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Production of Hepatotoxin by the Cyanobacterium *Scytonema* sp. Strain BT 23

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Abstract The preliminary screening of several cyanobacteria, using mice bioassay, revealed the production of a hepatotoxin by the cyanobacterium *Scytonema* sp. strain BT 23 isolated from soil. An intraperitoneal injection of the crude toxin (LD_{50} 56 mg/kg body wt) from this strain caused the death of the mice within 40 min, and the animals showed clinical signs of hepatotoxicity. The toxin was purified and partially characterized. The active fraction appears to be nonpolar in nature and shows absorption peaks at 240 and 285 nm. The purified toxin had an LD_{50} of 100 μg/kg body wt and the test mice died within 40 min of toxin administration. The toxin-treated mice showed a 1.65-fold increase in liver weight at 40 min and the liver color changed to dark red due to intrahepatic hemorrhage and pooling of blood. Furthermore, the administration of the toxin to test mice induced a 2.58, 2.63, and 2.30-fold increase in the activity of the serum enzymes alanine aminotransferase, lactate dehydrogenase, and alkaline phosphatase, respectively. Further experiments with the ^{14}C-labeled toxin revealed a maximum accumulation of the toxin in the liver. The clinical symptoms in the mice were similar to those produced by microcystin-LR. These results suggest that hepatotoxins may also be produced in non bloom-forming planktonic cyanobacteria.

Key words: Cyanobacteria, hepatotoxin, microcystin, *Scytonema* sp., toxin

Toxic cyanobacterial blooms have been reported to occur in a number of eutrophic and hypereutrophic lakes, ponds, and rivers throughout the world, and are known to be responsible for the worldwide killing of wild and domestic animals, fishes, and birds [2, 5, 21, 24]. Two types of cyanobacterial toxins are known: (1) alkaloid neurotoxins (anatoxin-a, -a(s), saxitoxin, and neosaxitoxin), which interfere with the functioning of the nervous system causing very quick death (within minutes) due to paralysis of the respiratory muscles, and (2) cyclic peptide hepatotoxins (microcystins and nodularin), which damage the liver and result in excessive blood pooling in the liver, ultimately leading to fatal circulatory shock within a few hours or death within a few days due to liver failure [4]. Of these, microcystins (hepatotoxins) are the most ubiquitous cyanotoxins that have been responsible for the widespread deaths of animals throughout the world. A generalized structure of microcystin is represented as cyclo(-D-Ala-X-D-MeAsp-Z-Adda-D-Glu-Mdha-) in which X and Z are variable L-amino acids, D-MeAsp is D-erythro-β-methylaspartic acid, Mdha is N-methyldehydroalanine, and Adda is a novel amino acid (2S, 3S, 8S, 9S)-3-amino-9-methoxy-2,6,8 trimethyl-10-phenyldeca-4,6- dienoic acid [4, 8, 21]. Over 50 microcystins have been identified to date, most of them showing only minor variations. *Microcystis aeruginosa* produces many microcystins simultaneously [19]. Microcystin-LR is the dominant microcystin produced by cyanobacteria in several ponds and lakes. The microcystins-producing cyanobacteria are strains of species within the genera *Microcystis*, *Anabaena*, *Oscillatoria*, and *Nostoc* [2, 8, 13, 19] and certain clinically undefined hepatotoxins have been demonstrated in *Aphanizomenon*, *Gloeotrichia*, and *Coelosphaerium* [2, 14, 16]. Until now, most of the research on cyanotoxins has been confined to the species *M. aeruginosa* despite the fact that several genera of cyanobacteria produce a variety of bioactive compounds [14, 18]. For example, several strains of *Scytonema* produce a variety of secondary metabolites including strongly cytotoxic scytophycins [6, 9, 11, 18]. The isolation of cyanobacterin and hydroxycyanobacterin, which show toxicity to algae and higher plants, has also been reported from this genus [6, 11]. However, no production of a microcystin-like compound has ever been recorded from this genus.

The main objective of this study was to screen cyanobacteria, other than *M. aeruginosa*, which are capable of producing microcystin(s)-like compounds. It was also hoped to establish whether cyanotoxins do indeed accumulate in certain vital organs of a test animal. Accordingly, this study shows that microcystin may in fact be present in

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cyanobacteria other than the bloom-forming planktonic species.

