New Acylated Triterpene Saponins from *Polygala arenaria*

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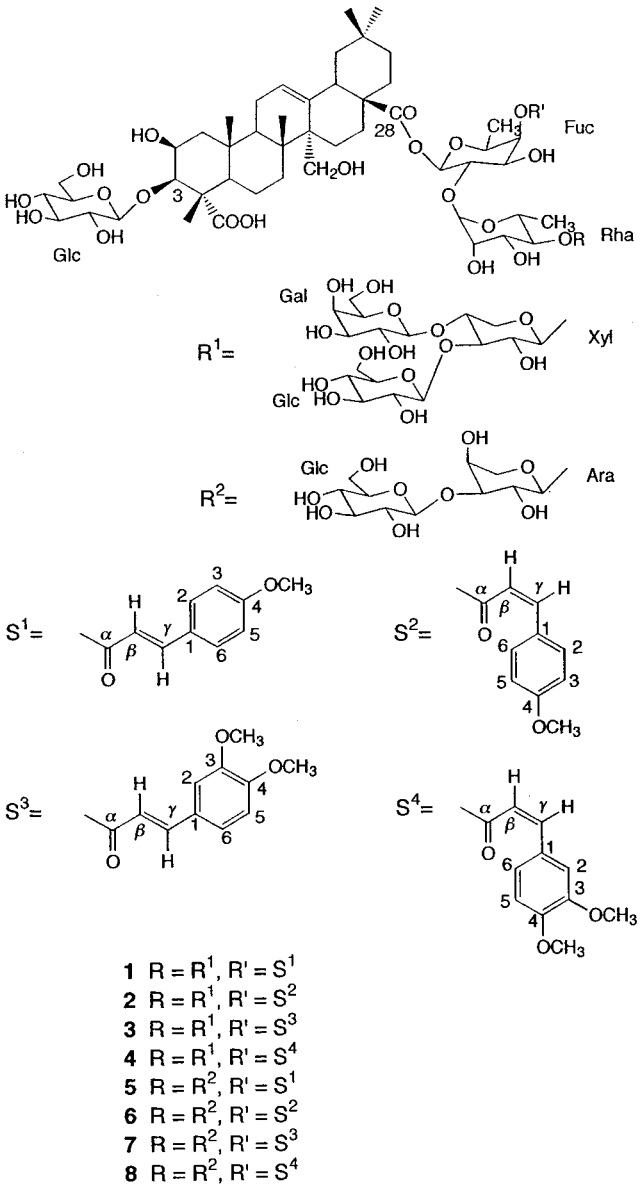
Eight new acylated triterpene saponins **1**–**8** were isolated from the roots of *Polygala arenaria* as four inseparable (*E*)/(*Z*) mixtures of the 4-methoxycinnamoyl and 3,4-dimethoxycinnamoyl derivatives by repeated MPLC over silica gel. Their structures were established mainly by 600-MHz 2D-NMR techniques (^{1}H,^{1}H-COSY, TOCSY, NOESY, HSQC, HMBC) as 3-*O*-(*β*-D-glucopyranosyl)presenegenin 28-(*O*-*β*-D-galactopyranosyl-(1→4)-*O*-[*β*-D-glucopyranosyl-(1→3)]-*O*-*β*-D-xylopyranosyl-(1→4)-*O*-*α*-L-rhamnopyranosyl-(1→2)-{4-*O*-[(*E*)-4-methoxycinnamoyl]}-*β*-D-fucopyranosyl) ester and its (*Z*)-isomer (**1**/**2**), 3-*O*-(*β*-D-glucopyranosyl)presenegenin 28-(*O*-*β*-D-galactopyranosyl-(1→4)-*O*-[*β*-D-glucopyranosyl-(1→3)]-*O*-*β*-D-xylopyranosyl-(1→4)-*O*-*α*-L-rhamnopyranosyl-(1→2)-{4-*O*-[(*E*)-3,4-dimethoxycinnamoyl]}-*β*-D-fucopyranosyl) ester and its (*Z*)-isomer (**3**/**4**), 3-*O*-(*β*-D-glucopyranosyl)presenegenin 28-(*O*-*β*-D-glucopyranosyl-(1→3)-*O*-*α*-L-arabinopyranosyl-(1→4)-*O*-*α*-L-rhamnopyranosyl-(1→2)-{4-*O*-[(*E*)-4-methoxycinnamoyl]}-*β*-D-fucopyranosyl) ester and its (*Z*)-isomer (**5**/**6**), and 3-*O*-(*β*-D-glucopyranosyl)presenegenin 28-(*O*-*β*-D-glucopyranosyl-(1→3)-*O*-*α*-L-arabinopyranosyl-(1→4)-*O*-*α*-L-rhamnopyranosyl-(1→2)-{4-*O*-[(*E*)-3,4-dimethoxycinnamoyl]}-*β*-D-fucopyranosyl) ester and its (*Z*)-isomer (**7**/**8**) (presenegenin = (2*β*,3*β*)-2,3,27-trihydroxyolean-12-ene-23,28-dioic acid). In our *in vitro* lymphocyte proliferation assay (*Jurkat* T-leukemia cells), a fraction containing **1**–**4** showed a concentration-dependent immunomodulatory effect. This effect was not found for the prosapogenin (tenuifolin = 3-*O*-(*β*-D-glucopyranosyl)presenegenin), underlining the importance of the acyl–oligosaccharidic moiety.

