Four new chamigrane sesquiterpenoids from the opistobranch mollusk *Aplysia dactylomela*

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Four new chamigrane sesquiterpenoids, (6*S*,10*S*)-10-bromo-3,11,11-trimethyl-7-methylidenespiro[5.5]undec-2-ene-4-one, (4*S*,6*S*,10*S*)-10-bromo-3,11,11-trimethyl-7-methylidenespiro[5.5]undec-2-ene-4-ol, (3*R*,4*S*,6*S*,10*R*)-10-bromo-3,11,11-trimethyl-7-methylidenespiro[5.5]undecane-3,4-diol, and (6*S*,7*S*,11*R*)-2-chloro-3,7,11-trimethyl-10-methylidenespiro[5.5]undec-2-ene-7-ol, were isolated from the sea hare *Aplysia dactylomela*. The chemical structures of new compounds were established by NMR spectroscopy and mass spectrometry. The cytotoxic activity of some of the obtained compounds against promyelocytic HL-60 and monocytic THP-1 leukemia cells was demonstrated.

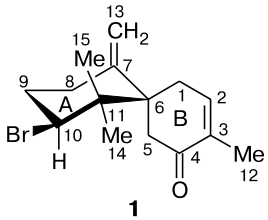
Key words: mollusks, sesquiterpenoids, spiro compounds, organobromine compounds, anticancer activity.

It is known that sea mollusks *Aplysia* feeding on red algae accumulate large amounts of terpenoids, which perform protective function[1] and exhibit bactericidal,[2] antifouling,[3] insecticidal,[4] and antitumor[5] activities. Compounds isolated from these objects often have unusual structures and contain functional groups that are seldom found in natural compounds. Therefore, studies of the chemical composition of the mollusks *Aplysia* and the algae *Laurencia* attract considerable attention of chemists as regards the search for new biologically active compounds.

Previously we reported the isolation of three chamigrane sesquiterpenoids from the sea hare *Aplysia dactylomela*[6—8] and studies of the chemical properties of one of them.[9] This paper is devoted to the isolation of four new sesquiterpenoids from the same material, elucidation of their structures and study of the biological activities of some of them.

Results and Discussion

A fraction of terpenoid compounds was isolated from the ethanol extract of the mollusk *A. dactylomela* by column chromatography on silica gel. High performance liquid chromatography (HPLC) of this fraction on an analytical column Ultrasphere Si gave four new chamigrane sesquiterpenoids **1**—**4**.



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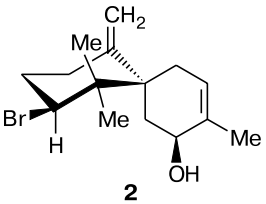

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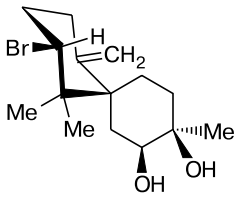

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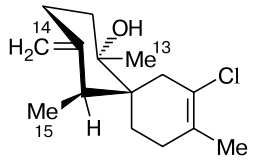

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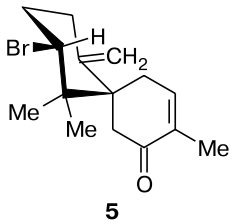
On the basis of ^{13}C NMR and high-resolution mass spectrometry data, the molecular formula of compound **1** was identified as $C_{15}H_{21}BrO$. The ^{1}H, ^{13}C NMR (Table 1), mass spectra, and CD spectra of this compound were closely similar to the spectra of the previously isolated[9] dactylone **5**, the absolute configuration of which was determined by X-ray diffraction.[6]



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Published in Russian in *Izvestiya Akademii Nauk. Seriya Khimicheskaya,* No. 10, pp. 2037—2042, October, 2007.