MATERIALS AND METHODS

Organisms and Growth Conditions

Scytonema sp. strain BT 23 was isolated from a soil sample from the Agriculture farm of Banaras Hindu University, employing standard microbiological techniques. Out of several synthetic media tested, the strain grew best in BG-11 (nitrogen-free) medium [20]. Cultures were routinely grown in a culture room at 25±1°C with a light intensity of 14.4 Wm^{-2} for 14 h daily, with hand shaking three times a day. A natural water bloom sample of *Microcystis aeruginosa* was taken from a heavily infested local pond. The other cyanobacterial strains used in this study were from the culture collection maintained in our laboratory for the last 10 years. Unless otherwise stated, only axenic cultures were used for testing toxicity against mice. Axenic cultures of *Scytonema* and other algal strains were raised after exposing the unialgal strain to antibiotic (streptomycin) or ultraviolet radiation.

Preparation of Crude Extract

Cyanobacterial cultures harvested in the late exponential phase of growth were used for the preparation of crude aqueous extract. The extracts were prepared in double distilled water by sonicating 1.0 g of wet algal cells in a Branson Sonifier-450 (Branson Ultrasonics Corp., Danburry, CT, U.S.A.) for 5 min at an 8 output control and 60% duty cycle. The sonicated suspension was stirred for 12 h at room temperature, and then centrifuged at 10,000 ×g for 15 min. If required, the resulting pellet was re-extracted 2-3 times. The supernatants were mixed, evaporated to dryness in vacuum, and weighed. A known amount of the dried material was dissolved in double distilled water to achieve the desired concentration of the crude toxin.

Isolation and Purification of Toxin

The separation and purification of the toxin from *Scytonema* sp. strain BT 23 was performed as per the method of Harada *et al.* [7] with certain modifications. Dried *Scytonema* sp. strain BT 23 cells were suspended in methanol:water (1:1) and sonicated in an ice bath for 15 min. The suspension was centrifuged at 10,000 ×g for 10 min and the pellet then re-extracted in the same solvent. Further partitioning of the suspension with the chloroform resulted in an aqueous methanol fraction and a chloroform fraction. Of the two fractions, the chloroform fraction showed toxicity to mice. The active constituent from the toxic chloroform fraction was separated and purified by thin layer chromatography (20×20 cm TLC plates, coated with silica-gel 60 G). After loading of the samples, the plates were developed in hexane:acetone (9:1, v/v). The spots were then visualized under short-wavelength UV radiation. A routine preparative TLC was employed for the enrichment of the putative toxic spot, which was then scratched from the silica gel and recovered in methanol. After centrifugation at 10,000 ×g for 10 min, spectroscopic analysis was performed using an ATI Unicam UV/Vis Spectrophotometer (Unicam Ltd., Cambridge, U.K.).

Toxicity Test

Known amounts of the cyanobacterial extracts or purified toxin, dissolved in normal saline (0.9% NaCl), were injected intraperitoneally (i.p.) into male mice (Albino Park's strain weighing 20±2 g) to evaluate the toxicity. Three mice per dose level and four different dose levels were selected. The mice injected with the toxin were closely observed over a period of 7 h. However, survival times of less than 5 h were taken to be due to cyanobacterial toxicity. The control mice received an injection of normal saline alone. The LD_{50} value was taken as the midway concentration between the doses at which 100% of the test mice survived and 100% died.

Assay of Serum Enzyme Activity

The activity of the serum enzymes, viz., alkaline phosphatase (APase), lactate dehydrogenase (LDH), and glutamate pyruvate transaminase/alanine amino transferase (GPT/ALT) was estimated according to Falconer *et al.* [5] with certain modifications [23]. The measurements were made using enzyme kits obtained from Ranbaxy Laboratories Ltd., New Delhi. Serum was collected from mice treated with known doses of toxin. The test mice were food-starved for 6 h prior to the sample collection. Heparin was used as the anticoagulant.

Alkaline Phosphatase

The APase was analyzed using *p*-nitrophenyl phosphate (PNP) as the substrate. The absorbance of the yellow-colored *p*-nitrophenol formed was measured at 405 nm in a spectrophotometer against a blank at different time intervals. One unit of the APase activity was defined as the amount which catalyzed the hydrolysis of 1 nmol of PNP per min at 25°C, pH 8.0.