Introduction. – In continuation of our studies of saponin constituents of medicinal plants of the Polygalaceae family [1–4], we have examined the saponin fraction of the roots of *Polygala arenaria* WILLD. This herbaceous plant of tropical Africa, used in treatment of smallpox [5], was reported to contain methyl salicylate and one major saponin derivative of presenegenin [6]. We describe in this paper the isolation and structural elucidation of eight new triterpene saponins **1**–**8**, which were obtained as four inseparable mixtures of their (*E*)- and (*Z*)-acylated derivatives (**1**/**2**, **3**/**4**, **5**/**6**, and **7**/**8**) from the aqueous ethanolic extract of the roots. Four of these compounds and their prosapogenins were tested for *in vitro* lymphocyte proliferation activities with *Jurkat* T-leukemia cells.

Results and Discussion. – A concentrated MeOH-soluble fraction of the EtOH (80%) extract of the roots of *P. arenaria* was purified by precipitation with Et_2O and treatment with charcoal to yield a crude saponin mixture [7]. The latter was further



1 R = R^1, R' = S^1
2 R = R^1, R' = S^2
3 R = R^1, R' = S^3
4 R = R^1, R' = S^4
5 R = R^2, R' = S^1
6 R = R^2, R' = S^2
7 R = R^2, R' = S^3
8 R = R^2, R' = S^4

fractionated by column chromatography (*Sephadex LH-20*) and repeated medium-pressure liquid chromatography (MPLC) over normal silica gel to yield compounds **1**/**2**, **3**/**4**, **5**/**6**, and **7**/**8** as four inseparable mixtures. Their structures were elucidated mainly by 600-MHz NMR spectroscopy, including 1D- and 2D-NMR experiments (^{1}H,^{1}H COSY, TOCSY, NOESY, HSQC, HMBC), and FAB- and APIES mass spectrometry. Acid hydrolysis of compounds **1**–**8**, obtained as white amorphous

powders, afforded an artifactual aglycon. Glucose, galactose, xylose, rhamnose, and fucose (in the case of **1**/**2** and **3**/**4**), and glucose, arabinose, rhamnose, and fucose (in the case of **5**/**6** and **7**/**8**) were identified by comparison with authentic samples (TLC). The NMR data of the prosapogenin of **1**–**8** obtained by alkaline hydrolysis were in good agreement with those of tenuifolin (= 3-*O*-(*β*-D-glucopyranosyl)presenegenin), commonly encountered in the Polygalaceae family [2]. The mild alkaline hydrolysis of **1**–**8** yielded compounds that presented a higher polarity than the native ones (lower R_f on TLC), indicating acylation of the saponins.

The negative-ion FAB-MS of compounds **1**/**2** exhibited a quasi-molecular-ion peak at *m*/*z* 1587 ($[M-H]^-$), indicating a molecular weight of 1588, compatible with a molecular formula of $C_{75}H_{112}O_{36}$. Other fragment ion peaks were observed at *m*/*z* 1425 ($[M-H-162]^-$) and 1263 ($[M-H-162-162]^-$), which revealed the elimination of two hexosyl moities. Their APIES-MS (negative-ion mode) showed a quasi-molecular ion peak at *m*/*z* 1587 ($[M-H]^-$), which confirmed the proposed molecular weight. On the basis of spectral evidence, mainly 2D-NMR, the structure of compounds **1**/**2** was elucidated as 3-*O*-(*β*-D-glucopyranosyl)presenegenin 28-(*O*-*β*-D-galactopyranosyl-(1 → 4)-*O*-[*β*-D-glucopyranosyl-(1 → 3)]-*O*-*β*-D-xylopyranosyl-(1 → 4)-*O*-*α*-L-rhamnopyranosyl-(1 → 2)-{4-*O*-[(*E*)-4-methoxycinnamoyl]}-*β*-D-fucopyranosyl) ester and its (*Z*)-isomer, new natural triterpene glycosides.

The ^{1}H,^{1}H-COSY experiment allowed us to identify the (*E*)- and (*Z*)-olefinic H-atoms of a 4-methoxycinnamoyl moiety, which appeared as two *doublets* at *δ* 7.80 and 6.36 (1 H each, *J* = 16), and *δ* 5.95 and 6.85 (1 H each, *J* = 13), respectively. Further investigations based on the HMBC and HSQC spectra allowed the complete assignments of all H- and C-atoms of the disubstituted benzene ring (*cf. Table 3*). These conclusions indicated that **1** and **2** are a mixture of (*E*)- and (*Z*)-4-methoxycinnamoyl triterpene glycosides, and all attempts to separate them were unsuccessful. Such a phenomenon, which could be explained by the tautomeric behavior of the 4-methoxycinnamoyl moiety in methanolic solutions under light, has already been described in saponins from *Silene jenisseensis* [8] and *S. fortunei* [9], senegasaponins from *Polygala senega* [10], and trimethoxycinnamoylated saponins from *Muraltia heisteria* [3].

