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Triterpene Saponins from the Aerial Parts of *Trifolium medium* L. var. *sarosiense*

Sylwia Pawelec,[†] Dariusz Jędrejek,[*,†] Mariusz Kowalczyk,[†] Łukasz Pecio,[†] Milena Masullo,[‡] Sonia Piacente,[‡] Francisco A. Macías,[§] Ana M. Simonet,[§] Wiesław Oleszek,[†] and Anna Stochmal[†]

[†]Department of Biochemistry and Crop Quality, Institute of Soil Science and Plant Cultivation, State Research Institute, ul. Czartoryskich 8, 24-100 Puławy, Poland

[‡]Department of Pharmaceutical Sciences, University of Salerno, via Ponte Don Melillo, 84084 Fisciano, Salerno, Italy

[§]Department of Organic Chemistry, Faculty of Science, University of Cádiz, C/Republic Saharaui s/n, 11510 Puerto Real, Cádiz, Spain

Supporting Information

ABSTRACT: Seven previously unreported triterpene glycosides (**1**−**7**) were isolated from methanol extract of the aerial parts of *Trifolium medium* var. *sarosiense* (zigzag clover). Their structures were established by the extensive use of 1D and 2D NMR experiments along with ESI-MS and HRMS analyses. Compounds **1**−**7** are oleanane derivatives characterized by the presence of a keto group at C-22 of an aglycone and a primary alcoholic function at C-24 and differing functions at C-30. Among these, compounds **1**−**3** and **6** showed a secondary alcoholic function at C-11, which is methoxylated in compounds **4** and **7**. Compound **5** was shown to possess a known aglycone, wistariasapogenol A; however, it is described here for the first time as a saponin constituent of the *Trifolium* genus. Some aspects of taxonomic classification of zigzag clover are also discussed.

KEYWORDS: *Trifolium medium var. sarosiense, zigzag clover, triterpene saponins*

INTRODUCTION

The Fabaceae (Leguminosae) is the third largest family of flowering plants of great importance for the food industry and agriculture. It consists of 727 genera and ca. 20000 species; the *Trifolium* L. genus, one of the largest genera in the family, is represented by ca. 300 species.[1,2] All *Trifolium* species are herbaceous perennials or annuals and are widely distributed in temperate and subtropical regions of both hemispheres.[3]

Clovers have interest mainly as fodder and pasture crops with the target for cattle feeding.[4,5] Several species of *Trifolium* are actively cultivated, such as *Trifolium pratense*, *Trifolium repens*, *Trifolium hybridum*, *Trifolium subterraneum*, and *Trifolium fragiferum*.[1] A very close relative of the former is *Trofolium medium*, which is a rhizomatous long-lived perennial found in Eurasia from the British Isles across Europe to Turkey and Iran.[2] The English name of *T. medium*, zigzag clover, comes from a characteristic zigzag stem branching pattern. Zigzag clover is very variable and has been divided into four varieties on the basis of flower structure and stem hairiness. Among these varieties are *T. medium* Grufb. var. *medium* and *T. medium* Grufb. var. *sarosiense* (Hazsl). The former can be found across Europe and Turkey, whereas the latter is more limited in distribution and occurs only in central Europe. However, according to some researchers, intraspecies variation in this case is far from resolved.[2,3,6,7] Some researchers[7,8] and plant databases[9,10] follow the classification of Zohary and Heller;[2] nevertheless, in other publications[6,11] and databases[12,13] *T. medium* var. *sarosiense* appears as a *T. sarosiense* that is classified as a taxon separate from *T. medium*. These two taxa nevertheless appear to be closely related and can be easily hybridized,[8] although some crossing barrier in the form of hybrid chlorosis exists between them, suggesting small genetic and cytoplasmic changes.[6,7] So far, the question of species rank for *T. sarosiense* is not resolved conclusively.

In recent years secondary metabolites of some clover species, especially phenolics, have been well recognized and reported.[14−16] Triterpene saponins were also researched in some *Trifolium* species, such as *T. pratense*, *T. repens*, *T. alexandrinum*, and *T. resupinatum*.[17−21] Earlier analyses of seeds of 57 *Trifolium* species[15] have revealed that two subspecies of *T. medium* produce soyasapogenol aglycones, but differ in saponin composition. This is the only report about saponin content in *T. medium* taxa. Simultaneously, phenolic profiles of the aerial parts of *T. medium* var. *medium* and *T. medium* var. *sarosiense* have proved to be significantly different.[16] On this basis, the phytochemical investigation of *T. medium* var. *sarosiense* aerial parts for saponin content and comparison between two varieties of *T. medium* taxa were carried out.

MATERIALS AND METHODS

Plant Material. Seeds of authenticated *T. medium* L. var. *sarosiense* were obtained from Genebank, Zentralinstitut für Pflanzengenetic und Kulturpflanzenforschung, Getersleben, Germany; referring to this source, we followed the classification of zigzag clover taxa. Plants were cultivated on the experimental field of the Institute of Soil Science and Plant Cultivation in Puławy. The aerial parts were harvested at the beginning of flowering in July 2010, lyophilized, and finely ground. A voucher specimen (KOWAL 080721) is deposited at our laboratory.

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Table 1. NMR Spectroscopic Data of the Aglycone Moieties of Compounds 1, 2, and 4−7 (500 or 600 MHz, δ, in CD_3OD)

