Novel Polyoxygenated Spirostanol Glycosides from the Rhizomes of *Helleborus orientalis*

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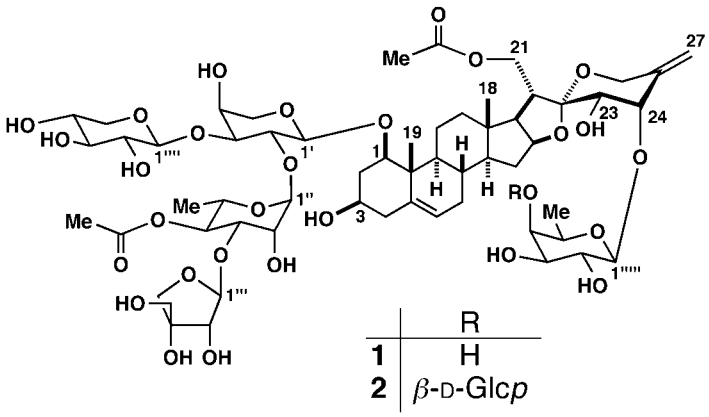
The two new polyoxygenated spirostanol bisdesmosides **1** and **2** and the new trisdesmoside **3**, named hellebosaponin A (**1**), B (**2**), and C (**3**), respectively, were isolated from the MeOH extract of the rhizomes of *Helleborus orientalis*. The structures of the new compounds were elucidated as (1*β*,3*β*,23*S*,24*S*)-21-(acetyloxy)-24-[(*β*-D-fucopyranosyl)oxy]-3,23-dihydroxyspirosta-5,25(27)-dien-1-yl *O*-*β*-D-apiofuranosyl-(1→3)-*O*-(4-*O*-acetyl-*α*-L-rhamnopyranosyl)-(1→2)-*O*-[*β*-D-xylopyranosyl-(1→3)]-*α*-L-arabinopyranoside (**1**), (1*β*,3*β*,23*S*,24*S*)-21-(acetyloxy)-24-{[*O*-*β*-D-glucopyranosyl-(1→4)-*β*-D-fucopyranosyl]oxy}-3,23-dihydroxyspirosta-5,25(27)-dien-1-yl *O*-*β*-D-apiofuranosyl-(1→3)-*O*-(4-*O*-acetyl-*α*-L-rhamnopyranosyl)-(1→2)-*O*-[*β*-D-xylopyranosyl-(1→3)]-*α*-L-arabinopyranoside (**2**), and (1*β*,3*β*,23*S*,24*S*)-24-[(*β*-D-fucopyranosyl)oxy]-21-{[*O*-*β*-D-glucopyranosyl-(1→2)-*β*-D-galactopyranosyl]oxy}-3,23-dihydroxyspirosta-5,25(27)-dien-1-yl *O*-*β*-D-apiofuranosyl-(1→3)-*O*-(4-*O*-acetyl-*α*-L-rhamnopyranosyl)-(1→2)-*O*-[*β*-D-xylopyranosyl-(1→3)]-*α*-L-arabinopyranoside (**3**), respectively, on the basis of detailed spectroscopic studies and chemical evidence.

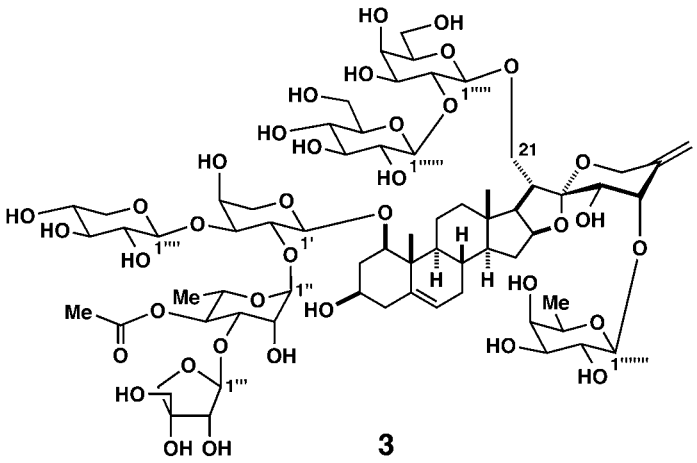
Introduction. – *Helleborus orientalis* LAM. (Ranunculaceae), known by the name 'Lenten Rose' in Europe, is indigenous to Greece and Turkey [1]. Its rhizomes contain several bufadienolide glycosides and were used as a cardiotonic agent [2]. However, presently, this plant is cultivated only for ornamental purposes because an extract prepared from the rhizomes produces harmful side effects on the heart such as heart block and arrhythmia. Plants containing cardiac glycosides, for example, *Digitalis purpurea* [3] and *Convallaria majalis* [4], concomitantly produce spirostanol and/or furostanol glycosides. We undertook a phytochemical work on *H. orientalis* rhizomes, focusing particularly on the steroidal glycoside constituents, which resulted in the isolation of the two new polyoxygenated spirostanol bisdesmosides **1** and **2** and the new trisdesmoside **3**, named hellebosaponin A (**1**), B (**2**), and C (**3**), respectively. This paper mainly reports the structure determination of the new spirostanol glycosides on the basis of detailed spectroscopic studies and chemical evidence.

Results and Discussion. – The fresh rhizomes of *H. orientalis* (2.7 kg) were extracted with refluxing MeOH, and the MeOH extract was passed through a porous-polymer-resin (*Diaion-HP-20*) column. The 80% MeOH eluate portion, in which steroidal glycosides were enriched, was subjected to column chromatography over silica gel and octadecylsilanized (ODS) silica gel to yield **1** (170 mg), **2** (70 mg), and **3** (89 mg).

Hellebosaponin A (**1**) was obtained as an amorphous solid with a molecular formula of $C_{58}H_{88}O_{29}$, as determined by data of the positive- and negative-ion FAB-MS



	R
1	H
2	β-D-Glc*p*



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showing an $[M+Na]^+$ at *m/z* 1271 and an $[M-H]^-$ at *m/z* 1247, respectively, ^{13}C-NMR spectrum (*Table 1*) with a total of 58 C-signals, and elemental analysis.

