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Study on Itching Substance and Mechanism in Chinese Yam (Dioscoreae Rhizoma)*

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Abstract Chinese yam is a traditional Chinese medicine. It has many benefits for people, such as antidiarrhea, anti-inflammation, antidiabetic, hypocholesterolemia, antioxidation, antitumor, and immunomodulation. However, in the process of contacting yam, it often causes itching. However, the pruritus compound in yam is very unclear. We extract allantoin crystal from fresh Chinese yam using ethanol extraction, membrane filtration, ion exchange chromatography, suspension drop method. The content of allantoin extracted from yam (origin from Jiaozuo, Henan) is about 3.567 mg/g. Allantoin is an important compound in plants and animals. Our results show that allantoin could induce more scratch numbers in mice than control group. Allantoin also directly actives dorsal root ganglia neurons, induces calcium influx and inward current in neurons.

Key words Chinese yam, allantoin, calcium influx, dorsal horn ganglion
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Yam, *Dioscorea batatas* belongs to the Dioscoreaceae family and has been widely used as food and traditional Chinese medicine in Asia which including many active components, such as amino acid, sapogenins, saponins, starch, protein, mucopolysaccharides, and others[1-3]. Chinese yam has many functions, such as antidiarrhea, anti-inflammation, antidiabetic, hypocholesterolemia, antioxidation, antitumor, and immunomodulation[4-5]. Many active components have been extracted and separated from yam. Due to their immunomodulatory and antitumor effects, yam polysaccharides have attracted increasing attention in the biochemical and medical fields[6]. Glycoprotein extracted from yam has been reported that it could be used as a potential immunostimulant *via* mitogen-activated protein kinases and NF-κB signal pathways[7]. In addition, numerous active constituents are present in Chinese yam tuber, such as allantoin[8], which promotes wound healing, speeds up cell regeneration, and exhibits a keratolytic effect[9-10].

The mucus of fresh Chinese yam could induce intense itch on the skin, as many plants, such as buttercups, nicotiana tabacum[11-12] . However, research on the itch induced by Chinese yam is rare up to now, and the pruritus compound in yam is very unclear, although there are many pruritic compounds. We extract allantoin from fresh Chinese yam (Jiaozuo,

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Henan province) using phytochemical methods. We obtain allantoin crystals from Chinese yam for the first time. Our further results show that allantoin could directly activate DRG neurons. It could induce calcium influx and inward current in DRG neurons. We speculate that allantoin induces itching by activating DRG neurons.

1 Materials and methods

1.1 Animals

C57BL/6 mice (8−10 weeks) were used for behavioral testing (Experimental Animal Center, Nanjing University of Chinese Medicine, Nanjing, China). Mice were housed, and behavior experiments were performed in a controlled environment of 20−24℃, humidity of 50%−60% with a 12-h day/night cycle. GCaMP3 and neomycin resistance genes were inserted into the Pirt locus using targeted homologous recombination[13]. Pirt-GCaMP3 heterozygotes were used in all Ca^{2+} imaging experiments.

1.2 Behavior tests

The neck of the mice was clipped and depilated with electric hair clippers 48 h before experiments. Mice were placed in a box for approximately 15 min for acclimatization. Subcutaneous injection of allantoin or saline into the neck back of experimental mice was adopted. Behavior of the testing mice was collected by camera for 1 h. A bout of scratching was defined as a continuous scratching movement with a hindpaw directed at drug injection site[14].

1.3 Calcium imaging and cell culture

DRG neurons, isolated from Pirt-GCaMP3 heterozygotes, were collected in DH10 medium on ice (90% DMEM/F-12, 10% FBS, 100 U/ml penicillin, 100 g/L streptomycin). Dissected DRGs were digested for 30 min at 37℃ in a protease solution (5 g/L dispase, 1 g/L collagenase type II in HBSS) DRG neurons were then triturated to free neurons and pelleted by centrifugation. Pelleted neurons were re-suspended in DH10 medium supplemented with NGF (20 μg/L) and plated onto glass coverslips (8 mm) coated with poly-D-lysine (0.5 g/L, Sigma) and laminin (10 g/L, Sigma). Neurons were cultured in an incubator (95% O_2 and 5% CO_2) for 24 h before they were used for calcium imaging.