The ^{1}H-NMR spectrum of **1**/**2** displayed signals for six anomeric H-atoms at *δ* 6.41 (br. *s*), 5.90 (*d*, *J* = 8.4), 5.04 (*d*, *J* = 7.7), 4.92 (*d*, *J* = 7.3), 4.87 (*d*-like), and 4.77 (*d*, *J* = 7.3), which gave correlations in the HSQC spectrum with ^{13}C signals at *δ* 100.4, 94.1, 104.3, 103.8, 105.6, and 101.9, respectively. The ring H-atoms of the monosaccharide residues were assigned starting from the readily identifiable anomeric H-atoms by means of the ^{1}H,^{1}H-COSY, TOCSY, HSQC, HMBC experiments (*Table 1*). Evaluation of spin–spin couplings and chemical shifts allowed the identification of one *α*-rhamnopyranose (Rha), one *β*-fucopyranose (Fuc), two *β*-glucopyranoses (Glc), one *β*-xylopyranose (Xyl), and one *β*-galactopyranose (Gal) units. The common D-configuration for Fuc, Glc, Xyl, and Gal, and the L-configuration for Rha were assumed to be those of the most commonly encountered analogues in the plant kingdom. Correlations observed in the HMBC spectrum between *δ*(H) 4.92 (*d*, *J* = 7.3, H–C(1)(Glc)) and *δ*(C) 86.5 ((C(3)(Agly)), and in the NOESY spectrum between *δ*(H) 4.92 (*d*, *J* = 7.3, H–C(1)(Glc)) and *δ*(H) 4.49 (H–C(3)(Agly)), confirmed the substitution at C(3) of the presenegenin by a *β*-D-glucopyranose. After subtraction of

the anomeric signals of the glucosyl moiety, the signals of five sugar moieties linked to the aglycon by an ester linkage remained. In the HMBC spectrum, a correlation between δ(H) 5.90 (d, $J = 8.4$, H–C(1)(Fuc)) and δ(C) 176.5 (C(28)(Agly)) established a glycosidic ester linkage to C(28) of the aglycon. A correlation between δ(C) 100.4 (C(1)(Rha)) and δ(H) 4.71 (t, $J = 8.6$, H–C(2)(Fuc)), and a reverse correlation between δ(C) 71.6 (C(2)(Fuc)) and δ(H) 6.41 (br. s, H–C(1)(Rha), revealed the (1 → 2) linkage between these two sugar moieties. The downfield shifts observed in the HSQC spectrum for the H–C(4)/C(4) resonances of the Fuc at δ 5.70/δ 74.7 established the secondary alcoholic function at C(4) of the Fuc to be acylated by a 4-methoxycinnamoyl acid. At this stage, we noticed that many saponins isolated from plants of the Polygalaceae family, which belong to genera *Polygala* [1][10–16], *Carpolobia* [2], *Muraltia* [3], and *Securidaca* [17], displayed the same sequence 3-*O*-(β-D-glucopyranosyl)presenegenin 28-[*O*-α-L-rhamnopyranosyl-(1 → 2)-β-D-fucopyranosyl] ester with additional substitutions of the 3-*O*-(β-D-glucopyranosyl) part and acylations at C(3) and C(4) of the Fuc moiety. This sequence may represent a chemotaxonomic marker for the Polygalaceae family.

Cross-peaks in the HMBC spectrum between δ(H) 4.14 (H–C(4)(Rha)) and δ(C) 105.6 (C(1)(Xyl)), and in the NOESY spectrum between δ(H) 4.14 (H–C(4)(Rha)) and δ(H) 4.87 (*d*-like, H–C(1))(Xyl)) indicated that the xylose was linked to the rhamnose by a (1 → 4) linkage. Moreover, other correlations between the deshielded δ(C) at 85.3 (C(3)(Xyl)) and an anomeric signal at δ5.04 (d, $J = 7.7$ Hz), and between δ(C) 71.1 (C(4)(Xyl)) and another anomeric signal at δ4.77 (d, $J = 7.3$), indicated a 3,4-disubstitution of this xylose by one terminal glucose (T-Glc) and one terminal galactose (T-Gal), respectively. These substitutions are confirmed by correlations in the NOESY spectrum between δ(H) 3.97 (H–C(3)(Xyl)) and δ(H) 5.04 (d, $J = 7.7$, H–C(1)-(T-Glc)), and δ(H) 4.32 (H–C(4)(Xyl)) and δ(H) 4.77 (d, $J = 7.3$, H–C(1)(T-Gal)).

The FAB- (negative-ion mode) and APIES (negative-ion mode) mass spectra of compounds **3**/**4** showed the same quasi-molecular ion peak at m/z 1617 ($[M-H]^-$), leading to the molecular formula as $C_{76}H_{114}O_{37}$. The other fragment ion peaks are at m/z 1427 ($[M-H-190]^-$), 1265 ($[M-H-190-162]^-$) in FAB and 1455 ($[M-H-162]^-$) in APIES. These results revealed the loss of a 3,4-dimethoxycinnamoyl moiety and two hexosyl moieties. The study of the 2D-NMR spectra of compounds **3**/**4** led to the establishment of their structures as 3-*O*-(β-D-glucopyranosyl)presenegenin 28-(*O*-β-D-galactopyranosyl-(1 → 4)-*O*-[β-D-glucopyranosyl-(1 → 3)]-*O*-β-D-xylopyranosyl-(1 → 4)-*O*-α-L-rhamnopyranosyl-(1 → 2)-{4-*O*-[(*E*)-3,4-dimethoxycinnamoyl]}-β-D-fucopyranosyl) ester and its (*Z*)-isomer, new natural compounds.

The ^{1}H- and ^{13}C-NMR signals of **3**/**4** assigned from the 2D spectra were almost superimposable on those of **1**/**2** except for the acyl moiety (*Tables 1* and *2*). The 4 position of Fuc was substituted with (*E*)- and a (*Z*)-3,4-dimethoxycinnamoyls in **3** and **4**, respectively, instead of (*E*)- and (*Z*)-4-methoxycinnamoyls in **1** and **2**, respectively. These two acyl moities appeared as two *doublets* at δ 7.82 and 6.47 (1 H each, $J = 16$), and δ 6.85 and 5.95 (1 H each, $J = 13$), respectively (*cf. Table 4*). The full assignments of the C- and H-atoms of the 3,4-dimethoxycinnamoyl units obtained by further 2D-NMR investigations were in good agreement with those described in the literature [10][14].

