Carbohydrate Research 375 (2013) 16–20

Contents lists available at SciVerse ScienceDirect

Carbohydrate Research

journal homepage: www.elsevier.com/locate/carres

Note

A new cytotoxic triterpene saponin from *Lysimachia nummularia* L.

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ARTICLE INFO

Article history:
Received 17 February 2013
Received in revised form 2 April 2013
Accepted 4 April 2013
Available online 12 April 2013

Keywords:
Lysimachia nummularia
Triterpene saponin
Cytotoxicity

ABSTRACT

A new glycosylated triterpene **1** (named nummularoside) was isolated from the underground parts of *Lysimachia nummularia* L. Its chemical structure was elucidated as 3-*O*-β-{{[β-D-xylopyranosyl-(1→2)]-[β-D-xylopyranosyl-(1→4)]-β-D-glucopyranosyl-(1→4)}-[β-D-glucopyranosyl-(1→2)-]-α-L-arabinopyranosyl]}, protoprimulagenin A on the basis of extensive NMR and MS spectral data. The saponin showed significant activity against prostate cancer cells DU145 and PC3 (EC_{50} 1.2 and 7.4 μg/mL, respectively), while it did not affect normal cells (EC_{50} 30 μg/mL), in contrast to the reference compound (mitoxanthrone, EC_{50} 0.45 μg/mL). Glioblastoma cells were also significantly affected by the tested saponin (EC_{50} 6.0 μg/mL), whereas the activity against melanoma cells was moderate (EC_{50} 17.5–23.2 μg/mL).

The genus *Lysimachia* L. comprises about 200 species, wild and cultivated, native to temperate regions of Eurasia, and is traditionally classified as one of the largest genera in Primulaceae.[1] However, results from recent phylogenetic analyses suggested its relocation to the family Myrsinaceae.[2] The medicinal value of many *Lysimachia* species is well known, there are reports on their use as, for example, analgesic,[3] anti-leishmanial,[4] anti-helminthic[5] agents and to treat cholecystitis.[6] Also *Lysimachia nummularia* L.—moneywort—has been used in medicine since antiquity for indications such as diarrhoea, fever, arthritis, tuberculosis, skin diseases and many other. It was listed in *Codex Medicamentarius sive Pharmacopoea Gallica* (Paris, 1818).[7] Extracts from *L. nummularia* herb were found active against a number of microorganisms, including *Streptococcus pyogenes*, *Staphylococcus aureus*, *Salmonella* sp. and *Shigella* sp.[8]

Phytochemical studies confirmed the presence of the following groups of compounds in this plant species: flavonoids[9,10] and phenolic acids[11] in herb, benzoquinones[12] and tannins[13] in whole plant. Genus *Lysimachia,* as well as other genera of the family Myrsinaceae (and/or Primulaceae), is characterized by the presence of saponins with oleanane-derived sapogenols, in this, fairly rare compounds possessing a completely saturated pentacyclic skeleton with 13β,28-epoxy bridge.[14,15]

Triterpene saponins have been reported to possess a wide range of pharmacological activities, including anti-inflammatory, expectorant, vasoprotective, immunoadjuvant, antileishmanial, gastroprotective and many other.[16,17] Also, their cytotoxic and antiproliferative potential has been attracting much interest in recent years,[18,19] and several compounds of the 13β,28-epoxy-oleanane group have exhibited such effects.[20–25]

In our on-going research on the cytotoxic activity of natural products and the chemical constituents of species of *Lysimachia*[12,26,27] we investigated *L. nummularia*, which is one of five representatives of this genus found on natural stands in Poland.

The present study deals with the isolation and structure elucidation of a new triterpene glycoside 3-*O*-β-{{[β-D-xylopyranosyl-(1→2)]-[β-D-xylopyranosyl-(1→4)]-β-D-glucopyranosyl-(1→4)}-[β-D-glucopyranosyl-(1→2)-]-α-L-arabinopyranosyl]}, protoprimulagenin A **1** (nummularoside) and evaluation of its cytotoxic activity as well as selectivity against a panel of five human cancer cell lines and normal cells of respective origin.

