Protoxylocarpins F−H, Protolimonoids from Seed Kernels of *Xylocarpus granatum*

Khanitha Pudhom,*[,†,‡] Damrong Sommit,[§] Paulwatt Nuclear,[⊥] Nattaya Ngamrojanavanich,[†,‡] and Amorn Petsom[†,‡]

Research Centre of Bioorganic Chemistry, Department of Chemistry, Faculty of Science, Chulalongkorn University, Bangkok, 10330, Thailand, Center for Petroleum, Petrochemicals, and Advanced Materials, Chulalongkorn University, Bangkok, 10330, Thailand, Department of Chemistry, Faculty of Science, Mahanakorn University of Technology, Bangkok, 10530, Thailand, and Program in Biology, Faculty of Science and Technology, Rajamangala University of Technology Krungthep, Bangkok, 10120, Thailand

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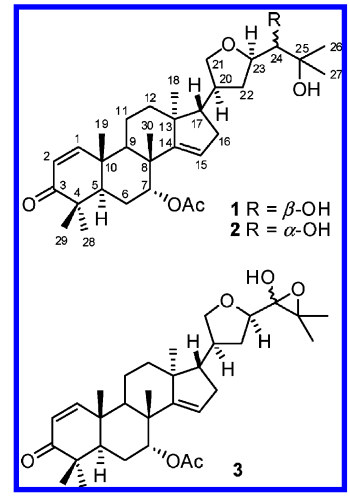
Three new protolimonoids, protoxylocarpins F−H (**1**−**3**), along with 11 known limonoids, were isolated from seed kernels of *Xylocarpus granatum*. Their structures were elucidated on the basis of extensive spectroscopic data analyses. All compounds isolated were evaluated for cytotoxic activity against five human tumor cell lines.

Research on limonoids from the Meliaceae family is of interest due to their range of biological activities, such as insect antifeedant and growth regulator, antibacterial, antifungal, antimalarial, anticancer, and antiviral activities.[1−3] Members of the genus *Xylocarpus*, in particular the cannonball mangrove *Xylocarpus granatum* Koenig. *X. granatum* (Meliaceae) are used as folk medicines in Southeast Asia for the treatment of diarrhea, cholera, viral diseases such as influenza, and malaria. They are also used as insect antifeedants or insecticides.[4] To date, more than 40 limonoid derivatives have been isolated from *X. granatum*, and these are classified as phragmalin, mexicanolide, and andirobin types.[5] In our search for structurally and biologically interesting metabolites from plant resources, we describe herein the isolation and structural elucidation of three new protolimonoids (**1**−**3**) along with 11 known limonoids from the kernel seeds of *X. granatum* collected from Samutsongkram Province, Thailand. The compounds were all evaluated for cytotoxicity against five human tumor cell lines.

The MeOH extract of *X. granatum* seed kernels was partitioned between EtOAc and H_2O to afford an EtOAc extract, which was subjected to silica gel column chromatography. Further separations by repeated normal column chromatography (CC) and preparative thin-layer chromatography (PTLC) gave three new protolimonoids, protoxylocarpins F−H (**1**−**3**), and 11 known limonoids, xyloccensins K (**4**),[5k] O (**5**), and P (**6**),[5i] xylogranatin C (**7**),[5d] mexicanolide (**8**),[6] methyl angolensate (**9**),[7] proceranolide (**10**),[8] 7-oxo-7-deacetoxygenudin (**11**),[9] 7-deacetylgenudin (**12**),[10] chisocheton F (**13**),[11] and 21-acetyloxy-21,23:24,25-diepoxy-7-hydroxy-4,4,8-trimethylcholest-14-en-3-one (**14**).[12] The structures of the known compounds were determined by comparison of their NMR spectroscopic data with those in the literature.

