54.5	55.6	55.6	62.9	62.9	63.1
18	16.6	16.1	15.2	16.0	16.1	15.2	15.2	16.0	15.2	11.2	11.2	16.7
19	13.4	12.8	19.4	12.8	12.9	19.4	19.4	12.8	19.3	13.5	13.7	13.8
20	42.0	42.6	43.0	42.6	42.6	43.0	42.9	41.1	41.2	43.0	43.0	42.0
21	15.0	13.9	13.7	13.9	13.8	13.7	13.7	15.0	14.7	14.3	14.3	15.0
22	109.2	109.3	109.5	109.3	109.3	109.5	109.4	112.7	112.9	109.5	109.5	109.2
23	31.8	31.8	31.8	31.7	31.8	31.8	31.8	30.7	30.5	31.9	31.9	31.9
24	29.2	29.2	29.2	29.2	29.2	29.2	29.2	28.2	28.2	29.3	29.3	29.3
25	30.6	30.5	30.5	30.5	30.5	30.5	30.5	34.2	34.2	30.6	30.6	30.6
26	66.8	66.9	67.0	66.9	66.9	67.0	67.0	75.2	75.2	66.9	66.9	66.9
27	17.3	17.3	17.3	17.3	17.3	17.3	17.3	17.1	17.1	17.4	17.3	17.3
OMe								47.3	47.4			
1′	104.1	103.2	103.2	103.2	103.4	103.3	103.2	103.2	103.2	102.1		
2′	72.3	72.5	72.5	72.5	72.7	72.7	72.5	72.5	72.5	76.1		
3′	75.3	75.5	75.5	75.5	75.5	75.5	75.5	75.5	75.5	76.5		
4′	70.2	79.4	79.5	79.4	80.9	81.0	79.4	79.4	79.5	70.8		
5′	77.2	75.7	75.8	75.7	75.5	75.5	75.7	75.7	75.7	76.9		
6′	62.3	60.6	60.6	60.6	60.4	60.4	60.6	60.6	60.6	62.2		
1″		104.7	104.7	104.7	105.1	105.1	104.7	104.7	104.7	101.7		
2″		81.2	81.2	81.2	85.9	86.0	81.2	81.2	81.2	72.5		
3″		87.0	87.1	86.7	78.5	78.5	86.7	87.0	87.0	72.8		
4″		70.4	70.4	70.4	71.8	71.8	70.4	70.4	70.3	74.1		
5″		77.6	77.6	77.5	78.2	78.2	77.5	77.6	77.6	69.3		
6″		62.9	62.9	62.9	63.2	63.2	62.9	62.9	62.9	18.5		
1‴		104.8	104.7	104.7	106.8	106.9	104.7	104.7	104.7			
2‴		76.0	76.0	76.0	76.6	76.6	76.0	76.0	76.0			
3‴		78.1	78.1	78.1	77.7	77.8	78.1	78.1	78.1			
4‴		71.4	71.4	71.3	70.6	70.5	71.3	71.3	71.3			
5‴		78.5	78.5	78.5	79.0	79.0	78.5	78.5	78.4			
6‴		62.7	62.7	62.7	61.9	61.8	62.7	62.7	62.7			
1⁗		104.9	104.9	104.7			10.4.7	104.9	104.9			
2⁗		75.1	75.1	75.2			75.2	75.1	75.1			
3⁗		78.7	78.7	74.7			74.8	78.7	78.7			
4⁗		70.7	70.8	76.1			76.1	70.7	70.7			
5⁗		67.3	67.3	64.0			64.1	67.3	67.3			
1‴′				99.8			99.8	104.9	104.9			
3‴′				72.4			72.5	75.2	75.2			
4‴′				72.5			73.9	78.5	78.4			
4⁗′				73.9			72.5	71.7	71.7			
5⁗′				69.9			69.9	78.6	78.6			
6⁗′				18.6			18.6	62.9	62.9			



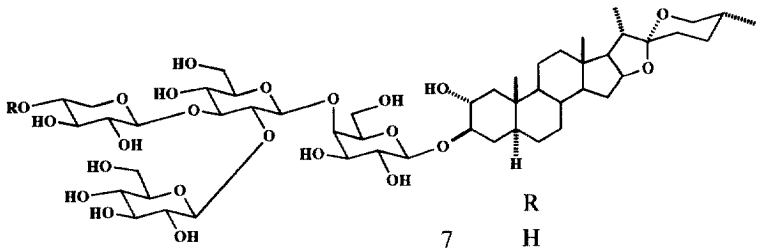
	R^1	R^2	R^3	R^4
1	OH	H	H	H
2	OH	H	α-L-Rha*p*	H
3	OH	H	α-L-Rha*p*	β-D-Glc*p*
4	H	H	α-L-Rha*p*	β-D-Glc*p*
18	OH	OH	α-L-Rha*p*	H