The ^{1}H-NMR spectrum ((D$_5$)pyridine; *Table 1*) of **1** contained signals for two angular Me groups at δ 1.38 and 1.04 (each *s*), and the ^{13}C-NMR spectrum showed an acetal C-atom at δ 111.0, suggesting **1** to have the spirostan skeleton [5]. Furthermore, the ^{1}H-NMR spectrum of **1** displayed signals for five anomeric protons at δ 6.43 (*d*, *J* = 0.9 Hz), 5.93 (*d*, *J* = 2.9 Hz), 5.14 (*d*, *J* = 7.9 Hz), 4.90 (*d*, *J* = 7.7 Hz), and 4.62 (*d*, *J* = 7.3 Hz), as well as two three-proton *d* at δ 1.47 (*J* = 6.4 Hz) and 1.37 (*J* = 6.2 Hz), which were associated with the Me signals at δ (C) 17.3 and 18.3, respectively, indicating that two of the five sugar moieties were 6-deoxyhexoses. The presence of two acetyl groups in **1** was shown by the signals at δ(H) 2.21 (3 H, *s*)/δ(C) 170.6 (C=O) and 21.1 (Me), and δ(H) 1.92 (3 H, *s*)/δ(C) 170.8 (C=O) and 20.9 (Me).

Acid hydrolysis of **1** with 0.2M HCl in dioxane/H_2O 1:1 gave D-apiose, L-arabinose, D-fucose, L-rhamnose, and D-xylose as the carbohydrate moieties, while the labile aglycone was decomposed under acidic conditions. The ^{1}H,^{1}H-COSY, 2D-TOCSY, and

Table 1. *^{1}H- and ^{13}C-NMR Data for Compound* **1**[a])

	δ(H)[b])	δ(C)		δ(H)[b])	δ(C)
H–C(1)	3.77 (*dd*, *J* = 12.0, 3.9)	84.3	H–C(1′)	4.62 (*d*, *J* = 7.3)	100.7
CH_2(2)	2.69 (br. *dd*, *J* = 12.0, 3.9, H_{eq}),	38.0	H–C(2′)	4.54 (*dd*, *J* = 9.0, 7.3)	72.6
	2.35 (*q*-like, *J* = 12.0, H_{ax})		H–C(3′)	4.00 (*dd*, *J* = 9.0, 3.1)	85.1
H–C(3)	3.88 (*m*, $w_{1/2}$ = 17.1)	38.0	H–C(4′)	4.39 (br. *s*)	69.7
CH_2(4)	2.62 (br. *dd*, *J* = 12.4, 4.0, H_{eq}),	43.8	CH_2(5′)	4.21 (br. *d*, *J* = 12.5, H_a),	67.1
	2.73 (*t*-like, *J* = 12.4, H_{ax})			3.65 (br. *d*, *J* = 12.3, H_b)	
C(5)	–	139.4			
H–C(6)	5.63 (br. *d*, *J* = 5.4)	124.8	H–C(1″)	6.43 (*d*, *J* = 0.9)	100.6
CH_2(7)	1.79 (H_{eq}), 1.50 (H_{ax})	31.8	H–C(2″)	4.90 (*dd*, *J* = 3.0, 0.9)	71.5
H–C(8)	1.50	33.0	H–C(3″)	4.72 (*dd*, *J* = 9.8, 3.0)	77.7
H–C(9)	1.52	50.3	H–C(4″)	5.88 (*dd*, *J* = 9.8, 9.8)	74.6
C(10)	–	42.8	H–C(5″)	4.90 (*dq*, *J* = 9.8, 6.2)	66.6
CH_2(11)	2.94 (H_{eq}), 1.54 (H_{ax})	24.0	Me(6″)	1.37 (*d*, *J* = 6.2)	18.3
CH_2(12)	1.79 (H_{eq}), 1.31 (H_{ax})	40.0	H–C(1‴)	5.93 (*d*, *J* = 2.9)	112.1
C(13)	–	40.9	H–C(2‴)	4.66 (*d*, *J* = 2.9)	77.9
H–C(14)	1.11	56.9	C(3‴)	–	80.0
CH_2(15)	1.82 (H_α), 1.41 (H_β)	32.4	CH_2(4‴)	4.52 (*d*, *J* = 9.5, H_a),	74.9
H–C(16)	4.63	83.6		4.23 (*d*, *J* = 9.5, H_b)	
H–C(17)	1.83 (*dd*, *J* = 8.6, 7.5)	58.7	CH_2(5‴)	4.03 (br. *s*, 2 H)	65.3
Me(18)	1.04 (*s*)	16.8			
Me(19)	1.38 (*s*)	15.0	H–C(1⁗)	4.90 (*d*, *J* = 7.7)	106.7
H–C(20)	3.24	42.7	H–C(2⁗)	3.86 (*dd*, *J* = 8.7, 7.7)	74.5
CH_2(21)	4.36 (H_a), 4.33 (H_b)	65.1	H–C(3⁗)	4.09 (*dd*, *J* = 9.5, 8.7)	78.5
C(22)	–	111.0	H–C(4⁗)	4.09	70.9
H–C(23)	4.15 (*d*, *J* = 4.0)	71.4	CH_2(5⁗)	4.25 (*dd*, *J* = 12.0, 4.3, H_a),	67.1
H–C(24)	4.77 (*d*, *J* = 4.0)	82.1		3.65 (*t*-like, *J* = 12.0, H_b)	
C(25)	–	143.5			
CH_2(26)	4.81 (*d*, *J* = 11.7, H_{eq}),	61.5	H–C(1⁗′)	5.14 (*d*, *J* = 7.9)	106.4
	3.97 (*d*, *J* = 11.7, H_{ax})		H–C(2⁗′)	4.42 (*dd*, *J* = 9.7, 7.9)	73.1
CH_2(27)	5.24 (br. *s*, H_a),	114.1	H–C(3⁗′)	4.06 (*dd*, *J* = 9.7, 3.2)	75.4
	5.10 (br. *s*, H_b)		H–C(4⁗′)	3.97 (*dd*, *J* = 3.2, 3.0)	72.8
			H–C(5⁗′)	3.77 (*qd*, *J* = 6.4, 3.0)	71.5
Me*C*O	–	170.8	Me(6⁗′)	1.47 (*d*, *J* = 6.4)	17.3
*Me*CO	1.92 (*s*)	20.9			
			Me*C*O	–	170.6
			*Me*CO	2.21 (*s*)	21.1

[a]) Spectra were measured in (D_5)pyridine. [b]) δ in ppm; *J* in Hz.

HMQC experiments disclosed the structural fragments of the aglycone moiety, which were connected by interpretation of the HMBC spectrum (optimized for *J*(C,H) = 8 Hz), leading to the structure of the aglycone with the polyoxygenated spirostanol skeleton. Analysis of the phase-sensitive NOESY spectrum made the aglycon configuration assignable. The ^{1}H,^{1}H-COSY and 2D-TOCSY experiments allowed the sequential assignment of the resonances for each monosaccharide, starting from the easily distinguished anomeric protons. The sugar sequences and their linkage positions to the aglycone were determined on the basis of the HMBC information and the transformation of **1** by Ac_2O/pyridine treatment to its tridecaacetate **1a**. The structure of **1** was established as (1β,3β,23*S*,24*S*)-21-(acetyloxy)-24-[(β-D-fucopyranosyl)oxy]-

3,23-dihydroxyspirosta-5,25(27)-dien-1-yl *O*-*β*-D-apiofuranosyl-(1 → 3)-*O*-(4-*O*-acetyl-*α*-L-rhamnopyranosyl)-(1 → 2)-*O*-[*β*-D-xylopyranosyl-(1 → 3)]-*α*-L-arabinopyranoside.

