A Preliminary Analysis of the Lipid Contents of a Biofilm from a

Yellowstone Hydrothermal Pool

by

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Abstract

The hot springs at Yellowstone National Park, such as Obsidian Pool, have been extensively studied as a source for previously unidentified microorganisms. Most of the previous studies focused on the genomic diversity of these environments, but recent interest in environmental samples for the study of lipid biomarkers has extended to these hot springs as well. In this preliminary study of Obsidian Pool, I used a modified Bligh and Dyer extraction process to create a total lipid extract (TLE), which was then further separated and processed by an acetone precipitation, mild alkaline methanolysis and column chromatography to isolate different fractions of the TLE. Compounds were then identified by gas chromatography-mass spectrometry (GCMS). The mild alkaline methanolysis allowed for the identification of decanoic acid methyl ester, a fatty acid methyl ester. From the column chromatography, only the saturated hydrocarbon fraction yielded interesting compounds; these were a series of n-alkanes (n-C_{17}, n-C_{20}, and n-C_{23} – n-C_{31}), two branched alkanes (5-methyloctadecane and 4-methylheptadecane), an isoprenoid (dibiphytane), a series of branched aliphatic alkanes with a quaternary substituted carbon atom (5,5 diethylalkanes), and a variety of hopanoids and steroids. These compounds indicate a strong community of bacterial and archaeal species in Obsidian pool. Some compounds that were indicative of eukarya were also identified, although a more in-depth study is necessary to determine whether these signals were from the pool or from external inputs. The lipids identified in this study indicate that the genomic diversity established by previous studies is reflected in the lipid diversity of Obsidian Pool. However, more study is required to fully categorize the lipids in the pool.
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Introduction

Yellowstone National Park, with a hydrothermal system of pools and vents, many of which also contain large quantities of sulfur or extremes in pH, is a spectacular natural laboratory for the study of microbial communities that have adapted to extreme conditions. Current theories about the earliest forms of life on this planet suggest that these microbes thrived in a high temperature environment with neutral pH and quantities of silica and sulfur. These theories are based on studies of simple microbes, Archaea and Bacteria, which, based on genetic analysis, are closest to the root on the single-subunit-RNA-based “tree of life,” and live in such environments. The study of these communities, therefore, can teach the scientific community invaluable lessons about our earliest ancestors. Obsidian Pool (Fig. 1) is a hot spring in Yellowstone National Park with a pH 5.1 that is fed by a subsurface vent with a temperature of 77.5° C. Extensive studies of the microbial ecology of Obsidian Pool have shown it to be the habitat for previously unknown species of bacteria and archaea and a few eukarya. An intriguing conclusion of many genomic and geochemical studies of the hot spring is that there is diverse community of hydrogen

Figure 1. Obsidian Pool. Located south of the Mud Volcano tourist area, the pool is surrounded by woods and on a bison trail. The pool is about 3X10 meters, and has a few boiling areas. The dark black color of the water comes from the course obsidian sand around the vents.
oxidizing species that have adapted to these extreme temperatures and low pH. This study involves an analysis of intact polar lipids in order to evaluate the relative abundances of various microbial inputs and determine the presence of taxa that genomic and geochemical studies would not have predicted.
Background

Many of the first studies of Obsidian Pool focused on the genomic diversity present in the community. Differences in the genome are what define individuals as unique and each species as distinct. Closely related species have genomic sequences that are similar and the more distant the relationship between two species, the more distinct their genomes. The genome of an individual consists of a unique sequence of DNA that, through RNA, encodes all of the enzymes necessary for life. A “tree of life” has been created from studies of the RNA that make up ribosomes, the universal protein-assembly mechanism, that places every species in relation to the others (Fig. 2). The main divisions of this tree are the three domains, Archaea, Bacteria and Eukarya, each of which have distinguishable ribosomal RNA, but are also defined by other features. Archaea are defined by a combination of characteristics, among them the lack of membrane-bound organelles, which differentiates them from the eukaryotic domain, Eukarya, which do have organelles. Also, archaeal membranes are composed of mostly isoprenoid glycerol diethers or
diglycerol tetraethers, unlike Bacteria, which have mostly diacyl glycerol diesters (Woese et al. 1990). Eukarya and Bacteria, which have been identified in environments as warm as 65°C and 95°C respectively, are generally not found in the extreme environments that are populated by, and are sometimes necessary, for some archaeal species, some of which grow optimally above 80°C and at temperatures as high as 121°C. Although Bacteria also live in extreme environments, the unique ether lipids that make up Archaeal membranes make them most able to function in conditions with extreme temperatures. Unlike Bacteria and Eukarya, which react to high temperatures by increasing the chain length of the membrane lipid chains, increasing the branching or increasing the saturation of the membranes, Archaea have been shown to respond by cyclization of lipid chains and transition from diether to tetraether lipids (Van de Vossenburg et al. 1998).