The negative-ion FAB-MS of **5**/**6** showed a quasi-molecular-ion peak at m/z 1425 ($[M-H]^-$), consistent with the molecular formula of $C_{69}H_{102}O_{31}$. Two other significant

Table 1. *^{1}H-NMR* (600 MHz) *Data of the Sugar Moieties of* **1**–**8** *in (D_5)Pyridine from 1D- and 2D-NMR Experiments* [a]). δ(H) in ppm, J in Hz.

	1/2	**3/4**	**5/6**	**7/8**
3-*O*-Glc H–C(1)	4.92 ($d, J = 7.3$)	4.92 ($d, J = 7.3$)	4.99 ($d, J = 7.3$)	4.99 ($d, J = 7.3$)
H–C(2)	3.86	3.86	3.88	3.88
H–C(3)	4.12	4.12	4.14	4.14
H–C(4)	3.91	3.91	4.00	4.00
H–C(5)	3.83	3.83	3.83	3.83
CH_2(6)	4.24[b])	4.24[b])	4.14, 4.30	4.14, 4.30
28-*O*-Sugars				
Fuc H–C(1)	5.90 ($d, J = 8.4$)	5.90 ($d, J = 8.4$)	6.02 ($d, J = 8.4$)	6.02 ($d, J = 8.4$)
H–C(2)	4.71 ($t, J = 8.6$)	4.71 ($t, J = 8.6$)	4.80 ($t, J = 8.5$)	4.80 ($t, J = 8.5$)
H–C(3)	4.50	4.50	4.55	4.55
H–C(4)	5.70	5.70	5.73	5.73
H–C(5)	4.22	4.22	4.21	4.21
Me(6)	1.30 ($d, J = 6.2$)	1.30 ($d, J = 6.2$)	1.30 ($d, J = 6.0$)	1.30 ($d, J = 6.0$)
Rha H–C(1)	6.41 (br. *s*)	6.41 (br. *s*)	6.56 (br. *s*)	6.56 (br. *s*)
H–C(2)	4.62	4.62	4.71	4.71
H–C(3)	4.44	4.44	4.56	4.56
H–C(4)	4.14	4.14	4.23	4.23
H–C(5)	4.40	4.40	4.50	4.50
Me(6)	1.67 ($d, J = 5.1$)	1.67 ($d, J = 5.1$)	1.74 ($d, J = 5.0$)	1.74 ($d, J = 5.0$)
Xyl H–C(1)	4.87 (*d*-like)	4.87 (*d*-like)		
H–C(2)	3.97	3.97		
H–C(3)	3.97	3.97		
H–C(4)	4.32	4.32		
H_α–C(5)	3.51 (br. $t, J = 10.4$)	3.51 (br. $t, J = 10.4$)		
H_β–C(5)	4.32	4.32		
Ara H–C(1)			4.93 (*d*-like)	4.93 (*d*-like)
H–C(2)			4.03	4.03
H–C(3)			3.88	3.88
H–C(4)			3.96	3.96
H_α–C(5)			3.43 (br. *d*)	3.43 (br. *d*)
H_β–C(5)			4.08	4.08
T-Glu H–C(1)	5.04 ($d, J = 7.7$)	5.04 ($d, J = 7.7$)	5.05 ($d, J = 7.7$)	5.05 ($d, J = 7.7$)
H–C(2)	4.01	4.01	4.07	4.07
H–C(3)	4.06	4.06	4.18	4.18
H–C(4)	3.93	3.93	3.90	3.90
H–C(5)	3.76	3.76	3.84	3.84
CH_2(6)	4.04, 4.23	4.04, 4.23	4.03, 4.39	4.03, 4.39
T-Gal H–C(1)	4.77 ($d, J = 7.3$)	4.77 ($d, J = 7.3$)		
H–C(2)	4.34	4.34		
H–C(3)	3.98	3.98		
H–C(4)	4.27	4.27		
H–C(5)	3.85	3.85		
CH_2(6)	4.05, 4.12	4.05, 4.12		

[a]) Overlapping signals are reported without designated multiplicity. [b]) Not determined.

ion peaks appeared at m/z 1263 ($[M - H - 162]^-$) and 1101 ($[M - H - 162 - 162]^-$), which suggested the loss of two hexosyl moities. The assignments of the ^{1}H- and ^{13}C-NMR signals of **5/6** led to the elucidation of their structures as 3-*O*-(*β*-D-glucopyranosyl)presenegenin 28-(*O*-*β*-D-glucopyranosyl-(1 → 3)-*O*-*α*-L-arabinopyra-

Table 2. *^{13}C-NMR* (150 MHz) *Data of the Sugar Moieties of* **1**–**8** *in (D_5)Pyridine from 1D- and 2D-NMR Experiments* [a]). δ(C) in ppm.

