New Acylated Triterpene Saponins from *Silene fortunei* that Modulate Lymphocyte Proliferation

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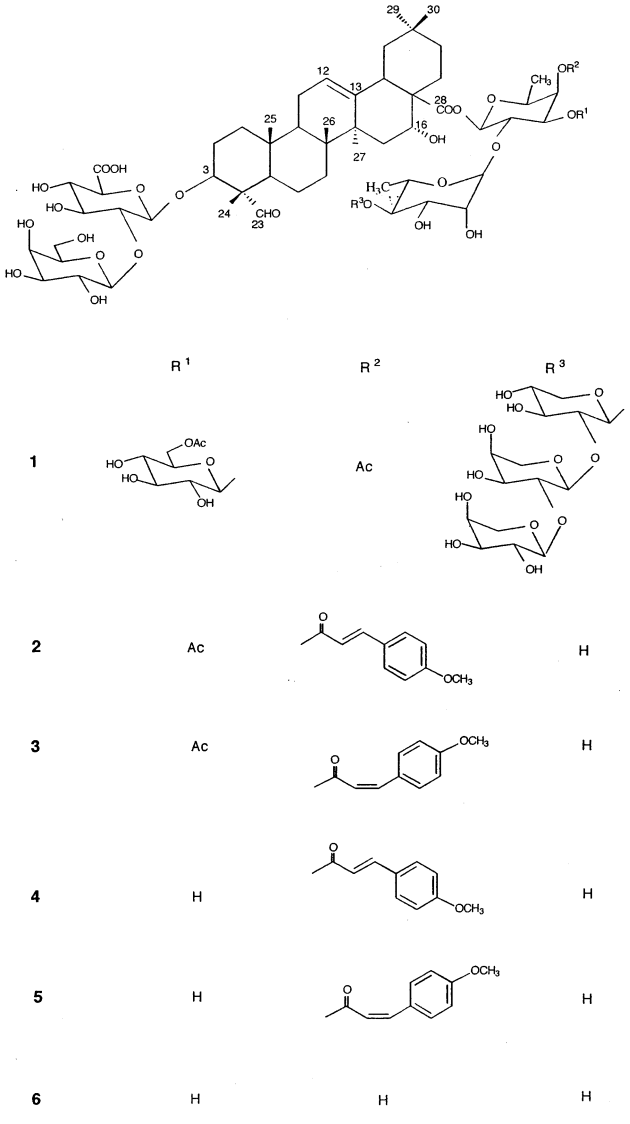
Three new acylated triterpene saponins **1**−**3**, with a quillaic acid as aglycon, were isolated from the roots of *Silene fortunei* together with a known phytoecdysteroid (20-hydroxyecdysone). The compounds were characterized mainly by a combination of 2D NMR techniques, mass spectrometry, and chemical methods. Saponins **1**−**3**, jenisseensosides C and D (**4**, **5**), and **6** (deacylated form of **2**/**3** and **4**/**5**) were found to stimulate the proliferation of the Jurkat tumor cell lines at low concentration. At high concentration, **2**/**3** and **4**/**5** inhibited the proliferation of the cells and suggested the induction of apoptosis.

Our previous phytochemical studies on the ethanolic extract of *Silene fortunei* Vis. (Caryophyllaceae) roots endemic to China led to the isolation of two acylated triterpene saponins named jenisseensosides C and D[1] and an acetylated triterpene octaglycoside.[2] A detailed further investigation of the same extract has led to the isolation of an additional acetylated triterpene octaglycoside, **1**, two acylated saponins named jenisseensosides E and F (**2**, **3**), and a known ecdysteroid, which was identified as 20-hydroxyecdysone. The effect of **1**−**3**, jenisseensosides C and D (**4**, **5**), and **6** (deacylated form of **2**/**3** and **4**/**5**) was investigated on the cell proliferation and apoptosis in Jurkat cells (human T-cell leukemia).

Results and Discussion

The ethanol extract of the dried roots of the plant was separately subjected to partitions between water and *n*-butanol. The concentrated *n*-butanol-soluble fraction was purified by precipitation with diethyl ether to yield the crude saponin mixture. A part of this mixture was subjected to multiple chromatographic steps over Sephadex LH-20 and medium-pressure liquid chromatography (MPLC) to afford **1** together with **2** and **3** (jenisseensosides E and F) as an inseparable mixture, giving only one spot by HPTLC but two peaks by HPLC. The soluble diethyl ether fraction was fractionated by MPLC, yielding the known phytoecdysteroid. All structures were elucidated mainly by FABMS and HRESIMS and by 600 MHz NMR analysis including 1D and 2D NMR (^{1}H−^{1}H COSY, TOCSY, NOESY, HSQC, and HMBC).

