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A Preliminary Investigation of Furanocoumarin Metabolism by *Aspergillus niger*

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Fungi metabolize polycyclic aromatic hydrocarbons by a number of detoxification processes. Prevalent fungal detoxification pathways for aromatic compounds include the formation of sulfated and glycosylated conjugates. Furanocoumarins, a class of aromatic compounds, are important for their diverse biological activities and clinical applications. The 6′,7′-dihydroxybergamottin (DHB), 6′,7′-epoxybergamottin (EB), and bergamottin (BM) are three major furanocoumarins in grapefruit, and their metabolism in humans is involved in the "grapefruit/drug interactions." The metabolism by fungi of these furanocoumarins has received little research attention. In our study, the metabolism by *Aspergillus niger* of DHB, EB, and BM has been investigated. *Aspergillus niger* metabolized EB, DHB, and BM into a water-soluble sulfate conjugate possibly using etherase- and sulfotransferase-like enzyme activities. In addition, *A. niger* showed a specificity in metabolizing furanocoumarins, in which the fungus metabolized bergaptol and xanthotoxol into sulfate conjugates but did not metabolize 5-methoxypsoralen and 8-methoxypsoralen. These sulfate conjugates, i.e., bergaptoyl sulfate and xanthotoxoyl sulfate, were characterized by ultraviolet spectroscopy, mass spectrometry, and acid hydrolysis.

Furanocoumarins (FCs) produced in plants have been extensively studied for their diverse biological activities and clinical applications. FCs play critical roles in the inhibition of cytochrome P450, in DNA photocrosslinking, and in plant defense mechanisms (Afek et al., 1999; Guo et al., 2000; Oliva et al., 2003; Santana et al., 2004; Weimin et al., 2003). Grapefruit juice contains three major FCs, bergamottin (BM), 6′,7′-dihydroxybergamottin (DHB), and 6′,7′-epoxybergamottin (EB), and other minor-occurring FCs including homo- or hetero-dimers of BM, EB, and DHB (Manthey and Buslig, 2005: Manthey et al., 2006). These grapefruit FCs are involved in the grapefruit–drug interactions, in which they act as inhibitors of the human intestinal cytochrome P450 3A4 isozyme, an enzyme responsible for the metabolism of a number of prescribed medications (Guo et al., 2000; Maskalyk, 2002; Wangensteen et al., 2003). Other common FCs in the human diet include xanthotoxol (XT), 5-methoxypsoralen (5-MOP), and 8-methoxypsoralen (8-MOP), which occur in a variety of plants including parsnips, bergamot and lime oils, and celery (Murray et al., 1982).

Biotransformation of natural products using fungi has been extensively used to produce novel compounds (Farooq and Tahara, 1999; Papagianni, 2004). Fungi metabolize polycyclic aromatic hydrocarbons by a number of detoxification processes (Pothuluri et al., 1998). Prevalent fungal detoxification pathways for aromatic compounds include the formation of sulfated and glycosylated conjugates, and such fungal metabolism has been extensively studied as models of mammalian metabolism (Coughtrie, 2002). To date, fungal metabolism of the major grapefruit FCs has not been explored, although the biotransformations of other non-grapefruit FC derivatives, using mammalian, insect, and fungal systems, have been reported (Mao et al., 2006; Mays et al., 1987; Spencer et al., 1990; Teng et al., 2004).

The objectives of this paper were to screen fungi capable of metabolizing EB, DHB, and BM and to identify novel metabolites. Of particular interest were the findings made with *Aspergillus niger*, which converted EB, DHB, and BM into a water-soluble bergaptoyl sulfate (BS) conjugate. *Aspergillus niger* was also capable of metabolizing bergaptol (BT) and XT to BS and xanthotoxoyl sulfate (XS), respectively. Spectral characterizations of the novel sulfate conjugates were carried out, and a metabolic pathway involving fungal intracellular etherase and sulfotransferase-like activities is proposed.

Materials and Methods

FURANOCOUMARINS. The EB, BM, BT were isolated as previously described (Tatum and Berry, 1979). 5-MOP (Sigma, St. Louis, MO), 8-MOP (Sigma), and XT (Indofine, Hillsborough, NJ) were commercially purchased.