One of the first genomic studies performed on sediments from Obsidian Pool (Barns et al., 1994) discovered an unprecedented diversity of Archaea. This study used polymerase chain reaction (PCR) amplification of the DNA sequences for small subunit ribosomal RNA (ssuRNA) on an environmental sample from the sediment, without culturing of individual taxa. Individual taxa contain slightly different DNA sequences for ssuRNA, allowing differentiation and estimates of genetic biodiversity. The group was able to identify many new sequences from well-known families in the subdomain crenarcheota (the other subdomain is euryarcheota), the Desulfurococcccus/Pyrodictium clade, Pyrobaculum sp. Thermofilum pendens and Archaeoglobus fulgidus, the first three of which were first identified in environments with temperature ranges similar to this hot spring. A few new sequences were identified that showed no close relationships to cultured taxa but were found to diverge from the crenarchaeal stem closer to the root than any taxa previously studied.
A study in 1996 by the same group (Barns et al. 1996) identified more sequences from the established crenarcheota lines and sequences from more new families. As in the previous study, sequences were identified that diverged from the crenarcheotal line very early in its evolution. The large number of these sequences encouraged the team to suggest the idea of a third Archaeal kingdom, the Korarchaeota meaning “young archaeota” for the early divergence of this group during the evolution of the Archaea. The research presented by Barns et al. in both of these papers suggests a large diversity of archaeal species in Obsidian Pool.

Hugenholtz et al. (1998), using the same procedure, PCR amplification of ribosomal DNA on a mixed sample, were able to identify sequences from a diverse range of previously unknown bacteria, but very few archaeal sequences. This result was not unexpected however, as this team used Bacteria-specific primers that have a higher probability of matching, and therefore preferentially amplifying, bacterial sequences. The unexpected result of this study was the diversity of sequences identified by this team.

One of the most recent studies of the Yellowstone high-temperature pools (Spear et al., 2005) focused on the geochemical energy budgets for such ecosystems. Through a combination of phylogenic studies and chemical analyses, this team concluded that the organisms in these pools derive their energy from the oxidation of molecular hydrogen, a hypothesis supported by the high concentrations of hydrogen that was measured in these pools (325 nM for Obsidian Pool, versus 2 nM for a control sample). Although sulfide has long been considered an important source of energy for these communities, this study suggests that it plays only a minor role in these source pools, though it may be more
important in pools where cooler water will allow for a higher solubility of molecular oxygen.

The topic of interest to my study is the lipid contents of a biofilm identified from Obsidian Pool sediment. In this study, I extracted the total lipids from the sediment and analyzed both the total extract and fractions, with gas chromatography-mass spectrometry (GCMS). My hypothesis is that the lipids will be diverse and reflect genomic diversity already established by sequencing approaches. This study of lipids provides another approach to study the diversity in the pool.

Rütters et al. (2001) reported one of the first such studies to investigate the differences between the lipid compositions of the sulfate-reducing bacteria Desulfothricus variabilis and Desulfotomaculum amnigenus grown in culture. The team used liquid chromatography – mass spectrometry (LCMS) to identify intact lipids and verified the presence of saturated ether lipids with n-alkyl and branched chains using chemical degradation with hydroiodic acid and identification of alkyl iodides. These lipids had previously only been identified in thermophilic Bacteria and the deepest branches of the phylogenetic tree. Although it is possible to identify specific lipids present in an extract by understanding their characteristic mass spectra and chromatographic retention behaviors, if they have been previously observed and identified, complex structures become easier to determine when they are degraded into smaller components that can be analyzed individually, as they are in this study.