1.4 Patch clamp

In whole recordings, inward currents were recorded with an Axon 700B amplifier and the pCLAMP 10.1 software package (Axon Instruments). DRG neurons were cultured in normal solution: 140 mmol/L NaCl, 4 mmol/L KCl, 2 mmol/L $CaCl_2$, 2 mmol/L $MgCl_2$, 10 mmol/L HEPES, 5 mmol/L Glucose, pH 7.4 in NaOH to adjust. Pipette resistance ranged from 3−4 MΩ. The internal solution was 35 mmol/L KCl, 3 mmol/L MgATP, 0.5 mmol/L Na_2ATP, 1.1 mmol/L $CaCl_2$, 2 mmol/L EGTA, 5 mmol/L Glucose, pH 7.4 in KOH to adjust, and osmolarity was adjusted to 300 mOSmol/L with sucrose. Electrodes were pulled (Sutter, model P-97) from borosilicate glass. All experiments were performed at room temperature.

2 Results

2.1 Purification and crystallization of allantoin

We speculated that the compounds in yam could induce itch. Followed work was finding the pruritic compounds in yam. Multidimensional chromatographic separation methods including membrane filtration, ion exchange chromatography, C18 reverse phase chromatography, silica column and preparation liquid phase, mass spectrometry analysis were used to purify the active compounds. Fresh Chinese yam was extracted by 85% ethanol (material liquid ratio 1∶2). Ethanol extract was obtained by filtration of extract from gauze. It was concentrated and dried at 55℃ using rotary evaporator. Behavior tests and calcium imaging *in vitro* were used to test the activation of the ethanol extract.

Ethanol extract contained saccharides or glycol-proteins and polar secondary metabolites[15]. To separate these complex ingredients, technics were adopted including membrane separation technology, C18 reverse phase chromatography, silica column, preparative liquid phase for fast preparation and purification of interested targets. The ethanol extract was separated by molecular weights of 10 ku and 100 ku and brought to three fractions with low molecular weight (LMW, <10 ku), middle (MMW,10−100 ku) molecular weight and high molecular weight (HMW, >100 ku). LMW fraction was separated into five fractions by preparative C18 column in a 30-min cycle. Each fraction was accurately collected and tracking detected by HPLC. Five fractions were dried in room temperature or freeze-dried before volatilizing the methanol. The third fraction of the five extracts grows out crystallization (Figure 1a).

Crystals were cultured by suspension drop method with water as crystallizing solvent. Crystallization temperature was controlled at 20−25℃. The crystal growth under polarizing microscope was observed. Dual-wavelength single crystal diffractometer was used to confirm the structure of allantoin. X-ray crystal diffraction pattern of allantoin showed in Figure 1b. The crystal data of allantoin was showed in Table 1.

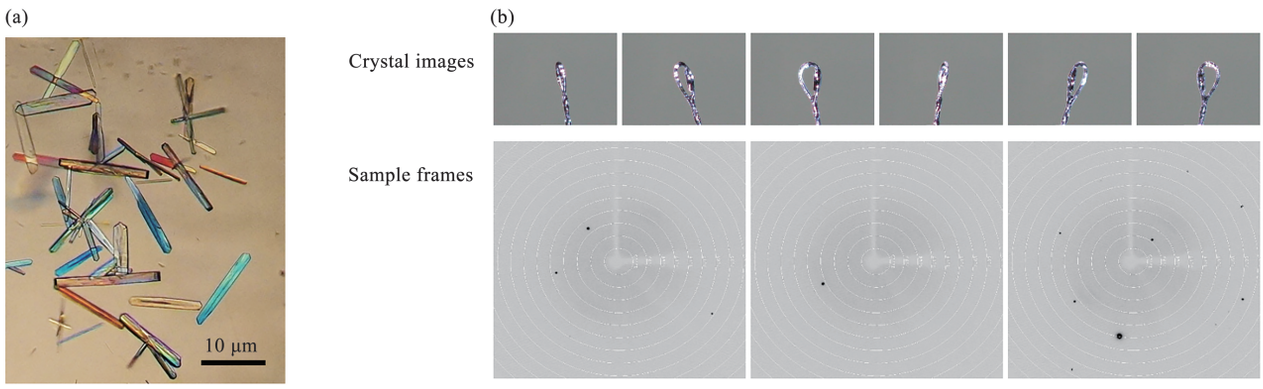

Fig. 1 Allantoin crystal and X−ray crystal diffraction pattern

（a）Allantoin crystal in 0.2 g discolourant silica gel. （b）X-ray crystal diffraction pattern of allantoin.