Compound **1** was obtained as a white amorphous powder. The high-resolution ESI mass spectrometry (HRESIMS) (positive-ion mode) of **1** exhibited a pseudomolecular ion peak at *m*/*z* 1781.6907 $[MNa]^+$ (calcd 1781.6893), consistent with a molecular formula of $C_{78}H_{118}O_{44}Na$. Its FABMS (negative-ion mode) exhibited a quasi-molecular ion peak at *m*/*z* 1757 $[M - H]^-$, indicating a molecular weight of 1758.



The ^{1}H and ^{13}C NMR data of **1** (Table 1) assigned from TOCSY, HSQC, and HMBC experiments were similar to those of 3-*O*-[*β*-D-galactopyranosyl-(1→2)-*β*-D-glucuronopyranosyl]quillaic acid-28-*O*-[α-L-arabinopyranosyl-(1→2)-α-

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Table 1. ^{13}C and ^{1}H NMR Spectral Data of Sugar Moieties for Compounds **1**−**3** (in DMSO-d_6, ppm)[a]

	1			**2**		**3**	
position	δ_C	δ_H	position	δ_C	δ_H	δ_C	δ_H
3-*O*-			3-*O*-				
Glc-A1	101.7	4.10 (d, 7.4)	Glc-A1	101.4	4.12 (d, 7.5)	101.4	4.12 (d, 7.5)
2	82.1	3.14	2	81.4	3.13	81.4	3.13
3	75.8	3.33	3	75.4	3.32	75.4	3.32
4	70.4	3.06	4	71.6	3.06	71.6	3.06
5	77.3	3.13	5	76.5	3.28	76.5	3.28
6	172.0		6	172.2		172.2	
Gal 1	105.1	4.22 (d, 7.1)	Gal 1	104.4	4.20 (d, 7.5)	104.4	4.20 (d, 7.5)
2	73.0	3.28	2	72.5	3.28	72.5	3.28
3	74.0	3.15	3	72.9	3.12	72.9	3.12
4	69.7	3.62	4	67.8	3.63	67.8	3.63
5	74.5	3.20	5	72.9	3.15	72.9	3.15
6	60.5	3.50, 3.58	6	59.8	3.48, 3.52	59.8	3.48, 3.52
28-*O*			28-*O*				
Fuc 1	93.7	5.36 (d, 7.9)	Fuc 1	93.0	5.33 (d, 7.5)	93.0	5.33 (d, 7.5)
2	73.1	3.66	2	69.4	3.52	69.4	3.52
3	81.1	3.94	3	73.0	4.75	73.0	4.75
4	73.0	5.11	4	72.9	5.02	72.9	5.02
5	69.6	3.80	5	67.4	3.66	67.4	3.66
6	16.1	0.90 (d, 5.8)	6	16.1	1.02 (d, 5.8)	16.1	1.02 (d, 5.8)
$COOCH_3$	20.5	1.99	$COOCH_3$	20.2	1.97	20.2	1.97
$COOCH_3$	171.1		$COOCH_3$	169.9		169.9	
Rha 1	100.2	5.14 (s)	Rha 1	99.8	5.22 (s)	99.8	5.22
2	69.6	3.82	2	70.2	3.70	70.2	3.70
3	71.4	3.66	3	71.9	3.62	71.9	3.62
4	81.8	3.76	4	72.1	3.85	71.8	3.85
5	67.9	3.58	5	69.2	3.52	69.2	3.52
6	18.6	1.10 (d, 6.6)	6	18.6	1.12 (d, 6.3)	18.6	1.12 (d, 6.3)
Glc 1	103.7	4.32 (d, 7.4)	*p*-methoxy cinnamoyl				
2	74.5	2.95	1″	126.7		126.9	
3	77.1	3.14	2″	130.5	7.70 (d, 7.2)	132.3	7.74 (d, 7.2)
4	70.4	3.02	3″	114.4	6.97 (d, 8.1)	113.5	6.92 (d, 8.5)
5	77.2	3.08	4″	161.2		160.2	
6	63.6	4.20, 4.00	5″	114.4	6.97 (d, 8.1)	113.5	6.92 (d, 8.5)
$COOCH_3$	21.3	2.07	6″	130.5	7.70 (d, 7.2)	132.3	7.74 (d, 7.2)
$COOCH_3$	170.2		α	115.2	6.52 (d, 15.8)	116.1	5.92 (d, 12.0)
Xyl 1	105.2	4.44 (d, 7.4)	*β*	144.7	7.62 (d, 15.8)	142.9	6.95 (d, 12.0)
2	74.2	3.18	CO	165.8		166.6	
3	84.6	3.36	OCH_3	55.4	3.79 (s)	55.2	3.77 (s)
4	68.2	3.37					
5	66.1	3.12, 3.74					
Ara 1	101.2	4.74 (d, 2.5)					
2	79.3	3.66					
3	70.8	3.64					
4	66.4	3.72					
5	63.6	3.42, 3.80					
Ara 1	105.0	4.32 (d, 7.4)					
2	71.8	3.38					
3	73.2	3.28					
4	66.3	3.70					
5	63.6	3.44, 3.80					

