Biologically Active Triterpene Saponins from Callus Tissue of *Polygala amarella*

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A new bioactive saponin (**1**), together with a known saponin (polygalasaponin XXVIII) has been isolated from the callus tissue culture of *Polygala amarella.* Based on spectroscopic data, especially direct and long-range heteronuclear 2D NMR analysis and on chemical transformations, the structure of **1** was elucidated as 3-*O*-β-D-glucopyranosyl presenegenin-28-*O*-β-D-galactopyranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 2)-[β-D-glucopyranosyl-(1 → 3)]-β-D-fucopyranoside. Both saponins showed significant immunological properties based on the enhancement of granulocyte phagocytosis in vitro.

Polygala amarella Crantz (Polygalaceae) is one of the 33 European species of the genus Polygala.[1] There are few reports on the chemical constituents of this species. We have previously reported the isolation of a novel tri-*O*-acetyl tri-*O*-benzoyl tetrasaccharide (amarelloside), two flavonoids, and one hydroxycinnamoyl ester from the aerial parts of this plant.[1] It was observed that this plant produced not only these metabolites but also saponins in small amounts. Thus, we have examined tissue culture of *P. amarella* to see whether they formed any triterpene glycosides. Tissue culture studies on saponins have aroused interest as a result of their production of bioactive compounds or from studies of biosynthesis, but there are few reports of this type. Some dammarane saponins have been obtained from the callus culture of *Panax ginseng*;[2,3] and oleanane saponins, from *Gypsophila* species[4] and from alfalfa.[5] This paper describes the isolation and structure elucidation of a new saponin (**1**), together with the known polygalasaponin XXVIII, from a methanolic extract of callus tissue culture of *Polygala amarella* seeds. As part of our ongoing investigation for biologically active saponins,[6−9] we also report on the immunological properties of these compounds.

Callus was induced from the seeds of *P. amarella* on Murashige−Skoog (MS) medium supplemented with indole acetic acid (IAA). After callus propagation, the cultures were extracted with MeOH. The MeOH extract was suspended in water and then partitioned successively with chloroform and *n*-BuOH. The resulting *n*-BuOH extract was purified by precipitation with diethyl ether and subjected to repeated column chromatography on Sephadex LH-20 and Si gel to yield five saponins, two of them in major amounts (**1** and polygalasaponin XXVIII).

The known polygalasaponin XXVIII (3-*O*-β-D-glucopyranosyl presenegenin 28-*O*-β-D-xylopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1 → 2)-β-D-fucopyranoside) was identified by comparing its physical and spectral data with literature values.[10] This compound was also present in the native plant, but only in small amounts.

Saponin **1** was obtained as an amorphous powder. The IR spectrum showed absorptions at 3400 (OH), 1750 (C=O of ester group), and 1707 cm $^{-1}$ (C=O of carboxylic acid group). The negative-ion FABMS of compound **1** showed a quasimolecular ion peak $[M - H]^-$ at *m/z* 1427, compatible with the molecular formula $C_{65}H_{104}O_{34}$. Other significant peaks visible at *m/z* 1265 $[(M - H) - 162]^-$, 1103 $[(M - H) - 162 - 162]^-$, 971 $[(M - H) - 2 \times 162 - 132]^-$, and 679 $[M - H - 162 - 162 - 132 - 2 \times 146]^-$ indicated the successive loss of one hexosyl, one hexosyl, one pentosyl, and two desoxyhexosyl moieties. Another fragment ion at *m/z* 517 corresponded to the pseudomolecular ion of the aglycon. Acid hydrolysis of **1** afforded an artifactual aglycon and rhamnose, fucose, xylose, galactose, and glucose (1:1:1:1:2) based on GLC analysis. The native aglycon was identified as presenegenin (2β,3β,27-trihydroxyolean-12-en-23,28 dioic acid) from the extensive 2D NMR study of **1**. Most of the signals were assigned through $^2J_{H-C}$ and $^3J_{H-C}$ couplings of the six methyls and were in good agreement with literature data.[11] Alkaline hydrolysis of **1** gave the prosapogenin **1a**. The FABMS (negative-ion mode; *m/z* 679 $[M - H]^-$), ^1H and ^{13}C NMR data of **1a** (Tables 1 and 2) were in good agreement with those of tenuifolin (3-*O*-β-D-glucopyranosyl presenegenin), a prosapogenin obtained from other saponins from *Polygala senega*,[12] *P. tenuifolia,*[12] and *Bredemeyera floribunda*.[13]