Table 1 Crystal data of allantoin

Empirical formular	$C_4H_6N_4O_3$	Volume/Å^3	601.6（2）
Formula weight	158.13	Z	4
Temperature / K	150.01（10）	ρ_{calc} mg/mm^3	1.746
Crystal systems	monoclinic	2θ range for data collection	11.12 to 115.26°
Space group	$P2_1/c$	μ/mm^{-1}	1.308
a/Å	7.9686（16）	F（000）	328.0
b/Å	5.1408（10）	Reflections collected	4352
c/Å	14.703（3）	Independent reflections	827 ［R(int) = 0.0454］
α/Å	90.00	Final R indexs［$I \geqslant 2\sigma(I)$］	R_1 = 0.0383，wR_2 = 0.0975
β/Å	92.838（19）	Final R indexs［all data］	R_1 = 0.0502，wR_2 = 0.1037
γ/Å	90	Goodness-of-fit on F^2	1.059

Analyses of allantoin were performed on a Waters Alliance 2695 High Performance Liquid Chromatography (HPLC) instrument (Waters, USA) consisting of a Waters Quaternary Pump, a Waters 2996 Photodiode Array Detector, and the Empower Pro software. The conditions were as follows: mobile phase CH3OH/H2O (10/90); flow rate 0.4 ml/min; UV detective wavelength 224 nm; and column temperature 30℃. Before injection, the samples were filtered through a 0.22 μm Millipore filter. The standards for allantoin were purchased from the Shanghai Aladdin Bio-Chem Technology Co., LTD, China. The results of HPLC for crystalline compounds were consistent with those of allantoin standard (Figure 2a, 2b).

NMR and LC－TOF-MS/MS methods were used to identify allantoin structure. The results showed short retention time and its peak almost mixed with that of the solvent in the UPLC system (Figure 3). The data was shown as: ^{13}C NMR (100 MHz, DMSO-d6) δ 174.0 (C-2), 157.2 (C-4), 62.9 (C-5), 157.8 (C-7)[16].

