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Biotransformation of 1-nitrobenzo[*e*]pyrene by the fungus *Cunninghamella elegans*

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Biotransformation of 1-nitrobenzo[*e*]pyrene (1-nitro-B*e*P), an environmental pollutant derived from the nitration of a non-carcinogen, benzo[*e*]pyrene, was studied using the fungus *Cunninghamella elegans* ATCC 36112. After 72 h incubation, 89% of 1-nitro-[^{3}H]B*e*P added had been metabolized to two major metabolites. These metabolites were separated by reversed-phase high performance liquid chromatography and identified by ^{1}H NMR, UV-visible, and mass spectral techniques as 1-nitro-6-benzo[*e*]pyrenylsulfate and 1-nitrobenzo[*e*]pyrene 6-*O*-β-glucopyranoside. Comparison of the fungal metabolism patterns of 1-nitro-B*e*P and B*e*P indicates that the nitro group at the C-1 position of B*e*P altered the regioselectivity of metabolism.

Keywords: nitro-PAHs; metabolism; *Cunninghamella elegans*; biotransformation

Introduction

Nitro-polycyclic aromatic hydrocarbons (nitro-PAHs) in the environment are formed during combustion processes or from atmospheric reactions of PAHs and nitrogen oxides, both of which are abundant environmental contaminants [10]. Nitro-PAHs have been detected in emissions from diesel engines and kerosene heaters, urban air, river sediments, and even in certain food products [15]. Nitro-PAHs are genotoxic and may pose a health risk to humans [15,23].

1-Nitrobenzo[*e*]pyrene (1-nitro-B*e*P), an environmental pollutant derived from a non-carcinogenic compound, benzo[*e*]pyrene (B*e*P), has been detected in diesel soot extract (2 μg g^{-1} of extracted organic matter) [12]. 1-Nitro-B*e*P is a structural isomer of the environmental contaminants 1-, 3-, and 6-nitrobenzo[*a*]pyrene [11]. Rat liver microsomal metabolism studies with 1-nitro-B*e*P [11] show weak mutagenicity to bacteria, which is dependent upon nitroreduction and transesterification. The major metabolites produced from rat liver microsomes incubated with 1-nitro-B*e*P are the *trans*-4,5-dihydrodiol and 6-hydroxy- and 8-hydroxy-1-nitro-B*e*P [11]. The phenolic metabolites of 1-nitro-B*e*P are mutagenic and the K-region *trans*-dihydrodiol is the most potent mutagenic derivative [11].

Microbial metabolism of some nitro-PAHs was recently reviewed [7,16,23]. The fungas *Cunninghamella elegans* metabolizes both PAHs and nitro-PAHs to products that are generally less mutagenic than the parent compounds [4,7,8,16], while mammalian systems show a greater tendency toward the more common bioactivation pathways [7,8,21,24]. The biological activities may be mediated by metabolic activation via oxidative and reductive pathways to form intermediates by mammalian microsomal enzymes to elicit their mutagenic, genotoxic, and carcinogenic properties [7]. The biological activities of nitro-PAHs include induction of mutations in prokaryotic and eukaryotic cells; neoplastic transformation of cultured normal human diploid fibroblasts; induction of DNA strand breaks; induction of sister chromatid exchanges; induction of DNA repair; and induction of chromosomal aberrations [7,23].

Previous studies with several nitro-PAHs using *C. elegans* has shown that, depending on the position of the presence of a nitro group on the PAH molecule, the metabolism pattern can shift which is indicative of a highly regioselective manner of oxidation [7,16,17,22]. *C elegans* transforms B*e*P to 3-benzo[*e*]pyrenylsulfate, 10-hydroxy-3-benzo[*e*]pyrenylsulfate, and benzo[*e*]pyrene 3-*O*-β-glucopyranoside [19]. In the present study, we report the first investigation of fungal metabolism of 1-nitro-B*e*P and the regioselective manner of oxidation due to the nitro group at the C-1 position of B*e*P.

Materials and methods

1-Nitro[^{3}H]B*e*P (specific activity, 2.47 mCi mmol^{-1}; radiochemical purity >98%), and unlabeled 1-nitro-B*e*P (purity 99%) were synthesized by nitration of B*e*P as described in Fu *et al* [11]. High-performance liquid chromatography (HPLC)-grade solvents were purchased from Fisher Scientific (Pittsburgh, PA, USA). All other chemicals were reagent grade and the highest purity available.