FUNGAL ORGANISMS. A culture of *Aspergillus niger* van Tieghem was obtained from the ARS Culture Collection at the National Center for Agricultural Utilization Research in Peoria, IL (NRRL No. 326). Cultures of *Phomopsis citri* (Sacc.) Bubák nom. cons. prop., *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl., and *Penicillium digitatum* (Pers.:Fr.) Sacc. were isolated from diseased fruit of sweet orange (*Citrus sinensis* L. Osbeck) and grapefruit (*Citrus paradisi Macf.*) The fungi were grown on potato dextrose agar (BD/Difco, Sparks, MD) plates for 5–7 d at 25 °C. The

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spores were removed from the plate surface using sterile water and a 0.1% Tween 20 solution and gently rubbing with a sterile, disposable transfer needle (Fisher Scientific, Pittsburgh, PA). The liquid from the plate was filtered through three layers of sterile cheesecloth. The resulting spore suspension was adjusted for inoculum concentration using a haemocytometer (Hausser Scientific, Horsham, PA).

Fermentation procedures. One milliliter of spore suspension (10^6 spores/mL) was used to inoculate 100 mL YED liquid medium (1% glucose and 1% yeast extract) in 250-mL flasks. Aliquots (100 µL) of 33 mg of EB, BM, BT, 5-MOP, 8-MOP, or XT were added to the YED liquid medium cultures. Flasks were maintained on a rotary shaker at 120 rpm and 25 °C for 5 d. The YED cultures were harvested 5 d after incubation and filtered through Miracloth, and the resulting filtrate and mycelial tissue were collected and subjected to ethyl acetate extraction. Since *A. niger* acidified the culture media, the pH of the control media in the absence of the fungus was adjusted with HCl to comparable pH of the media in the presence of the fungus during incubation.

Extraction and identification of metabolites. The filtrates from YED cultures incubated with *A. niger* were extracted three times with ethyl acetate (100 mL). Fungal material collected on the Miracloth was placed in 100 mL ethyl acetate for 1 h, shaken with added water (100 mL), and extracted three times with additional ethyl acetate (100 mL). The resulting ethyl acetate-soluble and non-soluble fractions from YED filtrates and the fungal tissues were dried using a rotary evaporator, and dissolved in 10–15 mL acetone for the ethyl acetate-soluble fraction and water : methanol (1:1) for the ethyl acetate-non-soluble (water-soluble) fraction, respectively. Aliquots (20–40 µL) were injected on a 4.6 × 150 mm 5 µ Eclipse XDB-C8 column (Agilent Technologies, Palo Alto, CA), using an Agilent 1100 quaternary pump system equipped with a Hewlett Packard 1050 photodiode array detector. Samples were eluted using a gradient of water : acetonitrile : 0.05% formic acid, which was started at 85:10:5, increased to 75:20:5 by 15 min, to 70:25:5 by 20 min, to 55:40:5 by 30 min, to 25:70:5 by 55 min, and to 0:95:5 by 67 min, and was held isocratically for 13 min. The flow rate was 0.75 mL·min^{-1}. Chromatograms were recorded at 320 nm and data were analyzed using ChemStation revision 09.01 (Agilent Technologies). For identification of the metabolites in samples, individual chromatographic peaks were compared with authentic standard compounds and characterized by UV spectroscopy and electrospray mass spectrometry (ESI–MS) as previously described (Manthey and Buslig, 2005; Manthey et al., 2006).

Bergaptoyl sulfate isolation and sulfate hydrolysis. The water : methanol (1:1 v/v) sample containing BS was applied to DE-52 anion-exchange resin (Whatman, Florham Park, NJ) in a glass column (15.0 × 2.8 cm), then rapidly eluted with 0.4 M KOH, and immediately neutralized with 5% acetic acid. The neutralized BS fraction was applied to pre-washed C18 SEP-PAK® cartridges (Millipore Corp., Milford, MA), where the salt (potassium acetate) was removed by extensive water washes (40 mL) of the sample-loaded cartridges. BS was eluted from the cartridges with methanol (10 mL), concentrated, and applied to a Sephadex® LH-20 column (Sigma) using 10% methanol as a mobile phase. The partially purified BS (>85%) was subjected to acid hydrolysis at pH 2 for 1 h; the products of the hydrolysis were monitored by HPLC as described above.

