

A Major Saponin from Leaves Extract of *Acer velutinum*

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A major triterpenoid saponin was isolated from the leaves extract of *Acer velutinum*. This compound was characterized as 21 β ,22 α -*O*-diangeloylprotoaescigenin 3-*O*-[β -D-glucopyranosyl-(1 \rightarrow 2)][β -D-glucopyranosyl-(1 \rightarrow 4)]- β -D-glucuronopyranosyl acid.

Its structure was elucidated by ¹H, gCOSY, ROESY, gHSQC, gHMBC NMR as well as ESI-MS experiments. Isolated saponin exhibited *in vitro* cytotoxicity against HL-60, B16-F0 and BALB/3T3 cell lines.

Key words: *Acer velutinum* (Boiss.), Aceraceae, Triterpenoid Saponin, Protoaescigenin, Cytotoxicity

Introduction

The family Aceraceae consists of two genera, *Dipteronia* and *Acer*, with approximately two and 180 species, respectively. The species of *Dipteronia* are endemic to China whereas the species of *Acer* are more widely distributed [1]. Maples (*Acer* L.) are one of the most important trees in the Northern Hemisphere, particularly in the temperate regions of eastern Asia, eastern North America and Europe. The most recent survey of *Acer* listed 156 species, including more than 20 recently described taxa [2].

Triterpenoid saponins of *Acer negundo* [3] were isolated as active antitumor compounds, and also an HIV-1 integrase inhibitor was obtained from *Acer okamotoanum* [4]. Some studies have revealed extracts from *Acer* sp. to have protective effects against X-ray-induced skin injuries and carbon tetrachloride-induced liver injuries [5, 6]. Antileukemic activity of water extracts and anti-inflammatory activity were also described [7, 8]. It is also interesting that some *Acer* extracts show antidiabetic activity [9]. Many species are also important sources of commercial products, for example syrup from *Acer saccharum* Marshall and timber from *A. saccharum*, *A. rubrum* L., and *A. pseudo-platanus* L. [2].

We present in this report the isolation and structure elucidation of a novel triterpenoid saponin from leaves of *A. velutinum* that shows cytotoxic activity. Except for our previous investigation [10], there were no other phytochemical studies on saponins of this species, and so far only the isolation of saponins from genus *Acer* (*Acer ginnala*) have been described [11]. In the paper dealing with a saponin from *Acer negundo* [3] the authors describe the structure of aglycones. Thus it is the first time that the full structure of a saponin from *Acer velutinum* is presented.

Materials and Methods

Plant material

The leaves of *A. velutinum* (Boiss.) were collected from the Arboretum of Warsaw Agricultural University (Rogów, Poland) in June 2003. Plant identification was confirmed by the Head of Arboretum MSc P. Banaszczyk. The herbarium voucher specimen was deposited at the Department of Pharmacognosy, Wrocław University of Medicine.

General procedures

The ¹H, gCOSY, ROESY, gHSQC, gHMBC NMR experiments were run under standard conditions on a Bruker DRX-600 spectrometer at 300 K. The ROESY spectrum

Table 1. ^{13}C and ^1H NMR spectroscopic data of acylated triterpenoid acersaponin I (CD_3OD ; δ in ppm, J in Hz in parentheses)^a.