This same team (Rütters et al. 2002) employed the Bligh-Dyer extraction and LCMS as an analytical procedure for the identification of microbial lipids in environmental samples, rather than in cultures. Understanding the connection between the occurrence or
particular phospholipids and individual microbial taxa allowed the team to identify the presence of microorganisms such as cyanobacteria from the lipids extracted from surface sediments in a sandy tidal flat. To facilitate interpretation of data from the environmental sample, they also analyzed three cultured strains of sulfate-reducing bacteria *Desulfbacter postgatei*, *Desulfomicrobium* sp. strain SAL and *Desulfococcus multivorans*.

Jahnke et al. (2004) performed a study of samples from Yellowstone National Park using a method of lipid extraction and study similar to the one used here, as well as the locale from which the samples were identified. This team studied environmental samples from bacterial mats in two locations and compared the results to a culture of a filamentous cyanobacterium, a species similar to those that created the mats. Using the comparison, Jahnke et al. were able to distinguish lipid biomarkers that are characteristic of cyanobacteria from others that are characteristic of the green non-sulfur bacterial species.
Methods

Sample Collection and Extraction

Three samples of sediment were collected from Obsidian Pool in Yellowstone National Park in August 2003 and August 2004. Two 2003 samples (YNP038 and YNP047) were taken from a surface at the edge of the pool and a third (2004) was a core of the sediment (YNP124). These samples were frozen immediately and subsequently freeze-dried upon return to the laboratory. The samples were extracted using a modified Bligh and Dyer extraction process (Bligh and Dyer 1959). The Bligh and Dyer extraction uses a chloroform, methanol and water mixture that can be harsh. Further, chloroform is also now considered a possible carcinogen, hence other methods are more appropriately used to disrupt the membranes and still extract the lipids effectively without any loss of complexity.

For the modified extraction, 10 g of freeze-dried sediment was suspended in 20 mL of a 3:1 mixture of dichloromethane (DCM) and methanol (MeOH). After mixing manually and by sonification, the mixture was centrifuged and the liquid layer, containing the lipids, was drawn off the top. The extraction was repeated three times. The DCM containing the lipids was then filtered and run through copper, which had been activated by HCl, to remove sulfur that would interfere with the GCMS analysis.

The products, or total lipid extract (TLE), yields from the three samples were: 5.1 mg of YNP038, 2 mg of YNP047 and 7.9 mg of YNP124. A small aliquot, 100 ng, taken by suspending the TLE in a known volume of DCM, was derivatized, and made more reactive, with 10 µl of N,O-bis (trimethylsilyl)-acetaminde (BSTFA) and 10 µl pyridine and heated at 60°C for 1 hour, diluted in ethyl acetate and run on the GCMS with an epiandrosterone
standard. A further aliquot of the TLE was separated into compound classes using a combination of acetone solubility and column chromatography (Fig 3).

Figure 3. Methods. Procedure designed for preliminary analysis of Obsidian Pool samples.

Polar Lipids

The major fraction of the TLE, 3.33mg of YNP038, 1.67mg of YNP047, and 5mg of YNP124 was suspended in cold acetone to precipitate polar lipids. Half of this polar lipid fraction was analyzed by mild alkaline methanolysis (MAM) using .2M methanolic KOH. This process results in transesterification where the ester-linked fatty acids are converted to fatty acid methyl esters (FAME). The organic phase yielded by this procedure contains the FAMEs, which are volatile and were suspended in hexane and run on GCMS with an epiandrosterone standard.
The other half of the polar lipid fraction was subjected to a cycle of oxidation with periodic acid and reduction with sodium borohydride, known as the Rohmer procedure. This procedure cleaves the complex side chains of bacteriohopanepolyols at the diol closest to the ring structure and yields a mixture of C$_{30}$ – C$_{32}$ bacteriohopanols. This mixture was then derivatized with 10 µl of N,O-bis (trimethylsilyl)-acetaminde (BSTFA) and 10 µl pyridine and heated at 60°C for 1 hour, and run on GCMS with an epiandrosterone standard.