The ^{1}H,^{1}H-COSY and 2D-TOCSY spectra of **1** afforded two partial structures for the aglycone (C(1) to C(4)) and (C(6) to C(12)/C(21)). The H−C(3) (*m* at *δ* 3.88) was coupled with two CH_2 groups at *δ* 2.69 (br. *dd*, *J* = 12.0, 3.9 Hz, H_{eq}−C(2)) and 2.35 (*q*-like, *J* = 12.0 Hz, H_{ax}−C(2)), and *δ* 2.73 (*t*-like, *J* = 12.4 Hz, H_{ax}−C(4)) and 2.62 (br. *dd*, *J* = 12.4, 4.0 Hz, H_{eq}−C(4)). The signals at *δ* 2.69 and 2.35 had spin-coupling links with the *dd* at *δ* 3.77 (*J* = 12.0, 3.9 Hz) arising from an OCH, while the other CH_2 resonances at *δ* 2.73 and 2.62 showed no additional correlation. This indicated the presence of O-atoms at C(1) and C(3). The signals for the olefinic H−C(6) and the OCH proton H−C(16) were identified at *δ* 5.63 (br. *d*, *J* = 5.4 Hz) and 4.63 (*m*), respectively. However, the *d* due to Me(21) of the spirostan skeleton could not be observed in **1** and was replaced by the OCH_2 signals at *δ* 4.36 and 4.33, which were correlated to the one-bond coupled *δ* (C) 65.1, compatible with an O-atom at C(21). These two partial structures, three quaternary C-atoms (C(5), C(10), and C(13)), and two tertiary Me groups (C(18) and C(19)) were established (A to E rings) by the HMBC data as shown by *Fig. 1*. The structure of ring F (C(22) to C(27)) was suggested by the following data. The protons of an

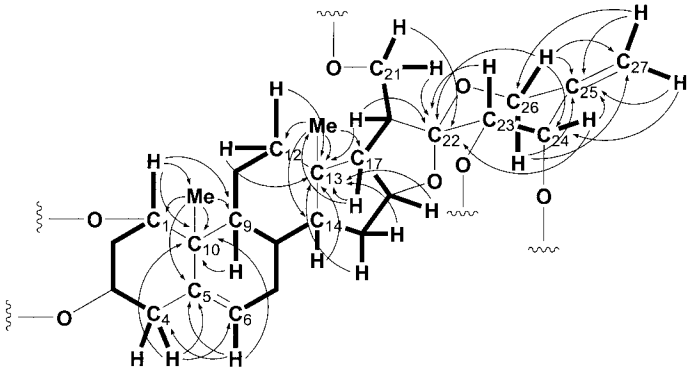

Fig. 1. *HMBC Correlations of the aglycone moiety of* **1**

OCH_2 group were observed at *δ* 4.81 and 3.97 as an *AB*('*q*') spin system with *J* = 11.7 Hz. The C(22) at *δ* (C)111.0 showed long-range correlations with one of the CH_2 protons at *δ* 3.97, along with H−C(20) at *δ* 3.24 (*m*). The proton at *δ* 3.97, in turn, displayed a HMBC with the olefinic C-atoms at *δ* 143.5 (C) and 114.1 (CH_2). On the other hand, two adjacent OCH signals were identified at *δ* 4.77 and 4.15. The *δ* 4.15 signal showed an HMBC with the signal of C(22), while another resonance at *δ* 4.77 was correlated not only with the C(22) signal but also with *δ* 143.5 of an olefinic C-atom. Thus, the presence of an O-atom at C(23) and C(24) and the bond C(25)=C(27) were established. All of these data were consistent with the spirosta-5,25(27)-diene structure for the aglycone of **1**, with an O-atom at C(1), C(3), C(21), C(23), and C(24). The NOE correlations in the phase-sensitive NOESY experiment, H−C(8)/Me(18) and Me(19), H−C(14)/H−C(9), H−C(16), and H−C(17), H−C(16)/H−C(17) and H_{ax}−C(26), and Me(18)/H−C(20) provided evidence for the usual B/C *trans*, C/D *trans*, and D/E *cis* ring fusions, and *α*-orientations at C(20) and C(22) (see *Fig. 2*). The *β*-configurations at C(1) and C(3) were established by the coupling constants of H−C(1) and H−C(3) (*δ* 3.77 (*dd*, *J* = 12.0, 3.9 Hz, H−C(1)) and 3.88 (*m*, $w_{1/2}$ = 17.1 Hz, H−C(3))) and were supported by NOEs between H−C(1) and H−C(9), and between H−C(1) and H−C(3). The NOEs from H−C(23) to H−C(20) and CH_2(21), and H−C(23) to H−C(24), and a small coupling constant between H−C(23) and H−C(24) (*J* = 4.0 Hz) allowed the assignments of the (23*S*) and (24*S*) configurations.

When the ^{1}H-NMR spectrum of the peracetate **1a** was compared with that of **1**, the signals due to H−C(3) and H−C(23) were shifted downfield by 0.94 and 1.23 ppm, respectively, while H−C(1), CH_2(21), and H−C(24) were almost unaffected. These findings indicated that C(3) and C(23) were substituted by a free OH group and that C(1), C(21), and C(24) were substituted. The ^{1}H,^{1}H-COSY experiment with **1** allowed the sequential assignments from H−C(1) to CH_2(5) and Me(6) of four monosaccharides. Their *m* patterns and

