Grayanane Diterpenoids from the Fruits of *Pieris formosa*

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Four new acylated grayanane diterpenoids, pierisformosins E–H (**1** – **4**), together with two known ones, pierisformosins B and C (**5**, **6**), were isolated from the fruits of *Pieris formosa*. The structures and relative configurations of the new compounds were elucidated by means of spectroscopic methods, including 1D and 2D NMR and mass spectrometry.

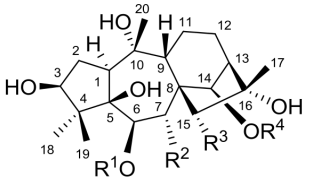
Key words: Grayanane Diterpenoids, *Pieris formosa*, Ericaceae

Introduction

Many species of the family Ericaceae, such as the plants belonging to the genera *Pieris*, *Rhododendron*, *Lyonia*, *Kalmia*, and *Leucothoe*, are usually rich in grayanane-type diterpenoids with a 5/7/6/5 ring system, which is responsible for the toxicity of these species [1, 2]. Recently, a series of novel highly acylated diterpenoids with specific 3,4-secograyanane skeleton have been reported from *Rhododendron molle* and *Pieris formosa* by Jian-Gong Shi and coworkers, Sheng-Hong Li and coworkers and our group [3 – 6]. In searching for new natural compounds with more new skeletons, we had investigated the leaves and stems of *Lyonia ovalifolia*, and obtained a novel polyesterified diterpenoid with a unique 9,10-*seco* grayanane skeleton, named lyonin A [7]. This finding made us interested in the biosynthetic pathway of *seco*-grayanane diterpenoids, and we further investigated the chemical constituents, especially diterpenoids, of the fruits of *Pieris formosa*. In the course of this study, four new acylated grayanane diterpenoids with 5/7/6/5 skeletons, named pierisformosins E–H (**1** – **4**), together with two known ones, pierisformosins B and C (**5**, **6**) [8], were isolated (Fig. 1). The isolation and structure elucidation of these four new compounds are described in this paper.

Results and Discussion

Compound **1**, obtained as a colorless amorphous powder, had the molecular formula $C_{27}H_{42}O_{11}$ as inferred from its HR-ESIMS (m/z = 541.2663, [M–H]$^-$). Two methyl groups [δ_H = 1.96 (s), δ_C = 21.4 (q), δ_H = 2.25 (s), δ_C = 21.7 (q)], belonging to two acetoxy groups, were observed in the 1D NMR spectrum of **1** (Tables 1 and 2). In addition, the presence of a methyl group [δ_H = 1.08 (t, J = 7.2 Hz), δ_C = 9.1 (q)] and a methylene unit [δ_H = 2.32 – 2.40 (m), δ_C = 28.3 (t)] suggested the presence of a propionyl unit. Aside from



1 R^1 = Ac, R^2 = OAc, R^3 = OH, R^4 = propionyl
2 R^1 = R^4 = Ac, R^2 = *O*-propionyl, R^3 = OH
3 R^1 = propionyl, R^2 = OAc, R^3 = OH, R^4 = Ac
5 R^1 = R^2 = R^3 = H, R^4 = propionyl
6 R^1 = R^3 = H, R^2 = OH, R^4 = propionyl

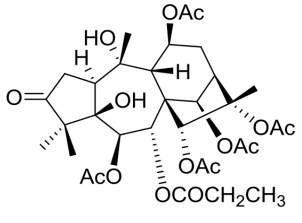


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Fig. 1. Structures of compounds **1** – **6**.

Table 1. ^{1}H NMR data of compounds **1** – **3** in [D$_5$]pyridine[a].