The fungal incubation conditions reported previously [20] were modified in this study as follows: after growth of *C. elegans* ATCC 36112 cultures in 30 ml of Sabouraud dextrose broth for 48 h, 3 mg of 1-nitro-B*e*P dissolved in 0.5 ml of dimethyl sulfoxide was added to each culture. All flasks were incubated for an additional 72 h in the dark, and then the contents of the flasks were pooled and filtered to separate the broth from the mycelium. The mycelium and the broth were then extracted with six equal volumes of ethyl acetate, which was dried over Na_2SO_4. The ethyl

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acetate was evaporated and the residue was dissolved in methanol for analysis by HPLC. Sterile control flasks dosed with 1-nitro-B*e*P showed no changes.

Kinetic experiments were conducted with 1.93 μCi of 1-nitro[^{3}H]B*e*P and 3 mg of unlabeled 1-nitro-B*e*P added to culture flasks. The percent metabolism to various products was quantified by liquid scintillation methods [20].

Fungal metabolites of 1-nitro-B*e*P were separated on a Perkin-Elmer (Norwalk, CT, USA) series 10 HPLC equipped with an LC-95 UV-visible absorbance detector operated at 254 nm. A Supelcosil LC-PAH C18 column (25 cm × 4.6 mm; Supelco, Bellefonte, PA, USA) was used to separate the metabolites. A 40-min linear gradient of methanol-water (from 30 : 70 to 95 : 5 [vol/vol]), at a flow rate of 1.0 ml min^{-1}, eluted the metabolites. 1-Nitro-B*e*P metabolites were collected from repeated injections. Fractions with similar HPLC retention times were pooled and concentrated. The metabolites obtained were further purified by reducing the flow rate to 0.8 ml min^{-1}.

UV-visible absorption spectra of the metabolites were determined in methanol with a Hewlett-Packard (Wilmington, DE, USA) 8453 UV-vis spectrophotometer. Mass spectral (MS) analyses were performed using a Finnigan MAT (San Jose, CA, USA) TSQ 700 mass spectrometer.

The samples were dissolved in methanol and analyzed by direct probe/electron ionization mass spectrometry (DEP/EI/MS). The first quadrupole of the mass spectrometer was scanned from 20 to 500 Da in 0.5 s. The ion source temperature was 150°C and the electron energy was 70 volts. The DEP current was ramped from 0 to 800 mA at 10 mA s^{-1}. For the suspected sulfate conjugate metabolite, the ion source was 175°C and was scanned from 50 to 700 Da in 0.5 s with DEP heating at 15 mA s^{-1}.

NMR spectra were recorded at 28°C in the ^{1}H configuration at 500.13 MHz on a Bruker (Billerica, MA, USA) AM500 spectrometer. Samples were dissolved in deuterated acetone and chemical shifts are reported on the δ scale by assigning the residual proton resonance of the deuterated solvent to 2.05 ppm.

The suspected sulfate conjugate metabolite was deconjugated by adding equal quantities to two test tubes, each containing 2 ml of 0.2 M Tris HCl buffer (pH 7.2). To one sample, 1.0 U (70 μl) arylsulfatase (type V, Sigma) was added. The second sample served as a control. Reaction mixtures were incubated for 48 h at 37°C. Activity of the enzyme was tested by adding 1.0 U *p*-nitrophenyl sulfate to a tube containing Tris-HCl buffer and 0.5 U arylsulfatase. Upon hydrolysis, *p*-nitrophenol formation was indicated by a yellow color. Samples were extracted with 15 ml ethyl acetate as described above.

Results

The HPLC elution profile of the ethyl acetate-soluble metabolites formed during 72 h incubation of 1-nitro-B*e*P with *C. elegans*, with the UV detector at 254 nm, shows two major peaks at 16.0 and 27.5 min and residual 1-nitro-B*e*P at 45.5 min (Figure 1). When *C. elegans* cultures were incubated with 1-nitro[^{3}H]B*e*P, about 40% of the total radioactivity added was recovered in the organic-soluble phase at time zero, while the remainder (60%) was bound to the mycelium. The radiolabeled fractions for the culture incubated for 72 h were separated by HPLC (Figure 1); radioactive fractions were collected every 0.5 min and detected by liquid scintillation methods. Insets above the major peaks, I and II, and the parent compound, 1-nitro-B*e*P, show the amount of radioactivity (dpm) recovered in each peak (Figure 1).