Table 1. ^{1}H and ^{13}C NMR spectra of compounds **1** and **2** ($CDCl_3$, δ, *J*/Hz)[a]

Atom	**1**			**2**			
	δH (*J*/Гц)	δC [b]	NOE	δH (*J*/Hz)	δC [b]	HMBC	NOESY
1	2.53 (dm, *J* = 18.9)	32.6 (t)		2.20 (m, 2 H)	30.8 (t)		H(2), H(13´)
1´	2.67 (dm, *J* = 18.9)						
2	6.63 (m)	142.9 (d)		5.51 (m)	128.2 (d)	C(6)	3 H(12), H(1)
3		134.6 (s)			133.6 (s)		
4		198.2 (s)		3.84 (br.dd, *J* = 11.3, *J* = 4.1)	69.3 (d)		3 H(12), H(13), H(13´)
5	2.81 (dd, *J* = 14.9, *J* = 2.3)	43.15 (t)		1.84 (dd, 14.1, 4.6)	34.5 (t)	C(4), C(6), C(7)	3 H(14)
5´	2.65 (d, *J* = 14.9)			2.47 (dt, *J* = 14.0, *J* = 1.8)		C(6)	H(10)
6		51.8 (s)			45.5 (s)		
7		144.3 (s)			150.4 (s)		
8a	2.42 (m)	31.7 (t)		2.73 (dddt, *J* = 14.0, *J* = 14.2, *J* = 5.6, *J* = 1.5)	34.5 (t)		
8e	2.15 (m)			2.28 (m)			
9a	2.03 (m)	35.4 (t)		2.08 (dddd, *J* = 13.7, *J* = 13.0, *J* = 13.0, *J* = 5.3)	35.6 (t)	C(7), C(11)	3 H(15)
9e	2.25 (m)			2.30 (m)		C(7)	
10	4.48 (dd, *J* = 12.4, *J* = 4.5)	63.6 (d)	H(5), 3 H(14)	4.66 (dd, *J* = 12.9, *J* = 4.4)	65.0 (d)	C(11), C(14)	H(5´), 3 H(14)
11		43.1 (s)			43.3 (s)		
12	1.71 (dt, *J* = 2.6, *J* = 1.4)	15.2 (q)		1.74 (q, *J* = 1.9)	20.6 (q)	C(2), C(3), C(4)	H(4), H(2),
13	5.00 (br.s)	114.6 (t)	H(8e), H(13´)	4.77 (t, *J* = 1.5)	114.1 (t)	C-6	H(4), H(8e), 3 H(15)
13´	4.54 (s)		H(1), H(13)	5.02 (t, *J* = 1.7)			H(1), H(4)
14e	1.20 (s)	24.8 (q)	H(5´), 3 H(15)	1.11 (s)	24.6 (q)	C(6), C(11), C(15)	H(1), H(5), H(10)
15a	1.00 (s)	17.3 (q)	2 H(1), H(9a), 3 H(14)	0.92 (s)	17.0 (q)	C(6), C(11), C(14)	H(1), H(9a), H(13)

[a] ^{1}H and ^{13}C NMR spectra were recorded at 300 and 75.5 MHz, respectively; the signals were assigned by COSY-45 and HSQC 2D NMR techniques.
[b] Signal multiplicities were determined by the DEPT spectrum.

Unlike dactylone **5**, compound **1** was oily rather than crystalline. The data of COSY-45, HSQC, HMBC and NOE NMR experiments (see Table 1) confirmed that compound **1** differs from dactylone **5** only by the configuration of the asymmetric center at C(10). The spin-spin coupling constants of the H(10) proton (*J* = 4.5, 12.4 Hz) in compound **1** attest to its axial position, and, hence, the Br atom occupies the equatorial position, as in dactylone **5**. However, NOE experiments revealed the difference in the conformations of ring A in compounds **1** and **5**. Thus in compound **1** correlation was detected between the H(10) proton and one of H(5) protons and between H(13´) and H(1), whereas in **5**, the H(10) proton is correlated with the H(1) proton, and H(13´) is correlated with H(5), which is possible only for two different chair conformations of ring A in structures **1** and **5**. The NOE data were also useful for establishing the relative configuration of the chiral centers at the C(10) and C(6) atoms. Although compounds **1** and **5** have different chair conformations of ring A and different configurations of the C(10) chiral centers, nevertheless, they belong to the same series of +β-chamigrenes,[10] which follows from the negative signs of the Cotton effect in the CD spectra of both compounds and determines the absolute configuration of the chiral centers. Hence, terpenoid **1** is (6*S*,10*S*)-10-bromo-3,11,11-trimethyl-7-methylidenespiro[5.5]undec-2-en-4-one.