Neutral Lipids

The neutral lipid fraction was analyzed by column chromatography using a silica-gel column as the solid phase and eluted by five solvents of increasing polarity, namely hexane, an 8:2 hexane: dichloromethane mixture, dichloromethane, an 8:2 dichloromethane: ethyl acetate mixture, and ethyl acetate. After removal of solvent, these fractions contained the saturated hydrocarbons, aromatics, ketones, alcohols and fatty acids, respectively. The saturated hydrocarbons and aromatics were suspended in hexane and analyzed by GCMS with an epiandrosterone standard. The ketones alcohols and fatty acids were first derivatized with 10 µl of N,O-bis (trimethylsilyl)-acetaminde (BSTFA) and 10 µl pyridine and heated at 60°C for 1 hour before being analyzed by GCMS with an epiandrosterone standard.

GCMS

Samples were analyzed by gas chromatography-mass spectrometry with a HP 6890 GC fitted with split/splitless injector and a Varian WCOT fused silica capillary column (60 m x 0.32 m; 0.25 um film thickness) attached to an Agilent 5973 mass selective detector.
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Results and Discussion

POLAR LIPIDS

Mild Alkaline Methanolysis

Fatty acid methyl esters, products of the MAM, were not abundant in any of the samples. In the organic phase there was a small signature of decanoic acid methyl ester (Fig. 4), a FAME in sample YNP047 and YNP124. This was the only peak to be observed on the chromatogram and can be recognized by a peak on the spectrum at 74 Da and 87 Da. FAMEs are characteristic of bacterial populations in the pool.

Figure 4. Decanoic acid methyl ester. The FAME detected in Obsidian Pool.

NEUTRAL LIPIDS

In the neutral lipid fraction there were many signals that appeared to be derived from the surrounding vegetation, not the microorganisms in the pool. Among these was a strong signal of a steroid that is a known biomarker for bison fecal matter; this compound has been found in many of the pools in the area. Numerous compounds identified are biomarkers for higher plants and indicate that material was introduced from outside the pool by species that could not have grown in the hyperthermophilic environment.

Saturated Lipids

A wide variety of lipids were identified in this study, but many of the most interesting molecules were found in the saturated hydrocarbon fraction that was isolated by liquid
chromatography using a silica gel column as the solid phase and hexane as the liquid mobile phase (See Fig. 3). These compounds were then identified using GCMS (Fig 5).

Figure 5. Total Saturated Hydrocarbon fraction for all three samples. Compounds with significant signal that were identified are labeled, those that were unable to be identified are labeled "U." Strength of signal is labeled on vertical axis.
n-alkanes: The simplest compounds identified in this fraction were the unbranched hydrocarbons, or n-alkanes. All three samples have C_{23} - C_{31} n-alkanes with a strong odd over even predominance that indicates a modern biogenic origin. The largest peak in the YNP038 chromatogram was a C_{17} n-alkane. The mass spectrum for this peak shows that it is n-heptadecane, recognized by a molecular ion of 240 Da and an exponential decay of saturated hydrocarbon peaks starting at 57 Da and increasing by 14 Da, the size of one CH\_2 (Fig 6). This peak was present in both YNP038 and YNP047, but nearly absent in YNP124. The C_{17} n-alkane signal indicates a presence of cyanobacteria, which is unusual for a pool this

Figure 6. n-alkane spectra. Spectra for major n-alkanes in all 3 samples. Molecular ion is marked at the right end of the spectrum.
temperature but is an unmistakable signal here. The presence of a signal for a non-
hyperthermophile indicates that either the edges of the pool are cool enough for the
proliferation of cyanobacteria, or that there is significant input of allochthonous
cyanobacterial biomass to this environment. Since thermophilic cyanobacteria thrive at
temperatures up to $65^\circ C$ in Yellowstone’s other springs, the former possibility is most
likely. In the YNP047 chromatogram there was also a $C_{20}$ n-alkane peak, recognized by a
molecular ion of 282 Da and the standard exponential decay of saturated hydrocarbon peaks.
This peak was identifiable, though small, in both the YNP038 and YNP124 samples.
Though this is the n-alkane form of $C_{20}$, the branched form, phytane, can indicate the
presence of photosynthetic organisms, as it can be derived from chlorophyll, or archaeal
species as it can be derived from the ether lipids.