The kinetics of the disappearance of 1-nitro[^{3}H]B*e*P and the appearance of the two labeled metabolites are shown in Figure 2. Since about 72% of the added radioactivity recovered in the organic phase at time zero was found in the 1-nitro-B*e*P peak, this absolute recovered radioactivity of 1-nitro-B*e*P was adjusted to 100% to correct for extraction efficiency. At 48 h, the 1-nitro-B*e*P peak had decreased to about 25% of the radioactivity at time zero, while metabolites I and II accounted for 54 and 21%, respectively. At 72 h, metabolite I accounted for 72% of the recovered radioactivity and at 96 h only 6% remained as 1-nitro-B*e*P (Figure 2). Sterile control flasks dosed with 1-nitro-B*e*P showed no changes in 72 h.

The structure of metabolite I, aided by NMR analyses (Table 1), including extensive homonuclear decoupling and nuclear Overhauser enhancement (NOE) experiments, and by comparison with the NMR assignments of 1-nitro-B*e*P, was determined as 1-nitro-6-benzo[*e*]pyrenylsulfate. The chemical shift of H5, which is *peri* to the sulfate substituent at 8.76 ppm is 0.40 ppm upfield of the H5 of 1-nitro-B*e*P. Similarly, the chemical shift of H7 which is *ortho* to the sulfate substituent is 0.26 ppm downfield of the H7 of 1-nitro-B*e*P. These chemical shifts are all in accordance with structural assignment [19]. The absence of aliphatic resonances eliminates the possibility of conjugation with glucose or glucuronic acid.

The DEP/EI mass spectrum of metabolite I (Table 1) shows an apparent molecular ion at *m/z* 313 and a characteristic fragment ion at *m/z* 283 (M^+-30). The sulfate moiety was lost upon sample heating and ionization.

The NMR data for metabolite I (Table 1) suggest the presence of a sulfate group. Therefore, metabolite I was treated with arylsulfatase and the HPLC, UV-visible, and mass spectral analyses were repeated. Metabolite I, after treatment with arylsulfatase, showed an increase in retention time from 16.0 min to 39.5 min (data not shown). The UV spectra of this metabolite before and after arylsulfatase treatment (not shown) were similar. The mass spectrum of the arylsulfatase-treated metabolite I (not shown) had a molecular ion at *m/z* 313 and a fragment ion at *m/z* 296 (M^+-17), which is characteristic of a hydroxy-1-nitro-B*e*P. Based on the increase in HPLC retention time after deconjugation with arylsulfatase, on analyses of the deconjugated metabolite by UV-visible spectroscopy and mass spectrometry, and on analysis of the conjugate by NMR, metabolite I was identified as 1-nitro-6-benzo[*e*]pyrenylsulfate.

The ^{1}H NMR spectrum of metabolite II (Table 1) contained 10 aromatic resonances and also aliphatic resonances. Based on NMR spectral analysis, the site of substitution was determined to be at C-6. The chemical shifts of the aliphatic protons were similar to those reported for B*e*P 3-*O*-β-glucopyranoside [19] with one exception (H′4 was not detected). The NMR results (Table 1) were essentially