Since the three other compounds were isolated from the same source as dactylone, one can suggest that they, like dactylone, would correspond to the +β-chamigrene series in absolute stereochemistry.[10]

The data of high resolution mass spectrometry and ^{13}C NMR spectra indicated that compound **2** had the molecular formula $C_{15}H_{23}BrO$. The ^{13}C NMR and DEPT spectra (see Table 1) showed the presence of three methyl groups, five methylene groups, three methine groups one of which is bound to oxygen, four quaternary carbon atoms, and four olefinic carbon atoms. The chemical shifts of the carbon atom at δ 65.0 and the corresponding proton at δ 4.66 and the presence of a doublet molecular ion at *m/z* 298/300 (1 : 1) in the mass spectrum attested to the presence of bromine in the molecule. The carbon signal at δ 69.3 in the ^{13}C NMR spectrum and the signal from the corresponding proton at δ 3.84 in the ^{1}H NMR spectrum suggest the presence of a CHOH group; this was confirmed by the ^{1}H NMR signal at δ 1.98 (in chloroform), which disappeared upon the addition of methanol, and by the IR absorption at 3560 cm^{-1}. The signals with δ_C 150.4 and 114.1 and δ_H 4.77 and 5.02 attested to the presence of an *exo*-methylidene group. All signals were assigned using COSY-45, HSQC, and HMBC NMR experiments (see Table 1).

The C(14)H_3/C(15), C(15)H_3/C(14) correlations and the correlations of C(14)H_3 and C(15)H_3 with C(11) in HMBC experiments indicated that the gem-dimethyl group occupies position 11. The C(10)H/C(11) and C(9)H_2/C(7) correlations and the correlations of C(13)H_2, C(14)H_3, and C(15)H_3 with C(6) in the HMBC spectra with allowance for the COSY-45 data made it possible to determine the structure of ring A, while the C(12)H_3/C(2),C(3),C(4) and C(5)H_2,C(2)H/C(6) HMBC coupling confirmed the chamigrene structure of compound **2**. The spin-spin coupling constants of the H(10) (J = 12.9, J = 4.4 Hz) and H(4) protons (J = 11.3, J = 4.1 Hz) were characteristic of axial protons in a six-membered ring and, hence, bromine is equatorial and the hydroxy group at C(4) is pseudoequatorial. These data and correlation of H(10) with H(5′); 3 H(14) with H(5); and H(4) with 2 H(13) in the NOESY spectra (see Table 1) allowed us to determine the ring conformations and the relative configuration of the asymmetric centers. These conformations were confirmed by MM2 minimization of the molecular energy. Since the compounds belong to the +β-chamigrene series, the absolute stereochemistry and the structure of compound **2** were proposed as (4*S*,6*S*,10*S*)-10-bromo-3,11,11-trimethyl-7-methylidenespiro[5.5]undec-2-en-4-ol.

According to ^{13}C NMR and high-resolution mass spectra, compound **3** had the molecular formula $C_{15}H_{25}BrO_2$ and a molecular mass greater than that of compound **2** by 18 units. Analysis of the COSY-45, HSQC, and HMBC data (Table 2) showed that the planar structure of ring A in compound **3** is the same as in compounds **1** and **2**. The signals with δ_C 68.1 (d) and δ_H 4.12 (dd) in the NMR spectra indicated the presence of a secondary hydroxy group, while the signal with δ_C 70.1 (s) is due to a tertiary alcohol group, which was confirmed by absorp-

Table 2. ^{1}H and ^{13}C NMR spectra of compound **3** (C_6D_6, δ, J/Hz)[a]

Atom	δH (J/Hz)	δC [b]	HMBC	1D NOE
1a	1.87 (td, J = 13.6, J = 3.3)	22.7 (t)		
1e	1.11 (m)			
2a	0.86 (br.t, J = 13.5)	33.9(t)	C(3), C(6)	3 H(12)
2e	1.47 (dt, J = 13.9, J = 3.3)			
3		70.1 (s)		
4	4.12 (dd J = 5.1, J = 12.3)	68.1 (d)		H(2a), 3 H(12), H(13)
5a	2.16 (dd, J = 12.0, J = 13.4)	35.5 (t)	C(4), C(6), C(7)	3 H(14), 3 H(15)
5e	2.01 (ddd, J = 13.4, J = 4.8, J = 2.9)		C(6)	
6		50.9 (s)		
7		146.9 (s)		
8a	1.69 (br.td, J = 12.5, J = 5.4)	33.5 (t)		
8e	1.58 (ddd, J = 12.8, J = 5.1, J = 2.1)			
9a	1.86 (qd, J = 12.3, J = 5.0)	36.3 (t)	C(7), C(8), C(11)	
9e	1.93 (m)			
10	4.12 (dd, J = 12.3, J = 5.1)	64.4(d)	C(11), C(14)	H(1e), 3 H(14) H(8a)
11		43.9 (s)		
12	1.05 (s)	28.7 (q)	C(2), C(3), C(4)	
13	4.46 (br.s)	113.3 (t)	C(6)	H(4), H(5e), H(13′)
13′	4.73 (t, J = 1.3)			H(8e), H(13)
14	1.07 (s)	23.7 (q)	C(6), C(10), C (11), C(15)	
15	0.87 (s)	17.5 (q)	C(6), C(10), C (11), C(14)	