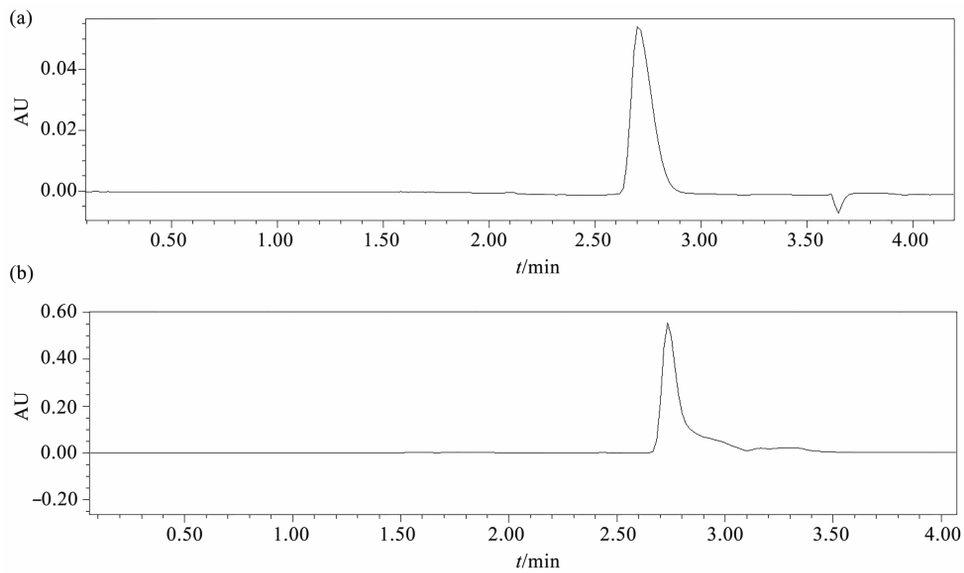

Fig. 2　HPLC of allantoin crystal（a）and standard product（b）

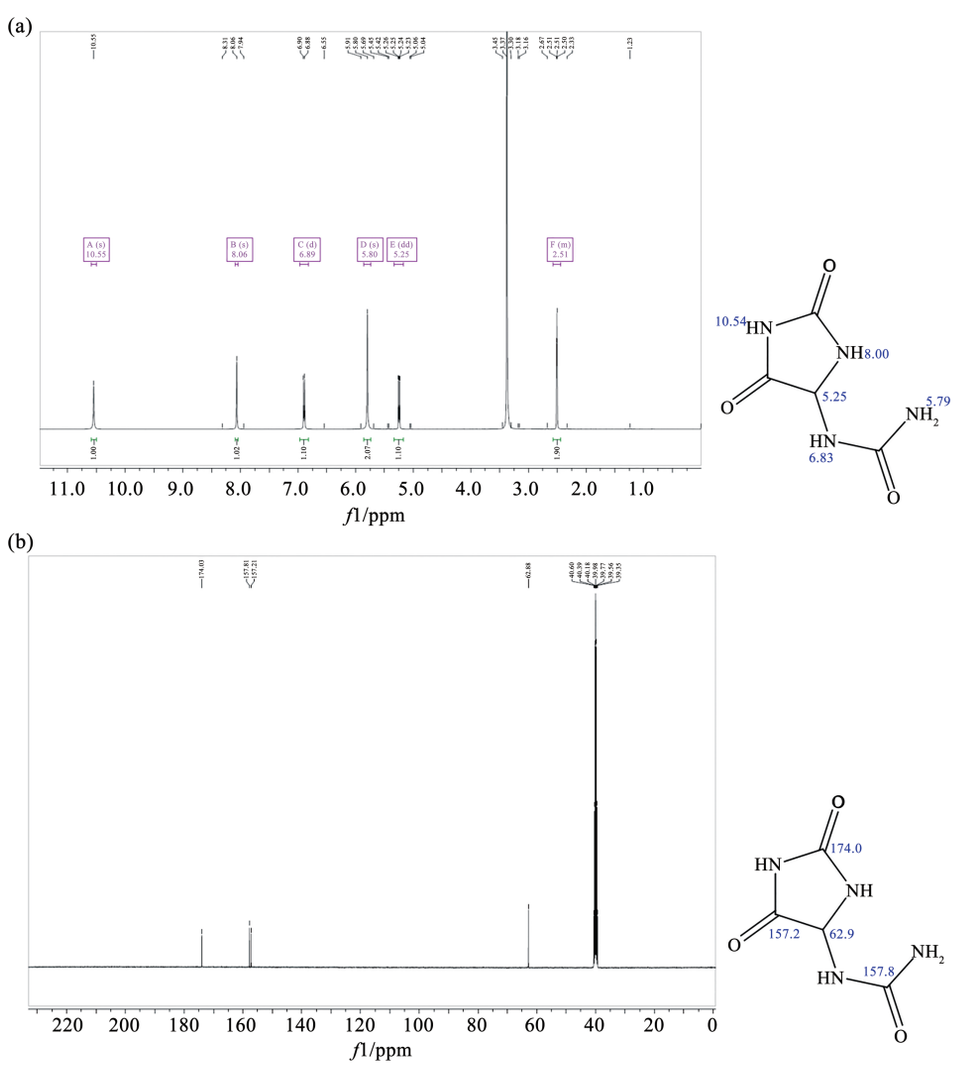

Fig. 3　H−NMR（a）and C−NMR（b）of allantoin extracted from Chinese yam

2.2 Allantoin directly excites DRG neurons

The content of allantoin from Chinese yam changed in volatility[17]. The content of allantoin extracted from yam (origin from Jiaozuo, Henan) was about 3.567 mg/g in our experiments. Allantoin was an important compound in many plants, such as Chinese yam, *Arabidopsis*. It could increase cadmium tolerance in *Arabidopsis via* activation of antioxidant mechanisms [18]. In clinic, allantoin was used as an independent marker associated with carotid intima-media thickness in subclinical atherosclerosis and a stable marker for *in vivo* free radical activity[19-20]. Calcium was a second messenger, playing an important role in excitable cells and signal transduction. Several versions of the original GCaMP sensor have been published[21-23]. We cultured DRG neurons from Pirt-GCaMP3 heterozygotes examined their responses to allantoin using calcium imaging techniques. Allantoin (1 mmol/L) was added into the perfusion system containing DRG neurons. DRG neurons were activated by allantoin (Figure 4a). However, when administered again, the response of DRG neurons was significantly reduced, because of desensitization (Figure 4b). The response curves of two cells were showed in Figure 4c. These results verified the allantoin could induce calcium influx in cultured DRG neurons.

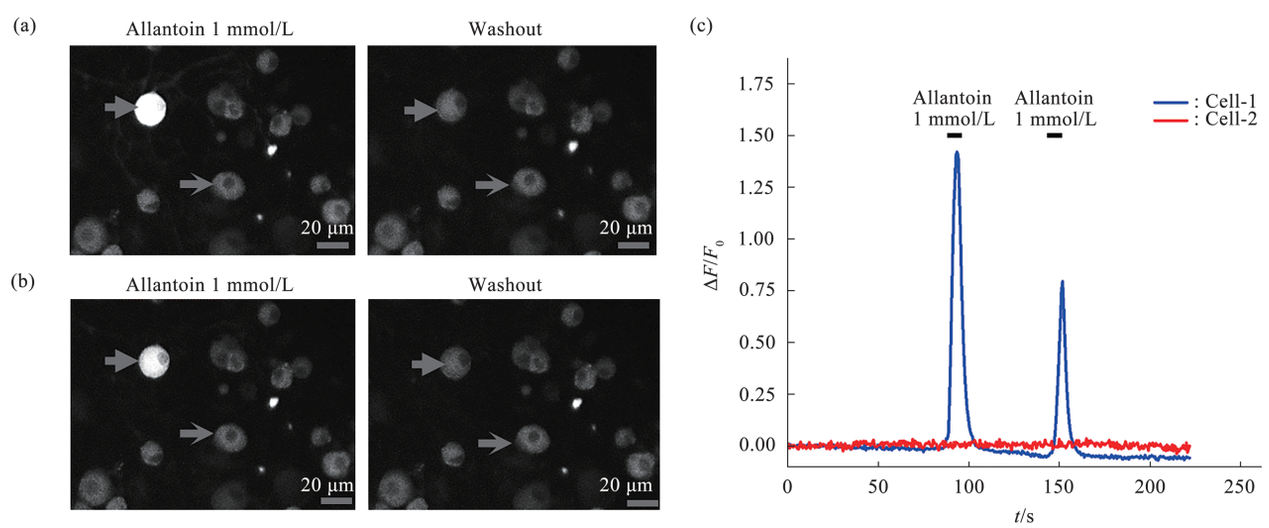

Fig. 4 Allantoin induced calcium ion influx in cultured DRG neurons *in vitro*