	1[a]		2[a,b]		4[a]		5[a]		6[a]		7[a]	
	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
1	41.3	2.22, 1.27, m	41.6	2.21, 1.30, m	40.4	1.92, 1.30, m	39.6	1.69, 1.06, m	41.2	2.23, 1.28, m	40.3	1.95, 1.31, m
2	27.0	2.18, 1.86, m	27.1	2.19, 1.86, m	26.9	2.18, 1.87, m	26.8	2.14, 1.86, m	26.8	2.08, 1.84, m	26.9	2.09, 1.84, m
3	91.9	3.44, dd (4.3, 12.3)	91.5	3.42, dd (4.3, 12.3)	92.0	3.45, m	92.2	3.43, dd (4.6, 11.7)	90.0	3.49, dd (4.3, 11.6)	90.0	3.49, dd (4.6, 11.6)
4	44.5		44.7		44.6		44.4		44.9		44.9	
5	57.4	1.01, m	57.4	1.00, m	57.1	1.03, m	57.1	1.00, m	57.1	1.02, m	57.2	1.04, m
6	19.4	1.68, 1.40, m	19.2	1.69, 1.42, m	19.4	1.70, 1.42, m	19.4	1.69, 1.43, m	19.3	1.71, 1.43, m	19.4	1.73, 1.45, m
7	34.7	1.56, 1.36, m	34.7	1.57, 1.37, m	34.4	1.56, 1.36, m	34.0	1.65, 1.61, m	34.6	1.57, 1.37, m	34.4	1.57, 1.37, m
8	43.4		43.6		43.8		40.8		43.5		43.4	
9	55.7	1.67, d (8.9)	55.8	1.69, d (8.9)	53.2	1.79, d (8.8)	48.5	1.66, m	55.6	1.70, d (8.9)	52.8	1.80, d (8.8)
10	38.4		38.5		38.5		37.5		38.4		38.7	
11	68.2	4.15, dd (3.2, 8.9)	68.2	4.17, m	77.5	3.87, dd (3.4, 8.8)	24.8	1.96 (2H), m	68.1	4.18, dd (3.0, 8.9)	77.4	3.89, dd (3.3, 8.8)
12	128.9	5.35, d (3.2)	129.3	5.36, d (3.2)	124.5	5.59, d (3.4)	125.2	5.42, dd (3.7, 4.5)	129.0	5.30, d (3.0)	124.1	5.52, d (3.3)
13	145.1		144.8		147.3		142.7		144.7		147.4	
14	43.0		42.8		42.8		43.0		42.8		42.8	
15	26.3	1.77, 1.11, m	26.0	1.75, 1.15, m	25.9	1.75, 1.15, m	26.2	1.81, 1.14, m	25.9	1.75, 1.16, m	25.9	1.76, 1.16, m
16	29.4	2.08, 1.15, m	28.0	2.14, 1.24, m	28.0	2.11, 1.24, m	28.1	2.10, 1.22, m	28.1	2.20, 1.16, m	28.1	2.22, 1.16, m
17	48.4		48.1		48.0		48.7		48.4		48.5	
18	48.5	2.44, dd (3.3, 13.6)	47.6	2.52, dd (3.4, 13.6)	47.8	2.55, dd (3.5, 13.7)	48.3	2.50, dd (4.1, 14.0)	48.3	2.38, dd (3.4, 13.4)	48.4	2.44, dd (3.5, 13.5)
19	45.0	2.08, 1.96, m	42.6	2.04, 1.73, m	42.9	2.04, 1.68, m	43.2	2.03, 1.64, m	46.8	2.23, 1.46, m	47.2	2.25, 1.42, m
20	47.7		39.3		39.3		39.4		35.2		35.0	
21	48.8	2.26, d (14.5)	46.6	2.36, d (14.5)	46.4	2.36, d (14.5)	47.4	2.41, d (14.5)	51.2	2.63, d (14.2)	51.6	2.64, d (14.2)
		2.21, d (14.5)		2.21, d (14.5)		2.20, d (14.5)		2.22, d (14.5)		2.01, d (14.2)		2.00, d (14.2)
22	219.2		219.8		219.8		219.8		219.4		219.3	
23	23.1	1.25, s	22.8	1.26, s	22.8	1.24, s	22.8	1.24, s	23.1	1.25, s	22.8	1.25, s
24	64.1	4.18, d (11.4)	64.1	4.17, d (11.3)	64.1	4.17, d (11.1)	64.2	4.15, d (11.5)	63.7	4.09, d (11.4)	63.8	4.09, d (11.4)
		3.26, d (11.4)		3.28, d (11.3)		3.27, d (11.1)		3.25, d (11.5)		3.37, d (11.4)		3.37, d (11.4)
25	17.4	1.02, s	17.1	1.03, s	17.4	1.02, s	16.1	0.93, s	16.8	1.05, s	17.1	1.04, s
26	18.1	1.03, s	18.1	1.04, s	18.5	1.03, s	17.3	1.00, s	18.4	1.06, s	18.4	1.05, s
27	25.0	1.35, s	25.1	1.36, s	24.6	1.34, s	25.7	1.28, s	25.1	1.39, s	24.7	1.37, s
28	21.3	1.03, s	21.2	1.00, s	21.1	1.00, s	21.4	1.00, s	20.9	1.01, s	20.9	1.02, s
29	27.1	1.21, s	26.6	0.99, s	26.3	0.99, s	26.9	0.97, s	31.9	1.06, s	31.9	1.06, s
30	183.8		68.7	3.32 (2H), m	68.7	3.34 (2H), m	68.8	3.29, d (11.0)	25.3	0.89, s	25.3	0.90, s
								3.32, d (11.0)				
OCH_3					54.2	3.28, s					54.1	3.28, s

[a]The assignments were based on $^1H-^1H$ COSY, 2D-TOCSY, HSQC, and HMBC experiments. [b]The chemical shift values of the aglycone moiety of **3** were superimposable on those reported for **2**.

Table 2. NMR Spectroscopic Data of the Sugar Portions of Compounds 1, 2, and 6 (500 MHz, δ, in CD_3OD)

	$1^{a,b}$		2^{a}		$6^{a,c}$	
	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
β-D-GlcA (at C-3)			β-D-GlcA (at C-3)		β-D-GlcA (at C-3)	
1′	104.6	4.50, d (7.9)	104.6	4.49, d (7.5)	105.6	4.46, d (7.5)
2′	81.3	3.55, dd (7.9, 9.2)	81.4	3.35, dd (7.5, 9.2)	75.3	3.23, dd (7.5, 9.0)
3′	78.5	3.66, dd (8.9, 9.2)	78.1	3.65, dd (9.0, 9.0)	77.9	3.41, dd (9.0, 9.0)
4′	73.2	3.50, dd (8.9, 9.7)	73.1	3.50, dd (9.0, 9.0)	73.7	3.47, dd (9.0, 9.0)
5′	76.7	3.60, d (9.7)	76.5	3.59, d (9.0)	76.5	3.59, d (9.0)
6′	176.5		176.7		176.7	
β-D-Glc (at C-2_{GlcA})			α-L-Ara (at C-2_{GlcA})			
1″	104.2	4.80, d (7.5)	104.3	4.64, d (7.7)		
2″	75.5	3.24, dd (7.5, 9.0)	73.1	3.57, dd (7.7, 8.5)		
3″	77.8	3.37, t (9.0)	74.3	3.47, dd (3.0, 8.5)		
4″	70.2	3.45, t (9.0)	70.2	3.74, m		
5″	77.9	3.23, m	67.4	3.89, dd (2.0, 11.9)		
				3.58, dd (3.0, 11.9)		
6″	61.7	3.81, dd (3.5, 12.3)				
		3.74, dd (4.5, 12.3)				

[a]The assignments were based on $^1H-^1H$ COSY, 1D-TOCSY, HSQC, and HMBC experiments. [b]The chemical shift values for the sugar portion of **3**−**5** were superimposable on those reported for **1**. [c]The chemical shift values for the sugar portion of **7** were superimposable on those reported for **6**.