[a] ^{1}H and ^{13}C NMR spectra were recorded at 300 and 75.5 MHz, respectively; the signals were assigned by COSY-45 and HSQC 2D NMR techniques.

[b] Signal multiplicities were determined by the DEPT spectrum.

tion at 3572 cm^{-1} in the IR spectrum. In addition, NMR spectra exhibited signals for the methyl group at the hydroxy-substituted quaternary carbon atom (δ_H 1.05 (s), δ_C 28.7 (q)). The $C(1)H_2/C(2)H_2$ and $C(4)H/C(5)H_2$ proton correlations in the COSY-45 spectra and the $C(12)H_3/C(2),C(3),C(4)$ and $C(2)H_2,C(5)H_2/C(6)$ correlations in the HMBC experiments taking into account the HSQC data allowed us to determine the structure of ring B. The spin-spin coupling constants of the H(4) and H(10) protons are equal (J = 12.3 Hz, J = 5.1 Hz) and typical of axial protons; hence, bromine and secondary hydroxyl occupy equatorial positions.

Ring conformations and relative configurations of the chiral centers were determined by means of NOE experiments (see Table 2) and, taking into account that the compound belongs to the +β-chamigrene series, the absolute stereochemistry and structure of compound **3** were identified as (3*R*,4*S*,6*S*,10*R*)-10-bromo-3,11,11-trimethyl-7-methylidenespiro[5.5]undecane-3,4-diol.

According to ^{13}C NMR and high resolution mass spectra, compound **4** has the molecular formula $C_{15}H_{23}ClO$. The ^{13}C NMR and DEPT spectra (Table 3) showed the presence of three methyl (one at the double bond), five methylene, one methylidene, and one methine groups and also five quaternary carbon atoms, three of which are olefinic and one of which is bound to oxygen. The signals with δ_C 151.5 (s), 109.4 (t), and δ_H 4.65, 4.74 attested to the presence of an *exo*-methylidene group, while those with δ_C 125.8 (s) and 128.0 (s) corresponded to a tetrasubstituted double bond. The presence of a tertiary hydroxy group was evident from the signal with δ_C 74.2 (s) and the IR absorption band at 3450 cm^{-1}. All the carbon and proton signals (see Table 3) were assigned and the structure of isolated compound **4** was established on the basis of COSY-45, HSQC, and HMBC data. The ring conformations and the relative configurations of the asymmetric centers were determined by NOE experiments (see Table 3) and confirmed by MM2 calculations of the accessible conformations. In view of the correspondence to the +β-chamigrene series, the absolute stereochemistry and the structure of **4** were identified as (6*S*,7*S*,11*R*)-2-chloro-3,7,11-trimethyl-10-methylidenespiro[5.5]undec-2-en-7-ol.

Sesquiterpenoid **3** exhibited a cytotoxic activity against the HL-60 and THP-1 leukemia cells with IC_{50} = 102 and 152 μmol L^{-1}, respectively. Dactylone **5** was nontoxic with respect to the same cells up to a concentration of 200 μmol L^{-1}. However, dactylone being present in noncytotoxic concentrations initiated early apoptosis in the HL-60 and THP-1 cells (Fig. 1); 50% of the HL-60 cells passed to the early apoptosis state after 24 h at a dactylone concentration of 80 μmol L^{-1}, whereas for the THP-1 cells, the result was attained at 40 μmol L^{-1}. Thus, the replacement of the carbonyl group conjugated with the double bond in ring B of dactylone **5** by a diol group in terpenoid **3** results in a pronounced increase in the cytotoxic activity of this compound compared with dactylone.