	1/2	**3/4**	**5/6**	**7/8**
3-*O*-Glc CH(1)	103.8	103.8	104.0	104.0
CH(2)	74.2	74.2	74.2	74.2
CH(3)	76.4	76.4	76.7	76.7
CH(4)	70.4	70.4	70.3	70.3
CH(5)	76.9	76.9	77.2	77.2
CH_2(6)	61.2	61.2	61.2	61.2
28-*O*-Sugars				
Fuc CH(1)	94.1	94.1	94.0	94.0
CH(2)	71.6	71.6	71.3	71.3
CH(3)	74.2	74.2	74.0	74.0
CH(4)	74.7	74.7	74.5	74.5
CH(5)	70.5	70.5	70.2	70.2
Me(6)	16.0	16.0	15.8	15.8
Rha CH(1)	100.4	100.4	100.6	100.6
CH(2)	70.6	70.6	70.6	70.6
CH(3)	71.6	71.6	71.5	71.5
CH(4)	85.0	85.0	84.4	84.4
CH(5)	67.1	67.1	66.9	66.9
Me(6)	17.6	17.6	17.9	17.9
Xyl CH(1)	105.6	105.6		
CH(2)	75.1	75.1		
CH(3)	85.3	85.3		
CH(4)	71.1	71.1		
CH_2(5)	64.8	64.8		
Ara CH(1)			105.8	105.8
CH(2)			74.5	74.5
CH(3)			86.0	86.0
CH(4)			67.6	67.6
CH_2(5)			65.9	65.9
T-Glu CH(1)	104.3	104.3	104.4	104.4
CH(2)	74.7	74.7	74.6	74.6
CH(3)	76.8	76.8	76.6	76.6
CH(4)	69.2	69.2	70.0	70.0
CH(5)	77.8	77.8	77.2	77.2
CH_2(6)	61.4	61.4	61.4	61.4
T-Gal CH(1)	101.9	101.9		
CH(2)	69.8	69.8		
CH(3)	73.4	73.4		
CH(4)	68.9	68.9		
CH(5)	76.3	76.3		
CH_2(6)	61.2	61.2		

[a]) Multiplicities were assigned from DEPT spectra.

nosyl-(1 → 4)-*O*-α-L-rhamnopyranosyl-(1 → 2)-{4-*O*-[(*E*)-4-methoxycinnamoyl]}-β-D-fucopyranosyl) ester and its (*Z*)-isomer, new natural saponins.

The study of the 2D-NMR spectra showed that the same sequence 3-*O*-(β-D-glucopyranosyl)presenegenin 28-(*O*-α-L-rhamnopyranosyl-(1 → 2)-{4-*O*-[(*E*)-4-methoxycinnamoyl]}-β-D-fucopyranosyl) ester and its (*Z*)-isomer, already encountered

in **1**/**2**, was found in **5**/**6** (*Tables 1–3*). The difference was located at the sugar moieties linked to the 4-position of Rha. The assignments of the ^{1}H- and ^{13}C-NMR signals of a α-L-arabinopyranosyl (Ara) and a β-D-glucopyranosyl (Glc) were deduced from the 2D-NMR spectra. The common L-configuration for Ara and D-configuration for Glc were assumed. The $(1\rightarrow4)$ linkage of Ara to Rha was deduced, in the NOESY spectrum, by a correlation between δ(H) 4.23 (H–C(4)(Rha)) and δ(H) 4.93 (*d*-like, H–C(1)(Ara)). Moreover, a cross-peak in the NOESY spectrum between a H-atom at δ 3.88, which correlated with a deshielded C-atom at δ 86.0 in the HSQC spectrum, and an anomeric signal at δ 5.05 ($d, J=7.7$) established that this Ara is substituted at the 3 position with one T-Glc.

Table 3. *^{13}C-* (150 MHz)[a]) *and ^{1}H-NMR* (600 MHz) *Data of the 4-Methoxycinnamoyl Moiety of* **1**, **2**, **5**, *and* **6** *in (D$_5$)Pyridine from 1D- and 2D-NMR Experiments.* δ in ppm, *J* in Hz.

	1		**2**		**5**		**6**	
	δ(C)	δ(H)	δ(C)	δ(H)	δ(C)	δ(H)	δ(C)	δ(H)
C(α)	167.8		166.7		[b])		[b])	
CH(β)	115.0	6.36 ($d, J=16$)	116.2	5.95 ($d, J=13$)	115.0	6.40 ($d, J=16$)	116.2	5.90 ($d, J=13$)
CH(γ)	145.5	7.80 ($d, J=16$)	144.1	6.85 ($d, J=13$)	144.8	7.85 ($d, J=16$)	143.9	6.85 ($d, J=13$)
C(1)	126.0		126.4		126.2		126.3	
CH(2,6)	129.9	7.33 ($d, J=8$)	132.5	7.89 ($d, J=8$)	129.8	7.35 ($d, J=8$)	132.5	7.91 ($d, J=8$)
CH(3,5)	114.4	6.95 ($d, J=8$)	113.5	6.90 ($d, J=8$)	114.1	6.95 ($d, J=8$)	113.3	6.90 ($d, J=8$)
C(4)	161.5		160.9		[b])		[b])	
MeO–C(4)	55.2	3.77 (*s*)	55.0	3.72 (*s*)	55.1	3.72 (*s*)	55.0	3.68 (*s*)

[a]) Multiplicities were assigned from DEPT spectra. [b]) Not determined.