[a] Measured at 600 MHz for the ^{1}H and 150 MHz for the ^{13}C referenced to δ 39.5 in DMSO-d_6 for **1**−**3**. Assignments were made on the basis of COSY, TOCSY, NOESY, HSQC, HMBC, and DEPT experiments.

L-arabinopyranosyl-(1→3)-*β*-D-xylopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→2)]-[*β*-D-glucopyranosyl-(1→3)]-4-*O*-acetyl-*β*-D-fucopyranoside,[2] except for the appearance of an additional acetyl group. The site of linkage of the second acetyl moiety was determinated by the HMBC experiment, which showed a long-range correlation between δ_H (Glc-6) 4.20 and δ_C 170.2. The downfield shifts observed in the HSQC spectrum for the Glc H-$6_{a,b}$/Glc C-6 resonances at δ_H 4.20, 4.00/δ_C 63.6 confirmed that the Glc residue was substituted by an acetyl group at the position C-6. The common D-configuration for Fuc, Gal, GlcA, Xyl, and Glc and the L-configuration for Rha and Ara were assumed, being the most frequently encountered among the plant glycosides. On the basis of the above results, the structure of **1** was elucidated as 3-*O*-[*β*-D-galactopyranosyl-(1→2)-*β*-D-glucuronopyranosyl]quillaic acid-28-*O*-[α-L-arabinopyranosyl-(1→2)-α-L-arabinopyranosyl-(1→3)-*β*-D-xylopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→2)]-[6-*O*-acetyl-*β*-D-glucopyranosyl-(1→3)]-4-*O*-acetyl-*β*-D-fucopyranoside, a new natural compound.[1−8]

The mixture of compounds **2** and **3** was obtained as a white amorphous powder. The high-resolution ESI mass spectrometry (HRESIMS) (positive-ion mode) of **2** and **3** exhibited a pseudomolecular ion peak at *m*/*z* 1341.5902 $[MNa]^+$ (calcd 1341.5880), consistent with a molecular formula of $C_{66}H_{94}O_{27}Na$. Their FABMS (negative-ion mode) exhibited a quasi-molecular ion peak at *m*/*z* 1317 $[M - H]^-$, indicating a molecular weight of 1318. Other fragment ion peaks at *m*/*z* 1275 $[(M - H) - 42]^-$ indicated the loss of one acetyl group. The fragment ion peak at *m*/*z* 1275 corresponded to the pseudomolecular ion of **4** and **5**.[1] The mineral acid hydrolysis of **2** and **3** afforded quillaic acid