Lactate Dehydrogenase

The measurement of the LDH activity was determined by the decrease in the absorbance at 340 nm resulting from the oxidation of NADH. One unit caused the oxidation of one μmol of NADH per min at 30°C, pH 7.4, under the specified conditions.

Glutamate Pyruvate Transaminase/Alanine Aminotransferase Activity

The estimation of the GPT/ALT activity was based on the rate of the decrease of the NADH concentration estimated

by taking the absorbance at 340 nm at different time intervals. One unit of enzyme activity was defined as the amount of enzyme which caused the oxidation of one μmol of NADH per min at 37°C, pH 7.4.

Labeling of Toxin by ^{14}C

In order to obtain the ^{14}C-labeled toxin, 1.0 ml of $NaH^{14}CO_3$ (specific activity 5 μCi/ml) was added to 200 ml of actively growing cultures of *Scytonema* sp. strain BT 23 (OD=0.40 at 663 nm). The cotton plug was replaced by a stainless steel (SS) cap to avoid the escape of $^{14}CO_2$. After 24 h, the SS-caps were removed and 0.1 ml of $NaH_{14}CO_3$ was added at 3-day intervals upto 12 days. After 14 days of growth, the cultures were harvested and toxin was extracted as described earlier [23]. A TLC of the crude extract was performed to obtain ^{14}C-labeled toxins. To confirm the incorporation of the ^{14}C in toxin, individual spots from the TLC plate were scratched and counts for each spot were taken using an LKB 1209 Rackbeta Liquid Scintillation Counter (LKB Wallac, Wallac OY, Turku, Finland).

Test for Accumulation of Labeled Toxin

The ^{14}C-labeled toxin, at a concentration of 100 μg/kg body weight, was injected into mice and the resulting ^{14}C incorporation in the liver and spleen was monitored at desired time intervals after sacrificing the mice.

Chemicals

Enzyme kits for the estimation of the serum enzymes were obtained from Ranbaxy Laboratories Ltd., New Delhi. $NaH_{14}CO_3$ was obtained from the Radio-isotope group, Bhabha Atomic Research Centre, Trombay, Mumbai. All other chemicals were of Analar grade available at the highest purity level from SISCO Research Laboratories, Mumbai.

RESULTS

Initial Screening of Toxic Cyanobacteria

A natural bloom sample of *M. aeruginosa* and a few other laboratory grown cyanobacteria, isolated from local rice fields, and belonging to genera such as *Anabaena*, *Scytonema*, *Tolypothrix*, and *Calothrix* (altogether 30 species) were screened for possible toxicity. Mice bioassay testing of the crude extracts of all these cyanobacteria revealed the toxic nature of *M. aeruginosa* and *Scytonema* sp. strain BT 23 only. An intraperitoneal injection of the crude extract of *Scytonema* sp. strain BT 23 caused death in mice after 40 min with a median lethal dose (LD_{50}) of 56 mg/kg body wt. Similarly, a dose of 60 mg/kg body wt of the aqueous extract of *M. aeruginosa* killed mice within 45 min. The administration of the toxins from both these cyanobacteria led to the appearance of clinical signs of poisoning such as restlessness, loss of co-ordination, slow movement, spasmodic leaping, fast breathing, and the splaying of hind limbs.

Isolation and Characterization of *Scytonema* sp. strain BT 23 Toxin

Once it became evident that *Scytonema* sp. strain BT 23 was indeed toxic, it was isolated and the toxic fraction characterized. Accordingly, the crude extract prepared in methanol:water (1:1) was partitioned with chloroform. Of the two partitioned fractions, the toxicity was routinely found to be associated with the chloroform fraction. Thin layer chromatography of the chloroform fraction resulted in the separation of 6 spots (ST-1 to ST-6) which were detected by short-wavelength UV radiation, iodine vapor, or by heating at 100°C after spraying with 30% H_2SO_4. From these six spots, the active fraction present in ST-6 proved toxic and was recovered in an appreciable amount. The pooled fraction of spot ST-6 (Rf 0.84), after rechromatography on TLC, showed the appearance of only one spot (ST-6) with an identical Rf value. The compound present in this spot showed absorption peaks at 240 and 285 nm in methanol (Fig. 1). The compound was soluble in nonpolar solvents (e.g. petroleum ether, diethyl ether, and ethyl acetate) and partially soluble in ethanol or methanol, yet completely insoluble in water. Based on its solubility character, this compound appears to be nonpolar in nature. Moreover, the compound was found to be heat-resistant and did not lose its toxicity after heat treatment (80°C for 2 h). The purified toxin (LD_{50} 100 μg/kg body wt), when injected into mice, caused death within 40 min and the clinical signs were similar to those observed with the administration of the crude toxin.