For compounds **7**/**8**, the negative-ion FAB-MS exhibited a quasi-molecular-ion peak at *m*/*z* 1455 ($[M-H]^-$), corresponding to the molecular formula of $C_{70}H_{104}O_{32}$. This spectra equally showed three other fragment ion peaks at *m*/*z* 1265 ($[M-H-190]^-$), 1293 ($[M-H-162]^-$) and 1103 ($[M-H-190-162]^-$), which revealed the loss of a 3,4-dimethoxycinnamoyl and two hexosyl moieties, respectively, as described for **3**/**4**. Based on the sudy of the 2D-NMR spectra, the structures of **7**/**8** were established as 3-*O*-(β-D-glucopyranosyl)presenegenin 28-(*O*-β-D-glucopyranosyl-$(1\rightarrow3)$-*O*-α-L-arabinopyranosyl-$(1\rightarrow4)$-*O*-α-L-rhamnopyranosyl-$(1\rightarrow2)$-{4-*O*-(*E*)-3,4-dimethoxycinnamoyl]}-β-D-fucopyranosyl) ester and its (*Z*)-isomer, new natural compounds.

The ^{1}H- and ^{13}C-NMR signals of **7**/**8** were almost superimposable on those of **5**/**6** except for the acyl moiety (*Tables 1* and *2*). As was observed for **3**/**4**, the 4 position of Fuc was substituted by a (*E*)- and a (*Z*)-3,4-dimethoxycinnamoyl unit, which appeared as two *doublets* at δ 7.87 and 6.48 (1 H each, $J=16$), and δ 6.85 and 5.90 (1 H each, $J=13$), respectively (*Table 4*).

Saponins have been reported to present immunostimulating activities [18], and structure–activity relationships were deduced underlining the importance of the acylation of the sugars, especially by hydroxycinnamic acids for saponins' cytotoxicity [9]. Thus, a MPLC fraction of the crude saponin extract containing a mixture of **1**/**2** and **3**/**4** was tested in an *in vitro* lymphocyte proliferation assay. The cellular proliferation was measured by [^{3}H]thymidine incorporation in *Jurkat* T-leukemia cells [9]. These saponins showed an immunomodulatory effect in a concentration-dependent manner.

Table 4. *^{13}C-* (150 MHz)[a]) *and ^{1}H-NMR* (600 MHz) *Data of the 3,4-Dimethoxycinnamoyl Moiety of* **3**, **4**, **7**, *and* **8** *in (D_5)Pyridine from 1D- and 2D-NMR Experiments.* δ in ppm, J in Hz.

	3		**4**		**7**		**8**	
	δ(C)	δ(H)	δ(C)	δ(H)	δ(C)	δ(H)	δ(C)	δ(H)
C(α)	167.8		166.7		[b])		[b])	
CH(β)	115.0	6.47 (d, J = 16)	116.2	5.95 (d, J = 13)	115.0	6.48 (d, J = 16)	116.2	5.90 (d, J = 13)
CH(γ)	146.0	7.82 (d, J = 16)	144.1	6.85 (d, J = 13)	145.2	7.87 (d, J = 16)	143.9	6.85 (d, J = 13)
C(1)	127.2		127.5		127.0		127.5	
CH(2)	110.1	6.95 (s)	113.8	7.80 (s)	110.1	7.00 (s)	113.9	7.90 (s)
C(3)	149.5		148.4		[b])		[b])	
C(4)	151.3		150.6		151.4		150.4	
CH(5)	111.5	6.95 (d, J = 8)	111.0	6.87 (d, J = 8)	111.3	6.93 (d, J = 8)	111.0	6.88 (d, J = 8)
CH(6)	123.1	7.02 (d, J = 8)	125.5	7.39 (d, J = 8)	122.7	7.05 (d, J = 8)	125.0	7.41 (d, J = 8)
MeO–C(3)	55.5	3.83 (s)	55.5	3.83 (s)	55.2	3.78 (s)	55.2	3.78 (s)
MeO–C(4)	55.5	3.86 (s)	55.5	3.80 (s)	55.2	3.80 (s)	55.2	3.76 (s)

[a]) Multiplicities were assigned from DEPT spectra. [b]) Not determined.

Compounds **1/2** and **3/4**, in the concentration range $10^{-4}-1$ μM, stimulated weakly *Jurkat* T cells proliferation with a maximal stimulation index (SI) of 1.25, and, in the range of $5-10$ μM, inhibition of lymphocyte proliferation by these compounds was observed (SI = 0.14). To show the role of the acylated oligosaccharidic part of these molecules in the immunomodulatory activity, we have tested their prosapogenin, tenuifolin. It displayed a proliferative activity with a SI of 1.37, but was not cytotoxic up to a concentration of 10 μM. In addition, the hemolytic activity of **1/2** and **3/4** was evaluated on sheep erythrocytes and compared with tenuifolin. The MPLC fraction of the crude saponin extract containing a mixture of **1/2** and **3/4** showed good hemolytic activity (HC_{50} = 50 μg/ml), whereas tenuifolin was not hemolytic up to 400 μg/ml. These results corroborated those obtained by *Gaidi et al.* [9], who showed that a 4-methoxycinnamoyl ester unit linked to a fucosyl residue of triterpene saponins isolated from *Silene fortunei* (Caryophyllaceae) might be responsible for cellular toxicity.