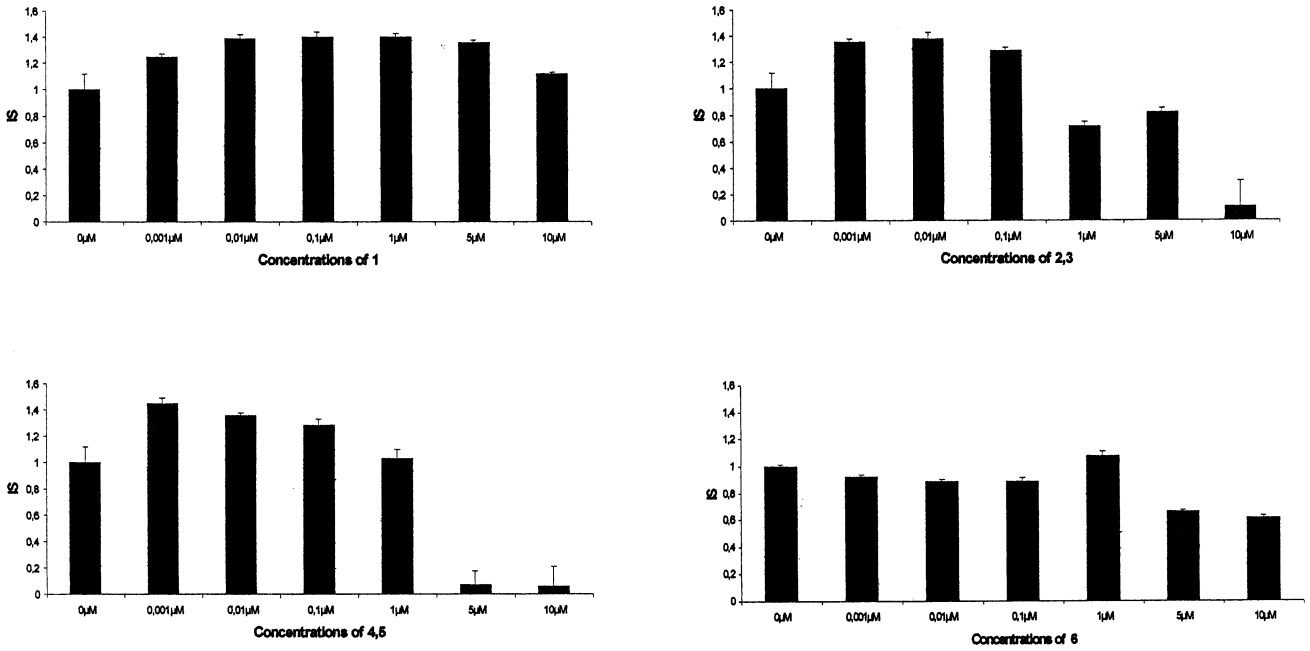

Figure 1. Effects of saponins **1**−**3**, **4**/**5**, and **6** on the proliferation of Jurkat cells. The proliferation was measured by uptake of ^{3}H thymidine. Each column represents the mean ± SE mean (n = 4).

and sugars identified as galactose, rhamnose, fucose, and glucuronic acid (co-TLC), respectively. The alkaline hydrolysis of **2** and **3** performed with 5% KOH yielded the same prosapogenin as in **1**.[1,2] Mild alkaline hydrolysis of this mixture with KOH 1% (30 min at room temperature) gave the previously isolated **4** and **5**[1] by TLC, and after 90 min yielded *trans*- and *cis*-*p*-methoxycinnamic acids and a deacylated saponin **6**[1] (co-TLC, HPLC). The above results indicated that **2** and **3** must be the acetylated compounds **4** and **5**. This mixture was homogeneous by HPTLC but was separated into *trans*- and *cis*-isomers by HPLC. Finally, the mixture of **2** and **3** was subjected to semi-preparative HPLC on a reversed-phase (C18) silica gel column to give compounds **2** and **3**. However after separation, the methanolic solution of each compound led, under light, to an equilibration resulting from the *trans*/*cis* isomerization to yield two mixtures, **2**/**3** and **3**/**2**. Such a phenomenon has been previously observed and explained in compounds **4**/**5**[1] and *E*- and *Z*-mixtures of senegasaponins from *Polygala senega*.[9]

