Novel Antileukemic Sterol Glycosides from *Ajuga salicifolia*

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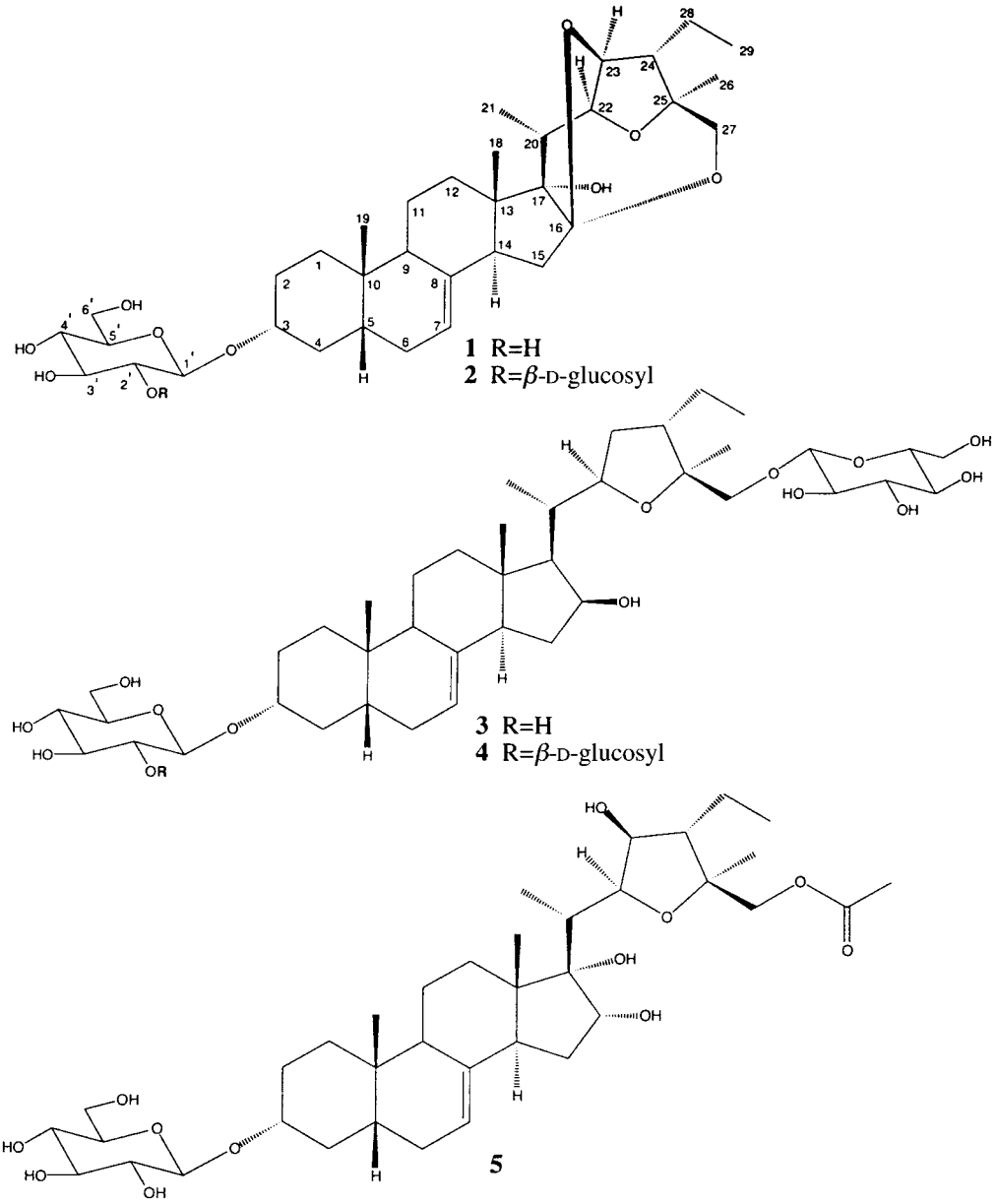
Two novel and three new sterol glycosides were isolated from the MeOH extract of the aerial parts of *Ajuga salicifolia* (L.) SCHREBER. The structures of the compounds were elucidated as (3*R*,16*S*,17*S*,20*R*,22*S*,23*S*,24*S*,25*S*)-16,23:16,27:22,25-triepoxy-3-(β-D-glucopyranosyloxy)coprostigmast-7-en-17-ol (**1**), (3*R*,16*S*,17*S*,20*R*,22*S*,23*S*,24*S*,25*S*)-16,23:16,27:22,25-triepoxy-3-{[β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl]oxy}coprostigmast-7-en-17-ol (**2**), (3*R*,16*S*,17*R*,20*S*,22*R*,24*S*,25*S*)-22,25-epoxy-3,27-bis(β-D-glucopyranosyloxy)coprostigmast-7-en-16-ol (**3**), (3*R*,16*S*,17*R*,20*S*,22*R*,24*S*,25*S*)-22,25-epoxy-3-{[β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl]oxy}-27-(β-D-glucopyranosyloxy)coprostigmast-7-en-16-ol (**4**), and (3*R*,16*R*,17*S*,20*R*,22*S*,23*S*,24*S*,25*S*)-22,25-epoxy-3-(β-D-glucopyranosyloxy)coprostigmast-7-ene-16,17,23,27-tetrol 27-acetate (**5**) by means of 1D and 2D NMR spectroscopy and HR-MALDI mass spectrometry. The novel compounds, which consist of three additional ring systems at the coprostigmastane skeleton, were named ajugasalicioside A (**1**) and B (**2**), and the new compounds C (**3**), D (**4**) and E (**5**). In our cytotoxicity assays (HeLa cells, Jurkat T cells, and peripheral mononuclear blood cells), ajugasaliciosides A–D specifically inhibited the viability and growth of Jurkat T-leukemia cells at concentrations below 10 μM. Ajugasalicioside A (**1**; (IC_{50} = 6 μM) and C (**3**; IC_{50} = 3 μM) were the most active compounds. Ajugasalicioside A (**1**) induced cell-cell contact, inhibited Jurkat T cell proliferation, and up-regulated mRNA levels of the cell-cycle regulator cyclin D1, which might be an indication for cell differentiation. Furthermore, **1** down-regulated the mRNA levels of the NF-κB subunit p65 in a concentration-dependent manner. These effects were not found for ajugasalicioside B (**2**), which has an additional glucose unit, and the onset of cytotoxicity of **2** (IC_{50} = 10 μM) was delayed by 24 h.

Introduction. – More than one hundred species, including fifty varieties and subspecies, of *Ajuga L.* (Lamiaceae) are unevenly distributed over the world. The genus *Ajuga L.*, commonly named bugle, is abundant in China, Korea, and Japan and widespread in Europe [1]. In the flora of Turkey, *Ajuga* is represented by 11 species [2], some of which are traditionally used in wound healing and as a diuretic, as well as against diarrhea and high fever [3]. There have been many phytochemical investigations on *Ajuga* species, focusing mainly on the isolation of phytoecdysteroids and diterpenes and on their antifeedant and insect-growth-inhibiting activities [1][4]. To date, there is only one report on *Ajuga salicifolia* (L.) SCHREBER, concerning the isolation of a diterpene [5]. Our investigations on the MeOH extract of the dried aerial parts of *Ajuga salicifolia*, which was collected in Turkey, resulted in the isolation of two novel and three new sterol glycosides (see **1**–**5**). We report here the chemical and biological characterization of these compounds.