Protoxylocarpin F (**1**) was isolated as a colorless gum, and its molecular formula was assigned as $C_{32}H_{48}O_6$ on the basis of the $[M + Na]^+$ peak at *m/z* 551.3348 (calcd 551.3349) in the HRESIMS, requiring nine degrees of unsaturation. IR absorptions implied the presence of α,β-unsaturated ketone (1733 cm^{-1}) and OH (3543 cm^{-1}) groups. The ^1H NMR spectrum (Table 1) showed signals of seven tertiary methyls [δ 1.24, 1.12, 1.10 (Me × 2), 1.00 (Me × 2), and 0.91], an olefinic proton (δ 5.23), an acetyl methyl (δ 1.88), and an α,β-unsaturated ketone moiety indicated by a pair of doublets at δ 7.10 and 5.97. A combined



analysis of ^{13}C NMR (Table 1) and HSQC spectra revealed 32 nonequivalent carbon resonances due to one carbonyl (δ 204.8), four olefinic carbons (δ 159.2, 158.5, 125.4, and 119.0), seven methyl carbons (δ 27.4, 27.0, 26.3, 22.4, 21.3, 20.0, and 19.0), and the acetyl carbons (δ 170.2 and 21.2) together with six methylenes and seven methines, accounting for four double-bond equivalents. The remaining five degrees required **1** to be pentacyclic. These data suggested that the structure of **1** possessed a protolimonoid skeleton. The NMR data (Table 1) of **1** were similar to those of hilstinone C,[13b] a protolimonoid isolated from *Turraea holstii* (Meliaceae), except for the presence of additional methylene [δ_H 3.44 (dd, J = 2.8, 13.6 Hz), 3.54 (d, J = 12.4 Hz); δ_C 64.2] and acetyl groups [δ_H 1.88 (s); δ_C 21.2 and 170.2] and the absence of the OCH_3 and C-21 hemiacetal methine resonances present in hilstinone. The HMBC cross-peak from H-7 (δ_H 5.16, s) to the acetyl carbon (δ_C 170.2) (Figure 1) indicated that the above acetoxyl group was attached to C-7 and suggested that **1** was a 7-acetyl-21-demethoxy analogue of hilstinone C. The full assignments (Table 1) were determined by COSY and HMBC correlations (Figure 1).

The relative configuration of **1** was assigned on the basis of NOESY data as depicted in Figure 2. In the tetracyclic nucleus, the NOESY correlations of Me-29/Me-19, Me-19/Me-30, and Me-30/H-7 indicated their β-orientations, while those of H-5/H-9 and H-9/Me-18 confirmed that H-5, H-9, and Me-18 were α-oriented. Since H-17 was assigned to the β-form, as observed in all protolimonoids,[5a,13] the NOE correlations of H-20/Me-18, H-20/H-23, and H-23/H-24 combined with the noninteraction of H-17/H-20 allowed assignment of the α-orientation to Me-18, H-20, H-23, and H-24. The absolute configuration at C-24 of **1** was assigned using the modified Mosher method.[15] The difference in

* To whom correspondence should be addressed. Tel: 66-2-218-7639. Fax: 66-2-218-7598. E-mail: Khanitha.P@chula.ac.th.
† Department of Chemistry, Chulalongkorn University.
‡ Center for Petroleum, Petrochemicals, and Advanced Materials, Chulalongkorn University.
§ Mahanakorn University of Technology.
⊥ Rajamangala University of Technology Krungthep.

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Table 1. ^{1}H and ^{13}C NMR Spectroscopic Data for Compounds **1**−**3** in $CDCl_3$