Statistical procedures. All experiments in this study were conducted with three replicates. Student *t* test was used to compare the differences in changes between controls and treatments if applicable. Two-tailed *P* values were calculated to report significant differences in the mean values.

Results and Discussion

Degradation of 6′,7′-epoxybergamottin in the absence or presence of *Aspergillus niger*. Four fungi were screened for their ability to metabolize EB, including *Aspergillus niger*, *Phomopsis citri, Lasiodiplodia theobromae*, and *Penicillium digitatum.* Although no EB metabolism occurred with the last three fungi (data not shown), significant metabolism of EB occurred with *A. niger* (Fig. 1). When EB was incubated for 5 d with *A.*

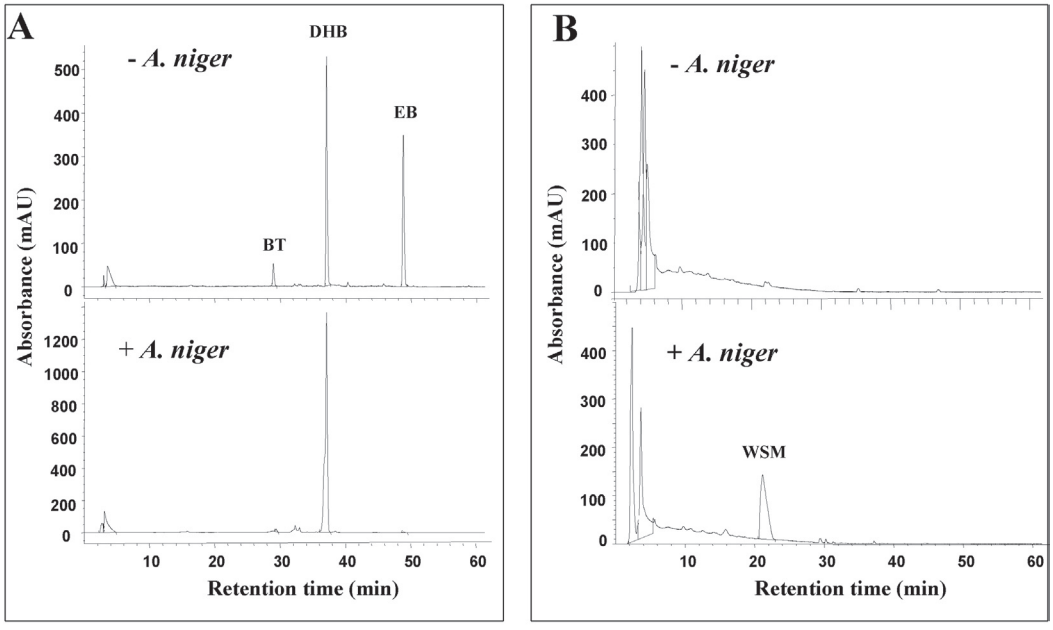

Fig. 1. HPLC elution profiles of metabolites formed from 6′,7′-epoxybergamottin (EB) in ethyl acetate-soluble (**A**) and water-soluble (**B**) fractions extracted from YED liquid medium in the absence (–) or presence (+) of *A. niger* after 5 d of incubation. Peaks were identified as EB, 6′,7′-dihydroxybergamottin (DHB), bergaptol (BT), and an unknown water-soluble metabolite (WSM).

niger, the level of EB in the culture dropped precipitously, while the concentration of DHB increased sharply (Fig. 1A). In the absence of *A niger*, less than 10% of EB was converted to DHB and BT after 5 d at 25 °C in YED liquid medium at pH 7.0 (data not shown). In the presence of *A. niger*, the pH of the medium was gradually lowered to 6.0 at 24 h after incubation, to 4.5 at 48 h, and to 4.0 and 3.5 at 72 and 120 h, respectively, and the pH of the control media was similarly adjusted to these changes over time. Due to these changes, the breakdown of EB into DHB and BT was accelerated (Fig. 1A), indicating that EB is susceptible to mild acid hydrolysis.