Compound **1** (Fig. 1) was isolated as a white amorphous powder. Its molecular formula, $C_{57}H_{94}O_{25}$, was deduced from the HR-ESI-TOF-MS ion at $m/z = 1201$, 60 ($[M+Na]^+$). This composition was supported by ^{13}C NMR spectral data and DEPT 135 experiments. The negative-ion FAB-MS showed fragment ions at m/z 1045 $[M-H-132]^-$ and 913 $[M-H-132-132]^-$ corresponding to the subsequent loss of two pentose units, at m/z 1015 due to the loss of hexose unit, at m/z 751 corresponding to the loss of a hexose and of two pentose units $[M-H-162-132-132]^-$. This fragmentation pattern indicated a branched sugar chain.

Acid hydrolysis on a TLC plate afforded arabinose, glucose and xylose, which were identified by co-chromatography with authentic sugar samples. To establish their absolute configurations further experiments were carried out, including acid hydrolysis of compound **1** (4 M HCl in dioxane and water 1:1, v/v; 3 h, 95 °C), followed by derivatization with

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http://dx.doi.org/10.1016/j.carres.2013.04.005

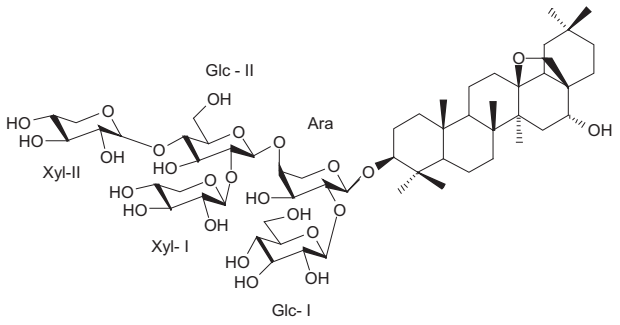

Figure 1. Structure of compound **1**.

L-cysteine methyl ester hydrochloride and *o*-tolyl-isothiocyanate, and direct HPLC analysis.[28] Thus, the monosaccharides were confirmed as L-arabinose, D-glucose and D-xylose.

The IR spectrum showed absorptions at 3334 (–OH), 2922 (CH) and at 1069 (C–O–C) cm^{-1}. Of the 57 carbons in the ^{13}C NMR spectrum, 30 were assigned to the triterpenoid skeleton and 27 to the oligosaccharide moiety. Among the triterpene skeleton carbons, two methine carbons bearing oxygen were found at δ 91.4 and 77.9 ppm. The spectrum lacked signals of the olefinic carbons C-12 and C-13 at δ ~122 and ~145 ppm characteristic of olean-12-enes, instead, an oxygenated quaternary carbon signal was observed at δ 88.4 ppm, which is the fingerprint resonance of pentacyclic triterpenes with a methyleneoxy bridge between C-13 and C-17.[29] This was supported by the ^1H NMR spectrum in which the C-28 protons appeared as two doublets at δ 3.12 and 3.49 (each 1H, AB system, J = 7.5 Hz) characteristic of the presence of a 13β,28-epoxide.[30] Moreover, the ^1H NMR spectrum revealed that **1** possesses seven tertiary methyl protons at δ 0.85 (3H, s, H-24), 0.91 (3H, s, H-25), 0.91 (3H, s, H-30), 0.95(3H, s, H-29), 1.05 (3H, s, H-23), 1.14 (3H, s, H-26), 1.23 (3H, s, H-27), and the corresponding methyl carbons were identified by an HSQC experiment. The structural assignment was initiated from the long-range coupling networks observed between the methyl protons and the adjacent carbons from the HMBC experiment.

Correlations were observed from singlets at δ 1.05 (H-23) and δ 0.85 (H-24) with carbon resonances C-3 (91.4), C-4 (40.6) and C-5 (56.8); from singlet at δ 0.91 (H-25) with C-1 (40.2), C-5 (56.8), C-9 (51.4), C-10 (37.8); from singlet at δ 1.14 (H-26) with C-7 (35.2), C-8 (43.3), C-9 (51.4), C-14 (45.4); from singlet at δ 1.23 (H-27) with C-8 (43.3), C-14 (45.4), C-15 (37.1), C-13 (88.4); from singlets at δ 0.91 (H-30) and 0.95 (H-29) with carbons C-20 (32.4), C-19 (39.8). Further diagnostic long-range correlation cross-peaks, which supported the presence of the 13β,28-epoxy oleanane skeleton,[31,32] were observed between H-28 (δ 3.12; 3.49) and C-16 (δ 77.9) and C-17(δ 45.4); between H-16 (δ 3.89) and C-14 (δ 45.4) and C-18 (δ 52.4).