Extraction and Isolation. The powdered plant material (182 g) was defatted with chloroform (3 × 2 L) in an ultrasonic bath for 30 min to remove chlorophyll and fatty acids. After drying, it was extracted under reflux for 2 h with 70% methanol (2 × 3 L). The extract was concentrated under reduced pressure; subsequently it was suspended in water and loaded onto a LiChroprep RP18 (40−63 μm, 60 mm × 100 mm; Merck, Darmstadt, Germany) short column equilibrated with 5% methanol. The column was washed first with water (1.5 L) to remove carbohydrates and subsequently with 40% MeOH (2 L) to elute phenolic compounds. The saponin fraction was eluted with 80% MeOH (2 L). Evaporation of the solvent, followed by lyophilization, yielded the crude saponin fraction (2.7 g).

Subsequently, this fraction was dissolved in 30% MeOH and applied on a preconditioned column filled with LiChroprep RP18 silica gel (40−63 μm, 40 mm × 500 mm; Merck). The gradient solvent system from 40 to 100% MeOH with 5% steps was used for saponin fractionation and resulted in five fractions.

Fraction 1 (16.9 mg) was separated into individual compounds on a LiChroprep Si 60 silica gel column (25−40 μm, 20 mm ×500 mm; Merck) and eluted with EtOAc/HOAc/H_2O (9:2:2 v/v/v). Fractions 2 (45 mg), 3 (90 mg), 4 (60 mg), and 5 (44 mg) were separated individually on LiChroprep RP18 (25−40 μm, 20 mm × 500 mm; Merck) columns using a gradient solvent system from 30 to 100% MeOH with 5% steps. The aforementioned analytical methods resulted in seven pure compounds. Fraction 1 yielded compound **1** (2.95 mg); fraction 2, compounds **2** (6.57 mg) and **3** (3.99 mg); fraction 3, compound **4** (3 mg); fraction 4, compound **5** (3 mg); and fraction 5, compounds **6** (5.61 mg) and **7** (11.54 mg).

Chromatographic and Mass Spectrometric Analysis. Exact masses were measured by an AB SCIEX Voyager DE mass spectrometer equipped with a 337 nm laser and delay extraction and operated in positive ion reflector mode. Samples were analyzed by MALDI/TOF mass spectrometry. A mixture of analyte solution and α-cyano-4-hydroxycinnamic acid (Sigma) was applied to the metallic sample plate and dried. Mass calibration was performed with the ions from adrenocorticotropic hormone (ACTH) fragment 18−39 human at 2465.1989 Da and α-cyano-4-hydroxycinnamic acid at 190.0504 Da as an internal standard.

The fragmentation patterns of saponins were obtained using a Surveyor HPLC system coupled with an LCQ Advantage Max (Thermo Fisher Scientific, Waltham, MA, USA) ion trap mass spectrometer. A reverse phase Waters Xbridge BEH C18 column (2.5 μm, 250 mm × 3 mm) was used. Compounds of interest were separated using a linear 45 min gradient from 20 to 50% MeCN in 0.1% formic acid with 0.3 mL/min flow, and the column temperature was held at 35 °C. The mass spectrometer was operated in the negative ion electrospray ionization (ESI^-) mode with the following ion source parameters: sheath gas flow and auxiliary gas flow set to, respectively, 65 and 10 arbitrary units; spray needle voltage, 3.9 kV; capillary temperature, 230 °C; capillary voltage, −47 V; tube lens offset, −60 V. Full scan spectra were acquired in the m/z range from 150 to 2000. MS/MS analyses were performed with the normalized collision energy of 35% and scanning range from the "cut-off" mass (approximately one-third of the parent mass) up to 10 mass units above the parent mass and the maximum ion inject time of 250 ms. Data acquisition was conducted using the Xcalibur data system (version 1.3 SR1, Thermo Fisher Scientific).

GC analysis was performed on a Thermo Finnigan Trace GC apparatus using a l-Chirasil-Val column (0.32 mm × 25 m).

TLC was performed on silica gel 60 F_{254} plates (Merck), and the solvent system EtOAc/HOAc/H_2O (7:2:2 v/v/v) was used for analysis. Spots were revealed by spraying with a Liebermann−Burchard agent, followed by heating at 150 °C. Column chromatography was carried out using silica gel (LiChroprep Si 60, 25−40 μm) (Merck) and RP-18 silica gel (Lichroprep RP18, 40−63 or 25−40 μm) (Merck).

Optical rotations were measured on a Jasco P 2000 digital polarimeter. IR measurements were performed on a Bruker IFS-48 spectrometer.

NMR Measurements. 1D and 2D NMR spectra were recorded either on a Bruker Avance-500 spectrometer equipped with a 5 mm indirect detection probe or on a Varian INOVA-600 spectrometer equipped with a 5 mm indirect detection probe. The ^1H and ^{13}C NMR spectra (at 500 or 600 and 125 or 150 MHz, respectively) were measured in methanol-d_4 at 300 K. Chemical shifts are given on the δ scale and referenced to residual methanol, δ ^1H 3.34 and ^{13}C 49.00.

Determination of Absolute Configuration of Sugars. The configuration of sugar units was established after hydrolysis of **1**−**7**, with 1 N HCl, trimethylsilation, and determination of the retention times by GC operating in the experimental conditions previously reported by De Marino et al.[28]

The peaks of silylated D-glucuronic acid (15.83 min) and silylated D-glucose (14.74 min) were detected in the hydrolysate of **1**. The peaks of silylated L-arabinose (8.94 and 9.81 min) and silylated D-glucuronic

acid (15.82 min) were detected in the hydrolysate of **2**. Retention times for authentic samples after being treated in the same manner with 1-(trimethylsilyl)imidazole in pyridine were detected at 8.92 and 9.80 min (silylated L-arabinose), 14.71 min (silylated D-glucose), and 15.81 min (silylated D-glucuronic acid).