Presumably, compound **1** is a by-product in the biosynthesis of dactylone **5** formed during bromination when the Br^+ ion attacks the reaction site at C(10) from the side opposite to that usual for dactylone biosynthesis. Most likely, sesquiterpenoid **2** results from reduction of the carbonyl group in terpenoid **1**. Compound **3** is formed apparently from dactylone **5** upon reduction of the carbonyl group and addition of a water molecule to the C(2)=C(3) double bond; terpenoid **4** is the product of the rearrangement described in our previous publication[7] and addition of a water molecule to the C(7)=C(13) double bond.

Table 3. ^1H and ^{13}C NMR spectra of compound **4** (C_6D_6, δ, J/Hz)[a]

Atom	δH (J/Hz)	δC [b]	HMBC	1D NOE
1	2.09 (dm, J = 16.9)	40.8 (t)		
1′	2.38 (br.d, J = 16.9)			
2		128.0 (s)		
3		125.8 (s)		
4	1.95 (m)	28.9 (t)		
4′	1.70 (m)			
5	1.76 (m)	23.2 (t)		
5′	1.28 (m)			
6		44.2 (s)		
7		74.2 (s)		
8a	1.32 (td, J = 13.3, J = 4.6)	37.9 (t)		H(1′), H(13)
8e	1.16 (ddd, J = 13.7, J = 5.1, J = 3.0)			
9a	2.60 (tdt, J = 13.5, J = 5.1, J = 1.8)	27.3 (t)		
9e	1.86 (ddd, J = 13.2, J = 2.9, J = 4.6)			
10		151.5 (s)		
11	2.44 (br.q, J = 7.4)	42.3 (d)		
12	1.74 (br.s)	19.8 (q)	C(2), C(4)	
13	0.74 (s)	26.1(q)	C(6), C(7), C(8)	H(1), H(5′), H(8a), H(8e)
14	4.65 (br.t, J = 2.2)	109.4 (t)	C(9), C(11)	H(9e)
14′	4.74 (t, J = 2.3)			H(11)
15	1.32 (d, J = 7.4)	17.5 (q)	C(6), C(10), C(11)	H(5), H(9a)

[a] ^1H and ^{13}C NMR spectra were recorded at 300 and 75.5 MHz, respectively; the signals were assigned by COSY-45 and HSQC 2D NMR techniques;
[b] Signal multiplicities were determined by the DEPT spectrum.