Effect of Varying Concentrations of Toxin on Death Time

Since a concentration as low as 100 μg/kg body wt caused death in mice within 40 min, it was considered desirable to

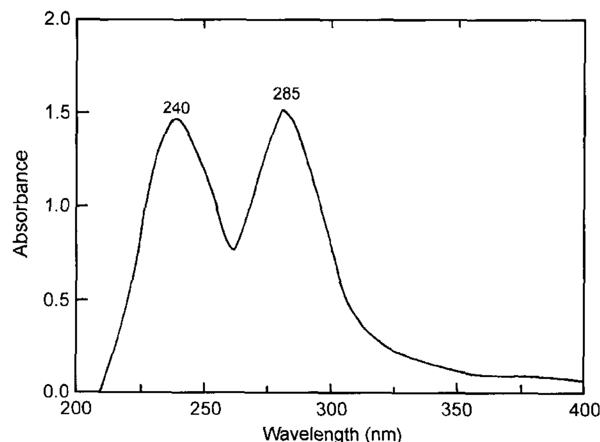

Fig. 1. UV absorption spectrum of purified toxin of *Scytonema* sp. strain BT 23 dissolved in methanol.

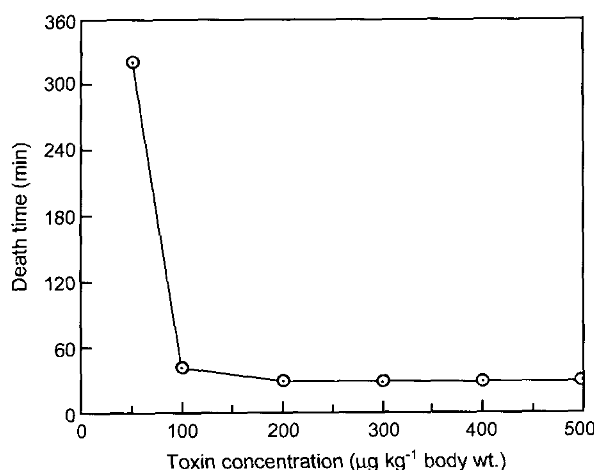

Fig. 2. Effect of varying concentrations of *Scytonema* sp. strain BT 23 purified toxin on death time of mice.
Each mouse was administered the indicated dose of toxin (i.p.). The response of the mice was observed upto 7 h.

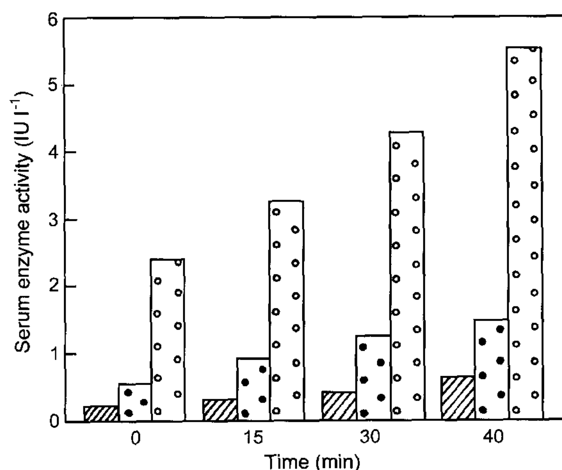

Fig. 3. Effect of toxin on activity of serum enzymes.
lactate dehydrogenase; alanine aminotransferase; and alkaline phosphatase. 100 µg purified toxin/kg body wt of mice was injected (i.p.) and thereafter the enzyme activity was measured at the indicated time intervals. The 0-time indicates the basal enzyme activity of the control mice which remained more or less unchanged upto 40 min. Data are means of three separate yet identical experiments.

study the effects of varying concentrations of the toxin on the death time. From the data represented in Fig. 2, it can be seen that the administration of increased doses of the toxin to mice showed a minimum survival time of 30 min which was independent of the amount of toxin administered. An increase in the concentration from 200 µg/kg body wt to 500 µg/kg body wt did not shorten the time until death.