Chemical Data of Saponins 1−7. *Saponin 1:* amorphous white solid; $C_{42}H_{64}O_{17}$; $[\alpha]^{25}_{D}$ −9.1° (*c* 0.1 MeOH); IR ν^{KBr}_{max} cm^{-1} 3430 (OH), 2925 (CH), 1680 (C═O), 1656 (C═C); for ^1H and ^{13}C NMR (CD_3OD, 500 MHz) data of the aglycone moiety and the sugar portion see Tables 1 and 2, respectively; ESI-MS *m/z* 839.4 $[M-H]^-$; ESI-MS/MS *m/z* 677.3 $[(M-H)-162]^-$, 501.2 $[(M-H)-162-176]^-$; HR-MALDI/TOF-MS $[M+Na]^+$ *m/z* 863.4043 (calcd for $C_{42}H_{64}O_{17}Na$, 863.4041).

Saponin 2: amorphous white solid; $C_{41}H_{64}O_{15}$; $[\alpha]^{25}_{D}$ +30.1° (*c* 0.05 MeOH); IR ν^{KBr}_{max} cm^{-1} 3440 (OH), 2935 (CH), 1670 (C═O), 1655 (C═C); for ^1H and ^{13}C NMR (CD_3OD, 500 MHz) data of the aglycone moiety and the sugar portion see Tables 1 and 2, respectively; ESI-MS *m/z* 795.4 $[M-H]^-$; ESI-MS/MS *m/z* 663.4 $[(M-H)-132]^-$, 487.3 $[(M-H)-132-176]^-$; HR-MALDI/TOF-MS $[M+Na]^+$ *m/z* 819.4146 (calcd for $C_{41}H_{64}O_{15}Na$, 819.4143).

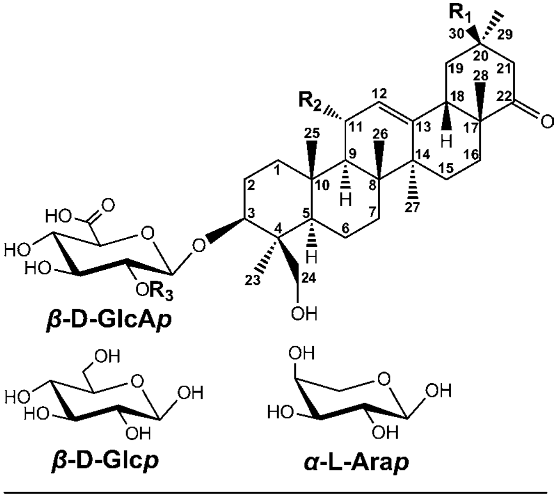
Saponin 3: amorphous white solid; $C_{42}H_{66}O_{16}$; $[\alpha]^{25}_{D}$ −8.3° (*c* 0.1 MeOH); IR ν^{KBr}_{max} cm^{-1} 3450 (OH), 2935 (CH), 1680 (C═O), 1660 (C═C); for ^1H and ^{13}C NMR (CD_3OD, 500 MHz) data of the aglycone moiety and the sugar portion see Tables and 2, respectively; ESI-MS *m/z* 825.5 $[M-H]^-$; ESI-MS/MS *m/z* 663.4 $[(M-H)-162]^-$, 487.4 $[(M-H)-162-176]^-$; HR-MALDI/TOF-MS $[M+Na]^+$ *m/z* 849.4253 (calcd for $C_{42}H_{66}O_{16}Na$, 849.4249).

Saponin 4: amorphous white solid; $C_{43}H_{68}O_{16}$; $[\alpha]^{25}_{D}$ −17.0° (*c* 0.1 MeOH); IR ν^{KBr}_{max} cm^{-1} 3430 (OH), 2948 (CH), 1670 (C═O), 1656 (C═C); for ^1H and ^{13}C NMR (CD_3OD, 600 MHz) data of the aglycone moiety and the sugar portion see Tables 1 and 2, respectively; ESI-MS *m/z* 839.4 $[M-H]^-$; ESI-MS/MS *m/z* 677.4 $[(M-H)-162]^-$, 501.3 $[(M-H)-162-176]^-$; HR-MALDI/TOF-MS $[M+Na]^+$ *m/z* 863.4409 (calcd for $C_{43}H_{68}O_{16}Na$, 863.4405).

Saponin 5: amorphous white solid; $C_{43}H_{68}O_{16}$; $[\alpha]^{25}_{D}$ +10.6° (*c* 0.1 MeOH); IR ν^{KBr}_{max} cm^{-1} 3435 (OH), 2925 (CH), 1670 (C═O), 1656 (C═C); for ^1H and ^{13}C NMR (CD_3OD, 600 MHz) data of the aglycone moiety and the sugar portion see Tables 1 and 2, respectively; ESI-MS *m/z* 809.4 $[M-H]^-$; ESI-MS/MS *m/z* 647.3 $[(M-H)-162]^-$, 471.2 $[(M-H)-162-176]^-$; HR-MALDI/TOF-MS $[M+Na]^+$ *m/z* 833.4252 (calcd for $C_{42}H_{66}O_{15}Na$, 833.4249).

Saponin 6: amorphous white solid; $C_{36}H_{56}O_{10}$; $[\alpha]^{25}_{D}$ −14.6° (*c* 0.05 MeOH); IR ν^{KBr}_{max} cm^{-1} 3443 (OH), 2935 (CH), 1670 (C═O), 1655 (C═C); for ^1H and ^{13}C NMR (CD_3OD, 500 MHz) data of the aglycone moiety and the sugar portion see Tables 1 and 2, respectively; ESI-MS *m/z* 647.4 $[M-H]^-$; ESI-MS/MS *m/z* 471.3 $[(M-H)-176]^-$; HR-MALDI/TOF-MS $[M+Na]^+$ *m/z* 671.3774 (calcd for $C_{36}H_{56}O_{10}Na$, 671.3771).