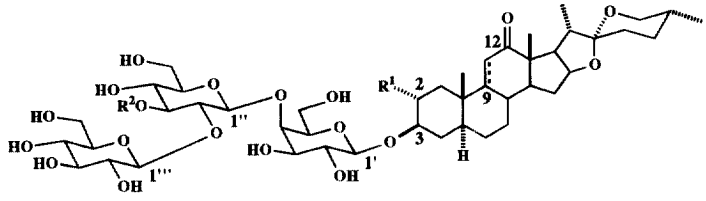
	R
5	H
6	β-D-Glc*p*

tetrol 3-*O*-{*O*-α-L-rhamnopyranosyl-(1→2)-*O*-β-D-galactopyranoside} (**5**) [4], (25*R*)-26-*O*-β-D-glucopyranosyl-22-*O*-methyl-5α-furostan-2α,3β,22ξ,26-tetrol 3-*O*-{*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[β-D-glucopyranosyl-(1→4)]-β-D-galactopyranoside} (**6**) [4], gitogenin 3-*O*-{*O*-β-D-glucopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside} (F-gitonin) (**7**) [7, 8, 9], gitogenin 3-*O*-{*O*-β-D-glucopyranosyl-(1→2)-*O*-[*O*-α-L-rhamnopyranosyl-(1→4)-β-D-xylopyranosyl-(1→3)]-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside} (**8**) [9], (25*R*)-3β-hydroxy-5α-spirostan-12-one (hecogenin) 3-*O*-{*O*-β-D-glucopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside} (**9**) [3], (25*R*)-2α,3β-dihydroxy-5α-spirostan-12-one (manogenin) 3-*O*-{*O*-β-D-glucopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside} (**10**) [10], (25*R*)-2α,3β-dihydroxy-5α-spirost-9-en-12-one (9,11-dehydromanogenin) 3-*O*-{*O*-β-D-glucopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside} (**11**) [3] and manogenin 3-*O*-{*O*-β-D-glucopyranosyl-(1→2)-*O*-[*O*-α-L-rhamnopyranosyl-(1→4)-β-D-xylopyranosyl-(1→3)]-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside} (**12**) [10], respectively. The physical and spectral data are consistent with the indicated literature values. Copies of the original spectra are obtainable from the authors.

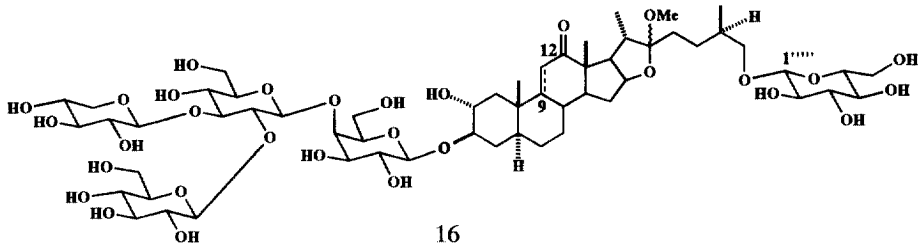
Compound **13** ($C_{45}H_{72}O_{20}$; negative-ion FAB mass spectrum *m/z* 931 $[M-H]^-$) was obtained as an amorphous solid, $[\alpha]_D$ −50.0° in a mixed solvent of chloroform and methanol (1:1). The ^1H NMR spectrum showed signals for four typical steroid methyls; two appeared as singlets at δ 1.07 and 0.75, and the other two as doublets at δ 1.35 (*J* = 6.9 Hz) and 0.70 (*J* = 5.7 Hz). Three anomeric proton signals were also noted at δ 5.28 (*d*, *J* = 7.4 Hz), 5.15 (*d*, *J* = 7.8 Hz) and 4.93 (*d*, *J* = 7.8 Hz). When **13** was submitted to acid hydrolysis with 1 M hydrochloric acid in dioxane-H_2O (1:1), it was hydrolysed to yield an aglycone, identified as manogenin, and D-glucose and D-galactose in a ratio of 2:1 as the carbohydrate compounds. On comparison of the whole ^{13}C NMR spectrum of **13** with that of **10**, the five signals assignable to the terminal xylopyranosyl moiety and the downfield shift by *O*-glycosylation at C-3 of the glucose attached to C-4 of the inner galactopyranosyl residue could not be recognized in the ^{13}C NMR spectrum of **13**. The above data indicated that **13** was a spirostanol saponin related to **10** but missing the terminal xylopyranosyl moiety, confirmative evidence for which was obtained by mild acid hydrolysis of **10** with 0.2 M hydrochloric acid at 100° for 30 min to provide **13**. The structure of **13** was formulated as manogenin 3-*O*-{*O*-β-D-gluco-



	R
7	H
8	α-L-Rha*p*



	R^1	R^2	
9	H	β-D-Xyl*p*	
10	OH	β-D-Xyl*p*	
11	OH	β-D-Xyl*p*	Δ^9
12	OH	α-L-Rha*p*-(1→4)-β-D-Xyl*p*	
13	OH	H	
14	OH	H	Δ^9
15	OH	α-L-Rha*p*-(1→4)-β-D-Xyl*p*	Δ^9



16

17 Δ^9

pyranosyl-(1→2)-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside}.

Acid hydrolysis of **14** ($C_{45}H_{70}O_{20}$) gave an aglycone, identified as 9,11-dehydromanogenin, and D-glucose and D-galactose in a ratio of 2:1 as the carbohydrate compounds. The ^{1}H NMR spectrum displayed three anomeric proton signals at δ 5.29 (*d*, J=7.3 Hz), 5.16 (*d*, J=7.9 Hz) and 4.90 (*d*, J=7.8 Hz), and the ^{13}C NMR proved **14** to have the same triglycoside sequence as **13**. These data were indicative of **14** being 9,11-dehydro derivative of **13**, which was confirmed by the fact that **13** could be prepared by partial acid hydrolysis of **11**. The structure of **14** was characterized as 9,11-dehydromanogenin 3-*O*-{*O*-β-D-glucopyranosyl-(1→2)-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside}.