The above data indicated that **1** was a bisdesmosidic glycoside with glucose linked to the C-3 position of the aglycon, and the other five monosaccharides were linked to the C-28 of the aglycon through an ester bond. Assignments of the ^1H and ^{13}C NMR signals obtained from $^1H-^1H$ COSY, HMQC, and HMBC spectra allowed the sequence of the oligosaccharide chain. The HMQC experiments revealed six correlations between the anomeric carbon signals in the δ 107−95 range and the anomeric protons signals resonating between δ 4.5 and 6.7. Thus, the anomeric ^{13}C NMR signals at δ 106.7, 106.0, 105.4, 103.1, 101.0, and 95.0 gave correlations with anomeric protons at δ 4.80 (d, $J = 8.2$ Hz), 4.95 (d, $J = 8.2$ Hz), 4.98 (d, $J =$

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Table 1. ^{13}C NMR Data of the Aglycons of Saponin **1** and of Compound **1a** (δ ppm, C_5D_5N as solvent)[a]

position	DEPT	**1**	**1a**
1	CH_2	44.5	44.5
2	CH_2	69.8	69.8
3	CH	87.7	86.5
4	C	53.2	53.2
5	CH	52.5	52.5
6	CH_2	21.9	21.9
7	CH_2	34.0	34.0
8	C	41.2	40.9
9	CH	49.4	49.3
10	C	36.9	36.9
11	CH_2	23.4	23.4
12	CH	128.3	128.0
13	C	139.0	139.7
14	C	48.4	46.9
15	CH_2	24.9	24.9
16	CH	24.7	24.7
17	C	47.1	47.1
18	CH	42.1	42.1
19	CH_2	45.4	45.4
20	C	30.8	30.8
21	CH_2	34.0	34.0
22	CH_2	32.4	32.0
23	C	180.8	180.9
24	Me	14.8	14.8
25	Me	17.7	17.7
26	Me	19.0	19.0
27	CH_2	64.1	64.1
28	C	176.6	180.0
29	Me	33.2	33.2
30	Me	23.8	23.8

[a] ^{13}C chemical shifts are referenced to C_5D_5N at δ 123.9, 135.9, 150.3. Multiplicities were assigned from DEPT spectra.

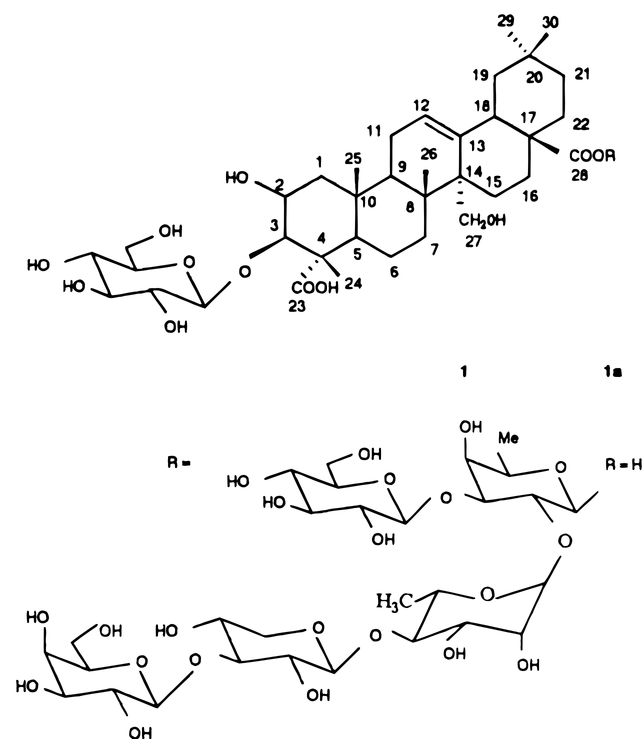

Table 2. ^{13}C and ^{1}H NMR Spectral Data of Sugar Moieties for Compounds **1** and **1a** (in C_5D_5N, ppm)[a]