	1		**2**		**3**	
position	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
1	7.10 d (10.0)	158.5	7.14 d (10.4)	158.5	7.14 d (10.0)	158.3
2	5.97 d (10.0)	125.4	5.80 d (10.4)	125.5	5.83 d (10.0)	125.4
3		204.8		204.8		204.8
4		44.1		44.2		44.1
5	2.10 m	46.2	2.16 m	46.4	2.14 m	46.1
6	1.64 m; 1.70 m	23.8	1.80 m; 1.90 m	24.0	1.76 m	23.8
7	5.16 br s	74.7	5.20 br s	74.8	5.19 br s	74.6
8		42.7		42.8		42.6
9	2.13 m	38.5	2.20 m	38.7	2.16 m	38.4
10		39.8		39.7		39.8
11	1.58 m; 1.83 m	16.7	1.55 m; 2.00 m	16.8	1.49 m; 1.73 m	16.7
12	1.60 m; 1.83 m	34.0	2.26 m	34.9	1.92 m; 2.16 m	33.9
13		46.3		46.2		46.5
14		159.2		159.0		158.7
15	5.23 br d (2.4)	119.0	5.30 br d (2.4)	119.6	5.25 br s	119.2
16	1.93 m; 2.21 m	35.0	2.26 m	35.0	2.14 m	29.8
17	1.84 m	54.2	2.00 m	52.3	1.40 m	57.1
18	0.91 s	20.0	0.90 s	20.4	1.04 s	20.4
19	1.09 s	19.0	1.14 s	19.1	1.13 s	19.0
20	1.84 m	36.3	1.88 m	35.8	1.76 m	34.5
21	3.44 dd (2.8, 13.6)	64.2	3.42 dd (2.0, 12.0)	70.0	3.60 m; 3.79 m	65.3
	3.54 br d (12.4)		3.98 br d (11.6)			
22	1.55 m; 1.91 m	37.9	1.52 m; 2.04 m	36.2	1.69 m	32.8
23	3.74 ddd (2.4, 8.8, 10.4)	68.0	3.86 ddd (2.8, 8.8, 13.2)	64.4	3.85 m	67.5
24	3.36 d (8.8)	80.7	2.98 d (8.8)	86.5		95.5
25		76.2		74.3		76.3
26	1.24 s	26.3	1.30 s	28.6	1.39 s	24.3
27	1.10 s	22.4	1.25 s	23.8	1.25 s	23.1
28	1.00 s	21.3	1.05 s	21.3	1.04 s	21.2
29	1.00 s	27.0	1.05 s	27.0	1.04 s	27.0
30	1.12 s	27.4	1.18 s	27.3	1.13 s	27.3
7-OCOCH_3	1.88 s	21.2	1.92 s	21.2	1.92 s	21.1
7-OCOCH$_3$		170.2		170.2		170.2

chemical shift values ($\Delta\delta$) for its diastereomeric esters, (*R*)-MTPA (**1a**) and (*S*)-MTPA (**1b**), indicated the *S*-configuration at C-24, as shown in Figure 3.

Protoxylocarpin G (**2**) had the molecular formula $C_{32}H_{48}O_6$, the same as that of **1**. The ^{1}H and ^{13}C NMR spectra of **2** were nearly identical to those of **1**. The obvious difference was observed in the carbon resonance of C-24 (δ_C 86.5 for **2** and 80.7 for **1**). The relative configuration of **2** was determined by NOESY correlations (Figure 2) and indicated that all of the chiral carbons had configurations the same as those of **1**, except for that of C-24. Due to the lack of an NOE cross-peak between H-23 and H-24, compound **2** was assigned as the C-24 epimer of **1**. This assignment was supported by the opposite signs of their specific rotations ($[\alpha]_D$ −57.0 for **1** vs +38.0 for **2**).

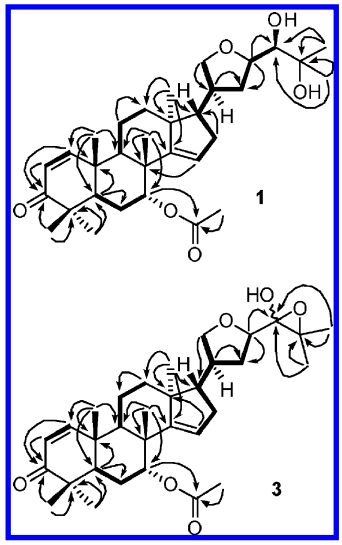

Figure 1. COSY (bold lines) and selected HMBC (arrows) correlations for **1** and **3**.

Protoxylocarpin H (**3**) was isolated as a colorless gum and had the molecular formula $C_{32}H_{46}O_6$ as determined by HRESIMS at *m/z* 526.3297 (calcd 526.3294), which indicated an additional double-bond equivalent relative to **1** and **2**. Similar to compounds **1** and **2**, the ^{1}H and ^{13}C NMR data of **3** were characteristic of protolimonoids, except for the presence of the hemiacetal quaternary carbon (δ 95.5) instead of the oxygenated methine carbon in **1** (δ 80.7) and **2** (δ 86.5) in the aliphatic part. An epoxide ring was assigned between C-24 (δ_C 95.5) and C-25 (δ_C 76.3) due to the additional degree of unsaturation and the HMBC correlations of H-23/C-24, Me-26/C-24, and Me-27/C-24 (Figure 1). The relative configuration of **3** was determined to be the same as **1** by NOESY data.