Results and Discussion. – *Isolation and Structure Elucidation*. Sequential percolation of the powdered aerial parts of *A. salicifolia* with petroleum ether, CH_2Cl_2, AcOEt, MeOH and MeOH/H_2O 1:1 yielded the crude extracts. The prefractionation of the MeOH extract by vacuum liquid chromatography (VLC) afforded eight

fractions. The fractions rich in sterol glycosides were further fractionated by open-column chromatography (silica gel), MPLC, and HPLC (*RP-18*), resulting in the isolation of compounds **1**–**5**.



The HR-MALDI-MS spectrum of compound **1** showed a pseudomolecular-ion peak at *m/z* 657.3614 ($[M+Na]^+$), which is compatible with the molecular formula $C_{35}H_{54}O_{10}$. The ^1H- and ^{13}C-NMR data (see *Tables 1* and *2*) of **1** indicated the presence

Table 1. *^{13}C-NMR Data* (75.5 MHz) *of Ajugasaliciosides A – E* (**1** – **5**). Chemical shifts δ in ppm.

	1[a])	**2**[a])	**3**[b])	**4**[b])	**5**[a])
C(1)	38.3 (*t*)	38.2 (*t*)	36.5 (*t*)	36.5 (*t*)	38.5 (*t*)
C(2)	30.4 (*t*)	30.4 (*t*)	29.1 (*t*)	29.1 (*t*)	30.4 (*t*)
C(3)	79.0 (*d*)	80.1 (*d*)	76.3 (*d*)	77.6 (*d*)	79.0 (*d*)
C(4)	35.2 (*t*)	35.3 (*t*)	33.9 (*t*)	33.9 (*t*)	35.2 (*t*)
C(5)	41.4 (*d*)	41.5 (*d*)	39.6 (*d*)	40.3 (*d*)	41.4 (*d*)
C(6)	30.8 (*t*)	30.7 (*t*)	29.3 (*t*)	29.2 (*t*)	30.9 (*t*)
C(7)	119.2 (*d*)	119.2 (*d*)	119.1 (*d*)	117.4 (*d*)	118.9 (*d*)
C(8)	140.0 (*s*)	139.9 (*s*)	139.6 (*s*)	138.6 (*s*)	140.7 (*s*)
C(9)	50.4 (*d*)	50.4 (*d*)	48.7 (*d*)	48.8 (*d*)	50.8 (*d*)
C(10)	35.5 (*s*)	35.4 (*s*)	33.9 (*s*)	33.9 (*s*)	35.5 (*s*)
C(11)	22.0 (*t*)	22.0 (*t*)	20.9 (*t*)	21.0 (*t*)	22.2 (*t*)
C(12)	33.7 (*t*)	33.7 (*t*)	39.2 (*t*)	39.8 (*t*)	34.0 (*t*)
C(13)	50.4 (*s*)	50.4 (*s*)	43.3 (*s*)	43.4 (*s*)	49.5 (*s*)
C(14)	45.3 (*d*)	45.3 (*d*)	52.1 (*d*)	52.1 (*d*)	49.0 (*d*)
C(15)	45.1 (*t*)	45.1 (*t*)	35.1 (*t*)	35.1 (*t*)	33.6 (*t*)
C(16)	110.2 (*s*)	110.2 (*s*)	70.3 (*d*)	70.4 (*d*)	82.2 (*d*)
C(17)	85.4 (*s*)	85.4 (*s*)	59.2 (*d*)	59.3 (*d*)	87.8 (*s*)
C(18)	16.7 (*q*)	16.7 (*q*)	12.9 (*q*)	12.9 (*q*)	13.6 (*q*)
C(19)	13.5 (*q*)	13.5 (*q*)	12.9 (*q*)	12.9 (*q*)	13.5 (*q*)
C(20)	34.8 (*d*)	34.8 (*d*)	34.4 (*d*)	34.5 (*d*)	35.3 (*d*)
C(21)	13.7 (*q*)	13.7 (*q*)	13.4 (*q*)	13.5 (*q*)	12.8 (*q*)
C(22)	81.3 (*d*)	81.3 (*d*)	78.7 (*d*)	78.8 (*d*)	81.8 (*d*)
C(23)	81.5 (*d*)	81.5 (*d*)	32.6 (*t*)	32.7 (*t*)	78.5 (*d*)
C(24)	55.4 (*d*)	55.4 (*d*)	44.9 (*d*)	44.9 (*d*)	56.2 (*d*)
C(25)	86.9 (*s*)	86.9 (*s*)	83.4 (*s*)	83.5 (*s*)	84.1 (*s*)
C(26)	19.6 (*q*)	19.6 (*q*)	17.6 (*q*)	17.5 (*q*)	18.0 (*q*)
C(27)	78.3 (*t*)	78.4 (*t*)	74.4 (*t*)	74.5 (*t*)	72.0 (*t*)
C(28)	21.3 (*t*)	21.3 (*t*)	23.0 (*t*)	23.1 (*t*)	22.9 (*t*)
C(29)	12.8 (*q*)	12.8 (*q*)	13.3 (*q*)	13.3 (*q*)	13.8 (*q*)
Me*C*O	–	–	–	–	172.7 (*s*)
*Me*CO	–	–	–	–	20.7 (*q*)
C(1′)	102.3 (*d*)	101.4 (*d*)	100.8 (*d*)	100.0 (*d*)	102.3 (*d*)
C(2′)	75.1 (*d*)	83.0 (*d*)	73.5 (*d*)	83.0 (*d*)	75.1 (*d*)
C(3′)	78.1 (*d*)	77.8 (*d*)	76.7 (*d*)	75.9 (*d*)	78.1 (*d*)
C(4′)	71.7 (*d*)	71.4 (*d*)	70.0 (*d*)	69.9 (*d*)	71.7 (*d*)
C(5′)	77.9 (*d*)	78.4 (*d*)	76.7 (*d*)	76.5 (*d*)	77.9 (*d*)
C(6′)	62.8 (*t*)	62.7 (*t*)	61.1 (*t*)	60.8 (*t*)	62.8 (*t*)
C(1″)	–	105.2 (*d*)	103.1 (*d*)	104.5 (*d*)	–
C(2″)	–	76.0 (*d*)	73.5 (*d*)	75.3 (*d*)	–
C(3″)	–	77.6 (*d*)	76.7 (*d*)	75.9 (*d*)	–
C(4″)	–	71.4 (*d*)	70.0 (*d*)	70.0 (*d*)	–
C(5″)	–	77.8 (*d*)	76.7 (*d*)	77.2 (*d*)	–
C(6″)	–	62.7 (*t*)	61.0 (*t*)	60.9 (*t*)	–
C(1‴)	–	–	–	103.2 (*d*)	–
C(2‴)	–	–	–	73.4 (*d*)	–
C(3‴)	–	–	–	76.8 (*d*)	–
C(4‴)	–	–	–	69.7 (*d*)	–
C(5‴)	–	–	–	76.8 (*d*)	–
C(6‴)	–	–	–	61.1 (*t*)	–

[a]) In CD_3OD. [b]) In $(D_6)DMSO$.

Table 2. *^{1}H-NMR Data* (500 MHz, CD_3OD) *of Ajugasalicioside A* (**1**) *and B* (**2**). δ in ppm, J in Hz.