	δ_{C}	δ_{H} (ppm)		δ_{C}	δ_{H} (ppm)
Protoaescigenin			21-Angeloyl		
1	39.0	1.65, 1.03, <i>m</i>	1	169.0	–
2	26.8	2.07, 1.85, <i>m</i>	2	129.0	–
3	92.2	3.42, <i>dd</i> (11.3, 4.2)	3	138.6	6.09, <i>qq</i> (7.3, 1.5)
4	44.1	–	4	15.9	1.94, <i>dq</i> (7.3, 1.5)
5	57.0	0.98, <i>m</i>	5	20.6	1.85, <i>q'</i> (1.5)
6	19.2	1.66, 1.39, <i>m</i>	22-Angeloyl		
7	33.7	1.65, 1.40, <i>m</i>	1	169.5	–
8	40.7	–	2	129.0	–
9	47.9	1.70, <i>m</i>	3	139.4	6.10, <i>qq</i> (7.4, 1.5)
10	37.3	–	4	15.9	1.96, <i>dq</i> (7.4, 1.5)
11	25.0	1.94, <i>m</i>	5	20.6	1.86, <i>q'</i> , (1.5)
12	125.0	5.41, <i>brt</i> (2.9)	glcA I (C-3)		
13	143.0	–	1	104.6	4.55, <i>d</i> , 7.8
14	42.2	–	2	<u>79.7</u> ^b	3.65, <i>dd</i> , 8.5, 7.8
15	34.5	1.72, 1.40, <i>m</i>	3	77.0	3.82, <i>t</i> , 8.5
16	69.5	4.02, <i>brs</i>	4	<u>82.5</u> ^b	3.70, <i>t</i> , 8.5
17	48.7	–	5	77.0	3.82, <i>d</i> , 8.8
18	40.9	2.69, <i>m</i>	COOH		
19	47.7	2.71, 1.22, <i>m</i>	glc II (C-2 glcA I)		
20	36.4	–	1	103.8	4.87, <i>d</i> , 8.5
21	79.8	6.02, <i>d</i> (10.1)	2	75.2	3.21, <i>dd</i> , 9.3 8.5
22	74.1	5.61, <i>d</i> (10.1)	3	77.6	3.39, <i>t</i> , 9.3
23	22.4	1.25, <i>s</i>	4	69.9	3.49, <i>t</i> , 9.3
24	63.9	4.14 3.24, <i>d</i> (11.7)	5	77.9	3.22, <i>m</i>
25	16.2	0.92, <i>s</i>	6	61.3	3.78, 3.78
26	16.7	0.95, <i>s</i>	glc III (C-4 glcA I)		
27	27.8	1.52, <i>s</i>	1	104.2	4.46, <i>d</i> , 7.7
28	64.3	3.29 2.98, <i>d</i> (11.3)	2	74.8	3.28, <i>dd</i> , 9.2 7.7
29	29.7	0.90, <i>s</i>	3	77.6	3.40, <i>t</i> , 9.2
30	20.2	1.12, <i>s</i>	4	70.9	3.33 <i>nd</i>
			5	77.9	3.34, <i>m</i>
			6	62.1	3.90, 3.69, 12.3 <i>nd</i>

^a *nd*, not determined; ^b substituted carbons are underlined.

was acquired with $t_{\text{mix}} = 400$ ms. The NMR sample was prepared by dissolving the saponin in CD_3OD (Sigma-Aldrich, 99.96 % D). The spectra were calibrated using the solvent signal as internal standard (^1H , $\delta = 3.34$ ppm; ^{13}C , $\delta = 49.0$ ppm). The NMR data were processed on a Silicon Graphics Indigo2 Workstation using UXNMR software.

The high-resolution mass spectra were obtained on a Bruker MicrOTOF-Q spectrometer (Bruker Daltonik, Germany), equipped with an Apollo II electrospray ionization source with ion funnel and operated in the negative and positive ion mode. The sample dissolved in 50:50 CH_3CN - H_2O containing 10 mM NH_4HCO_3 (negative ion mode) or CH_3OH containing 0.1 mM NaCl (positive ion mode) was infused at a flow rate of $3 \mu\text{L min}^{-1}$. The concentration of the sample was 0.05 mg mL^{-1} . Before each run the instrument was calibrated externally with the TunemixTM mixture (Bruker Daltonik, Germany) in quadratic regression mode. MS^2 spectra were obtained in negative ion mode for the par-

ent ion ($[\text{M}-\text{H}]^-$) at $m/z = 1169.6$ applying a collision energy of 45 eV. Optical rotation was measured on a Rudolph Autopol IV polarimeter (Rudolph Analytical, Hackettstonn, USA) in MeOH at 25°C .

Analytical HPLC conditions

The apparatus used for HPLC consisted of a Knauer Smartline series (Knauer, Berlin, Germany), a quaternary pump with vacuum degasser, and a Smartline PDA detector 2800. Separation was achieved on a Hypersil (Thermo Fisher Scientific Inc., MA, USA) Gold C-18 column ($250 \times 4.6 \text{ mm}^2$) of $5 \mu\text{m}$ particle size. The isocratic elution system consisted of aqueous 0.1 % formic acid and methanol + 0.1 % formic acid (35:65, v/v). The flow rate was set at 1.0 mL min^{-1} with UV/Vis/NIR detection at 190–1020 nm. The operating temperature was maintained at 25°C . The mobile phases were degassed by sonication before utilization.