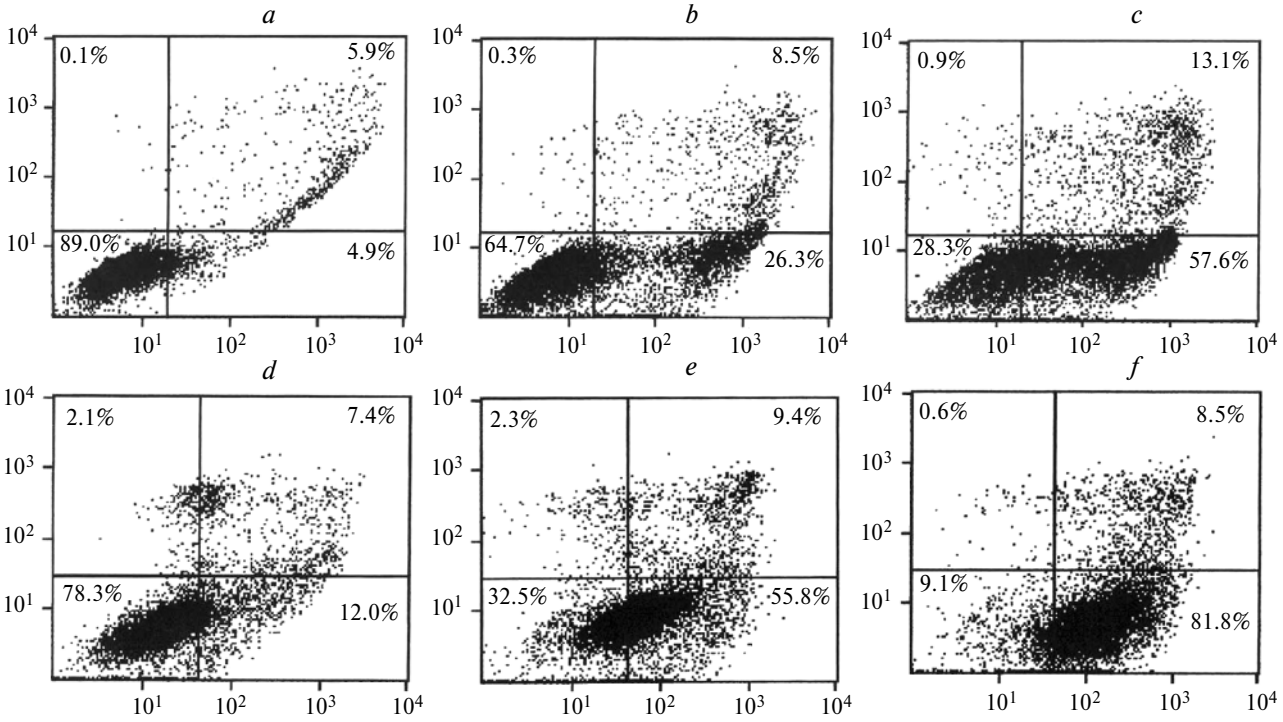

Fig. 1. Apoptosis in the HL-60 (*a*—*c*) and THP-1 (*d*—*f*) human leukemia cells in the control group (*a*, *d*) and dactylone **5** concentrations of 40 (*b*, *e*) and 80 μmol L^{-1} (*c*, *f*).

Experimental

^1H and ^{13}C NMR spectra were recorded on a Bruker DPX 300 spectrometer at 300.13 and 75.5 MHz, respectively, using $SiMe_4$ as the internal standard. The optical rotation was measured on a Perkin-Elmer 343 polarimeter; GLC/MS analysis was performed on a Hewlett Packard 5973 instrument (ionization energy 70 eV, helium as the carrier gas, an HP-5MS column). High-resolution mass spectra were run on an AMD-604S instrument. CD spectra were measured on a JASCO J-500A spectropolarimeter. IR spectra were recorded on a Bruker FT-IR Vector 22 spectrophotometer. HPLC was performed on a Du Pont 8800 chromatograph (with refractometer as the detector) with a Ultrasphere Si column (5 μm, 4.6×250 mm). The number of apoptotic cells was determined on a Becton Dickinson FACs Calibur Flow Cytometer (BD Biosciences, San Jose, CA, USA).

Column chromatography was carried out on silica gel KSK (50—160 μm, Sorbpolimer, Krasnodar, Russia); TLC was performed on Sorbfil plates (4.5×6.0 cm, Sorbpolimer, Krasnodar, Russia) with a foil-supported silica gel CTX-1A layer (5—17 μm).

The mollusks were collected in October 1986 at a 3—5 m depth near the Madagascar island by divers during the third scientific trip on the research ship entitled Academician Oparin. The mollusk species definition was carried out by A. V. Smirnov (Zoological Institute, Russian Academy of Sciences, St. Petersburg, Russia).

Isolation off compounds 1—4. The crushed mollusks (1 kg) were extracted twice with ethanol (2×2 L) at room temperature. The combined ethanol extract was concentrated *in vacuo* and chromatographed twice on a column with silica gel in the hexane—ethyl acetate system, 15 : 1. The terpenoid fraction (520 mg) was separated by HPLC on a Ultrasphere Si column (10 μm, 4×250 mm) with the hexane—ethyl acetate system, 25 : 1, as the eluent.

(6*S*,10*S*)-10-Bromo-3,11,11-trimethyl-7-methylidenespiro[5.5]undec-2-en-4-one (1). Colorless oil, yield 10 mg, $[\alpha]_D^{20}$ −39° (*c* 0.1, MeOH). The ^1H and ^{13}C NMR spectra are given in Table 1. CD (EtOH): $[\theta]_{305} = -3.5 \cdot 10^5$. MS (EI, 70 eV), *m/z* (I_{rel} (%)): 296/298 $[M]^+$ (each 6), 281/283 (each 1), 254/256 (each 7), 217 (90), 109 (100), 105 (90). HR MS: found: *m/z* 296.0731 $[M]^+$; $C_{15}H_{21}{}^{79}BrO$; calculated: 296.0776.