**Branched alkanes:** In YNP124 the two strongest signals were from the monomethyalkanes
(MMA), 5-
methyloctodecane and 4-
methylheptadecane (Fig
7a), which have a
molecular ion of 268 Da
and 254 Da respectively.
These compounds are
alkanes, and their mass
spectra show a series of
peaks beginning at 57 Da

![Figure 7. 4-methylheptadecane. 7a) Compound skeletal structure. 7b) Compound spectrum; molecular ion is marked at right end of spectrum. Ion sizes are the same as for n-alkanes but have different strengths.](image)
and increasing by 14 Da, but without the typical exponential decay shown by unbranched alkanes (Fig 7b). These compounds were present in YNP038 and YNP047, but with a much weaker signal. Short chain branched MMAs have been identified as biomarkers for modern cyanobacteria when found in sediments (Kenig et al. 1995), supporting the assignment of n-C\textsubscript{17} to an autochthonous but not prominent mat-forming community.

\textit{Branched aliphatic alkanes with a quaternary substituted carbon atom (BAQC):} Although difficult to see in the total ion signal, a series of BAQCs was identified in the saturated hydrocarbon fraction. These compounds can be identified by a specific pattern in the mass spectra, where an abnormally large peak disrupts the typical pattern of peaks of n-alkanes.

![Figure 8. Chromatographs for BAQCs. The mass differences selected for in each chromatograph and signal strengths are shown on the left side of the graphs. Stars label the series of significant peaks.](image-url)
To identify these compounds a specific-feature mass-spectrum analysis software (MassLynx) is used to create a chromatograph that shows only those compounds that contained mass difference of 14 Da: 169-155 Da, 155-141 Da, 141-127 Da, and 127-113 Da (Fig 8). The unique quaternary substituted carbon causes this mass difference. The series of peaks in the chromatograph of 127-113 Da, which indicates a series of 5,5 diethylalkanes (Fig 9), is significantly stronger than for the other mass differences. All of the identified series of BAQCs show an uneven distribution of even- and odd- numbered carbon chains, which suggests that these compounds were biosynthesized by addition of two-carbon units. These compounds have not yet been reported in any cultures of known modern organisms, but because of their common occurrence in sulfur-rich environments seem to be tied to microbial communities living on some component of the sulfur-cycle. Previous reports of these compounds are based on biogeochemical studies of microbial mats from the Proterozoic (Kenig et al. 2003, Logan et al. 1999)

Isoprenoids: A dibiphytane, the presumed degradation product of dibiphytanyl tetraether lipids, was detected in sample YNP038. The mass spectrum for this peak shows a molecular ion at 558 Da (Fig 10a). This breakdown of the sample indicates it is an ester-derived lipid
with two ring structures (Fig 10b). This compound was described by DeLong et al. (1998) as a lipid common to a group of non-thermophilic Crenarchaeota, though in this sample they must be representative of Archaea in general.

![Figure 10. Dibiphytane. 10a) Mass spectrum for dibiphytane. Molecular ion is at right end of spectrum and peak strength is labeled on vertical axis. 10b) Dibiphytane structure. In the organism two of these compounds are connected at each end by a glycerol.]

**Hopanes:** Four primary hopanes were identified in all three samples. Hopanes are the degradation products of hopanoids and have a pentacyclic ring system with four cyclohexane rings fused to one cyclopentane ring. Hopanes can be recognized by a strong peak at 191 Da in their mass spectra, formed by cleavage of the “C”-ring of the fused pentacyclic ring structure between C11 and C12 and C8 and C14 (Fig 11a). Some hopanoids are methylated on the A-ring, which gives an ion peak at 205 Da. In this study
the primary hopanes were identified by using MassLynx software to identify all peaks on the chromatogram that contained a 191 Da fragment in their mass spectra (Fig 11b). All three samples had few major peaks that were identified as common hopanes, and minor peaks that were determined to be not of the hopanoid family. A C_{27} hopane, trisnorhopane, has a molecular ion of 370 Da and was identified in all three samples. Diploptene, a C_{30} hopanoid with a double bond at between C22 and C29 has a molecular ion of 410 Da and was identified in YNP038 and YNP124. The saturated version of diploptene, a C_{30} hopane with
no double bonds has a molecular ion of 412 Da and was found in all three samples. A C$_{31}$ hopane was also identified in all three samples. This hopane had a distinctive peak at 191 Da, but also produced an ion of 205 Da at a much larger concentration, indicating that this is a methylated hopane. Hopanoids are biomarkers for aerobic bacteria and are particularly abundant in cyanobacteria.