Acid hydrolysis of **15** ($C_{56}H_{88}O_{28}$) furnished 9,11-dehydromanogenin, and D-glucose, D-galactose, D-xylose and L-rhamnose in a ratio of 2:1:1:1. The pentaglycoside structure of **15** was shown by the characteristic five anomeric proton signals at δ 5.59 (*d*, J=7.8 Hz), 5.46 (*br s*), 5.24 (*d*, J=7.7 Hz), 5.17 (*d*, J=7.7 Hz) and 4.91 (*d*, J=7.9 Hz) in the ^{1}H NMR spectrum. On comparison of the whole ^{13}C NMR signals of **15** with those of **11**, a set of additional six signals due to a terminal α-L-rhamnopyranosyl unit appeared and *O*-glycosylation-induced downfield shift could be recognized at C-4 of the xylose moiety

in **15**, indicating that **15** had the same pentaglycoside sequence as **8** and **12**. Partial acid hydrolysis of **15** yielded **11**. Thus, the structure of **15** was established as 9,11-dehydromanogenin 3-*O*-{*O*-*β*-D-glucopyranosyl-(1→2)-*O*-[*O*-α-L-rhamnopyranosyl-(1→4)-*β*-D-xylopyranosyl-(1→3)]-*O*-*β*-D-glucopyranosyl-(1→4)-*β*-D-galactopyranoside}.

Compound **16** ($C_{57}H_{94}O_{30}$) was shown to be a 22-methoxyfurostanol saponin by Ehrlich's test [11, 12], and the ^{1}H NMR [δ 3.26 (3H, *s*)] and ^{13}C NMR [δ 112.7 (C-22) and 47.3 (Me)] spectra [13]. The ^{1}H NMR spectrum showed five anomeric proton signals at δ 5.59 (*d*, J=7.8 Hz), 5.25 (*d*, J=7.8 Hz), 5.21 (*d*, J=7.9 Hz), 4.91 (*d*, J=7.9 Hz) and 4.85 (*d*, J=7.7 Hz), as well as four steroid methyls at δ 1.41 (*d*, J=6.8 Hz), 1.05 (*s*), 1.00 (*d*, J=6.6 Hz) and 0.73 (*s*). Enzymatic hydrolysis of **16** with *β*-glucosidase gave **10** and D-glucose. Thus, the structure of **16** was assigned as (25*R*)-2α,3*β*-dihydroxy-26-*β*-D-glucopyranosyloxy-22-methoxy-5α-furostan-12-one 3-*O*-{*O*-*β*-D-glucopyranosyl-(1→2)-*O*-[*β*-D-xylopyranosyl-(1→3)]-*O*-*β*-D-glucopyranosyl-(1→4)-*β*-D-galactopyranoside}.

Compound **17** ($C_{57}H_{92}O_{30}$) was also a 22-methoxyfurostanol saponin. The ^{1}H and ^{13}C NMR spectra, and enzymatic hydrolysis of **17**, which gave **11** and D-glucose, confirmed the structure of **17** to be (25*R*)-2α,3*β*-dihydroxy-26-*β*-D-glucopyranosyloxy-22-methoxy-5α-furost-9-en-12-one 3-*O*-{*O*-*β*-D-glucopyranosyl-(1→2)-*O*-[*β*-D-xylopyranosyl-(1→3)]-*O*-*β*-D-glucopyranosyl-(1→4)-*β*-D-galactopyranoside}.

The ^{1}H NMR spectrum of **18** ($C_{39}H_{64}O_{14}$) showed signals for two anomeric protons at δ 6.29 (*br d*, J=1.2 Hz) and 5.00 (*d*, J=7.8 Hz), and four steroid methyl protons at δ 1.43 (*d*, J=6.6 Hz), 1.08 (*s*), 0.93 (*s*) and 0.71 (*d*, J=5.2 Hz). Acid hydrolysis of **18** with 1 M hydrochloric acid gave an aglycone ($C_{27}H_{44}O_5$), and D-galactose and L-rhamnose. The ^{13}C NMR spectrum of **18a** exhibited seven signals between 60–110 ppm [δ 109.5 (C), 81.2 (CH), 79.2 (CH), 76.7 (CH), 73.0 (CH), 66.9 (CH_2) and 62.9 (CH)]. Four of them at δ 109.5, 81.2, 66.9 and 62.9 were assigned to the C-22, C-16, C-26 and C-17 positions in the spirostanol skeleton. Consequently, the remaining three signals at δ 79.2, 76.7 and 73.0 were hydroxy methine carbons. These data were indicative of **18a** being a spirostan derivative with three secondary hydroxyl groups. The ^{13}C NMR signals assigned to hexacyclic nucleus of **18a** featured a close similarity to those of gitogenin with the exceptions of the signals due to C-12 and its neighboring carbons. The methylene carbon signal at δ 40.2 assignable to C-12 in gitogenin was replaced by the oxymethine signal at δ 79.2 in **18a**, accompanied by downfield or upfield shifts of the signals due to C-11, C-13 and C-18 by +10.3, +5.7 and −5.5, respectively. This led to the assignment of the location of a hydroxyl group at C-12 in addition to C-2α and C-3*β*. The C-12*β* equatorial orientation of the hydroxyl group was confirmed by the spin-coupling constant between the proton signals of 12-H and 11-H_2 ($^3J_{12\text{-H},11\beta(ax)}$=11.1 and $^3J_{12\text{-H},11\alpha(eq)\text{-H}}$=4.8 Hz). From the above data, **18a** was shown to be (25*R*)-5α-spirostan-2α,3*β*,12*β*-triol. This was strongly supported by the fact that reduction of the aglycone of **10** with sodium borohydride furnished **18a**. Assignment of the ^{1}H and ^{13}C NMR signals due to the diglycoside moiety of **18** were established by analysis of the ^{1}H-^{1}H COSY spectrum combined with the HMQC data. The diglycoside sequence, rhamnosyl-(1→2)-galactose, and its linkage to the C-3 hydroxyl group of the aglycone was confirmed by the HMBC correlations between the signals δ 6.29 (anomeric proton of the rhamnose) and δ 76.1 (C-2 of the galactose), and δ 5.00 (anomeric proton of the galactose) and δ 85.5 (C-3 of the aglycone). Accordingly, the structure of **18** was determined to be (25*R*)-5α-spirostan-2α,3*β*,12*β*-triol 3-*O*-{*O*-α-L-rhamnopyranosyl-(1→2)-*β*-D-galactopyranoside}. Compounds **13**–**18** are new steroidal saponins.