Position	**1**	**2**	**3**
1	4.38 (br. s)	4.40 (br. s)	4.42 (br. d, 3.4)
2	2.77 (overlapped)	2.72 (m)	2.80 (m)
	2.57	2.57	2.59
	(dd, 14.8, 5.9)	(dd, 14.8, 5.7)	(dd, 14.8, 5.9)
3	4.06 (br. s)	4.06 (br. s)	4.09 (br. s)
3-OH	6.84 (s)	6.79 (s)	7.05 (s)
5-OH	5.36 (s)	5.36 (s)	5.44 (s)
6	5.95 (br. s)	5.94 (br. s)	5.98 (br. s)
7	6.98 (br. s)	7.00 (br. s)	7.05 (br. s)
9	2.98 (br. s)	3.00 (br. s)	3.01 (br. s)
10-OH	5.75 (s)	5.73 (s)	5.88 (s)
11	2.32 (m)	2.48 (m)	2.27 (m)
	1.75 (m)	1.84 (m)	1.80 (m)
12	2.54 (m)	2.50 (m)	2.54 (overlapped)
	1.69 (m)	1.67 (m)	1.71 (overlapped)
13	2.77 (br. s)	2.82 (br. s)	2.84 (br. s)
14	6.32 (s)	6.25 (s)	6.34 (s)
15	4.11 (s)	4.11 (s)	4.15 (s)
15-OH	5.49 (s)	5.46 (s)	5.53 (d, 4.0)
16-OH	6.03 (s)	6.10 (s)	6.18 (s)
17	1.62 (s)	1.62 (s)	1.67 (s)
18	1.00 (s)	0.98 (s)	1.01 (s)
19	1.42 (s)	1.42 (s)	1.45 (s)
20	1.96 (s)	1.96 (s)	2.00 (s)
6-OAc/Pro	1.96 (s)	2.21 (s)	1.34 (t, 7.5)
			2.68 (overlapped)
			2.49 (overlapped)
7-OAc/Pro	2.22 (s)	1.10 (t, 7.1)	1.98 (s)
		2.46 (m)	
14-OAc/Pro	1.08 (t, 7.2)	2.00 (s)	2.04 (s)
	2.32 – 2.40 (m)		

[a] ^{1}H NMR spectra of **1** and **2** were recorded at 600 MHz, the spectrum of **3** at 500 MHz; δ in ppm, multiplicities and J (Hz) in parentheses.

Table 2. ^{13}C NMR data of compounds **1** – **3** in [D$_5$]pyridine[a].

Position	**1**	**2**	**3**
1	49.0 (d)	49.0 (d)	49.5 (d)
2	34.3 (t)	34.4 (t)	34.7 (t)
3	81.1 (d)	81.2 (d)	81.5 (d)
4	50.8 (s)	50.5 (s)	51.3 (s)
5	83.6 (s)	83.6 (s)	84.0 (s)
6	67.7 (d)	67.8 (d)	68.1 (d)
7	70.7 (d)	70.5 (d)	71.0 (d)
8	57.2 (s)	57.2 (s)	57.6 (s)
9	49.7 (d)	49.6 (d)	50.1 (d)
10	78.9 (s)	78.8 (s)	79.3 (s)
11	20.9 (t)	20.9 (t)	21.4 (t)
12	26.3 (t)	26.3 (t)	26.8 (t)
13	50.8 (d)	50.5 (d)	51.1 (d)
14	83.1 (d)	83.6 (d)	83.7 (d)
15	87.0 (d)	87.0 (d)	87.4 (d)
16	78.9 (s)	78.8 (s)	79.2 (s)
17	23.5 (q)	23.4 (q)	24.0 (q)
18	23.3 (q)	23.4 (q)	23.8 (q)
19	17.0 (q)	16.9 (q)	17.5 (q)
20	27.8 (q)	27.9 (q)	28.2 (q)
6-OAc/Pro	21.4 (q)	21.5 (q)	9.7 (q)
	169.7 (s)	169.9 (s)	28.3 (t)
		173.7 (s)	
7-OAc/Pro	21.7 (s)	9.1 (q)	22.1 (q)
	169.9 (s)	28.4 (t)	170.3 (s)
		173.0 (s)	
14-OAc/Pro	9.1 (q)	21.7 (q)	22.1 (q)
	28.3 (t)	170.3 (s)	170.7 (s)
	173.5 (s)		

[a] ^{13}C NMR spectra of **1** and **3** were recorded at 125 MHz, the spectrum of **2** at 150 MHz; δ in ppm.