No EB metabolites were detected in the medium in the absence of *A. niger*, while an unknown water-soluble metabolite (WSM) was detected in the culture with *A. niger* (Fig. 1B). Since the WSM was not detected in fungal cultures without EB (data not shown), it was concluded that the WSM is either a metabolite of EB or an induced fungal derivative. It is noted that only a trace amount of BT was detected in the culture with *A. niger*. Overall, the data demonstrate that *A. niger* has the capability of producing a WSM in the presence of EB.

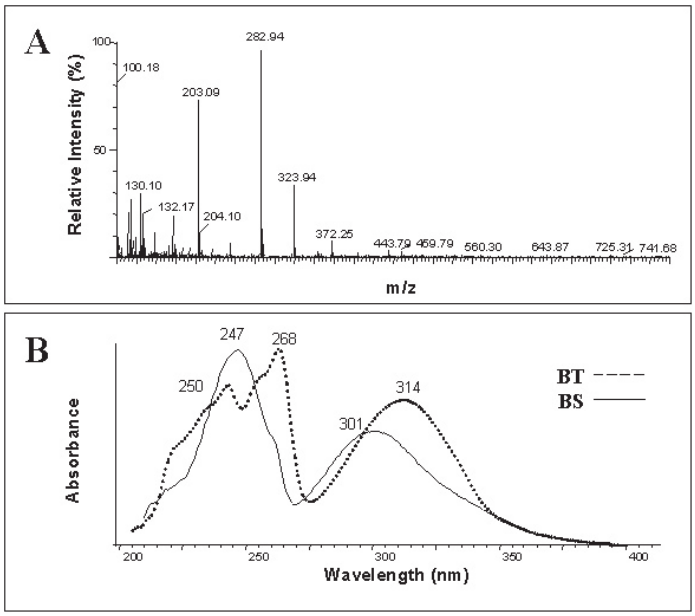

Fig. 2. Mass spectra (**A**) of water-soluble metabolite (WSM) and UV absorption spectra (**B**) of isolated bergaptol (BT, dashed line) and bergaptoyl sulfate (BS, solid line).

IDENTIFICATION AND CHARACTERIZATION OF BERGAPTOYL SULFATE, A NOVEL WATER-SOLUBLE METABOLITE OF 6′,7′-EPOXYBERGAMOTTIN. The mass spectrum of the WSM showed two major ions at 203 and 283 *m/z* (Fig. 2A). The ion at *m/z* 203 possibly corresponds to the protonated fragment ion $(M+H)^{+1}$ representing BT (mol. wt. 202.16 amu). We propose that the higher molecular weight ion at *m/z* 283 represents an addition of a sulfate group (SO_3^-) to BT. The neutral loss of 80 Da from the molecular ion is characteristic of the elimination of a sulfate group (El-Sharkawy et al., 1991; Golbeck et al., 1983; Plasencia and Mirocha, 1991; Sun et al., 2004; Yi et al., 2006). A minor peak with *m/z* 324 corresponds to $(M + Na + H_2O)^{+1}$. Hence, the mass spectral data suggest that the WSM is a sulfate conjugate of BT, i.e., bergaptoyl sulfate (BS). The UV spectrum of BT exhibits three absorption bands at 314, 268, and 250 nm, while the UV spectrum of the presumed BS showed two absorption bands at 247 and 301 nm (Fig. 2B). The slight shift observed from BS compared to BT is a property of phenolic sulfate conjugates as previously reported (Barron et al., 1988). Further support of the proposed BS structure is gained by the observed facile acid hydrolysis of this metabolite rendering BT, as detected by HPLC–MS and comparison with a known BT standard (Fig. 3).

BIOTRANSFORMATION OF BERGAPTOL AND BERGAMOTTIN TO BERGAPTOYL SULFATE BY *ASPERGILLUS NIGER*. To determine whether BT is also metabolized to BS, *A. niger* was incubated with BT for 8 d. The conversion of BT to BS was detected at 4 d, increased 4-fold at 6 d, but decreased at 8 d after incubation (Fig. 4). The results demonstrate that BS was synthesized from BT by the fungus. As expected, 80% of BT was rapidly removed from YED culture medium by the fungus and the portion of BT was recovered in fungal mycelium (Fig. 4), indicating that BT was taken up by the fungus and converted to BS inside the fungal mycelium, which was further secreted into the medium. The increased BT concentration in the fungus at 8 d after incubation could reflect a re-uptake of degraded BS, because a spontaneous rearrangement of BS into BT was observed under acidic condition (data not shown). Finally, when *A. niger* was incubated with BM for 5 d, trace amounts of BS were also detected in the YED culture medium (data not shown), indicating that the fungus can also metabolize BM to BS, although to a lower extent than with BT and EB.