Table 2. Cytostatic activity of the isolated saponins on leukaemia HL-60 cells

Compounds	Inhibition (%)[a]	IC_{50} (μg ml^{-1})
1	19.3	—[b]
2	94.6	3.0
3	98.8	2.8
4	62.6	4.5
5	65.2	5.9
6	98.6	3.0
7	54.1	3.0
8	58.7	6.5
9	55.4	7.7
10	57.7	7.2
11	53.9	8.2
12	39.3	—
13	0	—
14	27.2	—
15	54.0	8.2
16	29.8	—
17	45.9	—
18	8.7	—
etoposide[c]	100	0.30
methotrexate[c]	100	0.018

[a] Data expressed as percentage of cell growth inhibition at the sample concentration of 10 μg ml^{-1}.
[b] Not measured.
[c] Clinically applied antileukaemic agents.

Cytostatic activity of the isolated saponins on human promyelocytic leukaemia HL-60 cells were evaluated. The cells were continuously treated with the each sample for 72 hours, and the cell growth was measured with an MTT assay procedure. Percentage inhibition at the sample concentration of 10 μg ml^{-1} and the IC_{50} values are listed in Table 2. The activity of the saponins was less potent than that of the clinically applied antileukaemic agents, etoposide and methotrexate, however, the following structure-activity relationships were disclosed. Gitogenin diglycoside (**2**)

and tigogenin triglycoside with a 2,4-branched sugar sequence (**4**) exhibited cytostatic activity with the IC_{50} values of 3.0 and 4.5 $\mu g\,ml^{-1}$, respectively. Introduction of a hydroxyl group onto C-2 of the aglycone of **4** slightly increased the activity (**3**: 2.8 $\mu g\,ml^{-1}$). Removal of the rhamnosyl group from **2** and introduction of a hydroxyl group onto the aglycone C-12 of **2** caused the activity to fall (**1** and **18**: more than 10 $\mu g\,ml^{-1}$). Spirostanol saponins often possess common properties such as hemolytic activity and toxicity to fish, but bisdesmosidic furostanol saponins are inactive. With regard to the cytostatic activity against HL-60 cells, the furostanol saponins (**5** and **6**), which are the proto-type of **2** and **3**, showed considerable activity (**5**: 5.9 $\mu g\,ml^{-1}$; **6**: 3.0 $\mu g\,ml^{-1}$). Among the compounds **7**–**17**, which contained glucosyl-(1→2)-glucosyl-(1→4)-galactosyl moiety as the common saccharide sequence, **7** (gitogenin tetraglycoside) inhibited cell proliferation with an IC_{50} value of 3.0 $\mu g\,ml^{-1}$. Modification of the aglycone moiety with a C-12 carbonyl (manogenin) or a conjugated C-12 carbonyl group (9,11-dehydromanogenin), and glycosyl formation at the C-4 xylosyl moiety with a rhamnosyl group decreased the activity by half to one third (6.5–8.2 $\mu g\,ml^{-1}$) or more (more than 10 $\mu g\,ml^{-1}$). The manogenin triglycoside (**13**) and 9,11-dehydromanogenin triglycoside (**14**), and the furostanol saponins (**16** and **17**) were far less active than the others (more than 10 $\mu g\,ml^{-1}$).