Saponin 7: amorphous white solid; $C_{36}H_{56}O_{10}$; $[\alpha]^{25}_{D}$ −40.1° (*c* 0.08 MeOH); IR ν^{KBr}_{max} cm^{-1} 3430 (OH), 2938 (CH), 1670 (C═O), 1650 (C═C); for ^1H and ^{13}C NMR (CD_3OD, 500 MHz) data of the aglycone moiety and the sugar portion see Tables 1 and 2, respectively; ESI-MS *m/z* 661.3 $[M-H]^-$; ESI-MS/MS *m/z* 485.2 $[(M-H)-176]^-$; HR-MALDI/TOF-MS $[M+Na]^+$ *m/z* 685.3932 (calcd for $C_{37}H_{58}O_{10}Na$, 685.3928).



Compound	R_1	R_2	R_3
1	COOH	OH	β-D-Glc*p*
2	CH_2OH	OH	α-L-Ara*p*
3	CH_2OH	OH	β-D-Glc*p*
4	CH_2OH	OCH_3	β-D-Glc*p*
5	CH_2OH	H	β-D-Glc*p*
6	CH_3	OH	H
7	CH_3	OCH_3	H

Figure 1. Compounds **1**−**7** isolated from *Trifolium medium* var. *sarosiense* aerial parts.

RESULTS AND DISCUSSION

Structure Elucidation. The powdered aerial parts of *T. medium* var. *sarosiense*, after defatting with chloroform, were extracted with MeOH, and the obtained extract was fractionated by different chromatographic steps to yield seven compounds (**1**−**7**) (Figure 1).

The HR-MALDI/TOF mass spectrum of **6** supported a molecular formula of $C_{36}H_{56}O_{10}$. The negative ESI-MS spectrum showed the ion peak at *m/z* 647.4, which was assigned to $[M-H]^-$. The MS/MS of this ion showed fragmentation ion peak at *m/z* 471.3 $[(M-H)-176]^-$, corresponding to the loss of a hexuronic acid.

The ^{13}C NMR spectrum showed the presence of 36 carbon signals; thus, considering 6 carbon signals for a sugar unit, shown to be present from the MS fragmentation, 30 of them were assigned to the triterpene aglycone (Table 1).[22] Chemical shifts of two olefinic carbons at δ_C 129.0 (C-12) and 144.7 (C-13), characteristic for a Δ^{12}-β-amyrin skeleton, were observed.[23] Signals at δ_C 16.8, 18.4, 20.9, 23.1, 25.1, 25.3, and 31.9 ascribable to seven methyl groups, along with a signal at δ_C 63.7 due to a primary alcoholic function, could be also observed. Furthermore, the carbon spectrum displayed signals at δ_C 90.0 and 68.1 assigned to secondary alcoholic functions and at δ_C 219.4, suggesting the occurrence of a keto group. The ^1H NMR spectrum of **6** (Table 1) showed signals for seven tertiary methyl groups at δ_H 0.89, 1.01, 1.05, 1.06 (6H), 1.25, and 1.39, one olefinic proton at δ_H 5.30 (d, *J* = 3.0 Hz, H-12), two oxymethine protons at δ_H 3.49 (dd, *J* = 4.3, 11.6 Hz) and 4.18 (dd, *J* = 3.0, 8.9 Hz), and two geminal oxymethylene protons at δ_H 3.37 and 4.09 (each, d, *J* = 11.4 Hz). The secondary alcoholic function at δ_C 90.0 was attributed to C-3, and the coupling constants of the corresponding proton at δ_H 3.49 (dd, *J* = 4.3, 11.6 Hz), together with ROESY correlation between H-3 and the proton resonance at δ_H 1.02 (1H, *m*, H-5) allowed a 3β-OH orientation to be deduced. The downfield shift of C-3 (δ_C 90.0) suggested the occurrence of a glycosidation at this position. The additional secondary alcoholic group was located at C-11 on the basis of COSY correlations between the proton at δ_H 4.18 with the proton resonance at 5.30 (d, *J* = 3.0 Hz, H-12), and according to HMBC correlations between the proton signal at δ 4.18 and the carbon resonances at δ_C 55.6 (C-9) and 129.0 (C-12). ROESY experiment, which showed correlations

between H-11 and the protons at δ_H 1.05 (Me-25) and 1.06 (Me-26), allowed us to establish the α orientation of a secondary alcoholic function at C-11. This result was in agreement with literature data.[24,25] The proton resonances of a primary alcoholic function at δ_H 3.37 and 4.09 showed HMBC correlations with the carbon signals at δ_C 90.0 (C-3), 44.9 (C-4), 57.1 (C-5), and 23.1 (C-23) and identified the attachment site of a CH_2OH group as C-4. The 24-CH_2OH (δ_C 63.7) could be derived from the chemical shift of the methyl group (δ_C 23.1), which is reported as upfield shifted (δ_C 14.0) in the case of 23-CH_2OH.[24] ROESY correlations between the proton values of the secondary alcoholic function at C-24 (δ_H 3.37 and 4.09) with the proton resonance at δ_H 1.05 (Me-25) confirmed the occurrence of a 24-CH_2OH. The reciprocated correlations on the HMBC spectrum between two Me groups (δ_C 25.3 and δ_H 0.89, s; and δ_C 31.9 and δ_H 1.06, s), as well as HMBC correlations of their proton signals with the carbon resonances at δ_C 46.8 (C-19), 35.2 (C-20), and 51.2 (C-21), suggested the linkage of both methyl groups to C-20. Literature data report for aglycones possessing a methyl group at positions C-29 and C-30 the chemical shift of the Me-29 of ~32.0 ppm and ~25.0 ppm for Me-30.[26,27] ROESY experiment, in which correlations between the proton resonance at δ_H 2.38 (dd, J = 3.4, 13.4 Hz, H-18) and the proton at δ_H 0.89 (Me-30) and between the proton value at δ_H 1.06 (Me-29) and both H-19 protons (δ_H 1.46 and 2.23, each, m) could be observed, supported the aforementioned orientation of the methyl groups linked to C-20. The keto group was located at C-22 on the basis of the HMBC correlations between the carbon signal at δ_C 219.4 and proton resonances at δ_H 1.01 (Me-28), 2.01 (d, J = 14.2 Hz, H-21), and 2.63 (d, J = 14.2 Hz, H-21).

