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ORIGINAL PAPER

Joint occurrence of the bubble protein and mycophenolic acid in *Penicillium brevicompactum Dierckx*

Marcus Seibold · Peter Wolschann · Ole Olsen

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Abstract Colonies of the fungus *Penicillium brevicompactum Dierckx* contain exudate droplets on the surface of the mycelia when grown on Murashige and Skoog medium. It was previously shown that the exudate is enriched in the bubble protein. Here, we show that the exudate bubbles are also enriched in a fluorescent non-protein molecule, which upon isolation and characterisation was determined as mycophenolic acid. Based on this discovery, mycophenolic acid from exudate bubbles may constitute an alternative source for purification of the metabolite for clinical use.

Keywords Molecular modeling · NMR spectroscopy · Antifungal agent · Inhibition studies

Introduction

Like many fungi from *Penicillium* species, *Penicillium brevicompactum Dierckx* is well known for its ability to produce secondary metabolites [1, 2] and has been utilised for over 100 years to produce the antibiotic mycophenolic acid (MPA) [3]—its dominant metabolite [4]. MPA has a very broad range of biological activity (antibacterial, antifungal, antiviral, antitumor, antiparasitic, and immune modulating) [5]. Unfortunately, its application also promotes undesired side effects in human clinical therapy. Hence, from its broad range of activity, only the immune modulating effect of MPA is used in human medicine to prevent organ rejection after kidney transplantation (used as mycophenolate mofetil, an ester derivative of MPA). In this use, it inhibits the enzyme inosine monophosphate dehydrogenase (IMDP) of proliferating B and T lymphocytes [6]. This leads to cessation of the purine pathway and thereby decreases the titer of these immune cells and attenuates the rejection mechanism. This clinical use triggered establishment of production methods of MPA, including submerged or solid substrate fermentation [7–9]. The patented methods involve isolation by organic extraction of MPA from the whole biomass. However, in the present work we describe that under special culture conditions only, MPA is localised on the surface of the fungus in exudate droplets, also denoted bubbles, which are very easily harvested. This could inspire the development of new ways to produce MPA. In addition, the bubbles contain a second entity—the bubble protein (BP). Despite decade-long research efforts on *P. brevicompactum Dierckx*, the identification of BP was only reported recently [10]. This is remarkable since the concentration of the secreted bubble protein in the exudate bubbles is rather high (about 1 mg/cm^3). How exudates influence fungi is only beginning to be understood. In this respect, it has remained unnoticed that exudates of *P. brevicompactum Dierckx* contain a mixture of a defensin (bubble protein) and a clinically important small molecule (MPA). Additional insights into how exudates are formed could ideally prompt ideas for novel therapies. As a first step in this direction, this report describes the concomitance and separation of the two entities, and first considerations on their coordinate action with each other.

M. Seibold (✉) · P. Wolschann
University of Vienna, Institute of Theoretical Chemistry, Währinger Strasse 17, 1090 Vienna, Austria
e-mail: marcus.seibold@gmx.at

O. Olsen
Carlsberg Group Research, Gamle Carlsberg Vej 10, 1799 Copenhagen, Denmark

Results and discussion

Fungal growth

The fungus grows best on the Murashige and Skoog (MS) plant medium at room temperature, with no indications of a circadian rhythm. At 30°C development is very slow; at 37°C there is no growth at all. Two days after inoculation, a white fluffy mycelium structure appears with an approximate height of 1 mm; 2 days later the height is about 2 mm, but the size has not changed. At the same time, the color changes to green and production of exudate droplets begins. At 5 days after inoculation, the droplets have an average diameter of 2 mm and a corresponding volume of 10 mm^3. The height above the surface of the agar medium is now at its maximum of 3 mm, and below the surface the mycelia stretches about 2 mm down. After 7 days of growth, the fungus already has a "fur" of spores and dies. This is recognised because in this stage the mycelium is easily disturbed. Since the exudates evaporate easily, it is best to inoculate at 23°C and after 2 days decrease temperature to 20°C for the purpose of producing the BP containing bubbles. If bubbles are harvested at day 4, a second harvest at day 7 is possible, thereby increasing output of BP and MPA.