The mixture of **2**/**3** was investigated by TOCSY, COSY, NOESY, HSQC, and HMBC NMR experiments, and full assignments of all ^{1}H and ^{13}C resonances were obtained (Table 1). The ^{1}H NMR and COSY spectrum displayed characteristic signals in the range of δ_H 7.62 and 5.92 ppm, indicating the presence of a *p*-methoxycinnamoyl moiety in a *trans*−*cis* configuration. This evidence was confirmed by the coupling constants of 15.8 and 12.0 Hz for each pair of olefinic protons (δ_H 6.52, d; δ_H 7.62, d, J = 15.8 Hz) and (δ_H 5.92, d; 6.95, d, J = 12 Hz). The NMR spectral data of **2**/**3** at C-28 were similar to those of **4**/**5** except the additional signals due to the acetyl group at the position 3 of fucose (Fuc-3). The downfield shifts observed in the HSQC spectrum for the Fuc H-3/Fuc C-3 and Fuc H-4/Fuc C-4 resonances at δ_H 4.75/δ_C 73.0 and at δ_H 5.02/δ_C 72.9 proved the secondary alcoholic functions Fuc-3-OH and Fuc-4-OH to be acylated. The HMBC experiment showed long-range coupling between ^{1}H NMR signals at δ_H (Fuc-6) 1.02 and ^{13}C NMR signals at δ_C (Fuc-5) 67.4 (^2J) and δ_C (Fuc-4) 72.9 (^3J), confirming that Fuc C-4 was acylated with the *p*-methoxycinnamoyl groups. In the TOCSY spectrum we observed that the ^{1}H NMR signal of Fuc H-6 at δ 1.02 (d, J = 5.8 Hz) gave three correlations between the ^{1}H NMR signals at δ_{H-5} (3.66), δ_{H-3} (4.75), and δ_{H-2} (3.52), demonstrating that they belonged to the same spin system. The downfield signal of Fuc-3 at δ 4.75 gave a cross-peak with the downfield signal of Fuc C-3 at δ 73.0 in the HSQC spectrum and revealed the location of the acetyl group at this position. On the basis of the above results and the assumption that Fuc, Gal, and GlcA are members of the commonly found D-series and Rha of the L-series, the structures of **2** and **3** were determinated as 3-*O*-[β-D-galactopyranosyl-(1→2)-β-D-glucuronopyranosyl]quillaic acid-28-*O*-α-L-rhamnopyranosyl-(1→2)-3-*O*-acetyl-4-*O*-*trans*-*p*-methoxycinnamoyl β-D-fucopyranoside (**2**) and its *cis*-isomer (**3**), respectively.[1−8]

The known phytoecdysteroid was isolated and identified by analysis of its spectral data (FABMS and 2D NMR) as 20-hydroxyecdysone.[10]

Since triterpene saponins from plants in the Caryophyllaceae have been reported to exert immunostimulating activities,[11−14] the saponins **1**−**3**, **4**/**5**, and **6** (deacylated form of **2**/**3** and **4**/**5**) were tested in an in vitro lymphocyte proliferation assay.[15] The cellular proliferation was measured by ^{3}H-thymidine incorporation in Jurkat T-cells. Saponin **1** was not cytotoxic up to the concentration of 10 μM (Figure 1), but displayed a proliferative activity at low concentration (10^{-3}−5 μM) with a stimulation index (SI) of 1.56 (see Experimental Section). Saponins **2**/**3** showed an immunomodulatory effect dependent on the concentration (Figure 1). In the concentration range 10^{-3}−10^{-1} μM, compounds **2**/**3** stimulate weakly Jurkat proliferation with SI = 1.36, and from 1 μM an inhibition of lymphocyte proliferation by **2**/**3** was observed. Compounds **4**/**5** showed a significant inhibition of Jurkat cell proliferation from 5 μM and a proliferative activity in the concentration range 10^{-3}−10^{-1} μM with SI = 1.44. The deacylated form (**6**) of compounds **2**/**3** and **4**/**5** showed a lower proliferative activity and was found to be not cytotoxic to Jurkat cells from the concentration of 5 μM compared with its acylated form. These results suggested that the *p*-methoxycinnamoyl ester moiety, linked to the fucosyl residue, might be responsible

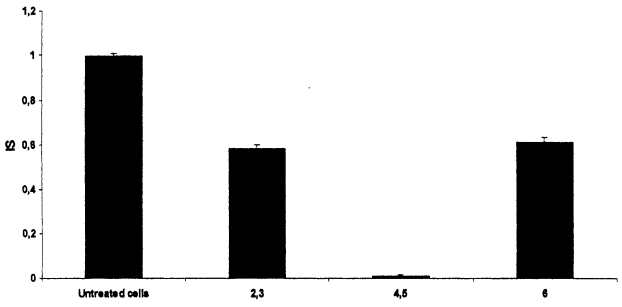

Figure 2. Effects of **2/3**, **4/5**, and **6** on DNA synthesis in Jurkat cells at 10 μM. DNA synthesis was measured by uptake of ^{3}H thymidine. Each column represents the mean of four replicates ± SE.