	1	**2**
CH_2(1)	1.86 (*m*)[a]); 1.15 (*m*)	1.85 (*m*)[a]); 1.10 (*m*)[a])
CH_2(2)	1.88 (*m*)[a]); 1.52 (*m*)[a])	1.92 (*m*)[a]); 1.46 (*m*)[a])
H–C(3)	3.71 (*m*)	3.72 (*m*)
CH_2(4)	1.86 (*m*)[a]); 1.30 (*m*)	1.88 (*m*)[a]); 1.32 (*m*)
H–C(5)	1.39 (*m*)	1.39 (*m*)
CH_2(6)	1.77 (*m*)	1.78 (*m*)
H–C(7)	5.09 (*m*)	5.09 (*m*)
H–C(9)	1.71 (*m*)[a])	1.68 (*m*)[a])
CH_2(11)	1.65 (*m*)[a]); 1.50 (*m*)[a])	1.65 (*m*)[a]); 1.46 (*m*)[a])
CH_2(12)	1.94 (*m*)[a]); 1.50 (*m*)[a])	1.94 (*m*)[a]); 1.48 (*m*)[a])
H–C(14)	2.47 (*m*)	2.47 (*m*)
CH_2(15)	1.98 (*dd*, J = 6.1, 12.8); 1.82 (*m*)[a])	1.98 (*dd*, J = 6.1, 12.8); 1.82 (*m*)[a])
Me(18)	0.74 (*s*)	0.73 (*s*)
Me(19)	0.83 (*s*)	0.83 (*s*)
H–C(20)	2.07 (*m*)	2.07 (*m*)
Me(21)	1.16 (*d*, J = 7.2)	1.16 (*d*, J = 7.2)
H–C(22)	4.14 (*dd*, J = 1.2, 5.6)	4.14 (*dd*, J = 1.1, 5.6)
H–C(23)	4.28 (*d*, J = 5.6)	4.27 (*d*, J = 5.6)
H–C(24)	2.20 (*dd*, J = 4.0, 11.4)	2.20 (*dd*, J = 4.0, 11.4)
Me(26)	1.11 (*s*)	1.11 (*s*)
CH_2(27)	4.25, 3.61 (2 *d*, J = 12.6)	4.26, 3.61 (2 *d*, J = 12.6)
CH_2(28)	1.63 (*m*)[a])	1.63 (*m*)[a]); 1.11 (*m*)[a])
Me(29)	1.05 (*t*, J = 7.2)	1.05 (*t*, J = 7.2)
H–C(1′)	4.40 (*d*, J = 7.8)	4.54 (*d*, J = 7.7)
H–C(2′)	3.14 (*dd*, J = 7.8, 9.1)	3.40 (*dd*, J = 7.8, 9.0)
H–C(3′)	3.35 (*t*, J = 9.1)	3.54 (*t*, J = 9.0)
H–C(4′)	3.27 (*m*)[a])	3.35 (*t*, J = 9.0)
H–C(5′)	3.26 (*m*)[a])	3.31 (*m*)[a])
CH_2(6′)	3.85 (*dd*, J = 1.6, 12.0); 3.65 (*dd*, J = 5.4, 11.9)	3.85 (*td*, J = 2.2, 11.8); 3.70 (*dd*, J = 5.0, 11.8)
H–C(1″)	–	4.57 (*d*, J = 7.8)
H–C(2″)	–	3.25 (*dd*, J = 7.8, 9.2)
H–C(3″)	–	3.36 (*m*)[a])
H–C(4″)	–	3.31 (*m*)[a])
H–C(5″)	–	3.28 (*m*)[a])
CH_2(6″)	–	3.85 (*dd*, J = 2.2, 11.8); 3.70 (*dd*, J = 5.0, 11.8)

[a]) Signals overlapped.

of a β-glucopyranosyl moiety connected to a C_{29}-sterol aglycone. ^{1}H,^{1}H-COSY, ^{13}C,^{1}H-HSQC, and ^{13}C,^{1}H-HMBC (see *Fig. 1*) allowed the complete assignment of all protons and C-atoms and the ROESY data of the configuration of compound **1**. Therefore, the structure of **1** was established as (3*R*,16*S*,17*S*,20*R*,22*S*,23*S*,24*S*,25*S*)-16,23 : 16,27 : 22,25-triepoxy-3-(β-D-glucopyranosyloxy)coprostigmast-7-en-17-ol[1]). The three epoxy groups provide the coprostigmastan skeleton with three additional, five-, six-, and eight-membered ring systems. To our knowledge, compound **1** is a novel sterol glycoside, which we named ajugasalicioside A (**1**).

[1]) Coprostigmastane = (5β)-stigmastane; for more systematic names, see *Exper. Part.*

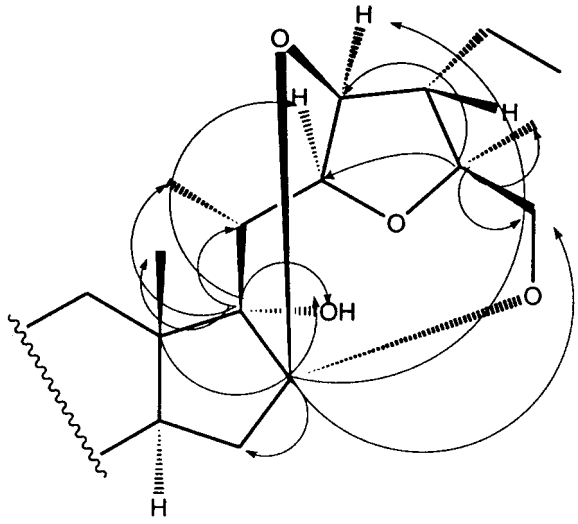


Fig. 1. *Main HMBC* (C → H) *correlations of ajugasalicioside A* (**1**)

Besides the anomeric proton resonance at δ(H) 4.40 (*d*, *J* = 7.8 Hz) and the signal of H–C(3) at 3.71 (*m*), the ^{1}H-NMR spectrum of **1** showed the presence of 5 Me groups at δ(H) 0.74, 0.83, and 1.11 as *s*, δ(H) 1.05 (*J* = 7.2 Hz) as *t*, and δ(H) 1.16 (*J* = 7.2 Hz) as *d*. Additional functionalities of the aglycone included the signal of an olefinic proton at δ(H) 5.09 (*m*, H–C(7)), as well as of 2 vicinal CH protons at δ(H) 4.14 (*dd*, *J* = 1.2, 5.6 Hz, H–C(22)) and 4.28 (*d*, *J* = 5.6 Hz, H–C(23)) and of 1 CH_2 group at δ(H) 4.25 (*d*, *J* = 12.6 Hz, H_a–C(27)) and 3.61 (*d*, *J* = 12.6 Hz, H_b–C(27)) on O-bearing C-atoms. In accordance, the ^{13}C-NMR spectrum showed signals of 35 C-atoms, confirming the presence of a highly oxygenated C_{29} sterol monoglucoside. The olefinic signals at δ(C) 119.2 and 140.0 corresponded to the endocyclic C=C bond between C(7) and C(8), and the signal at δ(C) 102.3 to the anomeric C-atom of the β-glucopyranosyl moiety. Oxygenated C-atoms resonated at δ(C) 79.0, 81.3, and 81.5 as oxymethine (C(3), C(22), and C(23) resp.) and at δ(C) 78.3 as an oxymethylene group (C(27)), and at δ(C) 110.2, 85.4, and 86.9 as quarternary C-atoms (C(16), C(17), C(25), resp.).

The sugar moiety of **1** was identified as β-glucose based on ^{1}H- and ^{13}C-NMR chemical shifts as mentioned above and confirmed by the correlations observed in HSQC-TOCSY spectrum. In the ROESY plot, 1′,3′-diaxial ROEs were also visible, whereas 2′,4′-diaxial ROEs could not unambiguously be identified due to spectral overlap and the presence of TOCSY artefacts in the ROESY.