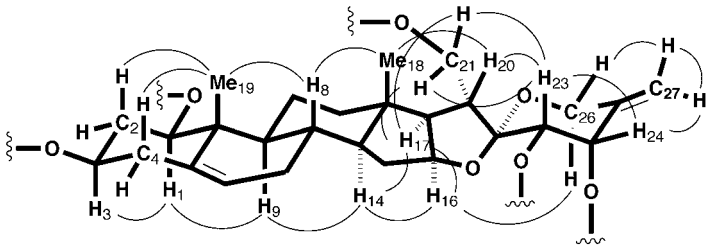

Fig. 2. *NOE Correlations of the aglycone moiety of* **1**

coupling constants allowed the identification of an α-L-arabinopyranosyl ($^4C_1$), β-D-fucopyranosyl ($^4C_1$), α-L-rhamnopyranosyl ($^1C_4$), and β-D-xylopyranosyl ($^4C_1$) unit. In addition, two pairs of *AB*('*q*') signals at δ 5.93 and 4.66 ($J = 2.9$ Hz), and δ 4.52 and 4.23 ($J = 9.5$ Hz), and a two-proton-broad *s* at δ 4.03, along with the results of acid hydrolysis, were indicative of an apiofuranosyl unit. The relatively large *J* values of the anomeric protons of the arabinosyl, fucosyl, and xylosyl moieties ($J = 7.3 - 7.9$ Hz) indicated α anomeric orientation for the L-arabinosyl, and β orientation for the D-fucosyl and D-xylosyl moieties. For the rhamnosyl residue, the large ^1J(C,H) value (175.2 Hz) and three-bond coupled strong HMBCs from the anomeric proton to C(3) and C(5) (dihedral angles between H–C(1) and C(3), and between H–C(1) and C(5) *ca.* 180°), indicated that the anomeric proton was equatorial thus possessing an α-L-pyranoid anomeric form [6] (see *Fig. 3*). The ^{13}C-NMR shift of the anomeric C-atom of the apiosyl unit at δ 112.1 gave evidence for a β-D anomer [7]. All the proton signals for the sugar moiety thus assigned were associated with the one-bond coupled C-signals by means of the HMQC plot. The apiosyl, fucosyl, and xylosyl residues were considered to be a terminal unit, as shown by the absence of any glycosylation shift for their δ(C), while the C(2) and C(3) positions of the arabinosyl unit and the C(3) and C(4) of the rhamnosyl unit were suggested to be substituted by comparison with those of authentic methyl glycosides [8]. The fucosyl residue was shown to be directly attached at C(24) of the aglycone by an HMBC between the signals of the anomeric proton of the fucosyl unit at δ 5.14 and C(24) of the aglycone at δ 82.1 (see *Fig. 3*). The anomeric proton of the apiosyl moiety at δ 5.93 showed a ^3J(C,H) correlation with C(3) of the rhamnosyl residue at δ 77.7, whose anomeric proton at δ 6.43, in turn, showed a long-range correlation with C(2) of the arabinosyl moiety at δ 72.6. The anomeric proton of the xylosyl unit at δ 4.90 was correlated to C(3) of the arabinosyl unit at δ 85.1. The latter was, thus, shown to be glycosylated at C(2) and C(3), and its anomeric proton at δ 4.62 exhibited an HMBC with C(1) of the aglycone at δ 84.3. The long-range correlations between the acetyl carbonyl signal at δ 170.8 and the CH_2(21) signals at δ 4.36 and 4.33, and between the carbonyl resonance at δ 170.6 and the H–C(4) signals of the rhamnosyl unit at δ 5.88 (*dd*, $J = 9.8$, 9.8 Hz) confirmed the positions of the two acetyl groups at C(21) of the aglycone and C(4) of the rhamnosyl moiety.

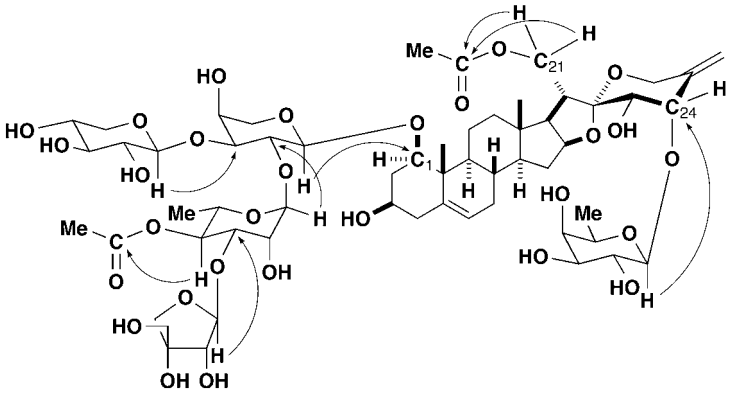

Fig. 3. *HMBC Correlations of the sugar moieties of* **1**

Hellebosaponin B (**2**) was shown to have the molecular formula $C_{64}H_{98}O_{34}$ on the basis of the FAB-MS (*m/z* 1433 ($[M+Na]^+$) and 1409 ($[M-H]^-$)), ^{13}C-NMR spectrum, and elemental analysis. The ^{1}H- and ^{13}C-NMR spectral data were very similar to those of **1**, except for the presence of the signals for six anomeric protons and C-atoms. Acid hydrolysis of **2** with 0.2M HCl in dioxane/H_2O 1:1 gave D-apiose, L-arabinose, D-fucose, D-glucose, L-rhamnose, and D-xylose as the carbohydrate moieties. Thus, **2** was shown to have one more hexose than **1**, identified as β-D-glucopyranose, which was linked to C(4) of the fucosyl residue attached at C(24) of the aglycone. The structure of **2** was assigned as (1β,3β,23*S*,24*S*)-21-(acetyloxy)-24-{[*O*-β-D-glucopyranosyl-(1 → 4)-β-D-fucopyranosyl]oxy}-3,23-dihydroxyspirosta-5,25(27)-dien-1-yl *O*-β-D-apiofuranosyl-(1 → 3)-*O*-(4-*O*-acetyl-α-L-rhamnopyranosyl)-(1 → 2)-*O*-[β-D-xylopyranosyl-(1 → 3)]-α-L-arabinopyranoside.

The anomeric protons and C-atoms of **2** appeared at δ(H) 6.43 (*d*, *J* = 1.4 Hz), 5.93 (*d*, *J* = 3.0 Hz), 5.14 (*d*, *J* = 7.9 Hz), 5.12 (*d*, *J* = 8.0 Hz), 4.89 (*d*, *J* = 7.4 Hz), and 4.62 (*d*, *J* = 7.7 Hz) and δ(C) 112.1, 106.9, 106.7, 106.1, 100.7, and 100.6. On comparison of the whole ^{13}C-NMR spectrum of **2** with that of **1**, a set of additional six signals corresponding to a terminal β-D-glucopyranosyl moiety appeared at δ 106.9 (C(1)), 76.2 (C(2)), 78.5 (C(3)), 71.5 (C(4)), 78.6 (C(5)), and 62.7 (C(6)), and the signals due to C(4) of the fucosyl moiety and its neighboring C-atoms varied, although all other signals remained almost unaffected. In the HMBC spectrum, correlation peaks between the anomeric proton of the glucosyl unit at δ 5.14 and C(4) of the fucosyl unit at δ 83.3, and between the anomeric proton of the fucosyl unit at δ 5.12 and C(24) of the aglycone at δ 82.2, confirmed that the additional glucosyl group was linked to C(4) of the fucosyl residue attached at C(24) of the aglycone.