Effect of Toxin on Liver Weight and Morphology

A significant increase in liver weight following injection of the purified toxin (100 µg/kg body wt) was observed. The liver showed an enlargement and there was 1.65-fold increase in fresh weight 40 min after the toxin administration (Table 1). Besides the weight increase, the color of the liver changed to a deep red probably due to hemorrhaging and blood pooling. Almost the same effects on mice livers were observed with the purified toxin of *M. aeruginosa* (microcystin-LR).

Table 1. Changes in the liver weight (in g) of the mice treated with *Scytonema* sp. strain BT 23 toxin.

Treatment	Time (min)			
	5	10	20	40
Control[a]	1.15	1.15	1.15	1.15
Scytonema sp. strain BT 23 toxin[b]	1.19 (1.04)	1.32 (1.15)	1.65 (1.44)	1.89 (1.65)

[a]Control mice (weighing 20±2 g) were injected with 0.5 ml normal saline (0.9%).
[b]Experimental mice were injected (i.p.) with 0.5 ml of the purified toxin (100 µg/kg body wt). At the indicated time intervals, the liver weight was taken after sacrificing the animal. The death of the mice occurred 40 min after the toxin administration. Values shown are means of three separate experiments.
Numbers in parentheses indicate-fold increase.

Apart from the increase in the weight of the liver following the toxin administration (100 µg/kg body wt), autopsies showed a marked congestion in the livers of the sacrificed mice. This was seen from a histopathological examination of the livers. Necropsy revealed a swollen liver and centrilobular to panlobular hemorrhagic necrosis. Altogether, the liver suffered gross changes in its normal structure.

Effect on Serum Enzyme Activity

The impact of the toxin on the level of serum enzyme activity in mice was studied in a time course experiment. The purified toxin caused a marked and gradual increase in the activity of the serum enzymes ALT, LDH, and APase (Fig. 3). The maximum increase in enzyme activity was found 40 min after the toxin administration. The ALT activity showed a 2.58-fold increase whereas LDH and APase showed 2.63 and 2.30-fold increases, respectively.

Accumulation of Toxin in Different Organs

Knowing that the *Scytonema* toxin severely affects the liver function and causes gross morphological and histological changes, the accumulation of the toxin in liver and some other organs, such as the spleen was investigated. The ^{14}C-labeled toxin was administered to mice and the resulting uptake of the labeled C in liver and spleen was estimated at desired time intervals. The data in Table 2 show that the toxin instantly accumulated in the liver, with a significant accumulation occuring within 20 min, whereafter the increase in the total count was negligible. In contrast, the accumulation of the toxin in the spleen was time dependent and the largest count was recorded after 30 min. Altogether,

Table 2. Distribution pattern of the ^{14}C-labeled toxin in the liver and spleen of the mice.

Time (min)	DPM g^{-1} fresh wt	
	Liver	Spleen
10	12,340	1,600
20	14,600	2,540
30	15,115	4,270
40	15,360	5,135

^{14}C-Labeled purified toxin (100 μg/kg body wt) was injected (i.p.) into mice and the ^{14}C incorporation in the liver and spleen was determined at desired time intervals after sacrificing the mice. The total initial count (0.5 ml toxin) was 1×10^5 DPM (disintegrations per minute). The results are based on averages of three separate yet identical experiments.

there was an approx. 15% incorporation of the ^{14}C toxin in the liver and only 5% in the spleen.

DISCUSSION

Serveral species of cyanobacteria have been reported to produce highly toxic bioactive secondary metabolites [1, 3, 18, 21, 24]. These metabolites include both cytotoxins and biotoxins, however, only biotoxins are of primary concern for animal and human health. Among biotoxins, the most common include hepatotoxic cyclic peptides (microcystins and nodularins) and the hepatotoxic alkaloid (cylindrospermopsin) [16]. Hepatotoxins have been isolated and purified from certain planktonic bloom-forming species which include both filamentous (*Anabaena, Oscillatoria, Nostoc, Hapalosiphon, Cylindrospermum*) and unicellular colonial (*Microcystis*) species [3, 16]. Until now, no hepatotoxins have been reported from any *Scytonema* species [3, 21].