In the HMBC spectrum of **1**, the long-range correlations between H–C(27) and C(16), H–C(23) and C(16), and H–C(22) and C(25) showed the presence of three epoxy groups. The long-range correlation observed in the HMBC spectrum (measured in DMSO) between the tertiary OH proton and C(13), C(16), C(17), and C(20) established the existence of OH–C(17). The linkage of the sugar moiety to C(3) was confirmed by the long-range correlation between H–C(1′) and C(3). The β-D-configuration at C(1′) is based upon the chemical shift of H–C(1′) (4.4 ppm), C(1′) (102.3 ppm), and the ^3J coupling (7.8 Hz). The relative configuration of the aglycone was determined by the ROE data. *Fig. 2* displays the important ROEs on the energy-minimized model of ajugasalicioside A (**1**). The ROESY correlations between Me(19) and H–C(5) established the *cis* A/B ring fusion and the cross-peak observed between H–C(5) and H–C(3) the α-glycosidation at C(3). The configuration at the other stereogenic centers was confirmed by the ROESY correlations between H–C(14)/Me(21)/H–C(27) and OH–C(17), Me(21) and H–C(12), Me(21)/H–C(23) and H–C(22), H–C(23) and Me(29), as well as between H–C(24) and H_a–C(27).

The molecular formula of compound **2** was deduced to be $C_{41}H_{64}O_{15}$ from the pseudomolecular-ion peak at *m*/*z* 819.4041 ($[M + Na]^+$) in the HR-MALDI-MS and from NMR data. The structure of **2** was established as (3*R*,16*S*,17*S*,20*R*,22*S*,23*S*, 24*S*,25*S*)-16,23 : 16,27 : 22,25-triepoxy-3-{[β-D-glucopyranosyl-(1 → 2)-β-D-glucopyra-

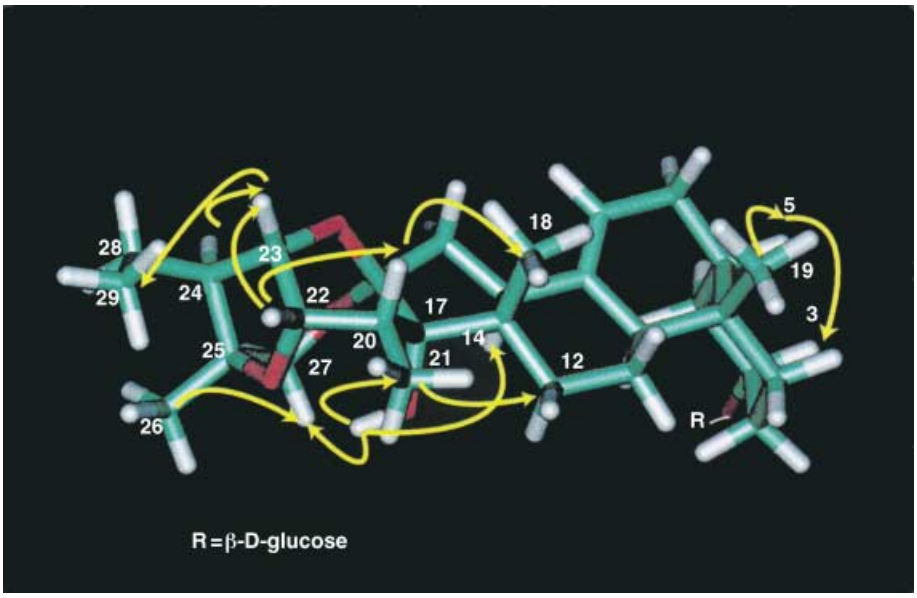


Fig. 2. *Key ROEs of ajugasalicioside A* (**1**)

nosyl]oxy}coprostigmast-7-en-17-ol[1]). Compound **2**, named trivially as ajugasalicioside B, is a diglycoside containing the same novel aglycone as compound **1**.

The ^{13}C-NMR (*Table 1*) and DEPT-135 spectra of **2** showed peaks of 41 C-atoms. The ^{1}H- and ^{13}C-NMR spectra of **2** resembled those of **1**, and the signals of the aglycone moiety appeared almost at the same shift values and with identical multiplicities. The assignment of the sugar signals in ^{1}H,^{13}C and ^{1}H,^{13}C-HSQC spectra suggested the presence of an additional β-D-glucopyranosyl moiety. This was confirmed by the COSY and HSQC-TOCSY data. The downfield shift at δ(C) 83.0 (C(2′)) and δ(H) 3.40 (*dd*, H−C(2′)) established the position of the interglycosidic linkage. The long-range correlation observed between H−C(1″) and C(2′) supported this deduction. The ROESY data of **2** showed the same correlations as those of **1**.

Ajugasalicioside C (**3**) had a pseudomolecular-ion peak $[M+\mathrm{Na}]^+$ at m/z 807.4499 in the HR-MALDI-MS, consistent with the molecular formula $C_{41}H_{68}O_{14}$. Besides the ^{1}H- and ^{13}C-NMR (see *Tables 1* and *3*) and DEPT-135 spectral data, COSY and HSQC-TOCSY experiments confirmed again the presence of a diglucoside with a C_{29}-sterol aglycone moiety. The structure of compound **3** was established as (3*R*,16*S*,17*R*,20*S*,22*R*,24*S*,25*S*)-22,25-epoxy-3,27-bis(β-D-glucopyranosyloxy)coprostigmast-7-en-16-ol[1]). A C_{27} ecdysteroid with an epoxy group between C(22) and C(25) has been reported previously [6]. Ajugasalicioside C (**3**) is a new sterol diglucoside, showing the same kind of epoxidation at a C_{29}-sterol skeleton.

The ^{13}C-NMR spectra of **3** displayed the signals of 3 oxymethine groups at δ(C) 76.3 (C(3)), 70.3 (C(16)), and 78.7 (C(22)), one oxymethylene group at δ(C) 74.4 (C(27)), and one oxygenated quaternary C-atom at δ(C) 83.4 (C(25)). The chemical shifts of three methine protons at δ(H) 3.55 (*m*, H−C(3)), 4.19 (*m*, H−C(16)), and 4.06 (*m*, H−C(22)) and two methylene protons at δ(H) 3.70 (*d*, J = 10.1 Hz, H_a−C(27)) and 3.31 (*d*, J = 10.1 Hz, H_b−C(27)) were in accordance with the ^{13}C-NMR data mentioned above, supporting the assignment of the oxygenated centers. The HMBC correlation between H−C(22) and C(25) indicated the

Table 3. *^{1}H-NMR (Data* (500 MHz) *of Ajugasalicioside C* (**3**) *and D* (**4**) *in (D_6)DMSO and Ajugasalicioside E* (**5**) *in CD_3OD.* δ in ppm, J in Hz.