The α-configuration of the hydroxyl group at C-16 was evident from the ^{13}C chemical shift in comparison to literature data (16α OH ca. 77 ppm; 16β OH ca. 74 ppm)[29] and was confirmed by the ROESY spectrum where cross-peaks were observed between H-16 (δ 3.89) and H-15 (δ 2.10), H-22 (δ 1.78) and H-28 (δ 3.49). The correlation of H_{ax}-3 with H-23 (δ 1.05) and H-5 (δ 0.73) indicated a β-configuration of the hydroxyl at C-3. The glycosidic linkage at C-3 was indicated by its downfield chemical shift (δ 91.4) and from the J value of the proton ascribable to C-3 at δ 3.14 (dd, J = 11.9 and 4.6 Hz).

The above extensive NMR analysis and the comparison of ^{13}C data with the literature for similar compounds[20,27,33–35] led us to establish that the structure of the aglycone of compound **1** was protoprimulagenin A.

In the sugar region of ^1H NMR spectrum, signals corresponding to five anomeric protons were found at δ 4.34 (d, J = 7.7 Hz, 1H), 4.40 (t, J = 6.5 Hz, 1H), 4.54 (d, J = 7.6 Hz, 1H), 4.56 (d, J = 7.8 Hz, 1H), 4.70 (d, J = 7.7 Hz, 1H). These were correlated by HSQC experiment to the corresponding carbon resonances at δ 105.2, 105.6, 107.3, 104.6, 104.3, respectively (Table 1). In the ^1H NMR spectrum the relatively large coupling constants $J_{1,2}$ of the anomeric protons for both glucose and both xylose moieties (7.6–7.8 Hz) indicated a β-configuration. The value of $J_{1,2}$ coupling constant for the anomeric proton of arabinose (6.5 Hz), which has been reported not to be diagnostic on its own,[36] together with NOE connectivities between Ara H-1, Ara H-3 and Ara H-5 indicated an α-orientation of this sugar unit. Complete assignments of the resonances of each monosaccharide unit were achieved by extensive NMR analyses (^1H–^1H COSY, TOCSY, HSQC, HMBC, ROESY) (Table 1). The sugar sequence of the oligosaccharide chain as well as the glycoside sites were subsequently determined by HMBC spectrum. The key cross-peaks (Fig. 2) were observed between Ara H-1 (δ 4.40) and C-3 (δ 91.4), between H-1 of Glc-I (δ 4.70) and C-2 of Ara (δ 79.5), between H-1 of Glc-II (δ 4.56) and C-4 of Ara (δ 80.2), between H-1 of Xyl-I (δ 4.54) and C-2 of Glc-II (δ 84.5), between H-1 of Xyl-II (δ 4.34) and C-4 of Glc-II (δ 79.9). ROESY correlations were observed between δ_H 4.70 (Glc-I H-1) and δ_H 3.80 (Ara H-2), δ_H 4.56 (Glc-II H-1) and δ_H 3.89 (Ara H-4), δ_H 4.54 (Xyl-I H-1) and δ_H 3.47 (Glc-II H-2), δ_H 4.34 (Xyl-II H-1) and δ_H 3.58 (Glc-II H-4).

Based on the above findings, the structure of compound **1** was elucidated as 3-*O*-β-{{[β-D-xylopyranosyl-(1→2)]-[β-D-xylopyranosyl-(1→4)]-β-D-glucopyranosyl-(1→4)}-[β-D-glucopyranosyl-(1→2)-]-α-L-arabinopyranosyl]}, protoprimulagenin A. This is a new triterpene saponin, trivially named nummularoside.