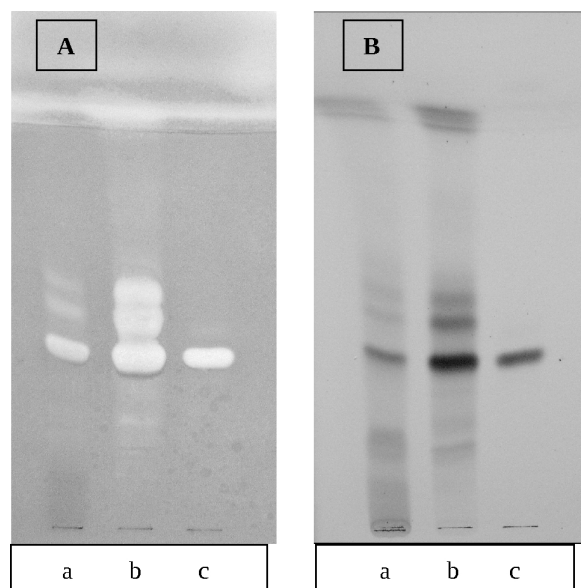


Fig. 1. TLC chromatograms of *Acer velutinum* saponins sprayed with water (A) and with 10 % sulfuric acid in MeOH (B); a) 80 % MeOH extract of *Acer velutinum* leaves; b) 80 % MeOH extract of *Acer velutinum* leaves after SPE purification; c) acersaponin I.

Clean-up with SPE, RP-18 column

For HPLC analysis the 80 % MeOH extract was purified by the SPE method. An SPE C18 Bakerbond cartridge was conditioned by passing through 10 mL of MeOH followed by 10 mL of H₂O. Thus after conditioning the SPE column the 80 % MeOH extract (10 mL) that was diluted earlier with water was applied on the column. After sample application the column was successively rinsed with 10 mL water, 40 % MeOH, 80 % MeOH, and finally with MeOH. After TLC inspection the eluate that was collected with 80 % MeOH was analyzed by HPLC in order to find the best eluting solvent for the saponin mixture.

TLC conditions

TLC was carried out on silica gel (Si 60 F₂₅₄, Merck) plates using the mobile system chloroform-methanol-water-formic acid (10:4:1:0.95, v/v/v/v). The spot was detected by spraying with water and 10 % H₂SO₄ in MeOH and heating at 120 °C for spot visualization.

Extraction and isolation

Air-dried and powdered leaves (500 g) of *A. velutinum* were extracted twice with 80 % MeOH (3 L) at r.t. The extracts were combined, and the methanol was evaporated under reduced pressure. The aqueous residue was passed

through a porous polymer gel column (Diaion HP-20, Supelco). The column was then eluted with water, 30 %, 40 %, 70 %, and 100 % MeOH. About 1 L of the 70 % MeOH eluates were combined, and the material from the combined fractions (170 mg dry weight) was chromatographed on a silica gel column eluting with CHCl₃-MeOH-H₂O (7:3:1-lower phase) to give 6 similar saponin fractions examined by TLC. From these fractions, after repeated HPLC [column, LiChrospher® 100, RP-18 (10 μm) 250 × 10 mm; solvent, CH₃OH-H₂O (65:35 – 100:0); flow rate, 6.0 mL/min; detection, UV 196 nm] the acersaponin I was obtained (12 mg).

Acersaponin I

Amorphous powder; $R_f = 0.3$ (TLC, Fig. 1); $R_t = 13.0$ (HPLC, Fig. 2). – $[\alpha]_D^{25} = -15$ ($c = 0.1$, MeOH). – HRMS ((–)-ESI): $m/z = 1169.5679$ (calcd. 1169.5749 for C₅₈H₈₉O₂₄, [M–H][–]). – ¹H and ¹³C NMR: see Table 1. – UV spectra: see Fig. 3.