		1		**1a**			**1**	
position		δ_C	δ_H	δ_C	position		δ_C	δ_H
3-*O*-Glc	1	105.4	4.98 (d, 7.9)	105.4	28-*O*-Fuc	1	95.0	5.95 (d, 8.0)
	2	75.4	3.90	75.4		2	72.5	4.76
	3	77.9	4.07	77.9		3	86.4	4.18
	4	71.5	4.15	71.5		4	73.5	3.95 (d, 3.0)
	5	78.2	3.84 (m)	78.6		5	72.5	3.93 (m)
	6	62.7	4.28	62.6		6	16.8	1.42 (d, 6.3)
			4.45					
					Rha	1	101.0	6.7 (br *s*)
						2	71.8	4.75
						3	72.8	4.59
						4	86.6	4.19
						5	67.5	4.44 (m)
						6	18.5	1.62 (d, 6.0)
					Glc	1	106.0	4.95 (d, 8.2)
						2	74.4	4.05
						3	78.2	3.85
						4	71.5	4.08
						5	77.9	3.70 (m)
						6	62.6	4.30
								4.46
					Xyl	1	106.7	4.80 (d, 8.2)
						2	75.8	3.96
						3	87.6	3.82
						4	69.8	4.15 (m)
						5	66.0	3.42
								4.44
					Gal	1	103.1	4.85 (d, 7.6)
						2	69.9	4.45
						3	75.7	4.13
						4	69.8	4.37
						5	77.5	4.27 (m)
						6	62.1	4.18
								4.25

[a] Measured at 500 MHz for ^{1}H and 125 MHz for ^{13}C with reference to δ 150.5 in C_5D_5N. Assignments were made on the basis of ^{1}H−^{1}H DQF COSY, HMQC, HMBC, and DEPT experiments: β-D-galactopyranose (Gal), β-D-xylopyranose (Xyl), β-D-glucopyranose (Glc), β-D-glucuronopyranose (Glc-A), α-L-arabinopyranose (Ara), α-L-rhamnopyranose (Rha), β-D-fucopyranose (Fuc).

7.9 Hz), 4.85 (d, J = 7.6 Hz), 6.70 (br s), and 5.95 (d, J = 8.0 Hz), respectively.

Evaluation of spin−spin couplings and chemical shifts allowed identification of one β-xylopyranose (Xyl), two β-glucopyranoses (Glc), one β-galactopyranose (Gal), one α-rhamnopyranose (Rha), and one β-fucopyranose (Fuc) unit, respectively. The common D configuration for Glc, Gal, Xyl, and Fuc and the L configuration for Rha were assumed, according to that most often encountered among the plant glycosides in each case. After subtraction of the anomeric signals of the glucosyl moiety linked at the C-3 position from the total NMR spectrum of **1**, the signals of five sugars linked to the aglycon by an ester linkage remained and will be assigned. The extensive 2D NMR spectra analysis of **1** showed that the Fuc was substituted at position C-2 and C-3, the Rha was substituted at position C-4, and the three remaining sugars were a terminal Gal (T-Gal), a terminal Glc (T-Glc), and a disubstituted 1,3-Xyl. A correlation in the HMQC spectrum at δ_C/δ_H 95.0/5.95 (d, J = 8.0 Hz) showed that the fucose residue was attached to the carboxylic group of the aglycon by an ester linkage. This conclusion was confirmed by the HMBC experiment, which showed a correlation between signals at δ_H (Fuc-1) 5.95 and δ_C (agly-C-28) 176.6.

Other HMBC correlations were observed between the following carbon and proton signals in the oligosaccharide ester moiety of **1**: δ_H (Rha-1) 6.7 (s) and δ_C (Fuc-2) 72.5, δ_H (Fuc-2) 4.76 and δ_C (Rha-1) 101.0, δ_H (Fuc-3) 4.18 and δ_C (Glc-1)106.0, δ_H (Rha-4) 4.19 and δ_C (Xyl-1) 106.7, δ_H (Xyl-3) 3.82 and δ_C (T-Gal-1) 103.15. These data proved that the 1,4-Rha and T-Glc were linked to the 1,2,3-Fuc by a 1 → 2 and a 1 → 3 linkage, respectively, and the T-Gal was bound to the 1,3-Xyl by a 1 → 3 linkage and the 1,3-Xyl to the 1,4-Rha by a 1 → 4 linkage. At this stage, we observed that the compound **1** was different from polygalasaponin XXVIII by the presence of two additional sugars (T-Gal at the C-3 of Xyl and T-Glc at the C-3 of Fuc). The comparison