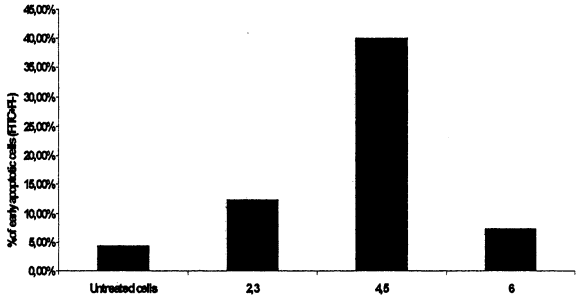

Figure 3. Effects of **2/3**, **4/5**, and **6** on the percentage of early apoptotic Jurkat cells at 10 μM.

for saponins' toxicity of T lymphocytes. It was recently reported that saponins securiosides A and B, isolated from the roots of *Securidaca inappendiculata*, having a 3,4-dimethoxycinnamoyl group, induced the apoptosis activity in macrophages.[16] So we decided to investigate the apoptosis-inducing activity of our compounds **2/3**, **4/5**, and **6** at 10 μM on Jurkat cells.[17] Apoptotic cells were detected by double labeling with propidium iodide (PI) and annexin V as described previously.[18,19] Cells beginning to undergo apoptosis expose the phosphatidylserine groups to the exterior of the cell membrane and annexin V-fluorescein isothiocyanate (FITC) can bind it, but these cells conserved cytoplasmic membrane integrity and are not labeled by PI. ($FITC^+$, PI^-) corresponded to early apoptotic cells. The result was analyzed with flow cytometry technique. We showed a good correlation between the inhibition of the proliferation and the induction of apoptosis (Figures 2 and 3). We observed that the presence of a *p*-methoxycinnamoyl group at 10 μM might induce apoptosis on Jurkat cells. It enhanced the proportion of early apoptotic cells ($FITC^+$, PI^-) by over 9-fold (**4/5**) and 2.8-fold (**2/3**) than untreated cells, respectively (Figure 3). The loss of this group (compound **6**) abrogated this activity. This result suggested that the *p*-methoxycinnamoyl ester moiety present in **2/3** and **4/5** might induce apoptosis. It was corroborated with the same results found for the saponins securiosides A and B.[16] Nevertheless, when the compounds **4/5** were attached with an acetyl group (**2/3**), the induction of apoptosis was more moderate. We can postulate that the acetyl group (CH_3-CO) produces a steric change in the saponin's conformation that hinders the availability of its methoxycinnamoyl group for induction of apoptosis. At this time, the present results must be considered as preliminary data in view of apoptosis potency assessment. This activity needs to be confirmed by additional assays, as has been done for other saponins.

Experimental Section

General Experimental Procedures. The 1D and 2D NMR spectra (^{1}H−^{1}H COSY, TOCSY, NOESY, HSQC, and HMBC) were performed using a UNITY-600 spectrometer at the operating frequency of 600 MHz on a Varian INOVA 600 instrument equipped with a SUN 4 L-X computer system (600 MHz for ^{1}H and 150 MHz for ^{13}C spectra). Conventional pulse sequences were used for COSY, HSQC, and HMBC. TOCSY spectra were acquired using the standard MLEV17 spin-locking sequence and 90 ms mixing time. The mixing time in the NOESY experiment was set to 500 ms. The carbon type (CH_3, CH_2, CH) was determined by DEPT experiments. All chemical shifts (δ) are given in ppm, and the samples were solubilized in DMSO-d_6 (δ 39.5). Fast-atom bombardment (FABMS) (negative-ion mode, glycerol matrix) was conducted on a JEOL SX 102. The HRESIMS was carried out on a Q-TOF 1-micromass spectrometer. Optical rotations were taken with a Perkin-Elmer 241 polarimeter. IR spectra (KBr disk) were recorded on a Perkin-Elmer 281 spectrophotometer. TLC and HPTLC employed precoated silica gel plates 60 F_{254} (Merck). The following TLC solvent systems were used: for saponins (a) $CHCl_3$−MeOH−AcOH−H_2O (15:8:3:2); for sapogenins (b) toluene−Me_2CO (4:1); for monosaccharides (c) $CHCl_3$−MeOH−H_2O (8:5:1); for *p*-methoxycinnamic acid (d) toluene−ether (1:1, saturated with AcOH 10%). Spray reagents for the saponins were Komarowsky reagent, a mixture (5:1) of *p*-hydroxybenzaldehyde (2% in MeOH) and H_2SO_4 50%; for the sugars, diphenylaminephosphoric acid reagent. Isolations were carried out using a medium-pressure liquid chromatography (MPLC) system [Gilson pump M 303, head pump 25 SC, manometric module M 802, Rheodyne 7125 injector, Büchi column (460 × 25 mm and 460 × 15 mm), Büchi precolumn (110 × 15 mm)]. Analytical and semipreparative HPLC of saponins: Gilson pumps M 305 and 306; head pump 25 SC; Rheodyne 7125 injector; Dynamic mixer 811 C; manometric module 805; UV-Vis-151 Gilson detector; Merck Hitachi D 7500 integrator. Analytical conditions: column, Lichrospher RP-18 (5 μm) 125-4; eluent, linear gradient from 30% to 50% MeCN−H_2O with 0.06% TFA during 30 min; flow rate, 1 mL/min; detection wavelength, 210 nm. Semipreparative HPLC conditions: column, Waters Prep Nova-Pak HR C18, 6 mm, 300−7.8 mm; eluent, linear gradient from 40% to 50% MeOH−H_2O during 30 min; flow rate, 3 mL/min; detection, 210 nm.