	3	**4**	**5**
CH_2(1)	1.75 (*m*)[a]) 1.02 (*m*)[a])	1.72 (*m*)[a]); 0.98 (*m*)[a])	1.84 (*m*)[a]); 1.10 (*m*)
CH_2(2)	1.78 (*m*)[a]); 1.30 (*m*)[a])	1.80 (*m*)[a]); 1.32 (*m*)[a])	1.86 (*m*)[a]); 1.50 (*m*)[a])
H–C(3)	3.55 (*m*)	3.51 (*m*)	3.71 (*m*)
CH_2(4)	1.78 (*m*)[a]); 1.16 (*m*)[a])	1.77 (*m*)[a]); 1.20 (*m*)[a])	1.85 (*m*)[a]); 1.33 (*m*)[a])
H–C(5)	1.26 (*m*)[a])	1.26 (*m*)[a])	1.37 (*m*)[a])
CH_2(6)	1.78 (*m*)[a]); 1.67 (*m*)[a])	1.74 (*m*)[a]); 1.65 (*m*)[a])	1.80 (*m*)[a])
H–C(7)	5.12 (*m*)	5.09 (*m*)	5.22 (*m*)
H–C(9)	1.68 (*m*)[a])	1.57 (*m*)[a])	1.65 (*m*)[a])
CH_2(11)	1.54 (*m*)[a]); 1.42 (*m*)[a])	1.52 (*m*)[a]); 1.38 (*m*)[a])	1.63 (*m*)[a]); 1.54 (*m*)
CH_2(12)	1.95 (*m*); 1.15 (*m*)[a])	1.87 (*m*); 1.15 (*m*)[a])	1.76 (*m*)[a])
H–C(14)	1.64 (*m*)[a])	1.60 (*m*)[a])	2.40 (*m*)
CH_2(15)	2.03 (*m*)[a]); 1.34 (*m*)	2.01 (*m*)[a]); 1.31 (*m*)[a])	2.12 (*ddd*, *J* = 4.8, 6.9, 8.3); 1.44 (*ddd*, *J* = 5.6, 5.7, 5.8)
H–C(16)	4.19 (*m*)	4.17 (*m*)[a])	3.97 (*dd*, *J* = 5.7, 8.4)
H–C(17)	1.01 (*m*)[a])	1.00 (*m*)[a])	–
Me(18)	0.69 (*s*)	0.68 (*s*)	0.84 (*s*)
Me(19)	0.74 (*s*)	0.72 (*s*)	0.82 (*s*)
H–C(20)	2.03 (*m*)[a])	2.01 (*m*)[a])	2.57 (*m*)
Me(21)	0.86 (*d*, *J* = 5.6)	0.85 (*d*, *J* = 6.0)	1.03 (*d*, *J* = 6.7)
H–C(22)	4.06 (*m*)	4.06 (*m*)	3.91 (*ddd*, *J* = 3.0, 3.3, 3.4)
H–C(23) or CH_2(23)	1.88 (*m*)[a]); 1.60 (*m*)[a])	1.88 (*m*); 1.56 (*m*)[a])	4.00 (*m*)
H–C(24)	1.81 (*m*)[a])	1.80 (*m*)[a])	1.83 (*m*)[a])
Me(26)	1.00 (*s*)	0.98 (*s*)	1.16 (*s*)
CH_2(27)	3.70, 3.31 (2*d*, *J* = 10.1)	3.69, 3.31 (2*d*, *J* = 10.1)	4.17, 4.03 (2*d*, *J* = 11.2)
CH_2(28)	1.42 (*m*)[a]); 1.09 (*m*)	1.41 (*m*); 1.08 (*m*)	1.48 (*m*)[a]); 1.33 (*m*)[a])
Me(29)	0.88 (*t*, *J* = 7.2)	0.87 (*t*, *J* = 7.2)	1.16 (*t*, *J* = 7.3)
MeCO	–	–	2.08 (*s*)
H–C(1′)	4.22 (*d*, *J* = 7.8)	4.37 (*d*, *J* = 7.8)	4.40 (*d*, *J* = 7.8)
H–C(2′)	2.89 (*ddd*, *J* = 4.5, 4.5, 4.7)	3.12 (*m*)[a])	3.14 (*dd*, *J* = 7.9, 9.1)
H–C(3′)	3.02–3.18 (*m*)[a])	3.34 (*m*)[a])	3.35 (*t*, *J* = 9.3)
H–C(4′)	3.02–3.18 (*m*)[a])	3.08 (*m*)[a])	3.28 (*t*, *J* = 9.6)
H–C(5′)	3.02–3.18 (*m*)[a])	3.08 (*m*)[a])	3.27 (*m*)[a])
CH_2(6′)	3.65 (*m*)[a]); 3.40 (*m*)[a])	3.63 (*m*)[a]); 3.50 (*m*)[a])	3.65 (*dd*, *J* = 5.3, 11.8); 3.87 (*dd*, *J* = 9.6, 11.8)
H–C(1″)	4.18 (*d*, *J* = 7.8)	4.31 (*d*, *J* = 7.8)	–
H–C(2″)	2.97 (*ddd*, *J* = 4.5, 4.5, 4.7)	2.98 (*m*)[a])	–
H–C(3″)	3.02–3.18 (*m*)[a])	3.15 (*m*)[a])	–
H–C(4″)	3.02–3.18 (*m*)[a])	3.02 (*m*)[a])	–
H–C(5″)	3.02–3.18 (*m*)[a])	3.05 (*m*)[a])	–
CH_2(6″)	3.65 (*m*)[a]); 3.43 (*m*)[a])	3.63 (*m*)[a]); 3.40 (*m*)[a])	–
H–C(1‴)	–	4.14 (*d*, *J* = 7.8)	–
H–C(2‴)	–	2.95 (*m*)[a])	–
H–C(3‴)	–	3.10 (*m*)[a])	–
H–C(4‴)	–	3.13 (*m*)[a])	–
H–C(5‴)	–	3.05 (*m*)[a])	–
CH_2(6‴)	–	3.63 (*m*)[a]); 3.40 (*m*)[a])	–

[a]) Signals overlapped.

presence of an epoxy group. Resonances for the anomeric protons of the sugar moiety were observed at δ(H) 4.22 (d, J = 7.8 Mz, H–C(1′), β-D-glucosyl), and δ(H) 4.18 (d, J = 7.8 Hz, H–C(1″), β-D-glucosyl). The HMBC correlations observed between H–C(1′) and C(3) and between H–C(1″) and C(27) assigned the linkage of the β-D-glucose moieties to the aglycone. The remaining O-bearing methine CH(16) was assumed to be hydroxylated. This was established by HMBC (measured in (D_6)DMSO) correlations between C(15)/C(16)/C(17) and OH–C(16). The ROESY data of **3** resembled those of **1** and **2**, showing the identical relative configuration at C(17) as well as at C(3). The configuration at C(16) was assigned by ROESY correlations between Me(18) and OH–C(16), H–C(16) and H–C(17), and H–C(17) and H–C(14).

The pseudomolecular ion m/z 969.5025 ($[M + Na]^+$) in the HR-MALDI-MS as well as the ^{13}C- and ^{1}H-NMR spectral data (see *Tables 1* and *3*) of ajugasalicioside D (**4**) were consistent with the molecular formula $C_{47}H_{78}O_{19}$. The ^{13}C-NMR data of the aglycone moiety were superimposable with those of **3**, suggesting an identical aglycone part and the presence of a sugar moiety consisting of two monosaccharides linked to the steroidal skeleton at C(3) and at C(27). The ^{1}H- and ^{13}C-NMR data of ajugasalicioside D (**4**), the most polar isolate, indicated the presence of three β-glucopyranose units. The structure of **4** was established as (3*R*,16*S*,17*R*,20*S*,22*R*,24*S*,25*S*)-22,25-epoxy-3-{[β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranosyl]oxy}-27-(β-D-glucopyranosyloxy)coprostigmast-7-en-16-ol[1]).