Evaluation of cytotoxic potential

Cells

Human promyelocytic leukemia cells (HL-60) were maintained in RPMI 1640 supplemented by 10 % heat-inactivated fetal bovine serum (FBS). Melanoma (B16-F0) cells were cultured in RPMI 1640 : Opti-MEM (1:1) supplemented by 5 % FBS. Mouse embryonic fibroblasts (BALB/3T3) were used as reference cell line and cultured in Dulbecco supplemented by 10 % FBS. The cells were grown in appropriate medium at 37 °C in 5 % CO₂ and a humidified air atmosphere. All lines were obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured in the Cell Culture Collection of the Institute of Immunology and Experimental Therapy.

Medium

RPMI 1640 and Dulbecco complete medium containing 100 μg/mL streptomycin, 100 U mL^{–1} penicillin, 2 mM L-glutamine, 1 mM sodium pyruvate and 4.5 g L^{–1} D-glucose (for HL-60, B16-F0) were used throughout the study.

Cytotoxic assay

All cells in the exponential growth phase were placed in 96-well flat-bottom microplates at a density of 1.0×10^5 cells per 100 μL in each well and grown for 24 h in appropriate medium. After that, 100 μL of fresh medium with various concentrations of test compounds was added. After 72 h of culture, cell growth was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method or the sulforhodamine B (SRB) method, respectively.

Saponin was dissolved in DMSO at a concentration of 1 mg per 100 μL and afterwards diluted with a nutrient

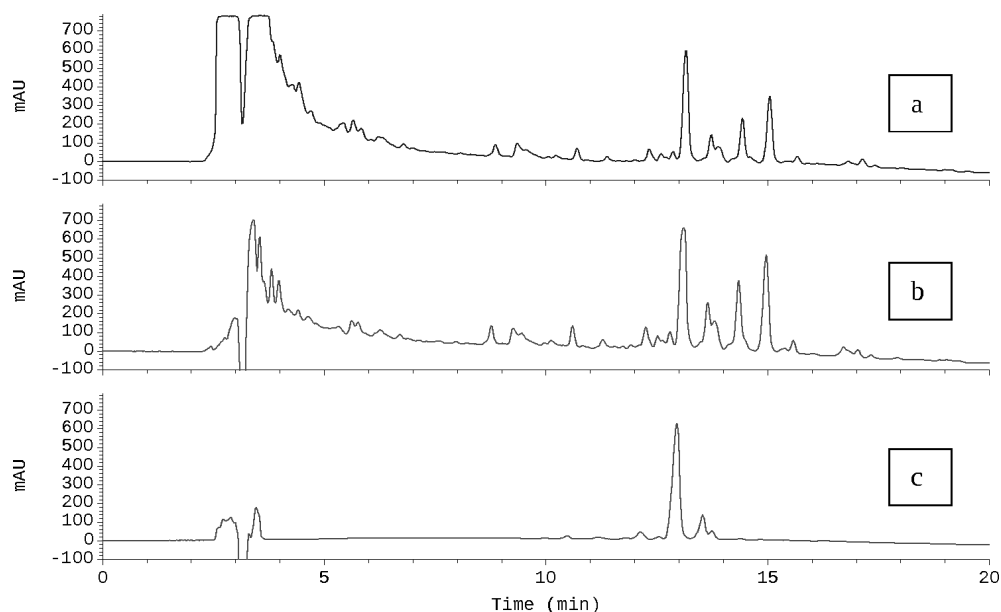


Fig. 2. HPLC chromatograms of *Acer velutinum* saponins recorded at 196 nm; a) 80 % MeOH extract of *Acer velutinum* leaves; b) 80 % MeOH extract of *Acer velutinum* leaves after SPE purification; c) acersaponin I.