EXPERIMENTAL

General

NMR (ppm, *J* Hz): 1D (Bruker AM-400, 400 MHz for ^{1}H NMR) and 2D (Bruker DRX-500 using XWIN-NMR 2.0 pulse programs, 500 MHz for ^{1}H NMR). CC: silica gel (Fuji–Silysia Chemical), ODS silica gel (Nacalai Tesque) and Diaion HP-20 (Mitsubishi–Kasei). TLC: precoated Kieselgel 60 F_{254} (0.25 mm thick or 0.5 mm thick, Merck) and RP-18 $F_{254}S$ (0.25 mm thick, Merck). HPLC: a Tosoh HPLC system (pump, CCPM; controller, CCP controller PX-8010; detector, UV-8000) equipped with a TSK-gel ODS-Prep column (Tosoh, 4.6 mm i.d. × 250 mm, ODS, 5 μm). Microplate reader: Immuno-Mini NJ-2300 (Inter Med, Japan). HL-60 cells: ICN Biomedicals, USA. RPMI 1640 medium: Gibco, USA. All other chemicals used were of biochemical reagent grade.

Plant material

The rhizomes of *H. sieboldii* used for this experiment were collected at Yamagata prefecture, Japan, in June 1993, and the plant specimen is on file in our laboratory.

Extraction and isolation

The plant material (fresh weight, 4.5 kg) was extracted with hot MeOH. The MeOH extract was concentrated under red. pres. and the viscous concentrate was partitioned between H_2O and *n*-BuOH. The *n*-BuOH-soluble phase was passed through a Diaion HP-20 column using gradients of MeOH in H_2O. The 80% and 100% MeOH eluate frs were combined and chromatographed on silica gel eluting with a stepwise gradient mixture of $CHCl_3$-MeOH system (9:1; 6:1; 4:1; 2:1), and finally with MeOH, gave five fractions (I–V). Fr. II was chromatographed on silica gel eluting with $CHCl_3$-MeOH-H_2O (60:10:1) and ODS silica gel with MeOH-H_2O (4:1) to give **1** (110 mg). Fr. III was also subjected to silica gel CC eluting with $CHCl_3$-MeOH-H_2O (40:10:1) and ODS silica gel CC with MeOH-H_2O (7:3) to result in the isolation of **2** (2.84 g). Fr. IV was further fractionated by a silica gel column eluting with $CHCl_3$-MeOH-H_2O (40:10:1) into two frs (IVa and IVb). Fr. IVa was subjected to silica gel CC eluting with $CHCl_3$-Et_2O-MeOH-H_2O (18:10:7:1) and ODS silica gel CC with MeOH-H_2O (7:3) to give **3** (3.57 g), **4** (171 mg), **13** (30.2 mg), **14** (37.1 mg) and **18** (39.5 mg). Fr. IVb was purified by CC on silica gel eluting with $CHCl_3$-Et_2O-MeOH-H_2O (5:5:4:1) and ODS silica gel with MeOH-H_2O (2:1) to yield **5** (2.71 g), **7** (1.25 g), **9** (452 mg), **10** (588 mg) and **11** (837 mg). Fr. V was chromatographed on silica gel eluting with $CHCl_3$-Et_2O-MeOH-H_2O (7:7:8:1) and ODS silica gel with MeOH-H_2O (2:1) and MeCN-H_2O (1:2; 1:3; 1:4) to give **6** (6.75 g), **8** (367 mg), **12** (29.1 mg), **15** (61.3 mg), **16** (362 mg) and **17** (230 mg).

Compound 1

Amorphous solid. $[\alpha]_D^{25}$ −120° ($CHCl_3$-MeOH, 1:1; *c* 0.10). Negative-ion FABMS *m/z* 593 $[M-H]^-$. IR $\nu_{max}^{KBr}\,cm^{-1}$: 3410 (OH), 2935 and 2870 (CH), 1445, 1375, 1235, 1205, 1170, 1155, 1120, 1085, 1065, 1045, 1005, 985, 980, 950, 920, 895, 860. ^{1}H NMR (pyridine-d_5): δ 4.99 (1H, *d*, *J* = 7.7 Hz, 1′-H), 1.13 (3H, *d*, *J* = 6.9 Hz, 21-Me), 0.81 (3H, *s*, 18-Me), 0.74 (3H, *s*, 19-Me), 0.70 (3H, *d*, *J* = 5.5 Hz, 27-Me).

Compound 13

Amorphous solid. $[\alpha]_D^{25}$ −50.0° ($CHCl_3$-MeOH, 1:1; *c* 0.10). Negative-ion FABMS *m/z* 931 $[M-H]^-$. IR $\nu_{max}^{KBr}\,cm^{-1}$: 3400 (OH), 2940 (CH), 1700 (C=O), 1455, 1430, 1375, 1345, 1300, 1260, 1240, 1155, 1075, 1040, 980, 920, 900, 865, 800. ^{1}H NMR (pyridine-d_5): δ 5.28 (1H, *d*, *J* = 7.4 Hz, 1‴-H), 5.15 (1H, *d*, *J* = 7.8 Hz, 1″-H), 4.93 (1H, *d*, *J* = 7.8 Hz, 1′-H), 1.35 (3H, *d*, *J* = 6.9 Hz, 21-Me), 1.07 (3H, *s*, 18-Me), 0.75 (3H, *s*, 19-Me), 0.70 (3H, *d*, *J* = 5.7 Hz, 27-Me).