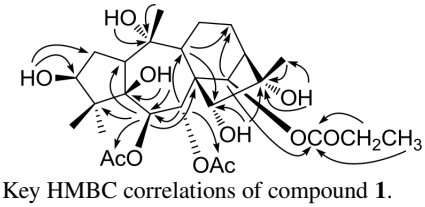

Fig. 2. Key HMBC correlations of compound **1**.

seven carbon atoms for two *O*-acetyls and one *O*-propionyl unit, the ^{13}C NMR and DEPT spectral data revealed the presence of four methyls, three methylenes, eight methines (five oxygenated ones), and five quaternary carbons (three of them oxygenated). The ^{1}H-^{1}H COSY experiment revealed the following fragments: $CHCH_2$-CH(OH)-, CH(OR)-CH(OR)-, and $CHCH_2CH_2$CH-CH(OR)-, which were all connected to quaternary carbon atoms at one or both ends. The above data suggested that compound **1** was a trisacylated grayanane diterpenoid with a 5/7/6/5 skeleton.

The spectra of **1** showed close similarities to two known grayanoids, pierisformosins B and C (**5**, **6**) [8]. Comparing the ^{13}C NMR spectral data of **1** with those of **5** and **6** suggested that **1** also had 3-, 5-, 10- and 16-hydroxyl units like **5** and **6**. These assignments were further confirmed by an HMBC experiment (Fig. 2), in which the correlations from 3-OH [δ_H = 6.84 (s)] to C-2 and C-3, from 5-OH [δ_H = 5.36 (s)] to C-5 and C-6, from 10-OH [δ_H = 5.75 (s)] to C-10, and from 16-OH [δ_H = 6.03 (s)] to C-16 and C-17, were observed. In the HMBC spectrum, the signal at δ_H = 595 (br. s H-6) showed cross-peaks with C-1, C-4, C-5, C-7, C-8, and one acetyl carbonyl carbon at δ_C = 169.7 (s), and the signal at δ_H = 698 (H-7, br. s) correlated with C-5, C-6, C-8, C-9, and another acetyl carbonyl carbon [δ_C = 169.9 (s)] demonstrating that the C-6 and C-7 hydroxyl groups were acetylated. The HMBC correlations from the signal at δ_H = 6.32 (s, 1H) to C-8, C-9, C-12, C-15, C-16, and the propionyl carbonyl at δ_C = 173.5 (s) assigned it as H-14. Furthermore, the location of the last hydroxyl group

Position	^{1}H NMR	^{13}C NMR	Position	^{1}H NMR	^{13}C NMR
1	3.49 (dd, 12.8, 7.7)	45.5 (d)	16		89.7 (s)
2	3.00 (dd, 18.2, 12.8)	37.5 (t)	17	1.79 (s)	22.7 (q)
	2.80 (dd, 18.2, 7.7)		18	1.26 (s)	21.7 (q)
3		219.2 (s)	19	1.52 (s)	19.5 (q)
4		58.0 (s)	20	2.16 (s)	28.6 (q)
5		81.3 (s)	6-OAc	2.13 (s)	20.7 (q)
5-OH	6.56 (s)				169.4 (s)
6	6.03 (d, 8.6)	74.2 (d)	7-OPr	1.12 (t, 7.5)	8.9 (q)
7	6.92 (d, 8.6)	66.2 (d)		2.58 (m)	27.9 (t)
8		53.1 (s)		2.46 (overlapped)	173.3 (s)
9	2.44 (overlapped)	61.6 (d)	11-OAc	2.15 (s)	21.0 (q)
10		74.4 (s)			169.6 (s)
10-OH	6.66 (s)		14-OAc	2.21 (s)	21.8 (q)
11	6.25 (br. d, 3.4)	68.1 (d)			170.2 (s)
12	2.50 (m)	31.9 (t)	15-OAc	2.16 (s)	21.9 (q)
	2.24 (m)				171.0 (s)
13	3.26 (d, 9.2)	44.9 (d)	16-OAc	2.03 (s)	22.4 (q)
14	6.77 (s)	78.5 (d)			169.8 (s)
15	5.68 (s)	88.5 (d)			

Table 3. ^{1}H and ^{13}C NMR data of compound **4** in [D_5]pyridine[a].

[a] The ^{1}H NMR spectrum was recorded at 400 MHz, the ^{13}C NMR spectrum at 100 MHz; δ in ppm, multiplicities and J (Hz) in parentheses.