Experimental Part

General. Column chromatography (CC): *Sephadex LH-20* (*Pharmacia*). Medium-pressure liquid chromatography (MPLC): silica gel *60* (*Merck*, 15–40 μm), *Gilson* pump *M 303*, head pump *25 SC*, manometric module *M 802*, *Rheodyne 7125* injector, *Büchi* glass column (460 × 25 mm and 460 × 15 mm), *Büchi* precolumn (110 × 15 mm). TLC and HPTLC: silica gel *60 F_{254}* (*Merck*); solvent systems: for saponins $CHCl_3$/MeOH/AcOH/H_2O 15:8:3:2 (*a*); for sapogenins CH_2Cl_2/MeOH 19:1 (*b*); for monosaccharides $CHCl_3$/MeOH/H_2O 8:5:1 (*c*); spray reagents: for saponins and sapogenins, *Komarowsky* reagent, 2% 4-hydroxybenzaldehyde in MeOH/50% H_2SO_4 soln. 5:1; for the sugars, 50% aq. H_2SO_4. IR Spectra: KBr disc; *Perkin-Elmer 281-IR* spectrophotometer; in cm^{-1}. 1D- and 2D-NMR Spectra: see [2]. Fast-atom bombardment (FAB) MS: negative-ion mode; *JEOL SX 102*; atmospheric-pressure ionization electrospray (APIES) MS: negative-ion mode; *Hewlett-Packard 1100 MSD* (100 and 150 eV).

Plant Material. The roots of *P. arenaria* WILLD. were collected in Democratic Republic of Congo, Africa, in June 1975, in fields near M'vuazi and Kisantu. A voucher specimen (Callens 2009) was deposited in the Herbarium of the Botanical Garden of Kisantu and in the Herbarium of the National Botanical Garden of Brussels, Belgium.

Extraction and Isolation. A crude saponin mixture (5 g) was obtained according to a method described in [7]. Thereof, 2 g was submitted to CC (*Sephadex LH-20*, MeOH) to give four main fractions. One of them

(300 mg), rich in saponins, was fractionated by successive MPLC (silica gel *60* (15–40 μm); $CHCl_3$/MeOH/H_2O 65:40:8): **1/2** (35 mg), **3/4** (35 mg), **5/6** (5 mg), and **7/8** (5 mg).

*(2β,3β)-3-(β-*D*-Glucopyranosyloxy)-2,27-dihydroxyolean-12-ene-23,28-dioic Acid 28-(O-β-*D*-Galactopyranosyl-(1→4)-*O*-[β-*D*-glucopyranosyl-(1→3)]-O-β-*D*-xylopyranosyl-(1→4)-O-α-*L*-rhamnopyranosyl-(1→2)-{4-O-[(*E*)-4-methoxycinnamoyl]}-β-*D*-fucopyranosyl) Ester and Its (Z)-Isomer* (**1/2**). White amorphous powder. TLC (*a*): R_f 0.33. IR (KBr): 3500–3300, 2930, 1725, 1710. ^{1}H- and ^{13}C-NMR ((D_5)pyridine): for aglycons see [2], for sugars and acids see *Tables 1–4*. FAB-MS (neg.): 1587 ($[M-H]^-$), 1425 ($[M-H-162]^-$), 1263 ($[M-H-162-162]^-$). APIES-MS (neg.): 1587 ($[M-H]^-$), 1425 ($[M-H-162]^-$).

*(2β,3β)-3-(β-*D*-Glucopyranosyloxy)-2,27-dihydroxyolean-12-ene-23,28-dioic Acid 28-(O-β-*D*-Galactopyranosyl-(1→4)-O-[β-*D*-glucopyranosyl-(1→3)]-O-β-*D*-xylopyranosyl-(1→4)-O-α-*L*-rhamnopyranosyl-(1→2)-{4-O-[(E)-3,4-dimethoxycinnamoyl]}-β-*D*-fucopyranosyl) Ester and Its (Z)-Isomer* (**3/4**). White amorphous powder. TLC (*a*): R_f 0.35. IR (KBr): 3500–3300, 2930, 1725, 1710. ^{1}H- and ^{13}C-NMR ((D_5)pyridine): for aglycons see [2], for sugars and acids see *Tables 1–4*. FAB-MS (neg.): 1617 ($[M-H]^-$), 1427 ($[M-H-190]^-$), 1265 ($[M-H-190-162]^-$). APIES-MS (neg.): 1617 ($[M-H]^-$), 1455 ($[M-H-162]^-$).

*(2β,3β)-3-(β-*D*-Glucopyranosyloxy)-2,27-dihydroxyolean-12-ene-23,28-dioic Acid 28-(O-β-*D*-Glucopyranosyl-(1→3)-O-α-*L*-arabinopyranosyl-(1→4)-O-α-*L*-rhamnopyranosyl-(1→2)-{4-O-[(E)-4-methoxycinnamoyl]}-β-*D*-fucopyranosyl) Ester and Its (Z)-Isomer* (**5/6**). White amorphous powder. TLC (*a*): R_f 0.66. IR (KBr): 3500–3300, 2930, 1725, 1710. ^{1}H- and ^{13}C-NMR ((D_5)pyridine): for aglycons see [2], for sugars and acids see *Tables 1–4*. FAB-MS (neg.): 1425 ($[M-H]^-$), 1263 ($[M-H-162]^-$), 1101 ($[M-H-162-162]^-$).

*(2β,3β)-3-(β-*D*-Glucopyranosyloxy)-2,27-dihydroxyolean-12-ene-23,28-dioic Acid 28-(O-β-*D*-Glucopyranosyl-(1→3)-O-α-*L*-arabinopyranosyl-(1→4)-O-α-*L*-rhamnopyranosyl-(1→2)-{4-O-[(E)-3,4-dimethoxycinnamoyl]}-β-*D*-fucopyranosyl) Ester and Its (Z)-isomer* (**7/8**). White amorphous powder. TLC (*a*): R_f 0.68. IR (KBr): 3500–3300, 2930, 1725, 1710. ^{1}H- and ^{13}C-NMR ((D_5)pyridine): for aglycons see [2], for sugars and acids see *Tables 1–4*. FAB-MS (neg.): 1455 ($[M-H]^-$), 1293 ($[M-H-162]^-$), 1265 ($[M-H-190]^-$), 1103 ($[M-H-190-162]^-$).