When grown on two standard fungal media (Czapek agar and malt extract agar), the time pattern and temperature preference are the same. But there are no bubbles produced and the color is green from the beginning.

Only when grown on the plant medium were bubbles containing bubble protein and MPA observed (Fig. 1).

Inhibition studies

Fungal growth is not inhibited by co-cultivation of any of the tested bacteria, and vice versa. No matter which temperature, media, or organisms were used, there was no difference in microorganism development compared to the controls. Further, applying only the produced bubbles of the fungus also had no effect on the growth of *E. coli*, *E. faecium*, or *L. acidophilus*, even when the bubbles were applied several times (in a second experimental setup).

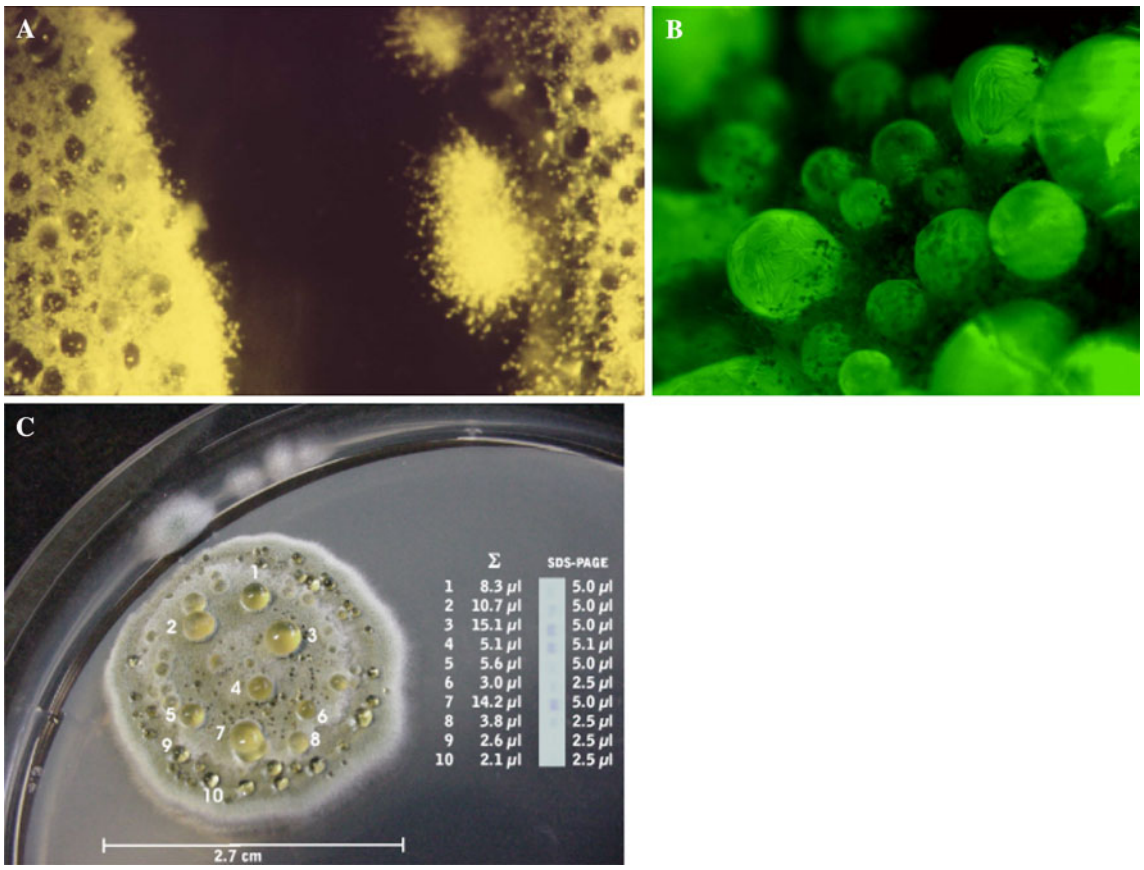

Fig. 1 **a** Two-day-old fungus showing emergence of the bubbles (daylight, ×5 magnitude); **b** 2-day-old fungus showing fluorescence of the bubbles under UV light (×15 magnitude); **c** 5-day-old fungus with indication about bubble size, the massive amount of bubbles compared to the overall biomass is visible