Hellebosaponin C (**3**) was deduced as $C_{68}H_{106}O_{38}$ by the positive-ion FAB-MS (*m/z* 1553 ($[M+Na]^+$)), ^{13}C-NMR spectrum, and elemental analysis. The NMR spectra established the presence of seven sugar moieties. Acid hydrolysis of **3** with 0.2M HCl in dioxane/H_2O 1:1 gave D-apiose, L-arabinose, D-fucose, D-galactose, D-glucose, L-rhamnose, and D-xylose. Comparison of the ^{13}C-NMR spectrum of **3** with that of **1** showed their considerable structural similarities. However, **3** differed from **1** in the lack of the acetyl group at C(21) and in the presence of an *O*-β-D-glucopyranosyl-(1 → 2)-β-D-galactopyranosyl unit. The diglycoside was shown to be attached at C(21) of the aglycone in place of the acetyl group by the HMBC spectrum. The structure of **3** was determined to be (1β,3β,23*S*,24*S*)-24-[(β-D-fucopyranosyl)oxy]-21-{[*O*-β-D-glucopyranosyl-(1 → 2)-β-D-galactopyranosyl]oxy}-3,23-dihydroxyspirosta-5,25(27)-dien-1-yl *O*-β-D-apiofuranosyl-(1 → 3)-*O*-(4-*O*-acetyl-α-L-rhamnopyranosyl)-(1 → 2)-*O*-[β-D-xylopyranosyl-(1 → 3)]-α-L-arabinopyranoside.

Analysis of the ^{1}H- and ^{13}C-NMR spectra of **3** immediately established that the aglycone of **3** was identical to that of **1** and **2**. However, the proton signals arising from the sugar moieties of **3** were much more complicated than those of **1** and **2**. To assign the sugar signals, the 1D-TOCSY method, which allowed a subspectrum of a single monosaccharide unit to be extracted from the crowded overlapped region, was applied. As a result, the subspectrum of each sugar residue was obtained with high digital resolution. Subsequent analysis of the ^{1}H,^{1}H-COSY and HMQC plots resulted in the sequential assignments of all of the H- and C-resonances for the individual monosaccharides (see *Table 2*). Comparison of the δ(C) thus assigned with those of **1** and the reference methyl glycosides, taking into account the known effects of *O*-glycosylation, indicated that **3** contained a terminal β-D-glucopyranosyl, a terminal β-D-

Table 2. *^{1}H- and ^{13}C-NMR Data for Glycosyl Moieties of Compound* **3**[a])

	δ(H)[b])	δ(C)		δ(H)[b])	δ(C)
H–C(1′)	4.57 (*d*, *J* = 7.6)	100.7	H–C(1‴′)	4.89 (*d*, *J* = 7.2)	103.4
H–C(2′)	4.51 (*dd*, *J* = 9.0, 7.6)	72.7	H–C(2‴′)	4.59 (*dd*, *J* = 9.5, 7.2)	81.3
H–C(3′)	3.96 (*dd*, *J* = 9.0, 3.0)	84.8	H–C(3‴′)	4.23 (br. *d*, *J* = 9.7)	75.2
H–C(4′)	4.38 (br. *s*)	69.8	H–C(4‴′)	4.51 (br. *s*)	69.6
CH_2(5′)	4.30 (*dd*, *J* = 12.9, 2.1, H_a),	66.9	H–C(5‴′)	4.02	76.8
	3.60 (br. *d*, 12.9, H_b)		CH_2(6‴′)	4.36 (H_a), 4.34 (H_b)	61.9
H–C(1″)	6.42 (br. *s*)	100.7	H–C(1⁗)	5.30 (*d*, *J* = 7.7)	105.8
H–C(2″)	4.91 (br. *d*, *J* = 2.8)	71.4	H–C(2⁗)	4.06 (*dd*, *J* = 8.4, 7.7)	76.5
H–C(3″)	4.70 (*dd*, *J* = 9.8, 2.8)	77.6	H–C(3⁗)	4.22 (*dd*, *J* = 9.7, 8.4)	78.0
H–C(4″)	5.86 (*dd*, *J* = 9.8, 9.8)	74.5	H–C(4⁗)	4.28 (*dd*, *J* = 9.7, 8.9)	71.1
H–C(5″)	4.89 (*dq*, *J* = 9.8, 6.1)	66.6	H–C(5⁗)	3.90	78.4
Me(6″)	1.39 (*d*, *J* = 6.1)	18.4	CH_2(6⁗)	4.54 (br. *d*, *J* = 11.6, H_a),	62.2
				4.42 (*dd*, *J* = 11.6, 4.0, H_b)	
H–C(1‴)	5.93 (*d*, *J* = 2.9)	112.0			
H–C(2‴)	4.65 (*d*, *J* = 2.9)	77.8	H–C(1‴′′)	5.13 (*d*, *J* = 7.8)	106.0
C(3‴)	–	80.0	H–C(2‴′′)	4.35 (*dd*, *J* = 9.5, 7.8)	73.0
CH_2(4‴)	4.55 (*d*, *J* = 9.7, H_a),	74.9	H–C(3‴′′)	4.03 (br. *d*, *J* = 8.1)	75.2
	4.23 (*d*, *J* = 9.7, H_b)		H–C(4‴′′)	3.91 (br. *s*)	72.7
CH_2(5‴)	4.02 (br. *s*, 2 H)	65.2	H–C(5‴′′)	3.68	71.4
			Me(6‴′′)	1.42 (*d*, *J* = 6.4)	17.2
H–C(1⁗)	4.91 (*d*, *J* = 6.7)	106.6			
H–C(2⁗)	3.90 (*dd*, *J* = 8.2, 6.7)	74.4	Me*C*O	–	170.7
H–C(3⁗)	4.11 (*dd*, *J* = 8.2, 7.9)	78.3	*Me*CO	2.23 (*s*)	21.1
H–C(4⁗)	4.10	70.9			
CH_2(5⁗)	4.26 (*dd*, *J* = 9.3, 3.0, H_a),	67.0			
	3.65 (*t*-like, *J* = 10.4, H_b)				

[a]) Spectra were measured in (D_5)pyridine. [b]) δ in ppm; *J* in Hz.

fucopyranosyl, and a 2-substituted β-D-galactopyranosyl unit, along with the branched tetraglycoside moiety with an acetyl group whose structure was the same as that of **1** and **2**.