While two glucopyranose residues attached to the 2- and 4-positions of arabinose is a sugar chain core that was previously reported in fungicidal avenacin A-1 from oat root[37] and later in ca. twenty other triterpene saponosides out of over 1500 described in years 1996–2007, as reviewed by Dinda et al.,[38] the additional substitution of a terminal glucose at positions 2 and 4 is uncommon, as compared to glycosylation at C-2 and C-3[23,39,40] or C-6.[41,42] A five unit sugar moiety branched in such a way was identified in *Anagallis arvensis* (anagallosaponin I),[43] however the monosaccharides attached to the terminal glucose at positions 2 and 4 were xylose and glucose, respectively. So, to the authors' knowledge the structure of the oligosaccharidic chain in nummularoside has not been reported so far in a triterpene saponin.

Literature reports on cytotoxicity of saponins similar in structure[27,34] as well as promising in vitro activity of the methanol extract from the underground parts of *L. nummularia* L. (50% dead cells at 70 μg/mL against murine melanoma B16) urged us to evaluate in this study the properties of compound **1**, isolated from this extract, against a panel of human cancer cell lines and normal cells of respective origin. The cytotoxicity assay was performed on glioblastoma astrocytoma (U375), two melanoma cell lines differing in metastatic potential (BLM and A375), two prostate cancer cell lines differing in metastatic potential (DU-145, PC-3) as well as on human normal skin fibroblasts (HSF) and human normal prostate cell lines (PNT2). Compound **1** showed cytotoxic activity against all cancer cell lines tested (Table 2). It was most active against DU-145 cells with an EC_{50} value of 1.2 ± 0.2 μg/mL. It is worth noting that while significant activity towards both prostate cancer cell lines of medium and high metastatic potential was seen, the compound did not affect normal prostate cells PNT2 (EC_{50} = 30.0 ± 3.2 μg/mL).

1. Experimental

1.1. General

Optical rotation was measured in MeOH at 20 °C on a P-2000 polarimeter. IR spectra were recorded on a Nicolet iS5 spectrometer.

Table 1
^{1}H and ^{13}C NMR data (δ ppm) of compound **1** in MeOD[a,b]

Position	Aglycone		Position	Sugar moiety	
	δ_H	δ_C		δ_H (J in Hz)	δ_C
1	1.75–0.95	40.2	3-*O*-Ara		
2	1.85 m–1.75	27.2	1′	4.40 t (6.5)	105.6
3	3.14 dd (11.9; 4.6)	91.4	2′	3.80	79.5
4	—	40.6	3′	3.80	74.4
5	0.73 d (11.1)	56.8	4′	3,89	80.2
6	1.51–1.44 m	18.7	5′	3.54	65.7
				4.20 dd (12.4; 2.8)	
7	1.54 d (5.1)–1.23	35.2	At C-2′ Glc-I		
8	—	43.3	1″	4.70 d (7.7)	104.3
9	1.26 m	51.4	2″	3.20	75.9
10	—	37.8	3″	3.28	77.8
11	1.63 d (12.2)–1.47	19.8	4″	3.19	72.0
12	2.05–1.29	33.3	5″	3.66	78.0
13	—	88.4	6″	3.60	63.3
				3.85	
14	—	45.4	At C-4′ Glc-II		
15	2.10 dd (14.6; 5.4)–1.22	37.1	1‴	4.56 d (7.9)	104.6
16	3.89	77.9	2‴	3.47	84.5
17	—	45.4	3‴	3.71	75.7
18	1.51	52.4	4‴	3.58	79.9
19	2.38 dd (13.9; 12.1)–1.19	39.8	5‴	3.43	76.4
20	—	32.4	6‴	3.68	61.7
				3.87 dd (11.3; 2.2)	
21	2.06–1.15	37.4	At C-2‴ Xyl-I		
22	1.78–1.52	32.1	1^{IV}	4.54 d (7.6)	107.3
23	1.05 s	28.4	2^{IV}	3.25	76.0
24	0.85 s	16.7	3^{IV}	3.38	77.6
25	0.91 s	16.7	4^{IV}	3.52	70.9
26	1.14 s	18.8	5^{IV}	3.32	67.4
				3.99 dd (11.3; 5.4)	
27	1.23 s	19.9	At C-4‴ Xyl-II		
28	3.49 d (7.5)–3.12 d (7.5)	78.7	1^{V}	4.34 d (7.7)	105.2
29	0.95 s	33.9	2^{V}	3.20	74.9
30	0.91 s	24.9	3^{V}	3.56	77.5
			4^{V}	3.52	70.9
			5^{V}	3.26	67.1
				3.91 dd (11.5; 5.5)	

[a] Assignments were confirmed by DQF-COSY, TOCSY, ROESY, HSQC and HMBC.
[b] Overlapped signals are reported without designating multiplicity.

