Cytotoxic Diacetylenic Spiroketal Enol Ethers from *Plagius flosculosus*

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Three new diacetylenic spiroketal enol ethers named flosculins A (**1**), B (**2**), and C (**3**), along with five known compounds (**4**−**8**) of the same structural class, were isolated from the leaves of *Plagius flosculosus*. The structures were deduced by extensive 1D and 2D NMR spectroscopy and mass spectrometry. All isolated compounds exhibited significant cytotoxic activity against leukemia cells (Jurkat T and HL-60). Compounds **5**−**8** induced apoptosis in HL-60 cells with corresponding IC_{50} values ranging from 4 to 6 μM.

The tribe Anthemideae of the plant family Asteraceae is a rich source of spiroketal-enol ethers, which show interesting biological properties, such as antifeedant, antiphlogistic, and cytotoxic activities.[1−4] Although the cytotoxicity of polyacetylene spiroketals has been reported previously,[1] little attention has been paid to the mechanism of these effects. In a continuing search for cytotoxic compounds from Sardinian endemic plants,[5] we focused on *Plagius flosculosus* (L.) S. Alavi & V. H. Heywood (Asteraceae), a plant species endemic to Sardinia and Corsica. In recent work, Calzado et al. reported the inhibition of NF-κB activation by three known polyacetylene spiroketals from *P. flosculosus*.[6] In the present contribution, we describe the cytotoxicity of three new (**1**−**3**) and five known (**4**−**8**) diacetylenic spiroketal enol ethers and their proapoptotic properties in leukemia cells. The compounds were isolated from the dichloromethane extract and elucidated by means of 1D and 2D NMR spectroscopy and mass spectrometry. The five known compounds (**4**−**8**) were identified by comparing their physical and spectroscopic data with those reported in the literature.[7−9]

The EIMS of **1** showed the molecular ion peak at *m/z* 308 with an isotopic peak $[M + 2]^+$ at *m/z* 310 (33% of the molecular ion), indicating the presence of chlorine in the molecule. In the mass spectrum the fragment at *m/z* 136, together with an isotopic peak at *m/z* 138, could be assigned to the ion $[C_8H_5Cl]^{+\bullet}$, further supporting the presence of a chlorine moiety. The presence of chlorine was confirmed by HRESIMS, which exhibited a pseudo-molecular ion at *m/z* 327.0631 $[M + H_2O + H]^+$, compatible with the molecular formula $C_{15}H_{13}ClO_5$. The FT-IR spectrum of **1** showed an absorption band at 3420 cm^{-1} due to a hydroxyl function. The ^{13}C NMR spectrum revealed 15 signals, which could be assigned to two CH_3, six CH, and seven quaternary carbons by the DEPT spectrum (Table 1). The ^1H NMR spectrum showed the presence of two methyl groups, one on an acetylene carbon atom (δ 1.48, s) and the other belonging to an acetoxyl group (δ 1.72, s), together with six methine protons and a hydroxyl proton (δ 1.98, br) (Table 1). A direct comparison of the ^{13}C NMR data of **1** with those of **6** suggested that the two compounds have the same basic structure, with the main differences due to the olefinic carbon signals at C-8 (δ 127.0) and C-9 (δ 133.5) in **6**, which disappeared in the spectrum of **1**. In the ^{13}C NMR spectrum of **1** appeared two methine carbons that were not present in the spectrum of **6**, at δ 57.7 and 87.7. A HSQC experiment was used to assign the protons to their attached carbons. HMBC correlations between the olefinic proton at δ_H/δ_C 5.39/87.7 and the acetylenic carbons at δ 65.7 (C-4′), 68.3 (C-2′), 81.6 (C-3′), and 82.5 (C-5′) established this proton at position 1′. Hence the C-1′ (δ 87.7) signal was shifted downfield about Δ +6 ppm compared with that of **6**. In C_6D_6, the broad signal at δ 4.73 changed to a doublet (J = 6.5 Hz) after adding one drop of D_2O, indicating the placement of the hydroxyl group on the CH at δ 81.4. This assignment was confirmed by DQF-COSY correlations between the hydroxyl proton at δ 1.98 (exchanged in D_2O) and the signal at δ 4.73. The locations of the methine protons at δ 4.69 and 4.73 were established by the $^1H-^1H$ COSY technique, indicating cross-peaks between H-8 and H-1′ and H-8 and H-9,