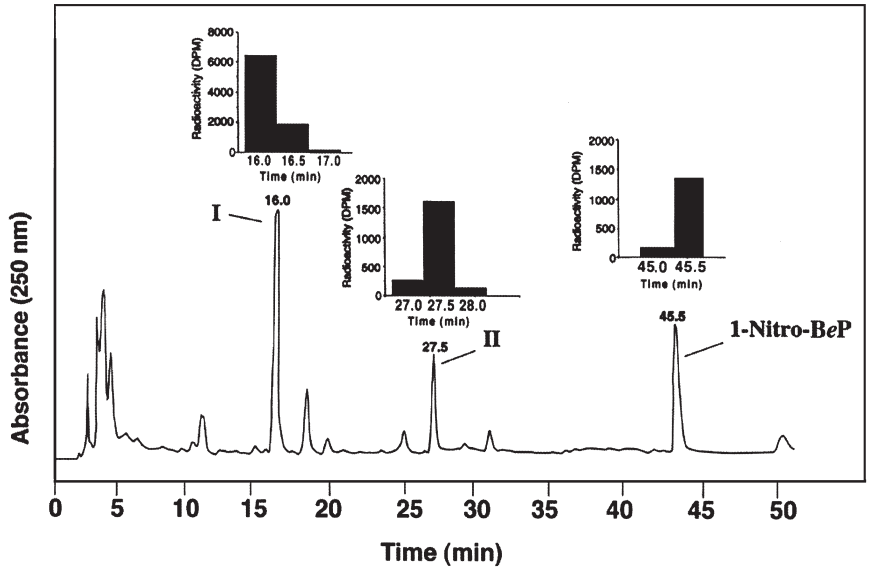

Figure 1 HPLC elution profile and radioactivity of the ethyl acetate-soluble metabolites formed from 1-nitro[^{3}H]B*e*P by *C. elegans*. Insets show the amount of radioactivity contained in each peak. Fractions eluting from the chromatograph were collected at 0.5-min intervals and their radioactivities were measured by liquid scintillation counting.

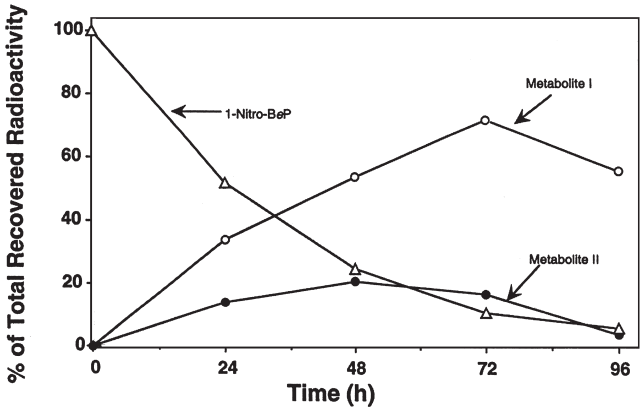

Figure 2 Metabolism of 1-nitro[^{3}H]B*e*P and formation of metabolites I and II over time by *C. elegans*.

the same as those previously reported for the B*e*P glucoside [19]. The DEP/EI/MS spectrum of metabolite II is consistent with a hydroxy-1-nitro-B*e*P, since the glucose moiety was lost thermally to yield the phenol (Table 1). Based on the NMR and MS analyses, metabolite II was identified as 1-nitro-benzo[*e*]pyrene-6-*O*-β-glucopyranoside.

Discussion

Cunninghamella elegans metabolizes PAHs and nitro-PAHs by phase I (oxidation) and phase II (conjugation) reactions [1,3,16,25]. We have previously reported that unsubstituted B*e*P is metabolized by *C. elegans* to the sulfate conjugates of 3-hydroxy-B*e*P and 3,10-dihydroxy-B*e*P [19]. A glucoside conjugate of 3-hydroxy-B*e*P is also formed [19]. In the present study, the oxidation of 1-nitro-B*e*P by *C. elegans* to a hydroxylated intermediate, which was then conjugated with sulfate or glucose, appears to be similar to the metabolism of other PAHs [1,8,16] and nitro-PAHs [7,16] by *C. elegans*. Therefore, by analogy to other studies on the fungal metabolism pattern of PAHs and nitro-PAHs [1–5,8,25], we propose that, in the transformation of 1-nitro-B*e*P by *C. elegans*, the initial attack is at the 6,7-positions of the aromatic ring to form the 6,7-oxide, presumably by a cytochrome P-450 monooxygenase reaction [2,3,9,25] followed by a non-enzymatic rearrangement via an NIH shift mechanism [1,2] to form a phenol (Figure 3). Subsequent conjugative pathways [25] involve sulfation and glycosylation. The formation of 1-nitro-6-benzo[*e*]pyrenylsulfate and 1-nitrobenzo[*e*]pyrene-6-*O*-β-glucopyranoside supports phase II conjugation reactions (Figure 3). In the metabolism of pyrene and 1-nitropyrene by *C. elegans*, oxidation occurs at the C-6 and C-8 positions of the pyrene molecule to form 6- and 8-hydroxypyrene and 6- and 8-hydroxy-1-nitropyrene [4,6]. Even though 1-nitro-B*e*P is similar to 1-nitropyrene in structural configuration, except for an additional aromatic ring, oxidation occured only at the C-6 position to form a phenol. Comparison of the metabolism patterns for 1-nitro-B*e*P and B*e*P indicates that in the metabolism of 1-nitro-B*e*P, which contains a nitro group at the C-1 position, the oxidation at the C-6 position of B*e*P is indicative of a highly regioselective manner of oxidation. In contrast, mammalian microsomes hydroxylate 1-nitro-B*e*P at both the C-6 and the C-8 positions [11].