（a）Allantoin （1 mmol/L） could active DRG neurons *in vitro*. Number 1 indicated by white flat end arrow was the responsive neurons. Number 2 indicated by pointed arrow was the negative cell. After washout in normal buffer， the responsive neurons could recover. （b）Ca^{2+} influx caused by the second addition of allantoin. （c）Allantoin induced fluorescence change in the cell-1 and cell-2 in figure（a）and cell desensitization induced by secondary dosage.

2.3 Allantoin induces inward current in DRG neurons and scratching behavior in mice

We also examined the electrophysiological characteristics of the neurons induced by allantoin. Neurons plated on cover slips were transferred into a chamber with the extracellular solution (Figure 5a). Patch pipettes had resistances of 3−4 MΩ. In wild type mouse, inward current was induced upon allantoin treatment (Figure 5b). In whole-cell voltage clamp recordings, inward current measurements were performed with an Axon 700B amplifier and the pCLAMP 10.1 software package (Axon Instruments). Neurons were perfused with 1 mmol/L allantoin for 20 sec. All experiments were performed at room temperature (~25℃). The diameters of allantoin sensitive neurons were about 8−20 μm. The data suggested that allantoin active DRG neurons directly and then play an important biological function

Although we already knew that allantoin could activate DRG neurons, what kind of behavioral responses did these neurons cause after stimulation was still unknown. To detect the role of allantoin in mice behavior, we intradermally injected allantoin (50 mmol/L) in the neck of the mice. The scratch

bouts induced by allantoin in mice more than control group (allantoin, (64 ± 11), *vs.* saline, (21 ± 5), n=10, $^{**}P$<0.01) (Figure 5c). This result suggests that allantoin could induce itch in mice.