Plant Material. As previously described.[2]

Extraction and Isolation. The 95% ethanolic extract (56 g), obtained by maceration of 500 g of the dried roots of *S. fortunei*, was suspended in H_2O (1 L) and partitioned with *n*-BuOH and H_2O to give the *n*-BuOH fraction (2.7 g). It was solubilized in MeOH (10 mL) and precipitated in Et_2O (3 × 250 mL), yielding 2 g of a crude saponin fraction. This residue was fractionated by column chromatography on Sephadex LH-20 eluted by MeOH, yielding 0.8 g of a white powder. It was first fractionated by MPLC on silica gel 60 (15−40 μm) using as eluent $CHCl_3$−MeOH−H_2O (15:7:2 → 8:5:1). Further separations were performed by successive MPLC on reversed-phase material, Lichroprep RP-18, Merck (40−63 μm) eluted with MeOH−H_2O (linear gradient 50−70%) to give compounds **1** (14 mg) and jenisseensosides E and F (**2/3**) as an inseparable mixture (10 mg). The phytoecdysteroid was obtained from the ether-soluble fraction. It was fractionated by column chromatography on silica gel 60 (15−40 μm) using as eluent $CHCl_3$−MeOH−H_2O (65:25:4) to give the phytoecdysteroid (16 mg).

Acid Hydrolysis. A solution of saponin (3 mg) in 2 N aqueous CF_3COOH (5 mL) was refluxed on a water bath for 3 h. After this period, the reaction mixture was diluted with H_2O (15 mL) and extracted with CH_2Cl_2 (3 × 5 mL). The combined CH_2Cl_2 extracts were washed with H_2O and then evaporated to dryness in vacuo. Evaporation of the solvent gave quillaic acid (co-TLC with an authentic sample). After repeated evaporations of the aqueous layer by adding MeOH to remove the acid, the sugars were analyzed by silica gel TLC in comparison with standard sugars (solvent system c).

Alkaline Hydrolysis. Saponin (3 mg) was refluxed with 5% aqueous KOH (10 mL) for 1 h. The reaction mixture was adjusted to pH 6 with dilute HCl and then extracted with H_2O-saturated *n*-BuOH (3 × 10 mL). The combined *n*-BuOH

extracts were washed (H_2O). Evaporation of the *n*-BuOH gave the prosapogenin. The acidic hydrolysis of prosapogenin in 2 N aqueous CF_3COOH for 2 h at 120 °C furnished quillaic acid and glucuronic acid and galactose for **1**−**3** (co-TLC with authentic samples).

Mild Alkaline Hydrolysis of 2/3. Compounds **2**/**3** (3 mg) were hydrolyzed with 1% aqueous KOH (10 mL) for 30 min at room temperature, yielding **4**/**5** (TLC with an authentic sample, solvent system a). The reaction mixture was extended to 1 h, then adjusted to pH 6 with dilute 1% HCl, and then extracted with Et_2O (3 × 10 mL). Evaporation of the Et_2O gave the *p*-methoxycinamoyl residue analyzed by co-TLC (solvent system d). The aqueous extracts were extracted with H_2O-saturated *n*-BuOH (3 × 10 mL). Evaporation of the *n*-BuOH gave the deacylated saponin (**6**) (solvent system a).