The ^{1}H-NMR spectrum of **3** showed signals for two angular Me groups at δ 1.32 and 0.95 (each *s*), exocyclic methylene protons at δ 5.13 and 4.96 (each br. *s*), and an olefinic proton at δ 5.58 (br. *d*, *J* = 5.4 Hz), as observed for **1** and **2**. In addition, seven anomeric protons and C-atoms were detected at δ(H) 6.42 (br. *s*), 5.93 (*d*, *J* = 2.9 Hz), 5.30 (*d*, *J* = 7.7 Hz), 5.13 (*d*, *J* = 7.8 Hz), 4.91 (*d*, *J* = 6.7 Hz), 4.89 (*d*, *J* = 7.2 Hz), and 4.57 (*d*, *J* = 7.6 Hz) and δ(C) 112.0, 106.6, 106.0, 105.8, 103.4, and 100.7 (2×). The tetraglycosyl and fucosyl residues of **3** were ascertained to be linked to C(1) and C(24), respectively, of the aglycone by long-range correlations from the anomeric-proton signal of the nodal arabinosyl moiety at δ 4.57 to the δ 84.2 resonance, and from that of the fucosyl residue at δ 5.13 to the δ 81.9 signal. The anomeric proton of the terminal glucosyl moiety at δ 5.30 displayed an HMBC with C(2) of the substituted galactosyl residue at δ 81.3, whose anomeric proton at δ 4.89 was correlated with C(21) of the aglycone at δ 69.6.

Compounds **1**–**3** are newly described bis- and trisdesmosides of polyoxygenated spirostanol with an O-atom at C(1), C(3), C(21), C(23), and C(24), among which **3** is considered to be one of the most polar spirostanol saponins and is unique in structure, bearing a new acetylated tetraglycoside moiety, a diglycoside, and a monosaccharide at C(1), C(21), and C(24) of the aglycone, respectively. Although some steroidal saponins,

along with cardiac glycosides, have been isolated from *H. odorus*, *H. multifidus*, *H. dumetorum*, *H. cyclophyllus*, *H. orientalis*, *H. bocconei* subsp. *siculus*, and *H. macranthus* [2][9], the structural features of **1**–**3** are quite different from those isolated previously from the other *Helleborus* species.

Since some of the steroidal saponins isolated recently have been reported to show cytotoxic activities against cultured tumor cell lines [10], **1**–**3** were evaluated for their cytotoxic activity against human oral squamous cell carcinoma (HSC-2) cells. Although **1** and **2** did not show any apparent cytotoxicity against HSC-2 cells even at the sample concentration of 100 μg ml^{-1}, **3** exhibited a moderate cytotoxic activity with an LD_{50} value of 15.8 μg ml^{-1}, when etoposide and doxorubicin used as the positive controls gave respective LD_{50} values of 24.4 μg ml^{-1} and 2.5 μg ml^{-1}.

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Experimental Part

General. The following reagents were obtained from the indicated companies: FBS (*JRH Biosciences*, Lenexa, KS, USA); *Dulbecco*'s modified eagle medium (DMEM) (*Gibco*, Grand Island, NY, USA); MTT, penicillin, and streptomycin (*Sigma*, St. Louis, MO, USA). All other chemicals used were of biochemical reagent grade. Column chromatography (CC): *Diaion HP-20* (*Mitsubishi-Chemical*, Tokyo, Japan), silica gel (*Fuji-Silysia Chemical*, Aichi, Japan), and ODS silica gel (*Nacalai Tesque*, Kyoto, Japan). TLC: Precoated silica gel *60* F_{254} (0.25-mm thick, *Merck*, Darmstadt, Germany) and *RP-18-*F_{254}*-S* (0.25-mm thick, *Merck*) plates; visualization by spraying with 10% H_2SO_4 soln., followed by heating. HPLC: *CCPM* pump (*Tosoh*, Tokyo, Japan), *CCP PX-8010* controller (*Tosoh*), *RI-8010* detector (*Tosoh*), *Shodex OR-2* detector (*Showa-Denko*, Tokyo, Japan), and *Rheodyne* injection port with a 20-μl sample loop; anal. HPLC, *Capcell-Pak-*NH_2*-UG80* column (4.6 mm × 250 mm, 5 μm, *Shiseido*, Tokyo, Japan); t_R in min. Optical rotations: *Jasco DIP-360* (Tokyo, Japan) automatic digital polarimeter. IR Spectra: *Jasco FT-IR 620* spectrophotometer. NMR Spectra: *Bruker DRX-500* (Karlsruhe, Germany) spectrometer using standard *Bruker* pulse programs; ^{1}H at 500 MHz; chemical shifts δ in ppm rel. to $SiMe_4$ as internal standard. MS: *Finnigan MAT-TSQ-700* (San Jose, CA, USA) mass spectrometer.

Plant Material. H. orientalis was purchased from a nursery in Heiwaen, Japan, in November, 1999, and identified by one of the authors, Prof. *Yutaka Sashida*. A voucher of the plant is on the file in our laboratory (99-11-7-HO).

Extraction and Isolation. The plant material (fresh weight, 2.7 kg) was extracted with hot MeOH twice. The MeOH extract was evaporated, and the viscous concentrate (300 g) was passed through a *Diaion-HP-20* column (30% MeOH, 80% MeOH, MeOH, EtOH, and AcOEt). The 80% MeOH eluate portion (90 g) was subjected to CC (silica gel, stepwise gradient $CHCl_3$/MeOH 9:1, 4:1, 2:1, and finally MeOH): *Fractions I–VIII*. *Fr. VI* was submitted to CC (silica gel, $CHCl_3$/MeOH/H_2O 30:10:1 and 20:10:1), followed by CC (ODS silica gel, MeOH/H_2O 1:1, MeCN/H_2O 1:4): **1** (170 mg) and **2** (70 mg). *Fr. VIII* was submitted to CC (silica gel, $CHCl_3$/MeOH/H_2O 20:10:1 and 70:40:9), followed by CC (ODS silica gel, MeCN/H_2O 1:4): **3** (89 mg).

Hellebosaponin A (= *(1β,3β,23*S*,24*S*)-21-(Acetyloxy)-24-[(β-*D*-fucopyranosyl)oxy]-3,23-dihydroxyspirosta-5,25(27)-dien-1-yl* O-*β*-D-*Apiofuranosyl-(1 → 3)*-O-*(4*-O-*acetyl-α*-L-*rhamnopyranosyl)-(1 → 2)*-O-*[β*-D-*xylopyranosyl-(1 → 3)]-α*-L-*arabinopyranoside*; **1**). Amorphous solid. $[\alpha]_D^{25} = -94.0$ ($c = 0.10$, MeOH). IR (film): 3400 (OH), 2976 and 2921 (CH), 1728 (C=O), 1449, 1250, 1042, 899, 836. ^{1}H-NMR (500 MHz, (D_5)pyridine) and ^{13}C-NMR (125 MHz, (D_5)pyridine): *Table 1*. FAB-MS (pos.): 1271 ($[M + Na]^+$). FAB-MS (neg.): 1247 ($[M - H]^-$). Anal. calc. for $C_{58}H_{88}O_{29} \cdot 9/2\ H_2O$ (1330.3707): C 52.36, H 7.35; found: C 52.46, H 7.50.