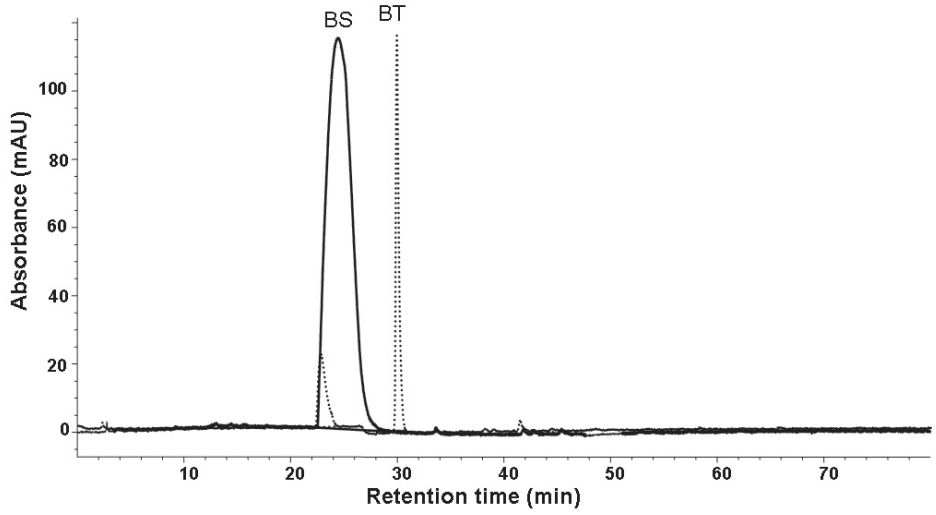

Fig. 3. HPLC elution profiles of bergaptoyl sulfate before acid hydrolysis (solid line) and after acid hydrolysis (dashed line).

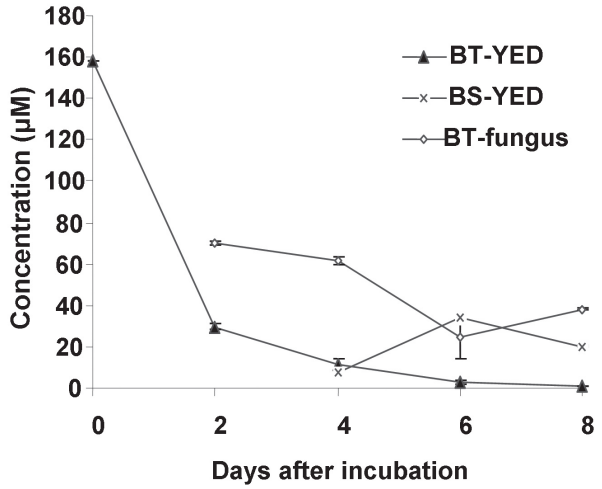

Fig. 4. Kinetics of metabolization of bergaptol (BT) by *A. niger* in 100 mL of YED liquid medium. The final concentration of BT added to each flask was 158 μM. Concentrations of BT in the YED (–▲–) and the fungus (–◊–) and bergaptoyl sulfate (BS) in the YED (–x–) were calculated from known amounts of authentic standards using HPLC. BS in the fungus is not shown in this figure as BS was not detected. Data represent means of three replications and bars indicate standard errors.