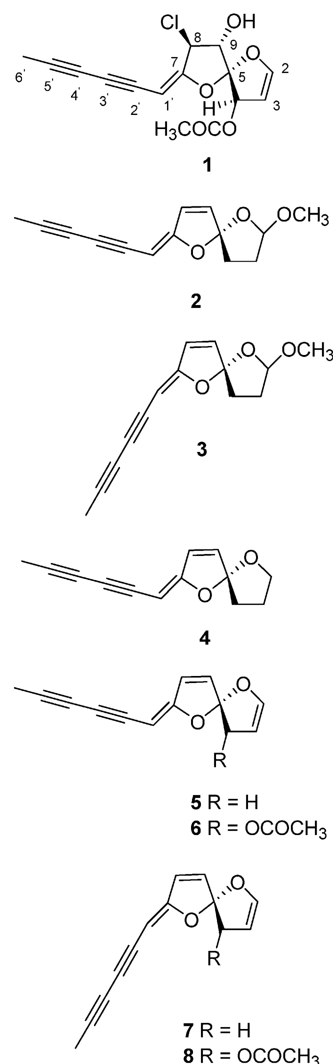

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Table 1. ^{1}H and ^{13}C NMR Spectral Data of Compounds **1−3** and $\Delta\delta$ ^{1}H Mosher Ester Derivatives of Compound **1** (δ ppm; *J*, Hz)[a]

	1			**2**		**3**	
C	δ_C	δ_H	$\Delta\delta^{1}H = \delta^{1}H_{1a} - \delta H_{1b}$	δ_C	δ_H	δ_C	δ_H
2	147.9, d	5.95, dd (2.1, 3.0)	+0.112	106.4, d	4.97, d (4.2)	106.6, d	4.97, d (5.1)
3	101.5, d	4.83, dd (2.1, 3.0)	+0.082	31.4, t	Ha: 2.02, m Hb: 1.72, m	31.2, t	Ha: 2.06.m Hb: 1.74, m
4	75.4, d	5.75, t (2.1)	+0.127	33.4, t	Ha: 1.99, m Hb: 1.76, m	33.5, t	Ha: 2.01, m Hb: 1.80, m
5	110.7, s			120.9, s		121.1, s	
7	164.9, s			169.4, s		169.6, s	
8	57.7, d	4.69, dd (6.5, 1.8)	−0.131	125.6, d	5.76, dd (5.4, 1.5)	127.0, d	5.61, d (5.7)
9	81.4, d	4.73,brs		137.0, d		136.1, d	5.75, d (5.7)
1′	87.7, d	5.39, brs	−0.079	80.3, d	6.54, d (5.4) 5.20, brs	79.5, d	4.55, brs
2′	68.3, s			67.0, s		67.2, s	
3′	81.6, s			79.8, s		80.0, s	
4′	65.7, s			66.1, s		66.0, s	
5′	82.5, s			81.8, s		80.9, s	
6′	4.0, q	1.48, s		4.1, q	 1.54, s	4.1, q	1.47, s
C=O	170.2, s						
OCH_3	19.9, q	1.72, s		55.0, q	3.18, s	55.0, q	3.20, s
OH		1.98, brs (exch.)					

[a] Compounds **1−3** were measured in C_6D_6, while the Mosher ester derivatives of compound **1** were measured in $CDCl_3$.

while H-9 correlated with H-8 but not with H-1′. Furthermore, a strong ROESY correlation between H-4 (δ 5.75) and H-9 (δ 4.73) was observed, confirming the hydroxyl group at position 9 of the diacetylene spiroketal. The position of the chlorine atom at C-8 was finally confirmed by the HMBC correlations between H-1′ and C-7 (δ 164.9) as well as C-8 (δ 57.7).