All compounds isolated (**1**−**14**) were tested for cytotoxicity toward CHAGO (lung carcinoma), SW-620 (gastric carcinoma), KATO-3 (colon carcinoma), BT-474 (breast carcinoma), and Hep-G2 (hepatocarcinoma). Compound **12** showed cytotoxic activity against CHAGO and Hep-G2 cells with IC_{50} values of 16.00 and 10.26 μM, respectively. Compound **7** was active against CHAGO cells with an IC_{50} value of 9.16 μM, and compound **11** was cytotoxic toward Hep-G2 cells with an IC_{50} value of 16.17 μM, whereas compounds **1**−**6**, **8**−**10**, and **13**−**14** were not cytotoxic to any of the cell lines tested.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 341 polarimeter using a sodium lamp at a wavelength 589 nm, and UV data were recorded on a Shimadzu UV-160 spectrophotometer. Melting points were measured using a Fisher-Johns melting point apparatus. IR spectra were recorded on a Perkin-Elmer model 1760X Fourier transform infrared spectrophotometer. HRESIMS

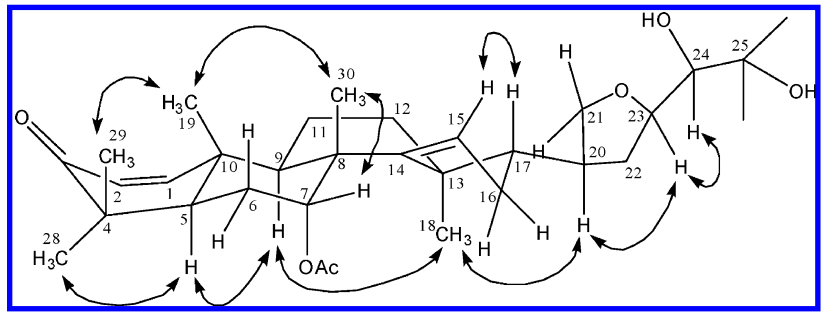

Figure 2. Selected NOESY correlations for **1**.

spectra were obtained using a Bruker micrOTOF mass spectrometer. The NMR spectra were recorded on a Varian YH400 spectrometer at 400 MHz for ^{1}H NMR and at 100 MHz for ^{13}C NMR using TMS (trimethylsilane) as an internal standard.

Plant Material. The fruits of *X. granatum* were collected from Samutsongkram Province, Thailand, in December 2008. A voucher specimen (BKF 159047) has been deposited at the Forest Herbarium, Royal Forest Department, Bangkok, Thailand.