**Steranes:** Although hopanoids were prominent in this fraction, the concentration of steranes

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**Figure 12.** Hopanoid and Steroid chromatograms. From top to bottom, samples are YNP038, YNP047 and YNP124 with steroids (compounds with 217 Da fragment) on top and hopanoids (compounds with 191 Da fragment) on bottom. Signal strength is labeled on left side of chromatogram. Significant peaks in sterane chromatographs correspond to peaks in hopanoid chromatograms.
was very low. Steranes have three cyclohexane rings fused to one cyclopentane ring and are
degraded and saturated forms of sterols. Both steroids and hopanoids are produced by the
cyclization of squalene, a universal isoprenoid hydrocarbon, through separate pathways in
the Bacterial and eukaryotic domains. Steranes are recognized by an ion at 217 Da in their
mass spectra, and can be identified by searching for chromatographic peaks that contain an
ion of this mass. The sterane concentration in these samples is one to two orders of
magnitude less than the hopane concentration (Fig 12). It also appears that many of the
compounds identified were not steranes, but other compounds that also produce an ion of
217 Da. This is to be expected as the steroids can be used as a proxy for eukarya, which has
a lower temperature limit for growth. The steranes in this sample must represent external
inputs.
Conclusion

The wide variety of biomarker lipids found in the three samples from Obsidian Pool suggests that the diversity that was found in the genomic studies is also indicated by the lipids of these species. The results indicate that Obsidian pool is a community of Bacteria and Archaea and perhaps types of Eukarya, but further study is needed to distinguish allochthonous inputs.

In this study, the biomarkers for the thermophilic archaea, as well as the crenarcheota and aerobic bacteria, were found in relatively equal concentrations throughout the samples, indicating that all are equi-abundant throughout the hyperthermophilic pool. These microorganisms may be important participants in the energy cycle described by Spear et al. and gain their energy from hydrogen oxidation. The biomarkers of cyanobacteria, which are photosynthetic bacteria, were also found throughout the pool. Environments near the vent feeding this pool are too extreme for photosynthesis, but it is likely possible that photosynthetic organisms thrive near the edges of the pool with their remains mixed in with the hyperthermophilic inhabitants.

The very low concentration of the steranes is unusual, since the pool is fully exposed to the eukaryotes that surround the area, but indicates either that there is a very low concentration of eukaryotes that live in Obsidian Pool, or that there is a relatively low allochthonous input and the lipids represent in-situ community. The presence of this biomarker at low concentration raises questions about the place of eukaryotes in the system as well as the breakdown of less stable compounds by the high temperatures of the pool. The biomarkers for the higher plants indicate that there is some input from the surrounding flora that likely enters the pool during storms and times of high run-off. These organisms
could not survive the high temperatures in Obsidian Pool and are not part of the hydrogen oxidizing energy cycle.

The most interesting biomarkers identified are the BAQCs. These compounds have not yet been linked to any known organisms, as they have not yet been identified in modern living samples. Their presence in Obsidian Pool indicates that the environment of a hot spring is ideal for these unidentified taxa, and further studies for this compound in other hyperthermophilic environments may yield positive results.

Although many biomarkers were identified in this study, it is certain that further studies of the lipids in the pool, using different procedures, would identify more. Using high performance liquid chromatography and mass spectrometry (LCMS), compounds that were unidentifiable in this study may be ultimately identified and quantified. Also, the culturing of different taxa from the pool could lead to identification of the specific organisms that synthesize BAQCs.

This study shows that the genetic diversity is echoed in the small fraction of lipid diversity of the species shown here. The findings also support the most recent study of the biochemical processes in Obsidian Pool that suggests chemical energy in this pool is derived mainly from hydrogen oxidation by simple microorganisms as many of the biomarkers found in the pool indicate the presence of organisms that participate in this process in other environments. There is much more work that may be done on Obsidian Pool and this study just begins to indicate the diversity of the biomarkers that may be found there.
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