The chemical shift of H-1′ in **1** was comparable with that of H-1′ in the known diacetylene spiroketals possessing an *E* geometry.[8,10] In accordance with these data, in the ROESY NMR spectrum no correlation between H-1′ and H-8 was observed, confirming an *E* geometry of the double bond at C-7. Additionally, H-11 showed a strong ROE to the proton at C-9, confirming the relative *syn*-disposition of the acetoxy group at C-11 with respect to the tetrahydrofuran oxygen atom and the β-oriented hydroxyl group at C-9. The absolute configuration of the C-9 hydroxyl group was determined by the modified Mosher ester methodology.[11] Compound **1** was treated with (*R*)- and (*S*)-(−)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (MTPA-Cl) to obtain the (*S*)-(−)- and (*R*)-ester C-6 analogues (**1a**, **1b**, respectively). Calculation of differences in chemical shifts of protons in **1a** and **1b**, displayed in Table 1, suggested the absolute configuration at C-9 to be *R*. The relative *anti* configuration of the hydroxyl group and chlorine was indicated by the coupling constant ($J = 6$ Hz) between H-8 and H-9. The lowest energy conformation of **1**, which was generated by the SPARTAN program based on the semiempirical AM1 potential, confirmed the configuration of **1** assigned by the ROESY spectrum. Hence, compound **1** was shown to be (4*R*,8*S*,9*R*)-8-chloro-7-[(*E*)-2,4-hexadiynylidene]-9-hydroxy-1,6-dioxaspiro[4.4]-non-2-en-4-yl acetate and accorded the trivial name flosculin A (**1**). Interestingly, relatively few terrestrial plant metabolites contain halogens.[12−15] On the other hand, many of the chlorinated compounds isolated from higher plants have been obtained from Asteraceae.[16] Compound **1** is not an artifact, since hydrochloric acid was not used during the isolation/purification process.

Compound **2** was isolated as a colorless semisolid, and the HREIMS gave an ion peak at *m/z* 230.2628, which together with the ^{13}C NMR data was compatible with the molecular formula $C_{14}H_{14}O_3$. The ^{1}H and ^{13}C NMR spectra of compound **2** showed that its structure is related to that of compound **4**, with the exception that the methylene proton signals at δ 3.59 (1H, q, $J = 8$, 15 Hz) and 3.89 (1H, m) were replaced by a singlet at δ 3.18 (3H, s), due to a methoxyl group, and a methine proton at δ 4.97 (1H, dd, $J = 4.2$ Hz) (Table 1). Hence, the only difference is the replacement of a methylene group (in **4**) with a methoxymethine moiety (in **2**). In compound **2**, the chemical shifts of the two CH_2 groups at δ 31.4 and 33.4 suggest that their position is not adjacent to the tetrahydrofuran oxygen atom. Therefore, the methoxyl group must be attached at position 2 of the diacetylene spiroketal. The assignments of the remaining methylene groups were deduced from the ^{1}H−^{1}H COSY spectrum, where H-2 at δ 4.97 showed a coupling to H_2-3 (δ 2.02, H-1a, m; δ 1.55, H-1b, m), which, in turn, coupled to H_2-4 (δ 1.98, H-1a, m; δ 1.78, H-1b, m). The structure of compound **2** was finally confirmed by 1D and 2D NMR experiments as 2-[(*E*)-2,4-hexadiynylidene]-7-methoxy-1,6-dioxaspiro-[4.4]non-3-ene (**2**) and trivially named flosculin B (**2**).

HREIMS of compound **3** gave a ion peak at *m/z* 230.2628, suggesting the same molecular formula of flosculin B (**2**). While the ^{13}C NMR spectrum was almost superimposable with that of **2**, the ^{1}H NMR spectrum showed upfield shifts for protons H-1′ (δ 4.55, s), H-8 (δ 5.61, d, $J = 5.7$ Hz), and H-9 (δ 5.75, d, $J = 5.7$ Hz) (Table 1), suggesting for compound **3** a *Z* geometry, whereas in **2** it was *E*. This finding was supported by ROESY experiments. In compound **3** a ROE cross-peak between H-1′ and H-8 was observed, whereas in **2** the same correlation was absent. Examination of a molecular model confirms that in compound **3** H-1′ is closer to H-8 than in compound **2**. On the basis of these spectroscopic data, compound **3** was assigned as 2-[(*Z*)-2,4-hexadiynylidene]-7-methoxy-1,6-dioxaspiro[4.4]non-3-ene (**3**) and named flosculin C (**3**). Flosculins B (**2**) and C (**3**) both possess one stereocenter at C-2, but additional experiments indicated that these compounds are racemic, since their $[\alpha]^{25}_{D}$ values were 0.