Extraction and Isolation. Air-dried and powdered seed kernels of *X. granatum* (1.5 kg) were extracted with MeOH (5 L × 3, each 2 days) at room temperature. After removing the solvent in vacuo, the combined MeOH crude extract was suspended in H_2O (250 mL), then partitioned with EtOAc (500 mL × 3) to afford the crude EtOAc extract (52.6 g). The EtOAc extract was chromatographed on a silica gel column eluted with a gradient of hexane−acetone (from 1:0 to 0:1) to yield nine fractions (I−IX). Fraction III was subjected to CC over silica gel eluting with MeOH−CH_2Cl_2 (1:19) to give 10 subfractions (IIIa−IIIj). Subfraction IIIc was subsequently separated on a silica gel column (EtOAc−hexane, 1:3 to 1:1) followed by PTLC (EtOAc−benzene, 1:8) to afford **12** (12.5 mg) and **13** (6.2 mg), while subfraction IIId was chromatographed on a silica gel column (acetone−hexane, 1:3) to yield **8** (322.6 mg). Subfraction IIIe was separated on a reversed-phase silica gel (C_{18}) column using a mixture of MeOH−H_2O (8:2) to afford **2** (86.4 mg). Fraction IV was subjected to silica gel CC (EtOAc−hexane, 1:2 to 1:1) to give 15 subfractions. Subfraction IVd was separated on a silica gel column (EtOAc−benzene, 1:6) to yield **11** (20.0 mg) and **7** (19.1 mg); subfractions IVe and IVj were separated in the same manner eluted with MeOH−CH_2Cl_2 (2:98) and acetone−benzene (1:5) to afford **9** (25.1 mg) and **1** (30.8 mg), respectively. Fraction V was chromatographed on a silica gel column using MeOH−CH_2Cl_2 (2:98 and 3:97) to furnish 13 subfractions (Va−Vl). Subfraction Vb was rechromatographed on a silica gel column (EtOAc−hexane, 1:2) to give **8** (6.7 mg). Subfraction Vc was subjected to silica gel CC (MeOH−$CHCl_3$, 1:99); then fraction Vc.2 was separated in the same manner, eluted with EtOAc−hexane (1:1), to yield **5** (3.2 mg) and **6** (14.7 mg). Subfraction Vh was separated into six fractions by CC over silica gel (acetone−hexane, 1:2), and fraction Vh.5 was further purified by reversed-phase (C_{18}) silica gel CC using a mixture of MeCN−H_2O (1:1) to afford **3** (62.8 mg). Fractions VI and VII were combined and then recrystallized from EtOAc to obtain **4** (12.6 g).

Protoxylocarpin F (1): colorless gum; $[\alpha]^{25}_D$ −57.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ϵ) 235 (4.10); IR (KBr) ν_{max} 3453, 2937, 1733, 1668, 1458, 1381, 1250, 1058, and 732 cm^{-1}; ^{1}H and ^{13}C NMR ($CDCl_3$), see Table 1; HRESIMS *m/z* 531.3348 (calcd for $C_{32}H_{48}O_6$ + Na, 531.3349).

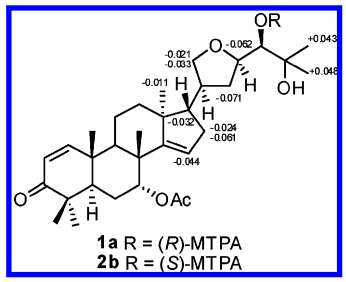

Figure 3. $\Delta\delta$ values (in ppm) = $\delta_S - \delta_R$ obtained for (*S*)- and (*R*)-MTPA esters **1b** and **1a**.

Protoxylocarpin G (2): colorless gum; $[\alpha]^{25}_D$ +38.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ϵ) 215 (4.08); IR (KBr) ν_{max} 3460, 2950, 1728, 1460, 1437, 1381, 1255, 1170, 1026, and 732 cm^{-1}; ^{1}H and ^{13}C NMR ($CDCl_3$), see Table 1; HRESIMS *m/z* 531.3345 (calcd for $C_{32}H_{48}O_6$ + Na, 531.3349).

Protoxylocarpin H (3): colorless gum; $[\alpha]^{25}_D$ +13.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ϵ) 230 (4.09); IR (KBr) ν_{max} 3454, 2941, 1736, 1667, 1461, 1381, 1250, 1028, and 826 cm^{-1}; ^{1}H and ^{13}C NMR ($CDCl_3$), see Table 1; HRESIMS *m/z* 549.3190 (calcd for $C_{32}H_{46}O_6$ + Na, 549.3192).