NMR spectra (^{1}H, ^{13}C, DEPT 135, ^{1}H–^{1}H DQF-COSY, ROESY, TOCSY, HMBC, HSQC) were measured on a Bruker AVANCE II 500 MHz and Bruker AVANCE III 600 MHz spectrometers using standard pulse sequences, in CD_3OD. ^{1}H were recorded at 500.13 and 600.20 MHz, ^{13}C at 125.77 and 150.94 MHz, respectively. All chemical shifts (δ) are given in ppm, and TMS was used as an internal standard. Coupling constants are reported in Hz. Spectra were analysed using MestRec magnetic resonance companion version 4.4 and CARA 1.8.4 (Computer Aided Resonance Assignment). FAB-MS spectra were recorded on a Finnigan MAT 95 mass spectrometer; glycerol as the matrix, Cs ions accelerated at 13 keV. HR-ESI-TOF-MS was obtained on a Waters SYNAPT G2-S HDMS spectrometer. Column chromatography was carried out on Merck Kieselgel 60 (70–230 mesh). Preparative TLC was performed on commercially precoated silica gel G plates (Analtech, 500 μ). Analytical TLC was carried out on Merck silica gel 60 aluminium plates and the spots were visualized by spraying with 5% sulfuric acid in MeOH followed by heating for 5–10 min at 105 °C.

1.2. Plant material

The underground parts of *Lysimachia nummularia* L. were collected from natural stands in southern Poland (near Cracow) in 2009 and were identified by dr Bożena Muszyńska PhD, Department of Pharmaceutical Botany, Jagiellonian University, Cracow, Poland. A voucher specimen (KFg/2009020) is deposited at the Department of Pharmacognosy, Jagiellonian University, Cracow, Poland.

1.3. Isolation

The air-dried plant material (350 g) was extracted with $CHCl_3$ (400 mL × 3) and next with MeOH with 0.5% pyridine (400 mL × 4). The combined methanolic extract was concentrated in vacuo, dissolved in water and eluted exhaustively with *n*-BuOH. The *n*-BuOH-soluble fraction, after evaporation of the solvent, was subjected to normal phase silica gel column chromatography using $CHCl_3$–MeOH–H_2O 23:12:2. Fractions were combined on the basis of TLC (SiO_2, $CHCl_3$–MeOH–H_2O 23:12:2; sulfuric acid + heating 5–10 min, 105 °C). Thus, six major fractions were obtained. Fraction III (containing mainly compound of R_f 0.32) was subjected to repeated preparative TLC using $CHCl_3$–MeOH–H_2O (23:12:2; 8:7:1) as solvents. Bands were removed from plates after spraying with distilled water, the silica was extracted with MeOH and solvent was evaporated in vacuo to yield **1** (37 mg).

1.4. Identification

3-*O*-β-{{[β-D-Xylopyranosyl-(1→2)]-[β-D-xylopyranosyl-(1→4)]-β-D-glucopyranosyl-(1→4)}-[β-D-glucopyranosyl-(1→2)-]-α-L-arabinopyranosyl]}, protoprimulagenin A (**1**, nummularoside):

White amorphous powder; mp 205 °C; $[\alpha]_D^{20}$ −3.96 (*c* 0.4, MeOH); IR (KBr); ν 3334 (OH), 2922 (CH), 1069 (C–O–C); HR-ESI-TOF-MS: *m*/*z* 1201, 60 $[M+Na]^+$; FAB-MS: *m*/*z* 1045 $[M-H-132]^-$, 913 $[M-H-132-132]^-$, 1015 $[M-162]^-$, 751 $[M-H-162-132-132]^-$; for ^{1}H and ^{13}C data see Table 1.