All compounds (**1−8**) displayed significant cytotoxicity against the Jurkat T and HL-60 leukemia cell lines (Table 2). The cytotoxicity seemed to be correlated with the presence or absence of double bonds in the tetrahydrofuran ring. In fact, compounds **5−8**, which possess a double bond in both rings, were more active than compounds **1−4**, containing only one double bond.

Compounds **5−8** were tested further for their mechanism of cell death. Flow cytometric measurements were performed to assess the ratio of apoptotic versus necrotic cells by FITC annexin and propidium iodide staining. Figure 1 shows that compounds **6** and **8** induced apoptosis in a concentration-dependent manner. Data in Table 2 suggest that apoptosis might be partly mediated by bcl-2. Anti-apoptotic proteins of the bcl-2 family inhibit the mitochondrial release of cytochrome *c* or/and the binding of cytochrome *c* to Apaf-1 in apoptotic processes.[17] Thus, cells overexpressing bcl-2 can decrease the cytotoxicity of compounds **5** and **7** by 40−50%.

Table 2. Cytotoxic Activities ($p < 0.05$) of Compounds **1**−**8** against Jurkat T and HL-60 Leukemia Cells (IC_{50}, ±SD), Determined after 20 h

compound	IC_{50} (μM)		
	Jurkat T	Jurkat T[a]	HL-60
1	13.2 ± 0.8	19.1 ± 1.0	18.9 ± 1.2
2	15.2 ± 0.6	19.2 ± 0.9	18.3 ± 0.9
3	10.8 ± 0.8	16.9 ± 1.2	16.7 ± 1.3
4	11.2 ± 0.5	14.1 ± 0.5	16.2 ± 1.4
5	4.7 ± 0.2	8.4 ± 0.4	6.1 ± 0.3
6	6.4± 0.3	6.9 ± 0.5	4.0 ± 0.4
7	3.8 ± 0.4	7.2 ± 0.3	5.6 ± 0.3
8	4.1 ± 0.1	5.2 ± 0.2	4.2 ± 0.6
helenalin[b]	0.46 ± 0.08	n.d.	0.7 ± 0.08

[a] Cells overexpressing the antiapoptotic factor bcl-2. [b] Positive control substance.

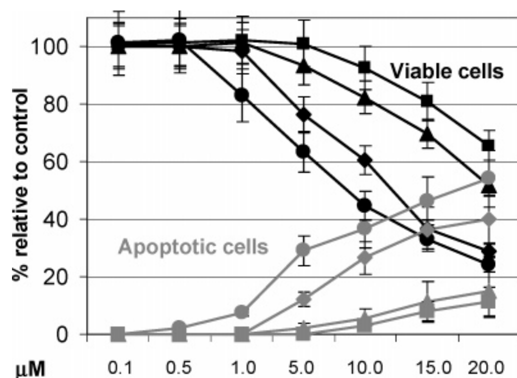

Figure 1. Cytotoxicity and induction of apoptosis in HL-60 cells. In this procedure, 10^6 cells were incubated with compounds for 24 h and then analyzed for viability (black lines) and apoptotic cells (gray lines). Squares: compound **5**; Diamonds: compound **6**; Triangles: compound **7**; Circles: compound **8**.

Therefore, diacetylenic spiroketal enol ethers appear to exert their activity, at least in part, via the mitochondrial apoptotic pathway.