Acid hydrolysis of 13

A soln of **13** (5 mg) in 1 M HCl (dioxane-H_2O, 1:1, 5 ml) was heated at 100° for 2 hr under an Ar

atmosphere. After cooling, the reaction mixture was neutralized by passing it through an Amberlite IRA-93ZU (Organo) column and chromatographed on silica gel eluting with a gradient mixture of $CHCl_3$-MeOH (19:1; 1:1) to give manogenin (2 mg) [3] and a mixture of monosaccharides (2 mg). The monosaccharide mixture was diluted with H_2O (1 ml) and treated with (−)-α-methylbenzylamine (5 mg) and $Na[BH_3CN]$ (8 mg) in EtOH (1 ml) at 40° for 4 hr, followed by acetylation with Ac_2O (0.3 ml) in pyridine (0.3 ml). The reaction mixture was passed through a Sep-Pak C_{18} cartridge (Waters) with H_2O-MeCN (4:1; 1:9, each 10 ml). The H_2O-MeCN (1:9) eluate fr. was further passed through a Toyopak IC-SP M cartridge (Tosoh) with EtOH (10 ml) to give a mixture of 1-[(*S*)-*N*-acetyl-α-methylbenzylamino]-1-deoxyalditol acetate derivatives of the monosaccharides [14, 15], which were then analyzed by HPLC under the following conditions: solvent, MeCN-H_2O (2:3); flow rate, 0.8 ml min^{-1}; detection, UV 230 nm. The derivatives of D-glucose and D-galactose were detected. R_t (min): 18.18 (D-galactose derivative); 21.46 (D-glucose derivative).

Preparation of ***13*** *by partial acid hydrolysis of* ***10***

Compound **10** (20 mg) was treated with 0.2 M HCl in dioxane-H_2O (1:1, 10 ml) at 100° for 30 min. After cooling, the reaction mixture was neutralized by passing it through an Amberlite IRA-93ZU column and chromatographed on silica gel eluting with $CHCl_3$-Et_2O-MeOH-H_2O (18:10:7:1) to give **13** (5.9 mg).

Compound ***14***

Amorphous solid. $[\alpha]_D^{25}$ −60.0° ($CHCl_3$-MeOH, 1:1; *c* 0.10). Negative-ion FABMS *m/z* 929 $[M-H]^-$. UV $\lambda_{max}^{dioxane-MeOH(1:1)}$ nm (log ε): 242 (3.81). IR ν_{max}^{KBr} cm^{-1}: 3400 (OH), 2940 (CH), 1660 (C=O), 1455, 1370, 1345, 1300, 1260, 1240, 1155, 1065, 980, 915, 895, 860, 795. ^1H NMR (pyridine-d_5): δ 5.95 (1H, *br s*, 11-H), 5.29 (1H, *d*, *J* = 7.3 Hz, 1‴-H), 5.16 (1H, *d*, *J* = 7.9 Hz, 1″-H), 4.90 (1H, *d*, *J* = 7.8 Hz, 1′-H), 1.41 (3H, *d*, *J* = 6.9 Hz, 21-Me), 1.01 (3H, *s*, 18-Me), 0.91 (3H, *s*, 19-Me), 0.70 (3H, *d*, *J* = 4.9 Hz, 27-Me).

Acid hydrolysis of ***14***

Compound **14** (3 mg) was subjected to acid hydrolysis as described for **13** to give 9,11-dehydromanogenin (1.2 mg) [3] and a mixture of monosaccharides (0.4 mg). The monosaccharides were identified as D-glucose and D-galactose by HPLC analysis of their corresponding 1-[(*S*)-*N*-acetyl-α-methylbenzylamino]-1-deoxyalditol acetate derivatives. R_t (min): 18.24 (D-galactose derivative); 21.64 (D-glucose derivative).

Preparation of ***14*** *by partial acid hydrolysis of* ***11***

Compound **11** (38 mg) was subjected to partial acid hydrolysis as described for **10** to give **14** (9.6 mg).

Compound ***15***

Amorphous solid. $[\alpha]_D^{25}$ −26.0° (MeOH; *c* 0.10). Negative-ion FABMS *m/z* 1207 $[M-H]^-$. Positive-ion FABMS *m/z* 1231 $[M+Na]^+$. UV λ_{max}^{MeOH} nm (log ε): 237 (4.04). IR ν_{max}^{KBr} cm^{-1}: 3400 (OH), 2925 and 2870 (CH), 1660 (C=O), 1450, 1370, 1340, 1295, 1260, 1240, 1155, 1060, 1040, 980, 920, 895, 865, 805. ^1H NMR (pyridine-d_5): δ 5.94 (1H, *br s*, 11-H), 5.59 (1H, *d*, *J* = 7.8 Hz, 1‴-H), 5.46 (1H, *br s*, 1⁗-H), 5.24 (1H, *d*, *J* = 7.7 Hz, 1⁗-H), 5.17 (1H, *d*, *J* = 7.7 Hz, 1″-H), 4.91 (1H, *d*, *J* = 7.9 Hz, 1′-H), 1.65 (3H, *d*, *J* = 6.3 Hz, 6⁗-Me), 1.40 (3H, *d*, *J* = 6.7 Hz, 21-Me), 1.00 (3H, *s*, 18-Me), 0.89 (3H, *s*, 19-Me), 0.70 (3H, *d*, *J* = 5.1 Hz, 27-Me).