For the sugar portion, compound **6** showed a signal corresponding to the one anomeric proton at δ_H 4.46 (d, J = 7.5 Hz) (Table 2). On the basis of 2D NMR data, a β-glucuronopyranosyl unit was identified. The determination of a linkage site was obtained from the HMBC correlation between the proton signal at δ_H 4.46 (H-1_{GlcA}) and the carbon resonance at δ_C 90.0 (C-3). The D configuration of the glucuronic acid unit was established after hydrolysis of **6** followed by GC analysis.[28] Thus, the structure of **6** was elucidated as the new 3-*O*-β-D-glucuronopyranosyl-3β,11α,24-trihydroxyolean-12-en-22-one, and the proposed trivial name is sarosiensin VI.

The HR-MALDI/TOF mass spectrum of **7** supported a molecular formula of $C_{41}H_{64}O_{15}$. The ESI-MS mass spectrum showed the major ion peak at *m/z* 661.3, which was assigned to $[M - H]^-$. Its MS/MS showed a fragmentation ion peak at *m/z* 485.2 $[(M - H) - 176]^-$, corresponding to the loss of a hexuronic acid.

Comparison of the ^{1}H and ^{13}C NMR spectroscopic data of **7** and **6** showed superimposable resonances, except for the presence of the signal at δ_H 3.28 (3H, s) with δ_C 54.1 corresponding to a methoxyl group. This function was located at C-11 on the basis of HMBC correlation between the proton value at δ_H 3.28 and the carbon resonance at δ_C 77.4 (C-11). The orientation of the 11-OMe group was established as α according to the ROESY spectrum, in which correlations between the proton signal at δ_H 3.89 (dd, J = 3.3, 8.8 Hz, H-11) with the proton resonances at δ_H 1.04 (Me-25) and 1.05 (Me-26) could be observed. This result was in agreement with reported data.[25] Consequently, compound **7** was identified as 3-*O*-β-D-glucuronopyranosyl-3β,24-dihydroxy-11α-methoxyolean-12-en-22-one, and the proposed trivial name is sarosiensin VII.

The HR-MALDI/TOF mass spectrum of **2** supported a molecular formula of $C_{41}H_{64}O_{15}$. The ESI-MS mass spectrum in the negative ion mode showed the major ion peak at *m/z* 795.4, which was assigned to $[M - H]^-$. The MS/MS fragmentation spectrum of this ion showed ions at *m/z* 645.2 $[M - H - 150]^-$, 487.3 $[M - H - 308]^-$, corresponding to losses of a pentose and a pentose-hexuronic acid disaccharide, respectively.

A detailed comparison of NMR spectroscopic data of the aglycone portion of **2** and **6** showed great similarity, and the only observed difference was the presence of an additional primary alcoholic function at C-30 (δ_C 68.7 and δ_H 3.32, 2H, m) in **2**, instead of the methyl group in **6**. The linkage of the CH_2OH group to C-20 was determined from the HMBC spectrum, in which correlations between the carbon resonance at δ_C 68.7 with the proton signals at δ_H 0.99 (Me-29) and δ 1.73 and 2.03 (each, m, H-19) could be observed. The ROESY spectrum showed correlations between the signal of Me-29 (δ_H 0.99) with both H-19 protons (δ_H 1.73 and 2.04), as well as correlations between the proton resonance at δ_H 3.32 (2H, H-30) with the proton signal at δ_H 2.52 (dd, J = 3.4, 13.6 Hz, H-18), thus allowing us to establish the β orientation of an additional primary alcoholic function. Our results are consistent with those reported in the literature.[26,29,30]

For the sugar portion compound **2** showed in the ^{1}H NMR spectrum signals corresponding to two anomeric protons at δ_H 4.49 (d, J = 7.5 Hz), and 4.64 (d, J = 7.5 Hz) (Table 2). On the basis of 2D NMR data one α-arabinopyranosyl (δ_H 4.64) and one β-glucuronic acid (δ_H 4.49) units were identified. The determination of sequence and linkage sites was obtained from the HMBC correlations between the proton signal at δ_H 4.64 (H-1_{Ara}) with the carbon resonance at δ_C 81.4 (C-2_{GlcA}) and between the signal at δ_H 4.49 (H-1_{GlcA}) with the carbon value at δ_C 91.5 (C-3). The D configuration of the glucuronic acid and the L configuration of the arabinose units were established after hydrolysis of **2** followed by GC analysis.[28] On this basis, the structure of **2** was determined as 3-*O*-[α-L-arabinopyranosyl-(1→2)-β-D-glucuronopyranosyl]-3β,11α,24,30-tetrahydroxyolean-12-en-22-one, and the proposed trivial name is sarosiensin II.

The molecular formula of **3** was established as $C_{42}H_{66}O_{16}$ by HR-MALDI/TOF mass spectrum. The ESI-MS mass spectrum in the negative ion mode showed the major ion peak at *m/z* 825.5, which was assigned to $[M - H]^-$. During MS/MS fragmentation, it produced ions at *m/z* 645.2 $[M - H - 180]^-$and at *m/z* 487.4 $[M - H - 338]^-$, corresponding to losses of a hexose and a hexose-hexuronic acid disaccharide.

A detailed comparison of NMR spectroscopic data for the aglycone portion of compounds **3** and **2** demonstrated superimposable resonances, and it was concluded that both compounds possessed the same aglycone moiety.

For the sugar portion, compound **3** showed signals corresponding to two anomeric protons at δ_H 4.50 (d, J = 7.9 Hz) and 4.80 (d, J = 7.5 Hz) (Table 2). On the basis of 2D NMR data, one β-glucopyranosyl (δ_H 4.80) and one β-glucuronopyranosyl (δ_H 4.50) units were identified. The determination of sequence and linkage sites was obtained from the HMBC correlations between the proton signal at δ_H 4.80 (H-1_{Glc}) and the carbon resonance at δ_C 81.3 (C-2_{GlcA}) and between the signal at δ_H 4.50 (H-1_{GlcA}) and the carbon resonance at δ_C 91.5 (C-3). The D configuration of the glucose

and glucuronic acid units was established after hydrolysis of **3** followed by GC analysis.[28] Therefore, compound **3** was identified as 3-*O*-[*β*-D-glucopyranosyl-(1→2)-*β*-D-glucuronopyranosyl]-3*β*,11*α*,24,30-tetrahydroxyolean-12-en-22-one, and the proposed trivial name is sarosiensin III.

The HR-MALDI/TOF mass spectrum of **4** showed a major ion peak at *m*/*z* 863.4409 $[M + Na]^+$ ascribable to the molecular formula $C_{43}H_{68}O_{16}$ (calcd for $C_{43}H_{68}O_{16}Na$, 863.4405). The negative ESI-MS spectrum showed the ion peak at *m*/*z* 839.4, which was assigned to $[M - H]^-$. The MS/MS of this ion was essentially identical to that of **3**.