Compound 1: white amorphous powder; $[\alpha]^{20}_D$ +13° (*c* 0.10, MeOH); IR ν_{max} 3398 (OH), 2928 (CH), 1735 (C═O ester), 1718 (CO carboxylic acid), 1615, 1386 cm^{-1}; ^{13}C NMR of quillaic acid,[1] ^1H NMR (DMSO-d_6, 600 MHz) and ^{13}C NMR (DMSO-d_6, 150 MHz) of sugar moieties, Table 1; HRESIMS positive mode *m*/*z* 1781.6907 $[MNa]^+$ (calcd for $C_{78}H_{118}O_{44}Na$, 1781.6893); negative FABMS glycerol matrix *m*/*z* 1757 $[M - H]^-$, 1583 $[(M - H) - 132 - 42]^-$, 1451 $[(M - H) - 2 \times 132 - 42]^-$, 1277 $[(M - H) - 3 \times 132 - 42 - 42]^-$, and 823 $[(M - H) - 3 \times 132 - 42 - 42 - 162 - 2 \times 146]^-$; TLC R_f 0.25 (system a); gray-violet spots by spraying with Komarowsky reagent.

Compounds 2/3: white amorphous powder; IR ν_{max} 3401 (OH), 2932 (CH), 1734 (C═O ester), 1715 (C═O carboxylic acid) cm^{-1}; ^{13}C NMR of quillaic acid,[1] ^1H NMR (DMSO-d_6, 600 MHz) and ^{13}C NMR (DMSO-d_6, 150 MHz) of sugar moieties, Table 1; HRESIMS positive mode *m*/*z* 1341.5902 $[MNa]^+$ (calcd for $C_{66}H_{94}O_{27}Na$, 1341.5880); negative FABMS (glycerol matrix) *m*/*z* 1317 $[M - H]^-$, 1275 $[(M - H) - 42]^-$; TLC R_f 0.53 (system a); violet spots by spraying with Komarowsky reagent; HPLC/UV **2**, t_R 10.33; λ_{max} nm 215, 230, (300 sh); **3**, t_R 10.63; λ_{max} nm (235 sh), 305.

Compounds 4−**6.** The retention time and TLC profile were in full agreement with previously published data.[1]

Bioassays. Proliferation Assay. Human T-cell leukemia Jurkat cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, GibcoBRL Life Technologies, Grand Island, NY) supplemented with 1 mM sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin (Gibco), 2 mM L-glutamin (Gibco), and 6% FCS (fetal calf serum) at 37 °C under humidified air with 5% CO_2. The cells were resuspended at 2 × 10^5 cells/mL, 1 mL of cells with 0% FCS were seeded in 24-well culture plates (NUNC Roskild, Denmark), and 30 μL of the appropriate concentration saponin sample solution was added. The cells were cultured at 37 °C under a 5% CO_2 atmosphere for 36 h. For the last 16 h, 200 μL aliquots of each well cell suspension were transferred in 96-well culture plates (NUNC Roskilde, Denmark), and four replicates were tested for each dilution of saponin. Lymphocyte proliferation was determined using ^3H-thymidine incorporation.

The assay was performed by adding 0.5 μCi/well of commercial solution (Perkin-Elmer Life Science, Boston), and the radioactivity incorporated into cells was measured using a liquid scintillation counter (Wallac, Finland).

Results were calculated as follow:

$$\text{SI (stimulation index)} = \frac{[^3\text{H}]\text{Thymidine uptake in cells + saponin}}{[^3\text{H}]\text{Thymidine uptake in cells alone}}$$

Apoptosis Induction. The in vitro Annexin V-FITC binding assay was performed according to a method described in ref 17. The cells were resuspended at 1 × 10^6 cells/mL, and 1 mL of cells with 0% FCS was seeded in 24-well culture plates (NUNC Roskild, Denmark) with 10 μM of saponin sample solution. The cells were cultured at 37 °C under a 5% CO_2 atmosphere for 18 h. After washing the cells in cold PBS (pH 7.4), they were treated at 37 °C by annexin V-FITC conjugate (TEBU, CA) at 1 μg/mL resuspended in binding buffer (10 mM NaOH, 140 mM NaCl, 2 mM $CaCl_2$) for 15 min in the dark. Cells were washed with the binding buffer and stained with PI (2.5 μg/mL). Data analyses were performed with a Beckman-Coulter EPICS-ELITE/ESP cytometer.

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