H–C(1′) of **4** resonated at δ(H) 4.37 (d, J = 7.8 Hz) and C(1′) at δ(C) 100.0, H–C(1″) at δ(H) 4.31 (d, J = 7.8 Hz) and C(1″) at δ(C) 104.5, and H–C(1‴) at δ(H) 4.14 (d, J = 7.8 Hz) and C(1‴) at δ(C) 103.2. The assignment of all sugar and aglycone signals was based on COSY, HSQC, and HSQC-TOCSY experiments. Acid hydrolysis and TLC comparison of the hydrolyzed components with authentic sugars confirmed the presence of three β-D-glucopyranose units. The linkage of the sugars was established by HMBC correlations. The long-range correlation observed between H–C(1″) and C(2′) revealed the interglycosidic connection of the two β-D-glucopyranoses, and the correlation between H–C(3) and C(1′) in the HMBC spectrum established the linkage at C(3).

The ^{1}H- and ^{13}C-NMR data (see *Tables 1* and *3*) of ajugasalicioside E (**5**) showed the presence of a β-D-glucopyranose moiety and a coprostigmast-7-ene skeleton substituted by an acetylated hydroxymethyl group at the tetrahydrofuran moiety. The HR-MALDI-MS provided a pseudomolecular ion $[M + Na]^+$ at m/z 719.4005, corresponding to the molecular formula $C_{37}H_{60}O_{12}$. With the help of COSY, HSQC-TOCSY, and HMBC data (see *Fig. 3*), compound **5** was determined as (3*R*,16*R*,17*S*,20*R*,22*S*,23*S*,24*S*,25*S*)-22,25-epoxy-3-(β-D-glucopyranosyloxy)coprostigmast-7-ene-16,17,23,27-tetrol 27-acetate[1]).

The ^{1}H- and ^{13}C-NMR data of the sugar moiety of **5** were identical to those of compound **1**. The long-range correlation between H–C(1′) and C(3) pointed out the existence of a β-D-glucose moiety at C(3). The remaining oxygenated C-resonances for the aglycone indicated the presence of three oxymethine groups (δ(C) 82.2, 81.8,78.5), one oxymethylene group (δ(C) 72.0), and two oxygenated quaternary C-atoms δ(C) 87.8, 84.1). The HMBC correlation observed between H–C(22) and C(25) (δ(C) 84.1) indicated the presence of an epoxy group. The signals at δ(C) 20.7 and 172.7 and δ(H) 2.08 (s, 3 H) were assigned to the acetyl group. The long-range correlations observed between the CH_2(27) signals and the carbonyl signal of the acetate at δ(C) 172.7 helped us to determine the position of the acetylation. The chemical shifts of two geminal methine protons (δ(H) 3.97 (dd, J = 5.7, 8.4) and 4.00 (m)) which were attributed to H–C(16) and H–C(23), respectively, suggested the presence of two OH groups. In the HMBC spectrum (measured in (D_6)DMSO), the long-range correlations observed between C(13)/C(17)/C(16) and OH–C(17) and between C(16)/C(15) and OH–C(16) confirmed the hydroxylation at C(16) and C(17), respectively. The relative configuration of **5** was identical to the other isolated sterol glycosides. The configuration at C(16), C(17), and C(23) was established by the

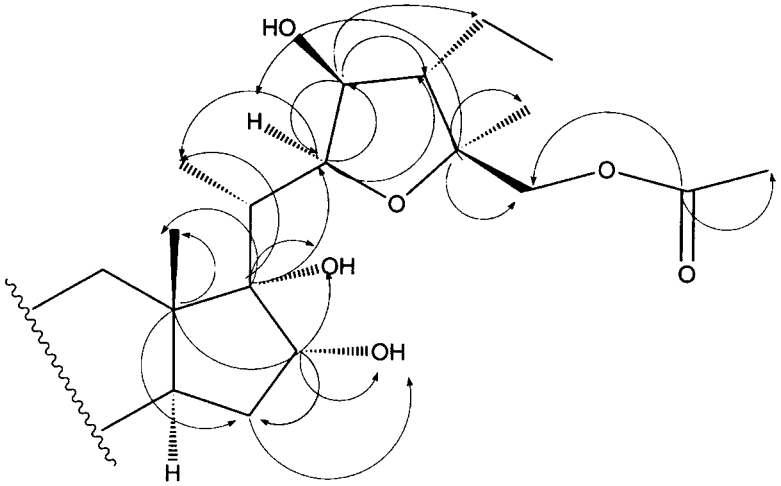


Fig. 3. *Selected long-range* (HMBC) *connectivities of ajugasalicioside E* (**5**)

ROESY correlations between H–C(14)/OH–C(17) and OH–C(16), Me(18)/H–C(21) and H–C(16), H–C(14)/OH–C(16) and OH–C(17), and Me(29) and H–C(23).

Biological Activity. The compounds **1**–**5** were tested for cytotoxicity against KB (HeLa), Jurkat (human T-cell leukemia), and human peripheral mononuclear blood cells (PMBC) (see *Table 4*). In KB cells, none of the compounds were active, whereas, in Jurkat T cells, ajugasalicioside A–D (**1**–**4**) showed significant to moderate activity ($IC_{50} \leq 10$ μM). Ajugasalicioside C (**3**) was the most active against Jurkat T cells ($IC_{50} = 3$ μM), followed by the novel compound ajugasalicioside A (**1**; $IC_{50} = 6$ μM). Ajugasalicioside C (**3**) also showed weak cytotoxicity against PMBC. An additional glucose unit led to weaker cytotoxicity against Jurkat T cells, as observed for ajugasalicioside B (**2**; $IC_{50} = 10$ μM) and D (**4**; $IC_{50} = 8$ μM). The lack of activity of ajugasalicioside E (**5**) against all cell types tested can be explained by the observed insolubility of this compound in the cell-culture medium. Interestingly, ajugasalicioside A (**1**) induced cell-cell contacts in Jurkat T cell populations similar to phorbol 12-myristate 13-acetate (PMA). Cell-cell interactions have been observed in cellular cytotoxicity and cell differentiation [7][8]. To follow this effect, we measured the possible modulation of ajugasalicioside A (**1**) on PMA-induced mRNA profiles in Jurkat T cells with reverse transcription real time PCR (RT-rt-PCR) [9]. No effect was

Table 4. *Cytotoxicity of Ajugasaliciosides A–E* (**1**–**5**) *against KB, Jurkat T, and Peripheral Mononuclear Blood Cells* (PMBC) *after 72 h*. Values given as IC_{50} in μM ($n = 4$).

	KB	Jurkat	PMBC
Ajugasalicioside A (**1**)	> 40	6	> 40
Ajugasalicioside B (**2**)	> 50	10	> 40
Ajugasalicioside C (**3**)	> 30	3	15
Ajugasalicioside D (**4**)	> 50	8	> 30
Ajugasalicioside E (**5**)	> 50	> 50	> 50

observed on mRNA levels of PMA-induced chemokine (IL-2, GM-CSF, INF-γ, data not shown) or house-keeping genes, but we discovered a significant up-regulation of cyclin D1 mRNA expression (see *Fig. 4*).