Comparison of the ^1H and ^{13}C NMR spectroscopic data for the aglycone portion and the sugar chain of **4** and **3** showed superimposable resonances, except for the additional presence of a methyl group (δ_C 54.2 and δ_H 3.28, 3H, s). In the HMBC spectrum the correlation between the methyl group at δ_H 3.28 and the carbon resonance at δ_C 77.5 (C-11) allowed us to locate a methoxyl group at C-11. The orientation of the $-OCH_3$ function was concluded to be α according to the ROESY correlations between the proton signal at δ_H 3.87 (H-11, dd, *J* = 3.4, 8.8 Hz) and the proton signals at δ_H 1.02 (Me-25), and 1.03 (Me-26) (Figure 2). This result was in agreement with literature data.[25] Thus, the structure of **4** was established as 3-*O*-[*β*-D-glucopyranosyl-(1→2)-*β*-D-glucuronopyranosyl]-3*β*,24,30-trihydroxy-11*α*-methoxyolean-12-en-22-one, and the proposed trivial name is sarosiensin IV.

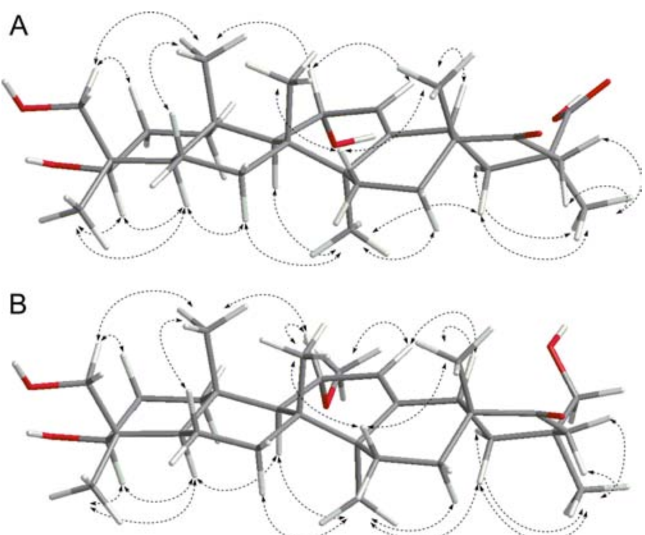


Figure 2. Selected ROESY correlations for the aglycone moieties of **1** (A) and **4** (B).

The HR-MALDI/TOF mass spectrum of **1** supported a molecular formula of $C_{42}H_{64}O_{17}$. The ESI-MS mass spectrum in negative ion mode showed the major ion peak at *m*/*z* 839.4, which was assigned to $[M - H]^-$. In its MS/MS fragmentation pattern, the base peak at *m*/*z* 659.2 $[M - H - 180]^-$ corresponds to the loss of a hexose. The peak at *m*/*z* 501.2 $[M - H - 338]$ likely represents the loss of the entire C-3 carbohydrate side chain, thus suggesting it is composed of a hexose and a hexuronic acid.

A detailed comparison of NMR spectroscopic data for aglycone moiety of compounds **1** and **6** demonstrated great similarity, except for the presence of a carboxylic function at C-30 (δ_C 183.8), instead of the methyl group in **6**. The HMBC correlations between the proton signal at δ_H 1.21 and the carbon resonances at δ_C 45.0 (C-19), 47.7 (C-20), 48.8 (C-21), and 183.8 (C-30) suggested the linkage of a $-COOH$ group to C-20. The chemical shift of the methyl group attached to C-20 (δ_C 27.1) supported a 30-COOH. Literature data report for an aglycone possessing Me-29 and COOH-30 the chemical shift of the methyl group at ∼27.0 ppm,[31,32] whereas for aglycones possessing Me-30 and COOH-29 the methyl group resonates at ∼20.0 ppm.[24,33] The ROESY experiment, in which the correlations between the proton signal at δ_H 1.21 (Me-29) and both H-19 protons (δ_H 1.96 and 2.08, each, m) and the absence of a correlation between the proton signal at δ_H 1.21 (Me-29) with the proton signal of H-18 (δ_H 2.44, dd, *J* = 3.3, 13.6 Hz) could be observed, confirmed the β orientation of the $-COOH$ (Figure 2).

For the sugar portion, compound **1** showed signals corresponding to the same sugar chain as compound **3**. Thus, the structure of **1** was established as 3-*O*-[*β*-D-glucopyranosyl-(1→2)-*β*-D-glucuronopyranosyl]-3*β*,11*α*,24-trihydroxyolean-12-en-22-oxo-30-oic acid, and the proposed trivial name is sarosiensin I.

The molecular formula of **5** was established as $C_{42}H_{66}O_{15}$ by HR-MALDI/TOF mass spectrum. The ESI-MS mass spectrum showed the major ion peak at *m*/*z* 809.4, which was assigned to $[M - H]^-$. Its MS/MS fragmentation pattern displayed ions at *m*/*z* 629.3 $[M - H - 180]^-$ and 471.2 $[M - H - 338]^-$, which correspond to the losses of a hexose and a hexose-hexuronic acid disaccharide, respectively. Also $^{1,5}X$ cross-ring fragmentation of a hexuronyl pyranose residue followed by a loss of water, which produced an ion at *m*/*z* 539.1 $[M - H - 270]^-$, was observed. The position of the glycosidic bond could be deduced from the $^{0,2}X$ cross-ring cleavage within the same residue, resulting in an ion at *m*/*z* 675 $[M - H - 135]$.[34] This indicated that this bond involves a hydroxyl at C-2 of a hexuronic acid.

The ^1H and ^{13}C NMR signals for the aglycone moiety of **5** were almost superimposable on those of **2** except for the absence of the secondary alcoholic function at C-11. Consequently, the aglycone portion was identified as 3*β*,24,30-trihydroxyolean-12-en-22-one, known as wistariasapogenol A, previously isolated from *Wisteria brachybotrys* (family Fabaceae).[30] However, it is reported here for the first time in *Trifolium* genus. The sugar portion of **5** was the same as of **1**, **3**, and **4**. Thus, **5** was identified as 3-*O*-[*β*-D-glucopyranosyl-(1→2)-*β*-D-glucuronopyranosyl]-3*β*,24,30-trihydroxyolean-12-en-22-one, and the proposed trivial name is sarosiensin V.