Acid Hydrolysis. A soln. of saponin (3 mg) in 2N aq. CF_3COOH (5 ml) was refluxed on a water-bath for 2 h. After extraction with $CHCl_3$, the aq. layer was repeatedly evaporated to dryness with MeOH until neutral and then analyzed by TLC by comparison with standard sugars (solvent system *c*).

Alkaline Hydrolysis. The saponin (3 mg) was refluxed with 5% aq. KOH soln. (10 ml) for 2 h. The mixture was adjusted to pH 7 with dil. HCl soln. and then extracted with H_2O-sat. BuOH (3 × 10 ml). The combined BuOH extracts were washed (H_2O) and concentrated to dryness: prosapogenin.

Mild Alkaline Hydrolysis. The saponin (3 mg) was hydrolyzed with 1% aq. KOH soln. (10 ml) at r.t. for 2 h. The mixture was adjusted to pH 7 with dil. HCl soln. and then extracted with H_2O-sat. BuOH (3 × 10 ml): deacylated saponin.

Bio-Assay. The *in vitro* lymphocyte proliferation assay on the *Jurkat* T-leukemia cells was realized according to the technique described in [9].

Hemolysis Assay: 100 μl of 2.5% sheep erythrocyte suspension (*Biomérieux*, France) was incubated with 150 μl of saponin dilutions in phosphate-buffered saline (PBS) at 37° for 30 min. After centrifugation (5 min at 1500 rpm), the hemoglobin content in the supernatant (duplicate of 75 μl) was measured at 450 nm with a multiwell spectrophotometer. Parallel measurements of saponin dilutions (without erythrocytes), erythrocytes (without saponin dilutions), and buffer (without both erythrocytes and saponin dilutions) were performed. The hemolytic concentration 50% (HC_{50}), *i.e.*, the concentration causing 50% of the maximal hemolysis observed with 1% *Triton X100*, was determined. The HC_{50} data (in μg/ml) of each tested compound were inferred from the hemoglobin absorbance *vs.* saponin concentration curve.

REFERENCES

[1] S. Desbène, B. Hanquet, Y. Shoyama, H. Wagner, M.-A. Lacaille-Dubois, *J. Nat. Prod.* **1999,** *62,* 923.
[2] A.-C. Mitaine-Offer, T. Miyamoto, I. A. Khan, C. Delaude, M.-A. Lacaille-Dubois, *J. Nat. Prod.* **2002,** *65,* 553.
[3] M. Elbandy, T. Miyamoto, B. Chauffert, C. Delaude, M.-A. Lacaille-Dubois, *J. Nat. Prod.* **2002**, *65*, 193.
[4] M. Elbandy, T. Miyamoto, C. Delaude, M.-A. Lacaille-Dubois, *Helv. Chim. Acta* **2002**, *85*, 2721.
[5] C. Delaude, *Bull. Soc. Roy. Sc. Liège* **1992**, *61*, 245.
[6] C. Delaude, *Bull. Soc. Roy. Sc. Liège* **1975**, *44*, 497.
[7] C. Delaude, *Bull. Soc. Roy. Sc. Liège* **1971**, *40*, 397.

[8] M.-A. Lacaille-Dubois, B. Hanquet, Z.-H. Cui, Z.-C. Lou, H. Wagner, *Phytochemistry* **1997**, *45*, 985.
[9] G. Gaidi, T. Miyamoto, V. Laurens, M.-A. Lacaille-Dubois, *J. Nat. Prod.* **2002**, *65*, 1568.
[10] M. Yoshikawa, T. Murakami, T. Ueno, M. Kadoya, H. Matsuda, J. Yamahara, N. Murakami, *Chem. Pharm. Bull*. **1995**, *43*, 350.
[11] D. Zhang, T. Miyase, M. Kuroyanagi, K. Umehara, H. Nogushi, *Chem. Pharm. Bull.* **1996**, *44*, 2092.
[12] D. Zhang, T. Miyase, M. Kuroyanagi, K. Umehara, A. Ueno, *Chem. Pharm. Bull.* **1996**, *44*, 810.
[13] T. Miyase, H. Saitoh, K. Shiokawa, A. Ueno, *Chem. Pharm. Bull.* **1995**, *43*, 466.
[14] D. Zhang, T. Miyase, M. Kuroyanagi, K. Umehara, H. Nogushi, *Phytochemistry* **1998**, *47*, 459.
[15] S. Sakuma, J. Shoji, *Chem. Pharm. Bull.* **1981**, *29*, 2431.
[16] S. Sakuma, J. Shoji, *Chem. Pharm. Bull.* **1981**, *30*, 810.
[17] M. Kuroda, Y. Mimaki, Y. Sashida, M. Kitahara, M. Yamazaki, S. Yui, *Bioorg. Med. Chem. Lett.* **2001**, *11*, 371.
[18] M.-A. Lacaille-Dubois, in 'Immunomodulatory Agents from Plants', Ed. H. Wagner, 'Progress in Inflammation Research', series Ed. M. J. Parnham, Birkhäuser Verlag, Basel, 1999, p. 243 – 272.

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