Cyclin D1 is the regulatory subunit of certain protein kinases, such as cdk5, which advances the G1 phase of the cell cycle. Cyclin D1 is expressed relatively early in the cell cycle and is crucial to commitment of DNA synthesis. Deregulated cyclin D1 expression has been implicated in several human neoplasms, including leukemia cells [10][11]. Cyclin D1 and cdk5 expression has been shown to play a role in cell differentiation, initiating the transit from the G1 phase to maturation [12]. Jurkat T cells do not up-regulate cyclin D1 when stimulated with PMA alone. Therefore, PMA does not induce complete differentiation in Jurkat T cells, although a T helper cell type 1 expression profile is favored. When the Jurkat T cells were pretreated with ajugasalicioside A (**1**; 10–30 μM), PMA stimulation resulted in a 2- to 4-fold up-regulation of cyclin D1 mRNA levels after 24 h (see *Fig. 4*). This is a possible indication that ajugasalicioside A (**1**) may stimulate differentiation processes. Since it has been

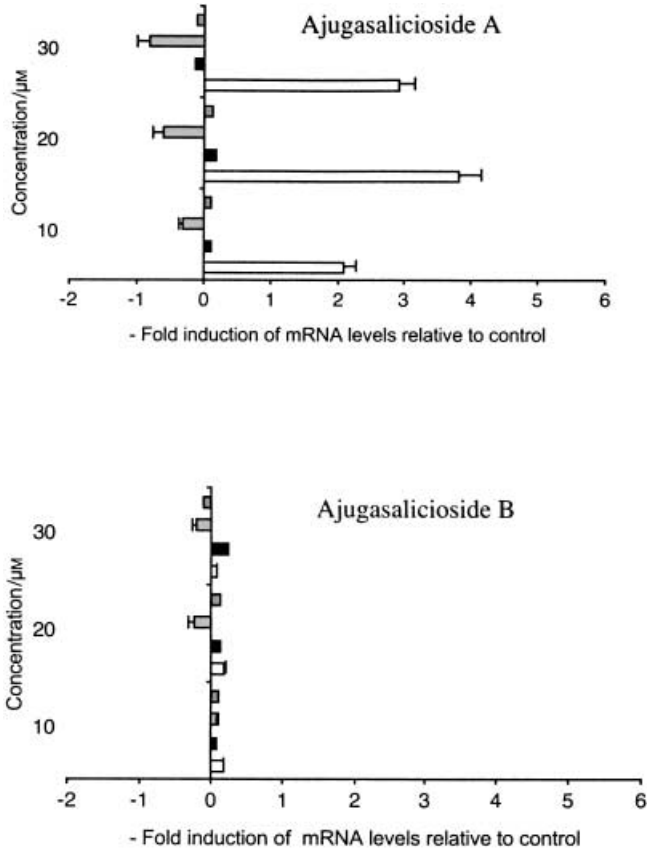


Fig. 4. *Induction of mRNA levels of cyclin D1* (white), *β-actin* (black), *p65* (light grey), *and I-κBα* (dark grey), *shown as fold induction* ± *S.E. at 3 concentrations given in* μM. $n = 3$.

shown that cyclin D1 can be co-activated by the transcription factor NF-κB [10], we also looked at the mRNA levels of NF-κB subunit p65 and I-κBα after 24 h incubation. Ajugasalicioside A (**1**) weakly inhibited PMA-induced p65 mRNA levels in a concentration-dependent manner (see *Fig. 4*) but did not influence I-κBα. Our results, therefore, suggest a NF-κB-independent induction of cyclin D1 by ajugasalicioside A (**1**). The down-regulation of p65 would not be observed under NF-κB-activated conditions and may rather represent an early sign of toxic action, but, on the other hand, the house-keeping gene β-actin was not modulated, and cell viability was not reduced (see *Fig. 4*). In contrast to ajugasalicioside A (**1**), no cytotoxic action was observed for ajugasalicioside B (**2**) within the first 48 h, nor did this compound induce cell-cell interactions. Ajugasalicioside B (**2**) was also tested for modulation of cyclin D1 mRNA levels and showed no effects at concentrations between 10 and 30 μM. Summing up, the additional glucose unit dramatically influenced the onset of the cytotoxicity against Jurkat T cells and abolished the cyclin D1 mRNA modulation, but altered the long-term cytotoxicity only moderately.

Antimicrobial activity of the isolated compounds was tested against *Gram*-positive (*Bacillus cereus*, *Staphylococcus epidermidis, Staphylococcus aureus*, and *Micrococcus luteus*) and -negative (*Pseudomonas aeruginosa*) bacterial strains and the yeast *Candida albicans*, but no activity was found.

Conclusions. – Two novel (ajugasalicioside A (**1**) and B (**2**)) and three new sterol glycosides (ajugasalicioside C–E (**3**–**5**)) were isolated from the aerial parts of *Ajuga salicifolia*. Ajugasalicioside A–D (**1**–**4**) showed significant to moderate cytotoxicity against Jurkat (human T-cell leukemia) cells. Ajugasalicioside A (**1**) and C (**3**) were the most cytotoxic compounds. An additional glucose unit led to a weaker cytotoxicity against Jurkat T cells, as observed for ajugasalicioside B (**2**) and D (**4**). Ajugasalicioside A (**1**) induced cell-cell contacts in Jurkat T cell population and significantly up-regulated cyclin D1 mRNA expression, which might be an indication for cell differentiation. It is known, that many hormones and growth factors influence cell growth through signal-transduction pathways that modify the activity of cyclin D1 [13], but there are no reports about such effects caused by plant-derived sterol glycosides. Induction of differentiation or antiproliferative therapies have been most effective in the treatment of promyelocytic leukemia, and hence our results might provide a basis for a potential therapeutic use of the novel structural features of ajugasalicioside A (**1**). However, further research will be necessary in order to focus on the biochemical nature of cytotoxicity of these compounds, and additional experiments are required to investigate whether ajugasalicioside A (**1**) induces apoptosis.

Experimental Part

General. Vacuum liquid chromatography (VLC): *RP-18 HL* (40–63 μm), silica gel *60* (15–40 μm). Open-column chromatography (CC): silica gel *60* (40–63 μm, 63–200 μm). MPLC: *Büchi 681* pump, *Büchi* glass column (45 mm × 450 mm); stationary phase *RP-18 HL* (15–35 μm); fraction collector *Büchi 684*. HPLC: *Merck-Hitachi-L-6200* pump, fraction collector *L-5200*, UV detector *L-4000* and chromato-integrator *D-2500*, *Spherisorb-S5-ODS-2* column (5 μm; 250 × 16 mm; *Knauer*), reversed phase-cartridge *Sep-Pak RP-18* (*Waters*). TLC: silica gel *60* F_{254} plates (*Merck*) and *RP-18* $F_{254}S$ plates (*Merck*); detection by spraying with 5% H_2SO_4 in EtOH and 1% vanillin in EtOH and heating at 100–110° for 5 min. Optical rotation: *Perkin-Elmer 241*

polarimeter at 20°. UV Spectra: *Uvikon 930* spectrophotometer. NMR Spectra: *Bruker DRX-500* (at 500.13 (^{1}H) and 125.77 MHz (^{13}C)), at 295 K, for all 1 D ^{1}H,^{1}H,^{1}H-COSY, ^{13}C,^{1}H-HSQC, ^{13}C,^{1}H-HSQC-TOCSY, ^{13}C,^{1}H-HMBC, and ^{1}H,^{1}H-ROESY experiments; *Bruker AMX-300* (at 75.47 MHz (^{13}C)), at 295 K, for all ^{13}C, DEPT-135 and DEPT-90 experiments; chemical shifts δ in ppm, referenced against residual non-deuterated solvent, and coupling constants J in Hz. HR-MALDI-MS: *Ionspec Ultima-FTMS* spectrometer, 2,5-dihydroxybenzoic acid (DHB) as matrix.