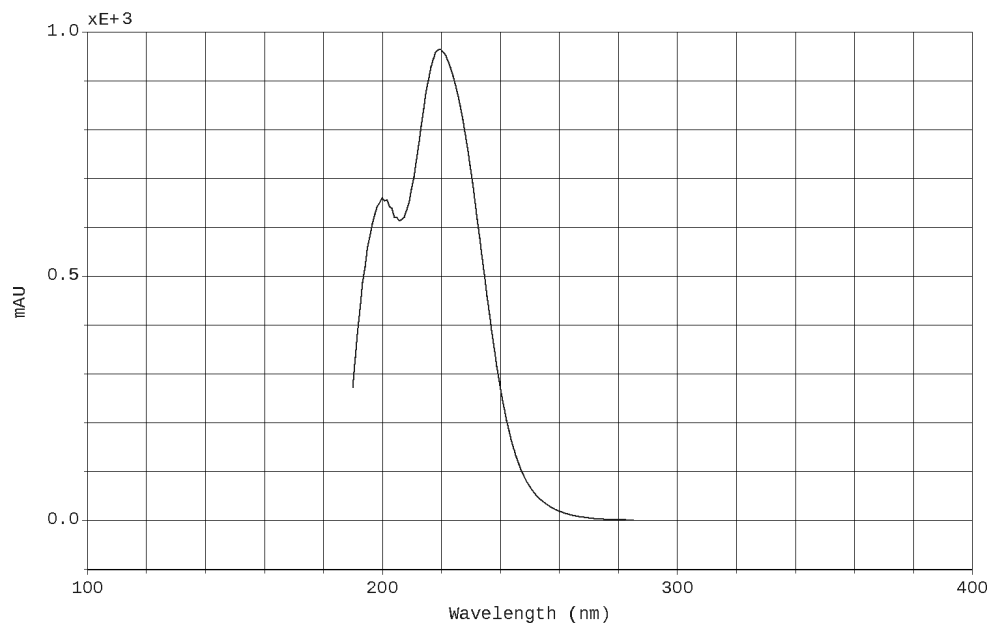


Fig. 3. UV spectrum of acersaponin I isolated from *Acer velutinum* with a HPLC-PDA detector.

medium and applied to target cells to obtain final concentrations ranging from $0.1 - 100 \mu\text{g mL}^{-1}$.

The final concentrations of DMSO (0.001–0.01 % in complete medium) did not influence the cell growth of the cell lines. Each experiment was repeated 3–5 times in 3 replicates. Only nutrient medium was added to the cells

in the control wells with corresponding concentrations of DMSO. The nutrient medium with corresponding concentrations of compounds, but without cells, was used as the blank reference.

As a reference substance non-alkaloid standard podophyllotoxin was used (Aldrich, USA).

Results and Discussion

The dried leaves of *A. velutinum* were extracted twice with 80 % aqueous methanol. The extracts were combined and methanol distilled off under reduced pressure. The aqueous crude saponin extract was subjected to column chromatography on a porous polymer polystyrene resin (Diaion HP-20) and silica gel followed by HPLC to give a novel saponin. The NMR data of the isolated saponin are shown in Table 1.

A sample of acersaponin was studied by ESI-MS. In the positive ion mode the ESI-MS gave a peak at $m/z = 1193.6$ corresponding to the pseudo-molecular ion $[M+Na]^+$ while in the negative ion mode a peak at $m/z = 1169.6$ ($[M-H]^-$) was detected. The molecular formula of the compound established by HRMS ((-)-ESI) was $C_{58}H_{90}O_{24}$ ($m/z = 1169.5679$; calcd. 1169.5749 for $C_{58}H_{89}O_{24}$, $[M-H]^-$). In the MS² experiment the parent ion $[M-H]^-$ at $m/z = 1169.6$ gave the following fragments: 1125.7 $[M-CO_2-H]^-$, 1007.6 $[M-Hex-H]^-$, 989.6 $[M-Hex-H_2O-H]^-$, 963.6 $[M-Hex-CO_2-H]^-$, 945.6 $[M-Hex-CO_2-H_2O-H]^-$, 845.6 $[M-2Hex-H]^-$, 809.6 $[M-2Hex-2H_2O-H]^-$, and 669.5 $[M-2Hex-GlcA-H]^-$. The fragmentation suggested that the saponin contained two hexose moieties (Hex, the neutral loss 162 Da), and a glucuronic acid unit (GlcA, the neutral loss 176 Da). The neutral loss of CO₂ from the glycone part of the saponin gave additional confirmation of the presence of glucuronic acid. The molecular weight of the aglycone was 670.5. The general pattern of fragmentation for the acersaponin was similar to that described previously by Ha [12] and Huhman [13].