Studies with 2- and 3-nitrofluoranthene using *C. elegans* also showed formation of sulfate conjugates of 8- and 9-hydroxy-2-nitrofluoranthene and 8- and 9-hydroxy-3-nitrofluoranthene, respectively [16–18]. The nitro group at C-3 shifts the oxidation to the C-8 and C-9 positions of the 3-nitrofluoranthene molecule compared to fluoranthene [17]. *C. elegans* also forms sulfate conjugates from 1- and 2-hydroxy 6-nitrochrysene [22]. Formation of sulfate conjugates from 1-hydroxy-6-nitrobenzo[*a*]pyrene has also been reported [14].

Table 1 HPLC retention times, mass spectral and UV spectrophotometric absorption maxima data, and ^{1}H NMR spectral parameters of metabolites formed from 1-nitrobenzo[*e*]pyrene by *C. elegans*

Compound	Assignment	HPLC retention time (min)	Mass spectral properties *m/z* (% relative abundance) [ion assignment]	UV-visible-light absorption maxima (nm)	Proton NMR assignments, chemical shifts (δ), and coupling constants (J)
I	1-nitro-6-benzo[*e*]pyrenylsulfate	16.0	313(28) [$M^{+\cdot}$][a], 284(16), 283(100), 281(12), 269(11), 268(49), 267(12), 266(25), 255(41), 254(12), 252(15), 250(11), 240(11), 239(32), 238(34), 237(52), 226(20), 119(30), 107(27)	225, 272, 309, 380	δH_2, 8.26; δH_3, 8.36; δH_4, 8.16; δH_5, 8.76; δH_7, 8.51; δH_8, 9.09; δH_9, 9.01; δH_{10}, 7.83; δH_{11}, 7.63; δ_{12}, 8.18 ppm; $J_{2,3} = 8.4$; $J_{4,5} = 8.9$; $J_{7,8} = 8.8$; $J_{9,10} = 8.4$; $J_{10,11} = 7.0$; $J_{11,12} = 8.4$ Hz
II	1-nitrobenzo[*e*]pyrene-6-*O*-β-glucopyranoside	27.5	313(27) [$M^{+\cdot}$][a], 284(19), 283(100), 282(24), 281(13), 267(11), 266(14), 255(25), 254(15), 252(11), 239(18), 238(15), 237(24), 227(12), 226(20), 225(15), 141(12), 135(28), 133(22), 126(14), 125(15), 123(10), 121(17), 113(16), 112(13), 111(12), 110(11), 109(12), 107(20), 98(28), 97(24), 96(13), 95(28), 91(17), 89(25), 84(13), 82(12), 77(11), 71(11), 70(42), 69(42), 68(13), 60(92), 56(33), 55(56), 53(16)	224, 270, 304, 375	δH_2, 8.30; δH_3, 8.41; δH_4, 8.23; δH_5, 8.82; δH_7, 8.07; δH_8, 9.12; δH_9, 9.00; δH_{10}, 7.85; δH_{11}, 7.67; δH_{12}, 8.20; $J_{2,3} = 8.4$; $J_{4,5} = 9.2$; $J_{7,8} = 8.8$; $J_{9,10} = 8.4$; $J_{10,11} = 7.0$; $J_{11,12} = 8.4$ Hz δ for glucose moiety: 1′, 5.42; 2′, 3.80; 3′, 3.65; 4′, not detected; 5′, 3.71; 6a′, 3.96; 6b′, 3.77 J for glucose moiety: $J_{1',2'} = 7.7$; The other coupling constants could not be accurately measured but were consistent with a glucose moiety
Parent	1-nitro-B*e*P	45.5	298(11), 297(60) [$M^{+\cdot}$], 268(13), 267(65) [M-30]$^+$, 266(14), 251(30), 250(100) [M-47]$^+$, 248(20), 240(17), 239(75), 237(13), 133(11), 125(49), 124(32), 120(24), 119(12), 112(14)	210, 271, 304, 375, 488	δH_2, 8.33; δH_3, 8.46; δH_4, 8.28; δH_5, 8.36; δH_6, 8.45; δH_7, 8.24; δH_8, 9.21; δH_9, 9.10; δH_{10}, 7.90; δH_{11}, 7.75; δ_{12}, 8.25 ppm; $J_{2,3} = 8.4$; $J_{4,5} = 8.9$; $J_{6,7} = 7.5$; $J_{7,8} = 8.0$; $J_{9,10} = 8.4$; $J_{10,11} = 7.0$; $J_{11,12} = 8.4$ Hz