(4*S*,6*S*,10*S*)-10-Bromo-3,11,11-trimethyl-7-methylidenespiro[5.5]undec-2-en-4-ol (2). Colorless oil, yield 12 mg, $[\alpha]_D^{20}$ +30° (*c* 0.2, MeOH). IR ($CHCl_3$), ν/cm^{-1}: 3560. The ^1H and ^{13}C NMR spectra are given in Table 1. MS (EI, 70 eV), *m/z* (I_{rel} (%)): 298/300 $[M]^+$ (each 2), 280/282 (each 12), 265/267 (each 8), 254/256 (each 7), 211/213 (each 11) 201 (47), 105 (100). HR MS: found: *m/z* 298.0964 $[M]^+$; $C_{15}H_{23}{}^{79}BrO$; calculated: 298.0932.

(3*R*,4*S*,6*S*,10*R*)-10-Bromo-3,11,11-trimethyl-7-methylidenespiro[5.5]undecane-3,4-diol (3). Colorless oil, yield 15 mg, $[\alpha]_D^{20}$ +18° (*c* 0.3, MeOH). IR, $CHCl_3$), ν/cm^{-1}: 3572. The ^1H and ^{13}C NMR spectra are given in Table 2. MS (EI, 70 eV), *m/z* (I_{rel} (%)): 316/318 $[M]^+$ (each 18), 298/300 (each 62), 281/283 (each 40), 237 (60), 201(90), 109 (100), 105 (94). HR MS: found: *m/z* 316.1083 $[M]^+$; $C_{15}H_{25}{}^{79}BrO_2$; calculated: 316.1038.

(6*S*,7*S*,11*R*)-2-Chloro-3,7,11-trimethyl-10-methylidenespiro[5.5]undec-2-en-7-ol (4). Colorless oil, 8 mg, $[\alpha]_D^{20}$ +41° (*c* 0.3, MeOH). IR ($CHCl_3$), ν/cm^{-1}: 3450. The ^1H and ^{13}C NMR spectra are given in Table 3. MS (EI, 70 eV), *m/z* (I_{rel} (%)): 254 $[M]^+$ (2), 236 (25), 201 (16), 171(80), 166

(100), 128 (82), 109 (90). HR MS: found: m/z 254.1484 $[M]^+$; $C_{15}H_{23}O^{35}Cl$; calculated: 254.1438.

Determination of cytotoxicity. The cytotoxic action of the sesquiterpenoid **3** and dactylone **5** on the HL-60 and THP-1 leukemia cells was estimated by the MTS method.[11] The cells were cultivated in 96-well plates (10 thousand cells per well in 50 μL of the medium) in the RPMI 1640 medium containing 10% FBS for 12 h. Then compound **3** or **5** in different concentrations in 50 μL of the medium was added to the corresponding wells and the cells were incubated with compound **3** or **5** for 22 h. Then 20 μL of MTS was added to every well. The result was measured after 2 h by spectrophotometry at 492 and 690 nm using a μQuant plate reader (Bio-Tek Instruments, Inc., USA).

Determination of apoptosis by flow cytometry. The induction of apoptosis in human leukemia cells by dactylone **5** was analyzed by flow cytometry after staining the cells by the annexin V-fluorescein isothiocyanate (FITC) and propidium iodide dyes according to the manufacturer´s protocol (BD Pharmingen, Flanklin Lakes, NJ, USA). The HL-60 or THP-1 cells (2×10^5 cells per well) were cultivated in six-well plates for 12 h in 10% FBS/RPMI 1640 and then treated with specified concentrations of dactylone for 24 h. Then the cells were washed in PBS and centrifuged for 5 min at 1000 rpm. The precipitate was resuspended in 500 μL of 1×binding buffer. Then 5 μL of annexin V-FITC and propidium iodide (500 ng mL^{-1}) were added to the cell suspension. The mixture was kept for 15 min at room temperature in the dark and analyzed on a flow cytometer.

This work was supported by the Russian Academy of Sciences (Program of the Presidium of the RAS "Molecular and Cell Biology", Project of the Far-East Division of the RAS No. 06-III-A-05-122), the Russian Foundation for Basic Research (Project No. 05-04-48246), Council on grants at President of the Russian Federation (Program for the Support of Leading Scientific Schools, Project 6491.2006.4), and the Korean Science and Engineering Foundation (KOSEF), which rendered financial support through the Medical Research Center for Cancer Molecular Therapy (MRCCMT) at the Dong-A University (grant R13-2002-044-02003-0).

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Received April 13, 2007