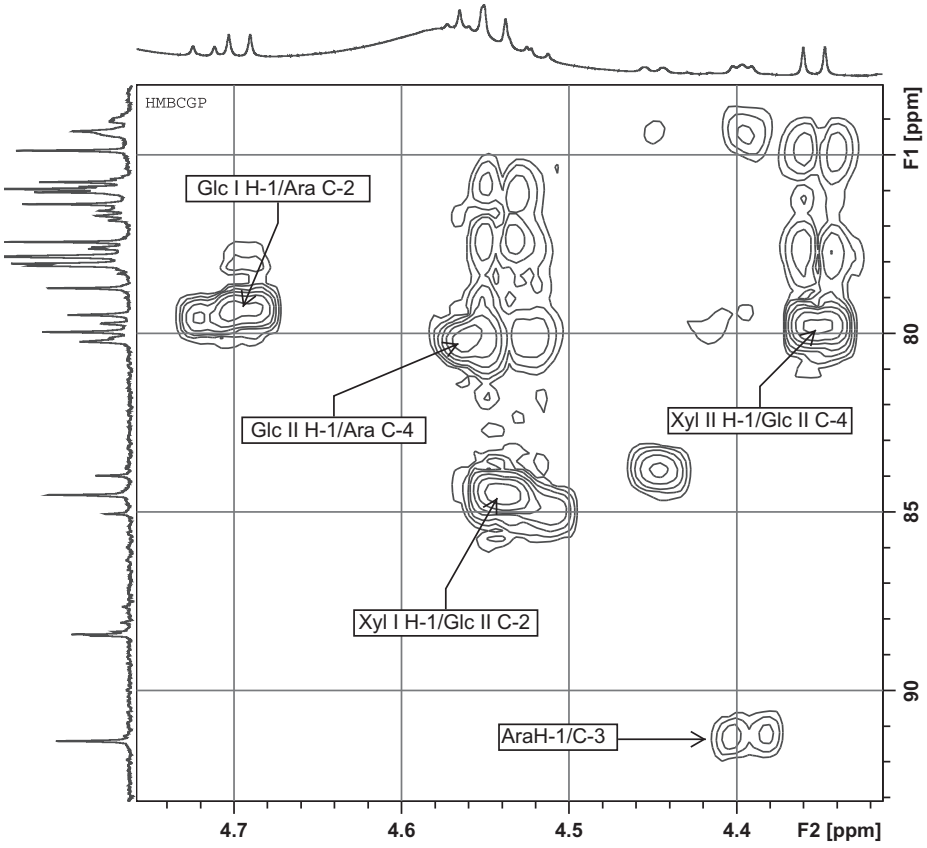

Figure 2. Detail (sugar region) of HMBC spectrum of compound **1**.

Table 2
In vitro cytotoxic activity of compound **1**

Cell line	Compound **1**[a] (EC_{50} μg/mL)	Mitoxantrone[b] (EC_{50} μg/mL)
Prostate panel:		
PC-3	7.4 ± 1.1*	2.0 ± 0.3*
DU-145	1.2 ± 0.3*	<5.0*
PNT2	30.0 ± 3.2	0.45 ± 0.12*
Skin panel:		
A-375	23.2 ± 1.2*	<5.0*
BLM	17.5 ± 1.6*	3.5 ± 0.5*
HSF	21.3 ± 1.8*	0.2 ± 0.04*
Glioblastoma U373	6.0 ± 1.3*	<5.0*

[a] The EC_{50} value is the concentration of compound that causes 50% cell death after 24 h of incubation. PC-3 = human prostate adenocarcinoma (high metastatic potential); DU-145 = human prostate carcinoma (medium metastatic potential); PNT2 = human normal prostate cells; A-375 = human malignant melanoma; BLM = human malignant melanoma (high metastatic potential); HSF = human skin fibroblasts; U373 = human glioblastoma astrocytoma.
[b] Positive control.
* Statistically significant.

1.5. Acid hydrolysis

1.5.1. Sugar identification

Total acid hydrolysis was performed on a TLC plate with gaseous HCl for 25 min according to procedure described by Janeczko et al.[44] Chromatograms were developed in a mobile phase $CHCl_3$–MeOH–H_2O 23:12:2 (twice) and visualized by spraying with aniline phthalate in *n*-BuOH followed by heating. R_f values were compared with authentic sugars. Thus, the presence of Glc (R_f 0.27), Ara (R_f 0.35) and Xyl (R_f 0.38) units was revealed.