Acid hydrolysis of ***15***

Compound **15** (5 mg) was subjected to acid hydrolysis as described for **13** to give 9,11-dehydromanogenin (1.1 mg) and a mixture of monosaccharides (2 mg). The monosaccharides were identified as D-glucose, D-galactose, D-xylose and L-rhamnose by HPLC analysis of their corresponding 1-[(*S*)-*N*-acetyl-α-methylbenzylamino]-1-deoxyalditol acetate derivatives. R_t (min): 16.96 (D-xylose derivative); 18.91 (D-galactose derivative); 22.32 (D-glucose derivative); 25.23 (L-rhamnose derivative).

Partial acid hydrolysis of ***15***

Compound **15** (10 mg) was subjected to partial acid hydrolysis as described for **10** to give **11** (3.9 mg).

Compound ***16***

Amorphous solid. $[\alpha]_D^{25}$ −60.0° (MeOH; *c* 0.10). Negative-ion FABMS *m/z* 1257 $[M-H]^-$. Positive-ion FABMS *m/z* 1281 $[M+Na]^+$. IR ν_{max}^{KBr} cm^{-1}: 3400 (OH), 2925 (CH), 1695 (C=O), 1445, 1415, 1370, 1255, 1155, 1065, 1030, 885, 795. ^1H NMR (pyridine-d_5): δ 5.59 (1H, *d*, *J* = 7.8 Hz, 1‴-H), 5.25 (1H, *d*, *J* = 7.8 Hz, 1⁗-H), 5.21 (1H, *d*, *J* = 7.9 Hz, 1″-H), 4.91 (1H, *d*, *J* = 7.9 Hz, 1′-H), 4.85 (1H, *d*, *J* = 7.7 Hz, 1⁗′-H), 3.26 (3H, *s*, OMe), 1.41 (3H, *d*, *J* = 6.8 Hz, 21-Me), 1.05 (3H, *s*, 18-Me), 1.00 (3H, *d*, *J* = 6.6 Hz, 27-Me), 0.73 (3H, *s*, 19-Me).

Enzymatic hydrolysis of ***16***

Compound **16** (10 mg) was treated with β-glucosidase (10 mg) in HOAc/NaOAc buffer (pH 5, 5 ml) at room temp. for 72 hr. The reaction mixture was chromatographed on silica gel eluting with $CHCl_3$-Et_2O-MeOH-H_2O (5:5:4:1) to yield **10** (6.1 mg) and D-

glucose. D-Glucose: TLC, R_f 0.40 (*n*-BuOH-Me_2CO-H_2O, 4:5:1).

Compound 17

Amorphous solid. $[\alpha]_D^{25}$ −24.0° (MeOH; *c* 0.10). Negative-ion FABMS *m/z* 1255 $[M-H]^-$. Positive-ion FABMS *m/z* 1279 $[M+Na]^+$. UV λ_{max}^{MeOH} nm (log ε): 237 (4.05). IR ν_{max}^{KBr} cm^{-1}: 3420 (OH), 2925 (CH), 1660 (C=O), 1455, 1370, 1300, 1260, 1160, 1070, 1040, 915, 890. ^1H NMR (pyridine-d_5): δ 5.93 (1H, *br s*, 11-H), 5.60 (1H, *d*, *J* = 7.8 Hz, 1‴-H), 5.25 (1H, *d*, *J* = 7.8 Hz, 1⁗-H), 5.21 (1H, *d*, *J* = 7.8 Hz, 1″-H), 4.92 (1H, *d*, *J* = 7.8 Hz, 1′-H), 4.85 (1H, *d*, *J* = 7.7 Hz, 1⁗′-H), 3.28 (3H, *s*, OMe), 1.46 (3H, *d*, *J* = 6.8 Hz, 21-Me), 1.00 (3H, *d*, *J* = 6.3 Hz, 27-Me), 0.99 (3H, *s*, 18-Me), 0.90 (3H, *s*, 19-Me).

Enzymatic hydrolysis of 17

Compound **17** (15 mg) was subjected to enzymatic hydrolysis as described for **16** to give **11** (5.2 mg) and D-glucose.

Compound 18

Amorphous solid. $[\alpha]_D^{25}$ −84.0° ($CHCl_3$-MeOH, 1:1; *c* 0.10). Negative-ion FABMS *m/z* 755 $[M-H]^-$. IR ν_{max}^{KBr} cm^{-1}: 3410 (OH), 2930 (CH), 1450, 1375, 1340, 1255, 1235, 1065, 1045, 975, 955, 915, 895, 860. ^1H NMR (pyridine-d_5): δ 6.29 (1H, *d*, *J* = 1.2 Hz, 1″-H), 5.00 (1H, *d*, *J* = 7.8 Hz, 1′-H), 4.85 (1H, *dq*, *J* = 9.5, 6.1 Hz, 5″-H), 4.82 (1H, *dd*, *J* = 3.4, 1.2 Hz, 2″-H), 4.65 (1H, *dd*, *J* = 9.2, 7.8 Hz, 2′-H), 4.62 (1H, overlapping, 16-H), 4.61 (1H, *dd*, *J* = 9.5, 3.4 Hz, 3″-H), 4.46 (1H, *br d*, *J* = 3.7 Hz, 4′-H), 4.45 (1H, *dd*, *J* = 11.1, 6.6 Hz, 6′a-H), 4.37 (1H, *dd*, *J* = 11.1, 5.3 Hz, 6′b-H), 4.28 (1H, *dd*, *J* = 9.5, 9.5 Hz, 4″-H), 4.27 (1H, *dd*, *J* = 9.2, 3.7 Hz, 3′-H), 4.12 (1H, *br dd*, *J* = 6.6, 5.3 Hz, 5′-H), 4.09 (1H, *ddd*, *J* = 11.7, 8.8, 4.6 Hz, 2-H), 3.89 (1H, *ddd*, *J* = 11.1, 8.8, 5.2 Hz, 3-H), 3.61 (1H, *dd*, *J* = 10.5, 2.3 Hz, 26a-H), 3.55 (1H, *dd*, *J* = 10.5, 10.5, 26b-H), 3.51 (1H, *dd*, *J* = 12.0, 5.4 Hz, 12-H), 1.60 (3H, *d*, *J* = 6.1 Hz, 6″-Me), 1.43 (3H, *d*, *J* = 6.6 Hz, 21-Me), 1.08 (3H, *s*, 18-Me), 0.93 (3H, *s*, 19-Me), 0.71 (3H, *d*, *J* = 5.2 Hz, 27-Me).