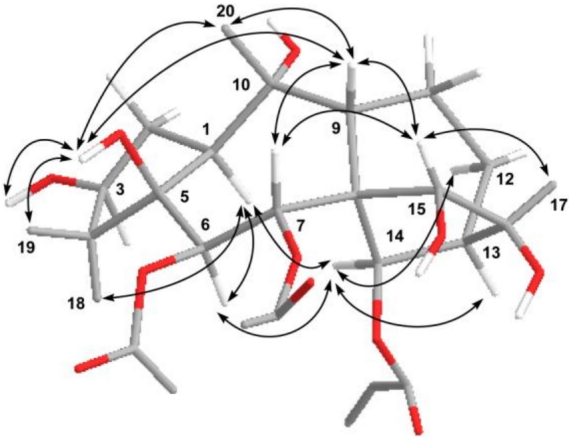

Fig. 3 (color online). Key ROESY correlations of compound **1**.

in **1** was deduced to be at C-15 because this extra oxygenated proton signal at δ_H = 5.49 (s) showed HMBC cross-peaks with C-15 and C-16. This deduction was confirmed by the HMBC correlation between H-9 and C-15.

The relative stereochemistry of **1** was mainly established using information from a ROESY spectrum (Fig. 3) and by comparison of its spectroscopic data to those of **5** and **6**. The ROESY correlations between H-1 with H-6, H-14, and Me-18, as well as between H-14 with H-6, H-12α, and H-13, indicated that H-1, H-6, H-13 and H-14 all possessed α-orientation. In addition, the presence of NOE correlations between 5-OH with Me-19, Me-20 and H-9, between H-15 with Me-17 and H-9, between H-9 with Me-20 and H-15, and between H-7 with H-9 and H-15, revealed that 5-OH, H-7, H-9, H-15, Me-17 and Me-20 were all β-directed. H-3 was assigned to be α-oriented based on the NOE correlation between 3-OH and 5-OH. Consequently, the structure of **1** was established doubtlessly and named pierisformosin E.

Both compounds **2** and **3** are colorless amorphous powders and have the same molecular formula ($C_{27}H_{42}O_{11}$) as **1** as revealed by respective HR-ESIMS (m/z = 541.2646 for **2** and 541.2655 for **3**, [M–H]$^-$). Detailed comparison of the 1D and 2D NMR spectra of **2** and **3** with those of **1** revealed that they share the same 5/7/6/5 grayanane diterpenoid skeleton and the same substituents, but have different substitution positions of the acetyl and propionyl groups. The presence of 6-*O*-acetyl, 7-*O*-propionyl and 14-*O*-acetyl groups in **2** were deduced from the HMBC correlations between H-6 [δ_H = 5.94 (br. s)] and C=O (δ_C = 169.9) of one acetyl group, between H-7 [δ_H = 7.00 (br. s)] and C=O (δ_C = 173.0) of the propionyl group, as well as between H-14 [δ_H = 6.25 (s)] and C=O (δ_C = 170.3) of another acetyl group. While the HMBC correlations from H-6 [δ_H = 598 (br. s)] to C=O (δ_C = 173.7) of the propionyl group, from H-7 [δ_H = 7.05 (br. s)] to C=O (δ_C = 170.3) of one acetyl group, and from H-14 [δ_H = 6.34 (s)] to C=O (δ_C = 170.7) of another acetyl group, revealed the 6-*O*-propionyl, 7-*O*-acetyl and 14-*O*-acetyl substitution pattern in **3**. Furthermore, the similar ROESY spectra of **2**, **3** and **1** showed that both compounds **2** and **3** possessed the same relative configurations as **1**. Therefore, compounds **2** and **3** were

isomers of **1** and named as pierisformosins F and G, respectively.

It is interesting that the three isomers pierisformosins E – G (**1**–**3**), differing only in substitution positions, were obtained from the same plant. To our knowledge, almost all grayanane diterpenoids with 5/7/6/5 skeleton are not oxygenated at C-15, thus compounds **1**–**3** are rare examples possessing a hydroxyl at C-15, and also the first trisacylated grayanane diterpenoids to be reported.