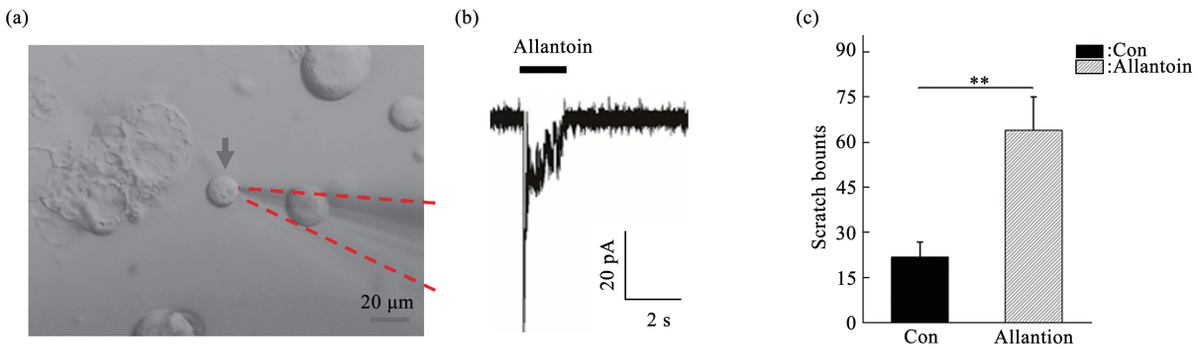

Fig. 5 The electrophysiology response of DRG neurons to allantoin（1 mmol/L）

（a）Bright-field image of a neuronal recording（arrow）with an extracellular electrode（outlined with dashed red lines）.（b）Inward current induced by allantoin in DRG neurons.（c）Scratch bouts induced by allantoin（7.9 mg/kg） were more than control group （allantoin，(64 ± 11)，*vs.* saline，(21 ± 5)，n=10，$^{**}P$<0.01）.

3 Conclusions

Yam has been widely used for the treatment of diabetes, diarrhea, asthma, and other ailments[24-25]. But in the process of contacting yam, it often causes itching. We chose fresh yam as raw material, and got the allantoin crystal by simple ethanol extraction. As far as we know, this is the first time to obtain allantoin crystal from fresh yam. This work provides an important reference for us to get allantoin from natural product

Allantoin is widely found in many natural plants, but the effect of allantoin on DRG neurons and animal itching behavior was the first time. Our research shows that allantoin not only activated small DRG neurons, but also caused itch in mice. Allantoin could induce the itching behavior of mice, which not only help us understand the cause of yam to induce itch, more importantly, we found a natural itch causing compound. This discovery will have implications for our future research on the mechanism of itch.

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山药中的致痒物质及致痒机制研究*

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摘要　山药是一种传统中药，对人体有许多益处，如抗腹泻、抗炎、抗糖尿病、低胆固醇血症、抗氧化、抗肿瘤和免疫调节. 山药黏液接触皮肤，常引起严重瘙痒. 而山药中存在的致痒物质成分尚不清楚. 我们采用乙醇提取、膜过滤、离子交换色谱、悬浮液滴法从新鲜山药中提取尿囊素晶体. 从河南焦作山药中提取的尿囊素含量约为3.567 mg/g. 活性实验结果表明，尿囊素引起的小鼠抓挠反应次数显著地高于对照组. 尿囊素直接激活背根神经节神经元，诱导钙流入，还可以诱导神经元内向电流产生. 我们首次在细胞水平证明尿囊素能够激活神经细胞，诱导痒觉信号传导.

关键词　山药，尿囊素，钙离子内流，背根神经节
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