Plant Material. Ajuga salicifolia (L.) SCHREBER was collected in Ankara, Beytepe, in July 1998. The plant was identified by Prof. *Zeki Aytac*, Gazi University, Ankara (Turkey). A voucher specimen (HU-98014) was deposited at the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Hacettepe University.

Extraction and Isolation. The dried and powdered aerial parts (548 g) of *A. salicifolia* were extracted with petroleum ether, CH_2Cl_2, AcOEt, MeOH, and MeOH/H_2O 1:1, resp. (sequential percolation with *ca.* 10–15 l of each solvent). The lyophilized MeOH extract yielded 137 g of residue. Thereof 40 g was subjected to VLC (*RP-18*, H_2O/MeOH 100:0 → 0:100) to give eight main fractions (*Fr. 1–8*). *Fr. 6* and *7* were rich in sterol glycosides. *Fr. 7* (3.7 g) was fractionated by CC (silica gel, CH_2Cl_2/MeOH/H_2O 90:10:1 → 40:60:4) yielding 13 fractions (*Fr. 7.1–7.13*). The purification of subfraction *Fr. 7.6* (49 mg) by CC (silica gel, CH_2Cl_2/MeOH 97:3) resulted in the isolation of *ajugasalicioside A* (**1**; 5 mg). *Fr. 7.7* (266 mg) was subjected to MPLC (*RP-18*, MeOH/H_2O 30:70 → 70:30, flow rate 5 ml/min). The fractions obtained with 70% MeOH were combined (116 mg). Ajugasalicioside B (**2**; 15 mg) was isolated by prep. HPLC (*RP-18*, MeOH/H_2O 75:25, flow rate 6 ml/min). Ajugasalicioside C (**3**; 194 mg) was isolated from *Fr. 7.9* (992 mg) by VLC (silica gel, CH_2Cl_2/MeOH/H_2O 84:16:1.6). *Fr. 7.10* (473 mg) yielded ajugasalicioside D (**4**; 75 mg) by CC (silica gel, CH_2Cl_2/MeOH/H_2O 82:18:1.8). Ajugasalicioside E (**5**; 20 mg) was isolated from *Fr. 6* (1.2 g) by MPLC (*RP-18*, MeOH/H_2O 60:40) and subsequent CC (silica gel, CH_2Cl_2/MeOH/H_2O 85:15:1.5).

Acid Hydrolysis of **4**. Ajugasalicioside D (**4**; 5 mg) was dissolved in 3 ml of aq. 2N HCl and heated at 100° for 5 h. The mixture was cooled and filtered. The filtrate was neutralized by passing it through *Dowex* (Cl^- form) and evaporated. The residue was analysed for sugars by TLC (cellulose, BuOH/pyridine/AcOH/H_2O 36:36:7:21, detection by spraying aniline phthalate).

Ajugasalicioside A (=*(3*R,*5β,16*S,*17*S,*20*R,*22*S,*23*S,*24*S,*25*S*)-16,23:16,27:22,25-Triepoxy-17-hydroxystigmast-7-en-3-yl β-*D*-Glucopyranoside*; **1**). Amorphous, colorless powder. $[\alpha]_D^{20} = +8.4$ ($c = 0.1$, MeOH). UV (MeOH): 212 (2.2). ^{1}H- and ^{13}C-NMR: *Tables 1* and *2*. HR-MALDI-MS (pos.): 657.3614 ($[M+Na]^+$, $C_{35}H_{54}O_{10}^+$; calc. 657.3618).

Ajugasalicioside B (=*(3*R,*5β,16*S,*17*S,*20*R,*22*S,*23*S,*24*S,*25*S*)-16,23:16,27:22,25-Triepoxy-17-hydroxystigmast-7-en-3-yl 2-O-β-*D*-Glucopyranosyl-β-*D*-glucopyranoside*; **2**). Amorphous, colorless powder. $[\alpha]_D^{20} = +16.5$ ($c = 0.1$, MeOH).UV (MeOH): 212 (2.3). ^{1}H- and ^{13}C-NMR: *Tables 1* and *2*. HR-MALDI-MS (pos.): 819.4041 ($[M+Na]^+$, $C_{41}H_{64}O_{15}^+$; calc. 819.4143).

Ajugasalicioside C (=*(3*R,*5β,16*S,*17*R,*20*S,*22*R,*24*S,*25*S*)-22,25-Epoxy-16-hydroxystigmast-7-ene-3,27-diyl Bis(β-*D*-glucopyranoside)*; **3**). Amorphous, white powder. $[\alpha]_D^{20} = -13.7$ ($c = 0.1$, MeOH). UV (MeOH): 214 (2.2). ^{1}H- and ^{13}C-NMR: *Tables 1* and *3*. HR-MALDI-MS (pos.): 807.4499 ($[M+Na]^+$, $C_{41}H_{68}O_{14}^+$; calc. 807.4507.

Ajugasalicioside D (=*(3*R,*5β,16*S,*17*R,*20*S,*22*R,*24*S,*25*S*)-22,25-Epoxy-27-(β-*D*-glucopyranosyloxy)-16-hydroxystigmast-7-en-3-yl 2-O-β-*D*-Glucopyranoside*; **4**). Amorphous, white powder. $[\alpha]_D^{20} = -16.6$ ($c = 0.1$, MeOH/CH_2Cl_2). UV (33% H_2O/MeOH): 210 (2.4). ^{1}H- and ^{13}C-NMR: *Tables 1* and *3*. HR-MALDI-MS (pos.): 969.5025 ($[M+Na]^+$, $C_{47}H_{78}O_{19}^+$; calc. 969.5035).

Ajugasalicioside E (=*(3*R,*5β,16*R,*17*S,*20*R,*22*S,*23*S,*24*S,*25*S*)-27-(Acetyloxy)-22,25-epoxy-16,17,23-trihydroxystigmast-7-en-3-yl β-*D*-Glucopyranoside*; **5**). Amorphous, colorless powder. $[\alpha]_D^{20} = -13.5$ ($c = 0.1$, MeOH). UV (MeOH): 213 (2.3). ^{1}H- and ^{13}C-NMR: *Tables 1* and *3*. HR-MALDI-MS (pos.): 719.4005 ($[M+Na]^+$, $C_{37}H_{60}O_{12}^+$; calc. 719.3983).

KB Cell Assay. The cytotoxicity of **1**–**5** against the KB (HeLa) cell line (ATCC CCL 17) was determined as described by *Heilmann et al.* [14].

Jurkat T Cell and PMBC Assay. The cytotoxicity of **1**–**5** against the Jurkat T cell line (ATCC TIB-152) and against PMBCs was determined as described by *Gertsch et al.* [9].

Cell Culture, RNA Extraction, and Real Time PCR for the Determination of Relative mRNA Amounts. Cell culture of Jurkat T cells (ATCC TIB-152), RNA isolation, and determination of relative mRNA expression profiles with real time PCR was performed as previously reported [9].

Antimicrobial Assays. Bacillus cereus (ATCC 10702, *Gram*-positive), *Staphylococcus epidermidis* (ATCC 12228, *Gram*-positive), *Staphylococcus aureus* (ATCC 25923, *Gram-positive*), *Micrococcus luteus* (ATCC 99431, *Gram*-positive), *Pseudomonas aeruginosa* (ATCC 27853, *Gram*-negative), and *Candida albicans* (ATCC

25790) were used as test organisms. Antimicrobial assays were carried out by the doubling dilution method by means of a modified procedure published previously [15]. Chloramphenicol (bacteria) and micronazole nitrate (yeast) were used as positive controls.

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