Experimental Section

General Experimental Procedures. Optical rotations were measured in MeOH at 25 °C using a Perkin-Elmer 241 polarimeter. UV spectra were recorded on a GBC Cintra 5 spectrophotometer. IR spectra were performed with a Perkin-Elmer system 2000 FT-IR spectrophotometer using KBr mulls. NMR spectra were recorded at 25 °C on a Varian Unity INOVA 400 MHz spectrometer, operating at 400 MHz for ^1H and 100 MHz for ^{13}C, respectively. Compounds were measured in C_6D_6 and $CDCl_3$ and the spectra referenced against residual nondeuterated solvents. EIMS were taken on a QMD 1000 instrument at 70 eV using a direct inlet system. HRESIMS were measured on a Micromass Q-TOF MICRO instrument. Column chromatography was carried out under TLC monitoring using silica gel (40−63 μm, Merck) and Sephadex LH-20 (25−100 μm, Pharmacia). For vacuum-liquid chromatography (VLC), silica gel (40−63 μm) (Merck) was used. TLC was performed on silica gel 60 F254 or RP-18 F254 (Merck). HPLC was conducted by means of a Hewlett-Packard 1050 instrument. The column was a 250 × 10 mm Spherisorb silica, particle size 5 μm (Waters), and the UV detection wavelength was 254 nm. For molecular modeling a PC Spartan Pro software program (Wavefunction Inc.) was used.

Plant Material. The leaves of *P. flosculosus* were collected in Fluminimaggiore, Sardinia, Italy, in July 2003. The plant material was identified by Prof. Bruno De Martis (Dipartimento di Scienze Botaniche, Università di Cagliari) and a voucher specimen (No. 0311) was deposited in the Herbarium of the Dipartimento Farmaco Chimico Tecnologico, Università di Cagliari.

Extraction and Isolation. The dried and powered leaves of *P. flosculosus* (400 g) were ground and extracted with CH_2Cl_2 (4.5 L) by percolation. After concentration, the resulting extract was suspended in 90% MeOH and partitioned with *n*-hexane to afford an *n*-hexane-soluble syrup (12.44 g). Then, the aqueous MeOH extract was concentrated and suspended in CH_2Cl_2 to give a CH_2Cl_2-soluble residue (11 g). The *n*-hexane-soluble syrup (12.44 g) was subjected to VLC (silica gel, 300 g, 40−63 μm) using a step gradient of *n*-hexane, *n*-hexane with increasing amounts of CH_2Cl_2 (25% each step), CH_2Cl_2, CH_2Cl_2 with increasing amounts of EtOAc (25% each step), EtOAc, EtOAc with increasing amounts of MeOH (25% each step), and MeOH (700 mL each step). The collected fractions were evaporated under vacuum and examined by TLC. Homogeneous fractions were pooled to give 13 major fractions (F1−F13). Fraction F2 (720 mg) was subjected to open column chromatography over silica gel (100 g) using *n*-hexane−EtOAc (9.75:0.25, 700 mL) for elution. Twelve subfractions (F2.1−F2.12) were obtained. Subfraction F2.5 (90 mg) was purified further by column chromatography on silica gel (12 g) and eluted with a mixture of *n*-hexane−EtOAc (9.5:0.5, 250 mL) to give compound **5** (35 mg). Subfraction F2.6 (40 mg) was subjected to column chromatography over silica gel (10 g) using *n*-hexane−EtOAc (9:1, 200 mL) as eluent, yielding compound **7** (2.5 mg). Fraction F4 (429 mg) was fractionated by column chromatography over silica gel (50 g) using *n*-hexane−EtOAc (9:1, 400 mL) as eluent to obtain seven subfractions (F4.1−F4.7). F4.2 (141 mg) was purified further by solid-phase extraction (silica gel) with *n*-hexane−EtOAc (9:1, 120 mL), as eluent, to give **4** (37.5 mg). Fraction F5 (700 mg) was separated by column chromatography on silica gel (90 g) eluting with *n*-hexane−EtOAc (9.25:0.75, 700 mL) to give seven subfractions (F5.1−F5.7). F5.3 (70.1 mg) was purified by normal-phase (silica gel) HPLC with *n*-hexane−EtOAc (9.25:0.75, 3.5 mL/min) as eluent to yield compounds **2** (1.2 mg, t_R 16.5 min) and **3** (3.1 mg, t_R 27.2 min). Subfraction F5.6 (90.5 mg) was subjected to open column chromatography over silica gel (11 g) using a mixture of toluene−EtOAc (9.95:0.05, 250 mL) to give **8** (40 mg).