1.5.2. Determination of absolute configuration

Absolute configuration was determined according to a slightly modified method, previously reported by Tanaka et al.[28] Compound **1** (5 mg) was dissolved in 1.0 mL of a mixture of 4 M HCl in dioxane and water (1:1; v/v) and was heated at 95 °C for 3 h. Then, 1.0 mL of water was added and the aglycone was extracted with ethyl acetate (3 × 3.0 mL). Subsequently, the aqueous phase was neutralized with 2 M ammonium hydride and concentrated under vacuum. After drying over P_2O_5 for 48 h, the residue was dissolved in anhydrous pyridine (1.0 mL) in an oven-dried screw-capped vial purged with argon. Then, L-cysteine methyl ester hydrochloride (5 mg) was added and the vial was purged with argon. The mixture was allowed to react at 60 °C for 1 h. Next, 5 μL of *o*-tolyl-isothiocyanate was added and the reaction mixture was heated at 60 °C for an additional 1 h. Samples of authentic sugars D-glucose, L-glucose, D-arabinose, L-arabinose, D-xylose and L-xylose were derivatized according to the same procedure.

Then, all samples were directly analysed by HPLC. The analysis was carried out on a Dionex apparatus equipped with a PDA 100 UV–vis detector, on a 250 × 4.6 mm i.d. Hypersil GOLD C-18 (Thermo EC) column at 35 °C with isocratic elution of 25% CH_3CN in 50 mM H_3PO_4 for 40 min, at a flow rate 1 mL/min, and subsequent washing for 15 min. The absolute configurations of the monosaccharides in **1** were confirmed to be D-glucose, L-arabinose and D-xylose by comparison of the retention times of monosaccharide derivatives with those of standard sugar samples: D-glucose (14.1 min), L-arabinose (15.0 min) and D-xylose (15.6 min), respectively.

1.6. Bioassay for cytotoxic activity

The human glioblastoma–astrocytoma cell line U375 was cultured in Eagle's minimum essential medium (EMEM) with 2 mM

L-glutamine, 1% non-essential amino acids (NEAA), 1 mM sodium pyruvate and 10% fetal bovine serum (FBS). Prostate cancer cells DU-145 and PC3 were cultured in Dulbecco's Modified Eagle's Medium (DMEM-F12 HAM) supplemented with 10% FBS. Malignant melanoma cell line A375 was cultured in DMEM high glucose medium supplemented with 10% FBS and 2 mM glutamine whereas cell line BLM was cultured in RPMI medium supplemented with 10% FBS. Normal prostate cells PNT2 were cultured in RPMI-1640 supplemented with 10% FBS. Human skin fibroblasts (HSF) were cultured in DMEM supplemented with 10% FBS. All reagents were from Sigma–Aldrich, St. Louis, MO, USA.

All cells were cultured in medium containing 100 i.u./mL penicillin, 10 μg/mL streptomycin and 10 μg/mL neomycin, in standard conditions, humidified atmosphere at 37 °C in the presence of 5% CO_2. Cells were seeded in 24-well plates at density 7.6×10^2 cells per well. After 24 h, the culture medium was replaced with the same medium containing different concentrations of **1** (from 0 up to 30 μg/mL). The controls, without tested compound, were incubated in the culture medium with 0.01% methanol. All cells were incubated at 37 °C for 24 h, and then cell viability was determined by the Trypan blue exclusion dye test and by the fluorescein diacetate and ethidium bromide test. In each experiment, for positive control, a standard drug mitoxantrone (Ebewe Pharma) was used, at concentrations from 0.1 to 1 μg/mL.

1.7. Statistical analysis

For each value measured, 300 cells were analysed. Each experiment was performed in triplicate. Each variable was expressed as the mean (±SD). The statistical significance was determined using Student's *t*-test or the non-parametric Mann–Whitney U-test, with $p < 0.05$ considered to indicate significant differences.

Acknowledgments

We acknowledge the financial support of the Grant No. N405 297636 from the Ministry of Science and Higher Education, Poland. We wish to thank dr Bożena Muszyńska for identification of the plant material and dr Edward Szneler for recording the NMR spectra.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carres.2013.04.005.

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