In the present work, seven triterpene glycosides never reported before, namely, sarosiensin I–VII, were isolated and characterized from the aerial parts of *T. medium* var. *sarosiense*. The base peak chromatogram obtained by negative-ion HPLC-ESI/MS of *T. medium* var. *sarosiense* saponins (Figure 3A) clearly shows that isolated compounds represent the most intense peaks in this fraction. Peaks at *m*/*z* 839 (**1**, **4**), 795 (**2**), 825 (**3**), 809 (**5**), 647 (**6**), and 661 (**7**) correspond to the compounds identified in this work. Furthermore, the chromatogram shows also an intense peak at *m*/*z* 941, which showed a fragmentation pattern identical to that of soyasaponin Bb (soyasaponin I[35,36]). Due to the fact that this compound is quite common, and its structure is well-known, it was not purified in this work. However, soyasaponin Bb is a common saponin occurring in most *Trifolium* species, and in many of them soyasapogenol glycosides were the only saponins present.[17−21] Therefore, *T. medium* var. *sarosiense* seems to be

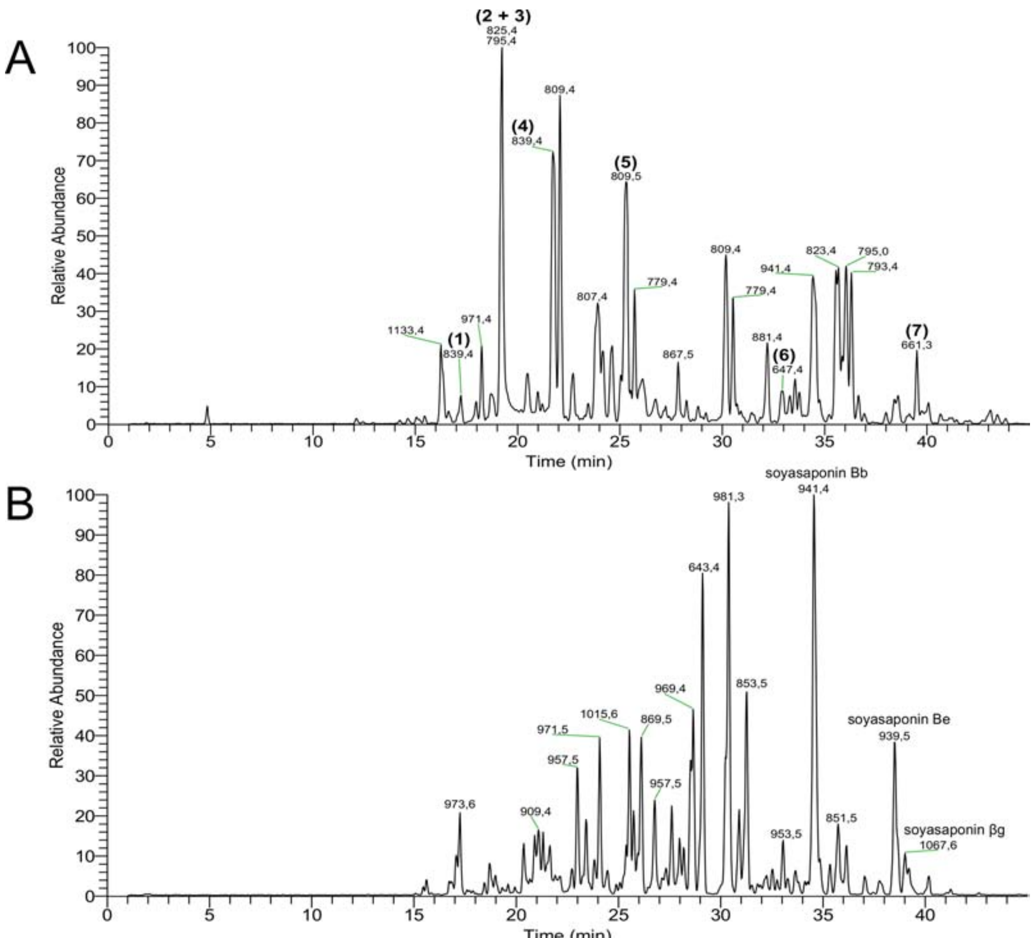


Figure 3. HPLC-ESI/MS base peak chromatograms of *T. medium* var. *sarosiense* (A) and *T. medium* var. *medium* (B) saponin fraction.

a unique species in the *Trifolium* genus, able to synthesize triterpene oleanane aglycones different from soyasapogenols.

Classification of zigzag clover taxa has not been conclusively resolved so far. Therefore, the phytochemical comparison of two mentioned subspecies of *T. medium* for their secondary metabolites might be of great interest, because it may contribute to resolving the doubts of species rank for *T. medium* var. *sarosiense*. Figure 3B shows the base peak chromatogram obtained by the negative-ion HPLC-ESI/MS of *T. medium* var. *medium* saponins and its comparison to the analogous chromatogram of *T. medium* var. *sarosiense*, which clearly shows that their saponin profiles are significantly different. Both subspecies produce few soyasapogenol B and soyasapogenol E derivatives, such as soyasaponin Bb (m/z 941, RT 34.5 min), Bc (m/z 911, RT 34.3 min, coeluting with Bb), and Be (m/z 939, RT 38.4 min) as well as DDMP-conjugated soyasaponin βg (m/z 1067, RT 38.9 min). However, a majority of peaks from *T. medium* var. *sarosiense* extract were not found in other subspecies. Our former[15,16] and present investigations of *T. medium* taxa show the existence of significant differences in a secondary metabolism of both zigzag clover subspecies, which may indicate their more distant relationship. Therefore, it seems to be more reasonable to follow databases and publications that classify *T. medium* var. *sarosiense* as a taxa species (*T. sarosiense*) separate from *T. medium*.

■ ASSOCIATED CONTENT

Supporting Information

NMR spectra (^{1}H and ^{13}C NMR, HSQC, HMBC, ^{1}H−^{1}HCOSY) for the new compounds (**1**−**7**), including ROESY spectra for compounds **1**, **2**, **4**, **6**, and **7**. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*(D.J.) E-mail: djedrejek@iung.pulawy.pl. Phone: +48 818863421, ext. 207. Fax: +48 818863421, ext. 295.

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Notes

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