Acid Hydrolysis of Hellebosaponin A (**1**). A soln. of **1** (15 mg) in 0.2M HCl (dioxane/H_2O 1:1, 3 ml) was heated at 95° for 30 min under Ar. After cooling, the mixture was neutralized by passage through an *Amberlite-IRA-93ZU* (*Organo*, Tokyo, Japan) column and chromatographed (*Diaion HP-20*, 40% MeOH followed by Me_2CO/EtOH 1:1) to give an aglycone fraction (5.0 mg) and a sugar fraction (3.3 mg). TLC Analysis of the aglycone fraction revealed several unidentified artifactual sapogenols. After the sugar fraction was passed through a *Sep-Pak-*C_{18} *cartridge* (*Waters*, Milford, MA, USA; with 40% MeOH) and *Toyopak-IC-SP-M-*

cartridge (*Tosoh*; with 40% MeOH), it was analyzed by HPLC (MeCN/H_2O 17:3, flow rate, 0.9 ml min^{-1}; detection, *RI* and *OR*): t_R 7.11 (D-apiose, pos. *OR*), 7.79 (L-rhamnose, neg. *OR*), 8.26 (D-fucose, pos. *OR*), 9.35 (L-arabinose, pos. *OR*), 9.73 (D-xylose, pos. *OR*).

Acetylation of Hellebosaponin A (**1**). Compound **1** (19.7 mg) was treated with a mixture of Ac_2O (1 ml) and pyridine (1 ml) in the presence of N,N-dimethylpyridin-4-amine (5.1 mg) as catalyst for 20 h. The mixture was chromatographed (silica gel, hexane/Me_2CO 5:3): 18.1 mg of *hellebosaponin A Tridecaacetate* (**1a**). Amorphous solid. $[\alpha]_D^{27} = -34.0$ ($c = 0.10$, MeOH). IR (film): 2939 and 2852 (CH), 1747 (C=O), 1434, 1371, 1227, 1048, 932. ^{1}H-NMR (500 MHz, (D_5)pyridine): 5.70 (br. *s*, H−C(1″)); 5.60 (br. *d*, $J = 5.8$, H−C(6)); 5.52 (*d*, $J = 3.3$, H−C(1‴)); 5.38 (*d*, $J = 4.0$, H−C(23)); 5.23, 5.12 (2 br. *s*, CH_2(27)); 5.13 (*d*, $J = 7.9$, H−C(1⁗)); 4.95 (*d*, $J = 7.9$, H−C(1‴)); 4.88 (*d*, $J = 4.0$, H−C(24)); 4.82 (br. *m*, $w_{1/2} = 20.2$, H−C(3)); 4.46 (*d*, $J = 7.8$, H−C(1′)); 4.35, 4.33 (2*m*, CH_2(21)); 3.62 (*dd*, $J = 12.1$, 4.2, H−C(1)); 2.36, 2.34, 2.31, 2.29, 2.22, 2.18, 2.11, 2.06, 2.04, 2.02, 2.00, 1.98, 1.96, 1.93 (2×) (15*s*, 15 MeCO); 1.29 (*s*, Me(19)); 1.09 (*s*, Me(18)).

Hellebosaponin B (= *(1β,3β,23S,24S)-21-(Acetyloxy)-24-[[*O-*β*-D-*glucopyranosyl-(1 → 4)-β*-D-*fucopyranosyl]oxy]-3,23-dihydroxyspirosta-5,25(27)-dien-1-yl* O-*β*-D-*Apiofuranosyl-(1 → 3)*-O-*(4*-O-*acetyl-α*-L-*rhamnopyranosyl)-(1 → 2)*-O-*[β*-D-*xylopyranosyl-(1 → 3)]-α*-L-*arabinopyranoside*; **2**). Amorphous solid. $[\alpha]_D^{28} = -54.0$ ($c = 0.10$, MeOH). IR (film): 3386 (OH), 2973 and 2917 (CH), 1728 (C=O), 1451, 1372, 1042, 895, 836. ^{1}H-NMR (500 MHz, (D_5)pyridine): 6.43 (*d*, $J = 1.4$, H−C(1″)); 5.93 (*d*, $J = 3.0$, H−C(1‴)); 5.63 (br. *d*, $J = 5.6$, H−C(6)); 5.21, 5.09 (2 br. *s*, CH_2(27)); 5.14 (*d*, $J = 7.9$, H−C(1⁗)); 5.12 (*d*, $J = 8.0$, H−C(1⁗)); 4.89 (*d*, $J = 7.4$, H−C(1⁗)); 4.82, 3.97 (2 *d*, $J = 11.9$, CH_2(26)); 4.75 (*d*, $J = 4.0$, H−C(24)); 4.62 (*d*, $J = 7.7$, H−C(1′)); 4.37, 4.34 (2 *m*, CH_2(21)); 4.13 (*d*, $J = 4.0$, H−C(23)); 2.21, 1.92 (2*s*, 2 MeCO); 1.53 (*d*, $J = 6.4$, Me(6⁗)); 1.38 (*d*, $J = 6.2$, Me(6″)); 1.39 (*s*, Me(19)); 1.06 (*s*, Me(18)). ^{13}C-NMR (125 MHz, (D_5)pyridine): 84.2 (C(1)); 38.0 (C(2)); 68.0 (C(3)); 43.8 (C(4)); 139.5 (C(5)); 124.8 (C(6)); 31.8 (C(7)); 33.1 (C(8)); 50.3 (C(9)); 42.8 (C(10)); 24.0 (C(11)); 39.9 (C(12)); 41.0 (C(13)); 56.9 (C(14)); 32.3 (C(15)); 83.6 (C(16)); 58.8 (C(17)); 16.8 (C(18)); 15.0 (C(19)); 42.7 (C(20)); 65.1 (C(21)); 111.0 (C(22)); 71.5 (C(23)); 82.2 (C(24)); 143.5 (C(25)); 61.5 (C(26)); 114.1 (C(27)); 170.8, 20.9 (MeCO); 100.7 (C(1′)); 72.6 (C(2′)); 85.1 (C(3′)); 69.6 (C(4′)); 67.0 (C(5′)); 100.6 (C(1″)); 71.5 (C(2″)); 77.8 (C(3″)); 74.6 (C(4″)); 66.6 (C(5″)); 18.3 (C(6″)); 112.1 (C(1‴)); 77.9 (C(2‴)); 79.9 (C(3‴)); 74.9 (C(4‴)); 65.3 (C(5‴)); 106.7 (C(1⁗)); 74.5 (C(2⁗)); 78.5 (C(3⁗)); 70.9 (C(4⁗)); 67.1 (C(5⁗)); 106.1 (C(1⁗⁗)); 73.8 (C(2⁗⁗)); 75.6 (C(3⁗⁗)); 83.3 (C(4⁗⁗)); 70.7 (C(5⁗⁗)); 17.5 (C(6⁗⁗)); 106.9 (C(1⁗⁗⁗)); 76.2 (C(2⁗⁗⁗)); 78.5 (C(3⁗⁗⁗)); 71.5 (C(4⁗⁗⁗)); 78.6 (C(5⁗⁗⁗)); 62.7 (C(6⁗⁗⁗)); 170.6, 21.1 (MeCO). FAB-MS (pos.): 1433 ($[M + Na]^+$). FAB-MS (neg.): 1409 ($[M - H]^-$). Anal. calc. for $C_{64}H_{98}O_{34} \cdot 5\ H_2O$ (1501.5189): C 51.19, H 7.25; found: C 51.20, H 7.42.