Preparation of (*R*)-MTPA Ester (1a) and (*S*)-MTPA Ester (1b). A reaction mixture of **1** (5 mg), (*S*)-MTPA Cl (10 μL), and DMAP (catalytic amount) in pyridine (0.5 mL) was stirred at room temperature overnight. After removing the solvent under reduced pressure, the (*S*)-MTPA ester (**1a**) was purified by mini-column chromatography on silica gel with EtOAc−hexane (1:8): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.12 (1H, d, J = 10.2 Hz, H-1), 5 0.98 (1H, d, J = 10.2 Hz, H-2), 5.26 (1H, br s, H-15), 5.18 (1H, m, H-7), 3.77 (1H, ddd, J = 2.7, 8.6, 10.0 Hz, H-23), 3.55 (1H, br d, J = 12.0 Hz, H-21a), 3.47 (1H, dd, J = 2.8, 12.9 Hz, H-21b), 3.40 (1H, d, J = 8.8 Hz, H-24), 2.14 (1H, m, H-9), 2.22 (1H, m, H-16a), 2.15 (1H, m, H-5), 1.99 (1H, m, H-16b), 1.97 (1H, m, H-22a), 1.87 (3H, s, 7-OCO*CH*$_3$), 1.88 (2H, m, H-17 and H-20), 1.84 (2H, m, H-11a and H-12a), 1.72 (1H, m, H-6a), 1.69 (1H, m, H-6b), 1.61 (2H, m, H-11b and H-12b), 1.54 (1H, m, H-22b), 1.22 (3H, s, Me-26), 1.18 (3H, s, Me-30), 1.11 (3H, s, Me-19), 1.10 (3H, s, Me-27), 1.05 (6H, s, Me-28 and Me-29), 0.94 (3H, s, Me-18).

Similarly, the reaction mixture of **1** (5 mg), (*R*)-MTPA Cl (10 μL), and pyridine (0.5 mL) was processed as described above for **1a** to afford **1b**; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.12 (1H, d, J = 10.2 Hz, H-1), 5.97 (1H, d, J = 10.2 Hz, H-2), 5.22 (1H, br s, H-15), 5.14 (1H, m, H-7), 3.71 (1H, ddd, J = 2.6, 8.9, 10.2 Hz, H-23), 3.53 (1H, br d, J = 12.4 Hz, H-21a), 3.44 (1H, dd, J = 2.6, 13.2 Hz, H-21b), 3.35 (1H, d, J = 8.8 Hz, H-24), 2.12 (1H, m, H-9), 2.20 (1H, m, H-16a), 2.09 (1H, m, H-5), 1.93 (1H, m, H-16b), 1.90 (1H, m, H-22a), 1.87 (3H, s, 7-OCO*CH*$_3$), 1.85 (2H, m, H-17 and H-20), 1.83 (2H, m, H-11a and H-12a), 1.70 (1H, m, H-6a), 1.66 (1H, m, H-6b), 1.59 (2H, m, H-11b and H-12b), 1.56 (1H, m, H-22b), 1.28 (3H, s, Me-26), 1.16 (3H, s, Me-30), 1.15 (3H, s, Me-27), 1.14 (3H, s, Me-19), 1.05 (6H, s, Me-28 and Me-29), 0.93 (3H, s, Me-18).

In Vitro Cytotoxicity Assays.[15,16] All stock cultures were grown in T-25 flasks. Freshly trypsinized cell suspensions were seeded in 96-well microtiter plates at densities of 5000 cells per well with compounds added from DMSO-diluted stock. To minimize potential solvent effects on cell growth, the final concentrations of DMSO in all culture wells were less than 0.05%. After three days in culture, attached cells were stained with MTT (3-[4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium] bromide). The absorbency at 540 nm was measured using a microplate reader after solubilizing the bound dye. The mean IC_{50} is the concentration of agent that inhibited cell growth by 50% under the experimental conditions and is the average from at least six independent determinations that were reproducible and statistically significant. The following human tumor cell lines were used in the assay: human breast ductol carcinoma ATCC No. HTB 20 (BT474), undifferentiated lung carcinoma (CHAGO), liver hepatoblastoma (Hep-G2), gastric carcinoma ATCC No. HTB 103 (KATO-3), and colon adenocarcinoma ATCC No. CCL 227 (SW-620). All cell lines were obtained from the Institute of Biotechnology and Genetic Engineering, Chulalongkorn University, and cultured in RPMI-1640 supplemented with 25 mM HEPES, 0.25%

(w/v) sodium bicarbonate, 5% (v/v) fetal bovine serum, and 100 μg/mL kanamycin.

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Supporting Information Available: ^{1}H and ^{13}C NMR spectra of compounds **1**−**3** are available free of charge via the Internet at http://pubs.acs.org.

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