Based on clinical symptoms and spectral properties, this study has demonstrated the presence of a hepatotoxic fraction(s) in the terrestrial cyanobacterium *Scytonema* sp. strain BT 23. The LD_{50} of the purified toxin (100 μg/kg body wt) from this strain compares well with the LD_{50} of purified hepatotoxin (microcystin-LR), as reported earlier [4, 8, 12, 19]. Furthermore, the clinical symptoms observed in test mice such as weak co-ordination, paralysis, muscle tremors, paddling, labored breathing, lethargy, and a coma-like stage before death are reminiscent of similar symptoms produced by hepatotoxic strains of *M. aeruginosa* [2, 19, 23, 24]. The resemblance of the *Scytonema* sp. strain BT 23 toxin to a microcystin-like compound is also apparent from its Rf value observed on TLC plates and absorbance at 240 nm. Purified microcystin shows absorbance at 230 nm which is very close to the absorbance observed with the *Scytonema* sp. strain BT 23 toxin. However, the solubility characteristics of the *Scytonema* toxin differ significantly from microcystins. The exact chemical nature of the newly reported toxin may be revealed only after its detailed characterization. Conceivably, the toxin isolated in this study may be a variant of a standard microcystin, or it could be a new class of hepatotoxins.

The ultimate cause of death by the *Scytonema* toxin appeared to be primarily hemorrhagic shock caused by the pooling of the blood in the liver [5, 15, 24]. Gross morphological and histopathological examinations of the toxin-treated mice did not reveal any other site of hemorrhage in the body. The animals treated with the toxin showed a swollen, blood-engorged liver with extensive centrilobular to panlobular hemorrhagic necrosis. The conclusion that death resulted from intrahepatic hemorrhage was based on the fact that an excessive increase in the liver weight associated with a hepatic hemorrhage, that could account for a blood loss high enough to induce irreversible shock, was observed in the treated mice. The liver in the toxin-treated mice comprised 8 to 10% of the body weight of the mice as compared to about 5% in the control animals [5]. This observation pertaining to an increase in the liver weight (upto 1.65-fold, ca. 65%) is in accordance with previous reports and points to the presence of a microcystin-like compound in *Scytonema* sp. BT 23. An increase in the hepatic hemoglobin and iron content that account for a sufficient blood loss in the liver has also been reported [5, 22]. Accumulation of ^{14}C-labeled toxin in liver also corroborated the conclusion that the toxin is hepatotoxic in nature.

The toxin administration also led to an increase in the activities of the serum enzymes, notably alanine aminotransferase, lactate dehydrogenase, and alkaline phosphatase. Such increases in the level of hepatic enzymes in plasma have been shown to be associated with liver malfunction [5, 22]. In human beings, an increase of alkaline phosphatase activity is generally observed in diseases of the liver and biliary tract, post-hepatic jaundice, as well as infective or toxic hepatitis [22]. Similarly, lactate dehydrogenase is widely distributed in all organs/cells of the body and shows a high activity in liver and skeletal muscles. An increase in LDH activity is also observed in liver diseases [3, 23] and is a useful indicator of microcystin-LR hepatotoxicity [5, 23]. Falconer *et al.* [5] noted an elevation of alanine aminotransferase in plasma specimens from humans whose source of drinking water was a reservoir infested with a heavy bloom of toxic *M. aeruginosa*.

The evidence presented in this study demonstrates that *Scytonema* sp. strain BT 23 is a toxic cyanobacterium that contains a hepatotoxic compound. Since this species grows in soil, the ecological impact of the toxin may be broad. The antialgal and damaging effects of certain *Scytonema* species have been well established [9, 11, 14, 17, 24]. Accordingly, taken together, it would appear that *Scytonema* sp. strain BT 23 toxin may prove to be a novel class of cyanotoxin when its chemical characterization is made in detail. Work is now currently in progress on the possible application(s) of this toxin as a microbiocide.

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