Pierisformosin D (**4**), isolated as a colorless amorphous powder, was shown to have the molecular formula $C_{33}H_{46}O_{15}$ from negative HR-ESIMS (m/z = 681.2761, calcd. 681.2758, $[M–H]^-$). The NMR data of **4** (Table 3) showed the existence of five acetyl groups and a propionyl unit. Furthermore four singlet methyls, two methylenes, eight methines (including five oxygenated ones), six quaternary carbons (including three oxygenated ones and one carbonyl carbon) were observed. The above data met the structural requirements of a grayanane diterpenoid with a 5/7/6/5 skeleton substituted by five acetyls and one propionyl group.

In the HMBC spectrum, the proton signals for Me-18 [δ_H = 1.26 (s)], Me-19 [δ_H = 1.52 (s)] and H-2 [δ_H = 3.00 (dd, J = 12.8, 18.2 Hz)] were correlated with the signal at δ_C = 219.2, indicating that the carbonyl carbon was located at C-3. Four acetoxyl groups were attached to C-6, C-11, C-14, and C-15 as deduced from the HMBC correlations of H-6 [δ_H = 6.03 (d, J = 8.6 Hz)], H-11 [δ_H = 6.25 (br. d, J = 3.4 Hz)], H-14 [δ_H = 6.77 (s)], and H-15 [δ_H 5.68 (s)] to four acetyl carboxyl carbons at δ_C = 169.4, 169.6, 170.2, and 171.0, respectively. The signal of H-7 [δ_H = 6.92 (d, J = 8.6 Hz)] showed HMBC correlation with the propionyl carbonyl carbon at δ_C = 173.3, which indicated that the *O*-propionyl group was attached to C-7. A literature survey showed that the oxygenated C-16 bearing a hydroxyl group, as in the cases of rhodomollein III, normally appeared at δ_C = 79.0, while the esterified C-16 generally appeared at δ_C = 88.0 [9, 10]. Thus, the chemical shift of C-16 at δ_C = 89.7 suggested the presence of an acetyl group at C-16 in **4**. In addition, the key HMBC correlations from one exchangeable proton [5-OH, δ_H = 6.56 (s)] to C-1, C-5 and C-6, as well as from another one [10-OH, δ_H = 6.66 (s)] to C-1, C-9, C-10 and C-20, indicated the presence of two hydroxyl groups at C-5 and C-10, respectively. Thus, the planar structure of **4** could be established.

The relative configuration of compound **4** was determined based on the ROESY spectrum. Cross-peaks between H-1 with H-6, 10-OH, H-11, H-14, and Me-18, H-6 with H-14 and Me-18, as well as H-14 with H-13, demonstrated that H-1, H-6, H-11, H-13, H-14, and 10-OH all took α-orientations. Key NOE correlations of H-15/H-7, H-15/H-9, H-15/Me-17, H-15/Me-20, Me-19/Me-20, Me-19/5-OH, and 5-OH/H-7, indicated that 5-OH, H-7, H-9, H-15, Me-17, and Me-20 were all in the same β-orientations. Therefore, the structure of **4** was identified unambiguously as pierisformosin H.

Up to date, most of the gayanane diterpenoids with 5/7/6/5 skeletons had no more than three acyl groups, except for those with a carbonyl group at C-3, such as compound **4**. On the other hand, almost all the grayanoids with *seco*-skeletons were highly acylated, containing five or six acylated groups, usually at C-6, C-7, C-11, C-14, C-15 and/or C-16. Therefore, a plausible biosynthetic pathway of the *seco*-skeleton might be derived from the high acylation of the normal 5/7/6/5 skeleton and the succesive cleavage of a carbon bond.

Experimental Section

General

Optical rotations were measured on a Jasco DIP-370 digital polarimeter (JASCO Corporation, Tokyo, Japan). 1D and 2D NMR spectra were recorded on Bruker AM 400, DRX-500 and AVANCE III instruments with tetramethylsilane (TMS) as an internal standard (Bruker BioSpin group, Germany). ESIMS and HR-ESIMS data were obtained on an API Qstar Pulsar instrument (Applied Biosystem Corporation, Canada). Semipreparative HPLC was carried out on an Agilent 1200 liquid chromatograph with a Zorbax SB-C_{18} (5 μm, 9.4 × 250 mm, Agilent, USA) column. Silica gel (200 – 300 or 100 – 200 mesh, Qingdao Marine Chemical Factory, P. R. China) and Sephadex LH-20 (Amersham Biosciences AB, Uppsala, Sweden) were used for column chromatography (CC). TLC was performed on silical gel GF_{254} plates (Qingdao Marine Chemical Factory, P. R. China), and visualized by spraying with 10 % H_2SO_4-EtOH, followed by heating.