of the ^{13}C NMR chemical shifts of these two molecules fully supported this observation. With regard to the oligosaccharide ester carbon region, ongoing from polygalasaponin XXVIII to **1**, the signals for C-3, C-2, and C-4 of Fuc were displayed downfield by +9.7 and upfield by −1.5 and − 0.3 ppm, respectively, as a consequence of the glycosylation shift at C-3. Similarly, the signals for C-3, C-2, and C-4 of Xyl were displayed downfield by +8.7 and upfield by −0.4 and −1.1 ppm, respectively, as a consequence of the glycosylation shift at C-3.

Based on the above results the structure of the saponin **1** was represented as 3-*O*-β-D-glucopyranosyl presenegenin-28-*O*-β-D-galactopyranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 2)-[β-D-glucopyranosyl-(1 → 3)]-β-D-fucopyranoside. This compound was similar to polygalasaponin XXX from *P. japonica*,[10] and polygalasaponin XLIV from *P. glomerata*,[11] except that it lacks the acyl moiety at C-4 of Fuc (methoxycinnamoyl and dimethoxycinnamoyl, respectively) and that the Gal was linked at C-3 of Xyl instead of at C-4. According to an updated literature search and previous reviews on triterpene-saponins[14,15] and on presenegenin glycosides from Polygalaceae species,[10,11,13,16−18] **1** is a new natural compound. It was not present in the saponin mixture of the natural plant. This finding showed that the callus tissue culture may possess a wider range of metabolic ability than the natural plant. This is the first report of presenegenin glycosides from the callus tissue culture in the Polygalaceae family.

Because triterpene saponins have been reported to exert immunostimulating activities,[19] the two saponins from *P. amarella* callus tissue were tested in the in vitro granulocyte phagocytosis assay according to a new flow cytometric technique.[20−23] In this assay, **1** and polygalasaponin XXVIII showed no cytotoxicity for the granulocytes up to 100 μg/mL. At the concentration range 10−100 μg/mL, **1** and polygalasaponin XXVIII, which should not contain lipopolysaccharide (LPS) according to the purification procedure, showed a significant enhancement of the granulocyte phagocytosis (70−60% and 49−77%, respectively). Results obtained from this in vitro bioassay probably indicate the immunostimulatory potency of both tested compounds. Such an assumption can be made according to the previous use of this bioassay for demonstrating immunostimulatory activity of saponins.[8] However, this activity needs to be confirmed by an in vivo assay as has been done for other saponins.[19] At this time, the present results must be considered as preliminary data in view of an immunostimulatory potency assessment.

Experimental Section

General Experimental Procedures. Optical rotations were taken with a Perkin−Elmer 241 polarimeter. IR spectra were measured with a Perkin−Elmer 881 spectrophotometer. UV spectra were recorded on a Kontron Uvicon 939 spectrophotometer. ^1H and ^{13}C NMR spectra were obtained on a Bruker DRX 500 spectrometer with standard pulse sequences, operating at 500 and 125 MHz, respectively. The chemical shifts (δ) were referenced to the solvent peaks (C_5D_5N). FABMS was conducted in the negative-ion mode (thioglycerol matrix) on a JEOL DX 300 instrument with a JMA-3500 system. TLC and HPTLC employed precoated Si gel plates $60F_{254}$ (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). 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, Büchi column (460 × 25 mm), Si gel 60 (15−40 μm, Merck)]. GLC analysis: Perkin−Elmer 900 B, glass column (200 × 0.3 cm) packed with 0V 225, carrier gas: Ar, 30 mL/min.

Plant Material. The seeds of *Polygala amarella* were collected in June 1989, in the Munich Botanical Garden and identified by Dr. G. Heubl. A voucher specimen (no. 5003) is deposited in the Herbarium of the Departmemt of Pharmacognosy, Faculty of Pharmacy, Dijon, France.

Preparation of the Callus Tissue. Seeds were washed with H_2O and sterilized with 1% of NaOCl solution for 10 min, and then with 70% of EtOH for 1 min. Seeds were bedded in agar medium. Seedlings were transferred to the MS medium supplemented with 1 mg/L of IAA and cultured at 25 °C under 16 h light for one month to give callus tissue. Callus tissues were subcultured on the same medium every month for the propagation of callus. Accumulated callus (1.19 kg) was homogenized with MeOH (1.5 L) and extracted three times. After evaporation of the solvent, 20.92 g of the MeOH extract was obtained.