Metabolism of other furanocoumarins by *Aspergillus niger*. To investigate whether *A. niger* can metabolize other structurally related furanocoumarins, such as XT, 5-MOP, and 8-MOP, these compounds were incubated with the fungus for 6 d. Even though they are not found in grapefruit, they are a class of furanocoumarins with basic psoralen structures similar to EB, DHB, and BT. We could not detect any metabolite from 5-MOP and 8-MOP (data not shown), suggesting that *A. niger* does not interact with the compounds. However, a sulfate conjugate was detected from cultures containing XT (Fig. 5A). The novel xanthotoxoyl sulfate (XS) had the same molecular mass (*m/z* 283) to BS but a different elution time (Fig. 1B, Fig. 2A, Fig. 5 A and B). Compared to the UV spectrum of XT, the wavelength maximum of XS was shifted to lower values (Fig. 5C), similar to BS (Fig. 2B), demonstrating that the shift is a property of the sulfate conjugates. Considering that XT is an isomer of BT, it appears that there is sulfotransferase-like enzyme activity that can catalyze both substrates. The 25.7% of XT was converted to XS at 6 d after incubation with the fungus, which was comparable to the biotransformation (21.7%) of BT to BS.

In summary, this is the first study to identify the capability of *Aspergillus niger* to metabolize an important set of grapefruit furanocoumarins into sulfate conjugates. We have detected the possible occurrence of novel enzymatic activities leading to the cleavage of an ether group and the transfer of a sulfate group. Since sulfated conjugates are unstable and expensive, the isolation of a sulfotransferase will provide opportunities to produce valuable bioactive compounds for a variety of studies. Our findings may also contribute to further understandings of sulfotransferase-like enzymes, and possibly to future commerical uses of these enzymes to modify FCs in foods, particularly in grapefruit, where these compounds cause the detrimental grapefruit/drug interactions.

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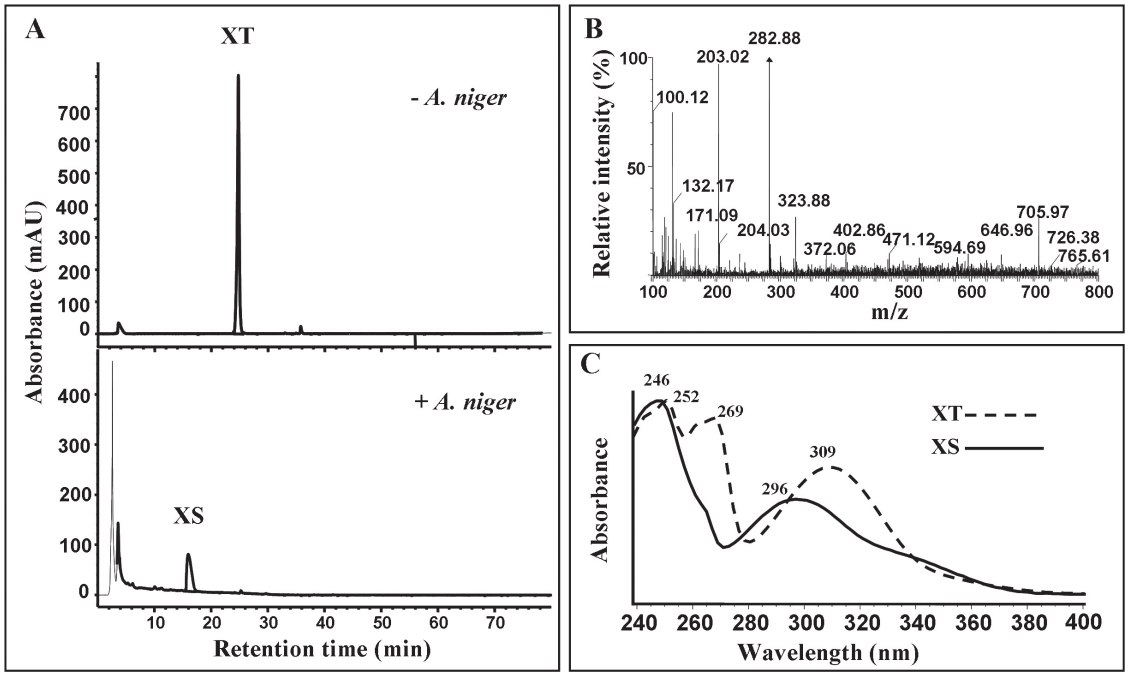

Fig. 5. HPLC elution profiles (**A**) of xanthotoxol (XT) and its metabolite, xanthotoxoyl sulfate (XS), mass spectra (**B**) of XS, and UV absorption spectra (C) of XT (dashed line) and XS (solid line).

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