Plant material

The fruits of *P. formosa* were collected in Jindian, Kunming, China, in October 2009 and identified by Dr. Yong-Peng Ma of Kunming Institute of Botany, Chinese Academy of Sciences. The voucher specimen (KMUST 2009100901) was deposited at the Faculty of Life Science and Technology, Kunming University of Science and Technology, China.

Extraction and isolation

Air-dried, powdered fruits of *P. formosa* (6 kg) were extracted with 75 % Me_2CO/H_2O (3 × 18 L, 24 h each) at r. t. The filtrate was concentrated *in vacuo* to give a crude extract, which was then partitioned between H_2O and EtOAc. The EtOAc fraction (350 g) was purified by CC over Sephadex LH-20 by using MeOH-H_2O (gradient 3 : 7, 6 : 4, 9 : 1, 1 : 0) as the eluent to give fractions I – IV. Fraction I (45 g) was subjected to a silica gel column ($CHCl_3$-MeOH 20 : 0, 19 : 1, 9 : 1, 8 : 2, 0 : 20) to afford fractions A – E. Fraction C was applied to Sephadex LH-20 ($CHCl_3$-MeOH 1 : 1), then chromatographed over silica gel (petroleum ether-Me_2CO, 9 : 1, 8 : 2, 7 : 3, 6 : 4) to get four subfractions (C1–C4). Compound **4** (18 mg) was obtained from subfraction C1 after repeated column chromatography over silica gel with $CHCl_3$-MeOH (200 : 1) and $CHCl_3$-Me_2CO (45 : 1), respectively. Subfraction C2 was further subjected to a silica gel column to afford fractions C2-1, C2-2 and C2-3. Compounds **1** – **3** were separated from C2-1 after repeated purification steps involving column chromatography over silica gel ($CHCl_3$-MeOH 60 : 1) and semipreparative HPLC (20 – 28 % MeOH-H_2O, 3 mL min^{-1}). Fractions C2-2 and C2-3 gave compounds **5** and **6**, respectively, after being purified by semipreparative HPLC with 18 – 27 % MeOH-H_2O and 15 – 25 % MeOH-H_2O (3 mL min^{-1}).

*Pierisformosin E (**1**)*

Colorless amorphous powder. – $[\alpha]_D^{16}$ = +36.8 (c = 0.21, MeOH). – ^{1}H and ^{13}C NMR data: see Tables 1 and 2. – MS ((–)-ESI): m/z = 541 [M–H]$^-$. – HRMS ((–)-ESI): m/z = 541.2663 (calcd. 541.2648 for $C_{27}H_{41}O_{11}$, [M–H]$^-$).

*Pierisformosin F (**2**)*

Colorless amorphous powder. – $[\alpha]_D^{15}$ = +40.9 (c = 0.15, MeOH). – ^{1}H and ^{13}C NMR data: see Tables 1 and 2. – MS ((–)-ESI): m/z = 541 [M–H]$^-$. – HRMS ((–)-ESI): m/z = 541.2646 (calcd. 541.2648 for $C_{27}H_{41}O_{11}$ [M–H]$^-$).

*Pierisformosin G (**3**)*

Colorless amorphous powder. – $[\alpha]_D^{15}$ = +30.5 (c = 0.20, MeOH). – ^{1}H and ^{13}C NMR data: see Tables 1 and 2. – MS ((–)-ESI): m/z = 541 [M–H]$^-$. – HRMS ((–)-ESI): m/z = 541.2655 (calcd. 541.2648 for $C_{27}H_{41}O_{11}$ [M–H]$^-$).

*Pierisformosin H (**4**)*

Colorless amorphous powder. – $[\alpha]_D^{16}$ = −9.1 (c = 0.25, $CHCl_3$). – ^{1}H and ^{13}C NMR data: see Table 3. – MS ((–)-ESI): m/z = 681 [M–H]$^-$. – HRMS ((–)-ESI): m/z = 681.2761 (calcd. 681.2758 for $C_{33}H_{45}O_{15}$ [M–H]$^-$).

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