[a]Apparent molecular ions after thermal deconjugation.

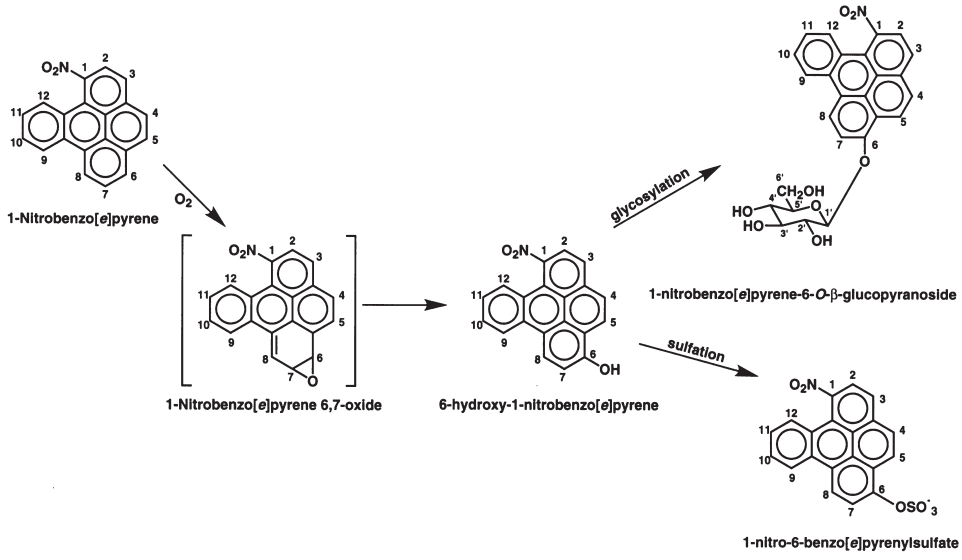

Figure 3 Proposed pathways for the metabolism of 1-nitro-B*e*P by *C. elegans*. The structure shown in brackets is a proposed intermediate that has not been detected.

The conjugated metabolic products of PAHs and nitro-PAHs produced by *C. elegans* are generally considered detoxification products [1,3,21]. The bacterial mutagenicity of 1-nitropyrene decreases with time and loss of mutagenicity of glucoside conjugates of 6- and 8-hydroxy-1-nitropyrene was consistent, indicating the detoxification potential of *C. elegans* [4]. This type of metabolism by *C. elegans* has been previously demonstrated for other xenobiotics [1,3,5,14,25]. However, mammalian sulfate conjugation could result in bioactivation of the xenobiotic [13].

Rat liver microsomal metabolism studies with 1-nitro-B*e*P show the formation of the *trans* 4,5-dihydrodiol and 6-hydroxy and 8-hydroxy-1-nitro-B*e*P as major metabolites [11]. The phenolic metabolites are weak mutagens but the *trans* 4,5-dihydrodiol is a potent mutagen [11]. Additionally, nitroreduction via cytochrome P-450 occurs [11]. In contrast to the activation pathway in mammalian metabolism, the formation of sulfate and glucoside conjugates from 1-nitro-B*e*P by *C. elegans* is likely a step towards detoxification.

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