Acid Hydrolysis of Hellebosaponin B (**2**). Compound **2** (15 mg) was subjected to acid hydrolysis as described for **1** to give a sugar fraction (3.5 mg). HPLC analysis (see above) showed the presence of D-apiose, L-arabinose, D-fucose, D-glucose, L-rhamnose, and D-xylose: t_R 7.08 (D-apiose, pos. *OR*), 755 (L-rhamnose, neg. *OR*), 8.34 (D-fucose, pos. *OR*), 9.19 (L-arabinose, pos. *OR*), 9.62 (D-xylose, pos. *OR*), 14.83 (D-glucose, pos. *OR*).

Hellebosaponin C (= *(1β,3β,23S,24S)-24-[(*O-*β*-D-*Fucopyranosyl)oxy]-21-[[*O-*β*-D-*glucopyranosyl-(1 → 2)-β*-D-*galactopyranosyl]oxy]-3,23-dihydroxyspirosta-5,25(27)-dien-1-yl* O-*β*-D-*Apiofuranosyl-(1 → 3)*-O-*(4*-O-*acetyl-α*-L-*rhamnopyranosyl)-(1 → 2)*-O-*[β*-D-*xylopyranosyl-(1 → 3)]-α*-L-*arabinopyranoside*; **3**). Amorphous solid. $[\alpha]_D^{22} = -66.0$ ($c = 0.10$, MeOH). IR (film): 3376 (OH), 2973, 2933 and 2905 (CH), 1731 (C=O), 1415, 1374, 1252, 1042, 898, 836. ^{1}H-NMR (500 MHz, (D_5)pyridine): 6.42 (br. *s*, H−C(1″)); 5.93 (*d*, $J = 2.9$, H−C(1‴)); 5.58 (br. *d*, $J = 5.4$, H−C(6)); 5.30 (*d*, $J = 7.7$, H−C(1⁗⁗)); 5.13 (*d*, $J = 7.8$, H−C(1⁗⁗)); 5.13, 4.96 (2 br. *s*, CH_2(27)); 4.91 (*d*, $J = 6.7$, H−C(1⁗)); 4.89 (*d*, $J = 7.2$, H−C(1⁗)); 4.78, 3.92 (2 *d*, $J = 11.3$, CH_2(26)); 4.78 (*d*, $J = 4.0$, H−C(24)); 4.57 (*d*, $J = 7.6$, H−C(1′)); 4.49 (*d*, $J = 4.0$, H−C(23)); 4.47, 3.90 (2 *m*, CH_2(21)); 2.23 (*s*, MeCO); 1.42 (*d*, $J = 6.4$, Me(6⁗⁗)); 1.39 (*d*, $J = 6.1$, Me(6″)); 1.32 (*s*, Me(19)); 0.95 (*s*, Me(18)). ^{13}C-NMR (125 MHz, (D_5)pyridine): 84.2 (C(1)); 37.9 (C(2)); 67.9 (C(3)); 43.7 (C(4)); 139.4 (C(5)); 124.9 (C(6)); 31.8 (C(7)); 33.0 (C(8)); 50.2 (C(9)); 42.8 (C(10)); 23.8 (C(11)); 39.9 (C(12)); 40.8 (C(13)); 56.8 (C(14)); 32.2 (C(15)); 83.2 (C(16)); 57.8 (C(17)); 16.5 (C(18)); 15.0 (C(19)); 43.7 (C(20)); 69.6 (C(21)); 111.1 (C(22)); 71.3 (C(23)); 81.9 (C(24)); 143.4 (C(25)); 61.3 (C(26)); 113.9 (C(27)); signals for the sugar moieties, *Table 2*. FAB-MS (pos.): 1553 ($[M + Na]^+$). Anal. calc. for $C_{68}H_{106}O_{38} \cdot 3\ H_2O$ (1585.5923): C 51.51, H 7.12; found: C 51.60, H 7.31.

Acid Hydrolysis of Hellebosaponin C (**3**). Compound **3** (17 mg) was subjected to acid hydrolysis as described for **1** to give a sugar fraction (5.0 mg). HPLC analysis (see above) showed the presence of D-apiose, L-arabinose, D-fucose, D-galactose, D-glucose, L-rhamnose, and D-xylose: t_R 7.07 (D-apiose, pos. *OR*), 7.82 (L-rhamnose, neg. *OR*), 8.03 (D-fucose, pos. *OR*), 8.75 (L-arabinose, pos. *OR*), 9.26 (D-xylose, pos. *OR*), 14.04 (D-galactose, pos. *OR*), 14.86 (D-glucose, pos. *OR*).

HSC-2 Cell-Culture Assay. HSC-2 cells were maintained as monolayer cultures at 37° in DMEM supplemented with 10% heat-inactivated FBS in a humidified 5% CO_2 atmosphere. Cells were trypsinized and inoculated at $6 \cdot 10^3$ per each 96-microwell plate, and incubated for 24 h. After washing once with phosphate-buffered saline (PBS; 0.01M phosphate buffer, 0.15M NaCl, pH 7.4) supplemented with 100 U ml^{-1} of penicillin and 100 μg ml^{-1} of streptomycin, they were treated 24 h without or with test compounds. They were washed once with PBS and incubated for 4 h with 0.2 $mg \cdot ml^{-1}$ of MTT in DMEM supplemented with 10% FBS. After the medium was removed, the cells were lysed with DMSO (0.1 ml), and the relative viable cell number was determined by measuring the absorbance at 540 nm of the cell lysate [11][12]. The LD_{50} value, which reduces the viable cell number by 50%, was determined from the dose-response curve.

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