Acid hydrolysis of 18

Compound **18** (16 mg) was subjected to acid hydrolysis as described for **13** to give **18a** (3.8 mg) and a mixture of monosaccharides (4.6 mg). The monosaccharides were identified as D-galactose and L-rhamnose by HPLC analysis of their corresponding 1-[(*S*)-*N*-acetyl-α-methylbenzylamino]-1-deoxyalditol acetate derivatives. R_t (min): 20.97 (D-galactose derivative); 28.24 (L-rhamnose derivative).

Compound 18a

Amorphous solid. $[\alpha]_D^{25}$ −99.0° ($CHCl_3$; *c* 0.20). EIMS *m/z* (rel. int.) 448 $[M]^+$ (6), 376 (15), 334 (21), 316 (53), 264 (16), 139 (100). IR ν_{max}^{KBr} cm^{-1}: 3320 (OH), 2930, 2885 and 2870 (CH), 1445, 1375, 1340, 1275, 1240, 1215, 1140, 1095, 1075, 1055, 1020, 1000, 975, 915, 890, 855. ^1H NMR (pyridine-d_5 + methanol-d_4): δ 4.60 (1H, *q*-like, *J* = 7.3 Hz, 16-H), 3.93 (1H, *ddd*, *J* = 11.5, 8.7, 4.7 Hz, 2-H), 3.75 (1H, *ddd*, *J* = 10.9, 8.7, 5.1 Hz, 3-H), 3.59 (1H, *dd*, *J* = 10.5, 3.5 Hz, 26a-H), 3.51 (1H, *dd*, *J* = 10.5, 10.5, 26b-H), 3.48 (1H, *dd*, *J* = 11.1, 4.8 Hz, 12-H), 1.37 (3H, *d*, *J* = 6.5 Hz, 21-Me), 1.04 (3H, *s*, 18-Me), 0.87 (3H, *s*, 19-Me), 0.72 (3H, *d*, *J* = 5.8 Hz, 27-Me).

*Preparation of **18a** by acid hydrolysis of **10** followed by reduction with $NaBH_4$*

A soln of **10** (100 mg) in 1 M HCl (dioxane-H_2O, 1:1, 15 ml) was heated at 100° for 2 hr under an Ar atmosphere. After cooling, the reaction mixture was neutralized by passing it through an Amberlite IRA-93ZU column and chromatographed on silica gel eluting with a gradient mixture of $CHCl_3$-MeOH (19:1; 1:1) to give an aglycone (manogenin) (29.5 mg). A mixture of the aglycone (29.5 mg) and $NaBH_4$ (20 mg) in MeOH (5 ml) was stirred at room temp. for 30 min. The reaction mixture was subjected to silica gel CC eluting with $CHCl_3$-MeOH (19:1) to yield **18a** (25 mg).

Cell culture and assay for cytostatic activity

HL-60 cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum supplemented with L-glutamine, 100 units ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin. The leukaemia cells were washed and resuspended in the above medium to 3×10^4 cells ml^{-1}, and 196 μl of this cell suspension were placed in each well of a 96-well flat-bottom plate. The cells were incubated for 24 hr at 37° in 5% CO_2/air. After incubation, 4 μl of EtOH-H_2O (1:1) soln containing the sample was added to give final concentrations of 0.01–10 μg ml^{-1}; 4 μl of EtOH-H_2O (1:1) was added into control wells. The cells were incubated for a further 72 hr in the presence of each agent, and then cell growth was evaluated with an MTT assay procedure [16]. The MTT assay was carried out according to a modified method of Sargent and Tayler as follows. After termination of cell culture, 10 μl of 5 mg ml^{-1} MTT in phosphate buffered saline was added to every well and the plate reincubated at 37° in 5% CO_2/air for a further 4 hr. The plate was centrifuged at 1500 *g* for 5 min to precipitate cells and formazan and then 150 μl of the supernatant was removed from every well, and 175 μl of DMSO was added to dissolve the formazan crystals. The plate was mixed on a microshaker for 10 min, and then read on a microplate reader at 550 nm. A dose response curve was plotted for the each sample which showed

more than 50% of cell growth inhibition at the sample concentration of 10 $\mu g\,ml^{-1}$, and a concentration giving 50% inhibition (IC_{50}) was calculated.

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