The presence of protoaescigenin as sapogenin in the structure of acersaponin I was deduced from the analysis of its ¹H and ¹³C NMR spectra (Table 1). The ¹H NMR spectrum showed for the aglycone signals characteristic of an olean-12-ene derivative. Besides, the deshielded vicinal H-21 and H-22 protons at $\delta = 6.02$ and 5.61 ppm, respectively, suggested that these positions were esterified. This consideration was confirmed by HMBC correlations observed between H-21 ($\delta_H = 6.02$) and the carbonyl C ($\delta_C = 169.0$) of the first angeloyl group, and between H-22 ($\delta_H = 5.61$) and the carbonyl C ($\delta_C = 169.5$) of the second angeloyl group.

The configuration of the stereogenic centers C-21 and C-22 were ascertained by observation of

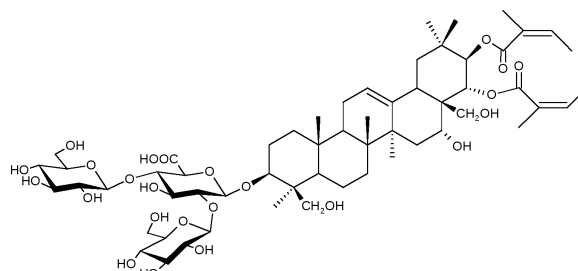


Fig. 4. Structure of acersaponin I isolated from *Acer velutinum*.

ROESY cross-peaks between H18-Me30, H18-H22, and H-21-Me29. The identification of the two angeloyl groups was also deduced from the presence of ROESY cross-peaks between H-3-Me5 for each angeloyl unit. This prosapogenin was already previously reported in *Harpullia ramiflora* [14], in *Harpullia austro-caledonica* [15], in *Aesculus chinensis* [16], and in *Aesculus pavia* [17]. Regarding the sugar portion in the HSQC spectrum, NMR data showed that the compound contained three monosaccharides, which were identified as one β -D-glucuronopyranosyl and two β -D-glucopyranosyl residues. The glucuronic acid and the two glucose units were determined to be in the pyranose form with a β -anomeric configuration from their ¹³C NMR spectroscopic data and ³J_{H1,H2} coupling constants (Table 1).

The attachment of the GlcA unit to C-3 of the aglycone was ascertained on the basis of the following HMBC correlations: H-3 ($\delta_H = 3.42$) of the aglycone and C-1 ($\delta_C = 104.6$) of GlcA, and H-1 ($\delta_H = 4.55$, d , 7.8 Hz) of GlcA with the downfield-shifted C-3 ($\delta_C = 92.2$) of the aglycone. An unambiguous determination of the linkage sites of the two glucoses to C-2 and C-4 of the GlcA unit was established by the following HMBC correlations: H-2 ($\delta_H = 3.65$) of GlcA with C-1 of Glc, H-1 ($\delta_H = 4.87$, d , $J = 8.5$ Hz) of Glc with C-2 of GlcA, H-4 ($\delta_H = 3.70$) of GlcA with C-1 of Glc, and H-1 of Glc ($\delta_H = 4.46$, d , $J = 7.7$ Hz) with C-4 of GlcA.

Thus, the structure of the new compound was established as 21 β ,22 α -O-diangeloylprotoaescigenin 3-O- $[\beta$ -D-glucopyranosyl-(1 \rightarrow 2)][β -D-glucopyranosyl-(1 \rightarrow 4)]- β -D-glucuronopyranosyl acid (Fig. 4).

Acersaponin I isolated from *Acer velutinum* differs from aesculioside D found in *Aesculus chinensis* by the presence of the two angeloyl instead of one angeloyl and one tigloyl groups. These findings indicate

a possible chemotaxonomic relationship between the two genera *Acer* and *Aesculus*, however, further investigations are needed.

The evaluation of the biological activity showed that acersaponin I is cytotoxic for HL-60, B16-F0 and BALB/3T3 cell lines *in vitro*. The IC₅₀ values

[$\mu\text{g mL}^{-1}$] were 30.79 ± 0.82 (0.026 ± 0.0007 mM), 32.09 ± 0.24 (0.027 ± 0.0002 mM) and 3.88 ± 0.71 (0.003 ± 0.0005 mM), respectively. The reference standard podophyllotoxin showed biological activity in this model against HL-60 cell lines at IC₅₀ at 0.035 ± 0.005 ng mL⁻¹.

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