The CH_2Cl_2-soluble residue (11 g) was subjected to VLC over silica gel (300 g, 40−63 μm) using the same step gradient mentioned above to yield 57 fractions. On the basis of the TLC similarities, identical fractions were combined to give a total of 14 fractions (G1−G14). Fraction G5 (85.6 mg) was fractionated by column chromatography over silica gel (10 g) using *n*-hexane−EtOAc (8:2, 230 mL) as eluent to give **6** (9 mg). Fraction G11 (800 mg) was subjected to open column chromatography over Sephadex LH-20 using MeOH (200 mL) as eluent to give four subfractions (G11.1−G11.4). G11.3 (440 mg) was purified by column chromatography on silica gel (60 g) and eluted with a mixture of toluene−acetone (9:1, 500 mL) to yield **1** (67.3 mg).

Flosculin A (1): colorless semisolid product; $[\alpha]^{25}_D$ +64.3 (*c* 0.25, MeOH); UV (MeOH) λ_{max} (log ϵ) 322 (2.32), 333 (1.76) nm; IR (Nujol) ν_{max} 3420, 2927, 2860, 2235, 2146, 1737, 1657, 1627, 1431, 1373, 1154, 1114, 954, 912, 699 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; EIMS (*m/z*) 310 (5), 308 (15), 248 (3), 219 (10), 185 (35), 136 (30), 101 (40), 76 (48), 43 (100); HRESIMS *m/z* 327.0631 $[M + H_2O + H]^+$, calcd for $C_{15}H_{13}ClO_5 \cdot H_2O$, 327.0630.

Flosculin B (2): colorless semisolid product; $[\alpha]^{25}_D$ 0 (*c* 0.02, MeOH); UV (MeOH) λ_{max} (log ϵ) 321 (1.95), 330 (1.24) nm; ^1H and ^{13}C NMR, see Table 1; EIMS (*m/z*) 230 (79), 215 (5), 198 (37), 170 (44), 159 (100), 141 (27), 115 (40), 102 (31), 91 (28), 77 (62), 71 (78), 43 (15); HREIMS *m/z* 230.2628 $[M]^+$, calcd for $C_{14}H_{14}O_3$, 230.2628.

Flosculin C (3): colorless semisolid product; $[\alpha]^{25}_D$ 0 (*c* 0.30, MeOH); UV (MeOH) λ_{max} (log ϵ) 325 (3.19), 331 (2.55) nm; ^1H and ^{13}C NMR, see Table 1; EIMS (*m/z*) 230 (100), 215 (6), 199 (43), 170 (55), 159 (82), 141 (31), 115 (43), 102 (29), 90 (25), 76 (56), 70 (62), 43 (29), 40 (75); HREIMS *m/z* 230.2628 $[M]^+$, calcd for $C_{14}H_{14}O_3$, 230.2628.

Cytotoxic Activity. The cytotoxicity of the pure compounds against CD4+ Jurkat human leukemia T-cells (ATCC TIB-152 and Bcl-2 transfected cells) and HL-60 cells (ATCC CCL-240 cells) was determined after 2.5, 20, and 72 h in a WST-1-based cell viability assay, as described previously.[18] All compounds were tested in a concentration range between 0.5 and 80 μM. Maximal standard deviation was 10% (absolute).

Determination of Apoptosis. FACS (fluorescence-activated cell sorting) experiments were carried out with HL-60 cells. In short, 10^6 treated and control cells were transferred from a culture well to a staining tube and washed with 4 mL of 4 °C PBS containing 1% (v/v) bovine calf serum. Cells were centrifuged 10 min at 200*g* at 4 °C, and the supernatant was removed. Then, 100 μL of 2 μg/mL annexin

V-FITC (Caltag Laboratories, Burlingame, CA) in annexin V-binding buffer [10 mM HEPES (LTI), 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, pH 7.4] was added to the cells, and the staining tubes were incubated for 10 min on ice in the dark. Propidium iodide (PI) single-color control cells were treated with 100 μL of annexin V-binding buffer alone. After adjusting the total volume of each tube to 0.5 mL with annexin V-binding buffer, 1 μg per tube of PI (Sigma) was added. The cells were analyzed within 20 min by flow cytometry on a FACSScan cytometer (BD Biosciences, Mountain View, CA), and data analysis was performed with CellQuest. The main HL-60 cell population was gated, and annexin V-FITC positive cells (F1-channel) were counted as apoptotic. Taxol (500 nM)-treated cells were used as positive control, and untreated cells were used as negative control.

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