Extraction and Isolation. The MeOH extract (20 g) was suspended in H_2O (1 L) and submitted to successive extractions by $CHCl_3$ and BuOH. After evaporation under reduced pressure of the solvent, 7 g of the BuOH extract were obtained. The BuOH extract was solubilized in MeOH (10 mL) and precipitated in Et_2O (3 × 250 mL) yielding 4 g of a saponin fraction containing phenolic compounds. This mixture was submitted twice to column chromatography on Sephadex LH-20 eluted by MeOH to afford 2 g of a crude saponin fraction. This mixture was separated by MPLC (normal-phase Si gel with $CHCl_3$−MeOH−H_2O, 8:5:1, as eluent) to yield six fractions. Fractions 3 (150 mg) and 6 (400 mg) were further purified by MPLC with the same solvent system, yielding polygalasaponin XXVIII (15 mg) and **1** (70 mg), respectively.

Acid Hydrolysis of 1. A solution of **1** (3 mg) in 2N CF_3-COOH (5 mL) was refluxed on a H_2O bath for 3 h. After extraction with $CHCl_3$, the aqueous layer was neutralized by repeated evaporation to dryness with MeOH and then analyzed on Si gel TLC by comparison with standard sugars (solvent system c). A 2-mg quantity of saponin **1** was refluxed in 2N CF_3COOH (2 mL) in a sealed serum vial at 100 °C for 3 h. Sugars in the hydrolysate were converted into the alditol acetates and then subjected to GLC analysis according to the method previously described.[24]

Alkaline Hydrolysis of 1. Compound **1** (15 mg) was refluxed with 5% aqueous KOH (10 mL) for 2.5 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 BuOH extracts were washed (H_2O). Evaporation of the BuOH gave the prosapogenin **1a** (4 mg).

Compound 1: $[\alpha]^{20}_D$ −3.0° (*c* 0.100, H_2O); UV (MeOH−H_2O) λ_{max} 210 nm; IR (KBr) ν_{max} 3500−3400 (OH), 2930 (CH), 1750 (CO ester), 1707 (CO carboxylic acid), 1634, 1564, 1384, 1070 cm^{-1}; ^1H NMR (C_5D_5N, 500 MHz) δ 0.78, 0.80, 1.10, 1.48, 1.88 (each 3H, *s*, Me at C-29, C-30, C-26, C-25, C-24); 5.85 (1H, *m*, H-12 of the aglycon), 3.27, 4.26 (2H, m, CH_2OH-27 of the aglycon); ^{13}C NMR (C_5D_5N, 125 MHz), see Tables 1 and 2; long-range correlations in the HMBC spectrum used for defining the aglycon of **1** δ 0.78 (Me-30) → C-20 (30.8), C-29 (33.2), C-21 (34.0), C-19 (45.4); δ 0.80 (Me-29) → C-30 (23.8), C-20 (30.8), C-21 (34.0); δ 1.10 (Me-26) → C-7 (34.0), C-8 (41.2), C-14 (48.4), C-9 (49.3); δ 1.48 (Me-25) → C-10 (46.5), C-20 (36.9), C-1 (44.5), C-9 (49.4), C-5 (52.5); δ 1.88 (Me-24) → C-5 (52.5), C-4 (53.6); negative FABMS (thioglycerol matrix) *m/z* 1427 $[M - H]^-$, 1265 $[(M - H) - 162]^-$, 1103 $[(M - H) - 2 \times 162]^-$, 971 $[(M - H) - 2 \times 162 - 132]^-$, 679 $[M - H - 2 \times 162 - 132 - 2 \times 146]^-$, 517 $[M - H - 2 \times 162 - 132 - 2 \times 146 - 162]$; TLC R_f 0.30 (system a), pink spot by spraying with Komarowsky reagent.

Compound 1a. The spectral data (see Tables 1 and 2) were almost completely superimposable with those described for the tenuifolin in previous reports.[12,13]

Polygalasaponin XXVIII. The spectral data (MS, ^{1}H NMR, and ^{13}C NMR) were in full agreement with those previously published data.[10]

Bioassay. A granulocyte phagocytosis assay was performed according to recent studies.[20–23]

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