Fungus identification

According to Glass [11], using the variability of the internal transcribed spacer (ITS) region of the 5.8S ribosomal DNA for comparison to other organisms was proposed. Therefore, genomic DNA was isolated from a 5-day-old fungus, and then the ITS region of the 5.8S rDNA was amplified by polymerase chain reaction (PCR). The PCR product was cloned into *E. coli*. The isolated plasmid of the *E. coli* was sequenced at the region where the PCR product was inserted. The obtained DNA sequences were analysed by BLASTN [12] for homology and similarity searches. The result revealed that the fungus was *Penicillium brevicompactum*, ultimately identified as subtype *P. brevicompactum Dierckx*.

Identification of MPA

Identification of MPA was performed on several levels. The melting point of the crystals was determined at 140°C, which is in accordance with the literature [13]. The fluorescence excitation-emission wavelength corresponds with the literature with 342 nm excitation and 425 nm emission [14]. The strong fluorescence is also used for determination of MPA in the exudate bubbles at an excitation wavelength of 410 nm and corresponding emission at 500 nm (Fig. 2).

NMR analysis of MPA

The *J*-modulated ^{13}C NMR spectrum of MPA showed the following peaks (100 MHz, $CDCl_3$, 300K): $\delta = 177.63$, 172.00, 154.08, 164.08, 144.44, 134.30, 123.36, 122.48, 117.11, 106.80, 70.44, 61.40, 34.67, 32.78, 23.01, 16.53, and 11.96 ppm. In addition, a ^{1}H NMR spectrum was recorded and heteronuclear multiple bond correlation (HMBC) experiments allowed assignment of all C and H atoms.

NMR calculation of MPA

The quantum chemical calculation with Gaussian 09 software was based on the HF/3-21G density function. Prior to NMR calculation the MPA molecule was energy minimised (Figs. 3, 4, Table 1).

Discussion

This work represents for the first time evidence that a clinically relevant small molecule is co-produced with a protein from the defensin family and deposited in exudate bubbles on the surface of the fungus. This observation became evident after identification of MPA by several methods. The production of MPA by *P. brevicompactum* is well known [5] and has been studied extensively (e.g., [15]). On the other hand, several fungi are under

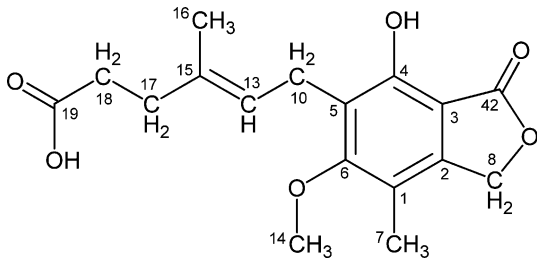

Fig. 3 Atom numbers for carbon atoms used by Gaussian 09

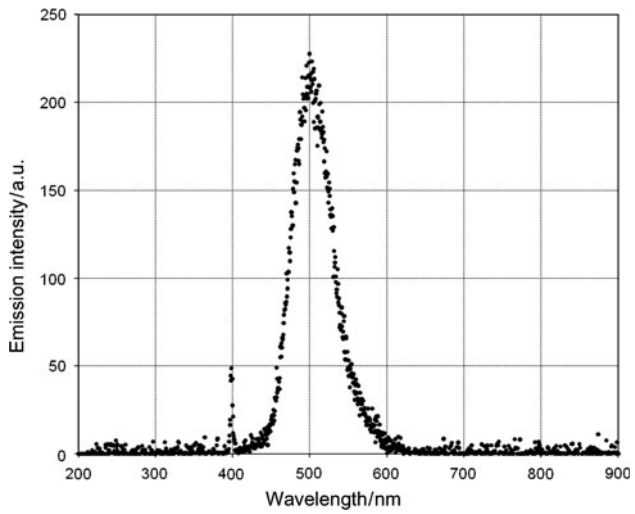

Fig. 2 Fluorescence of exudate bubbles (excitation at 400 nm)

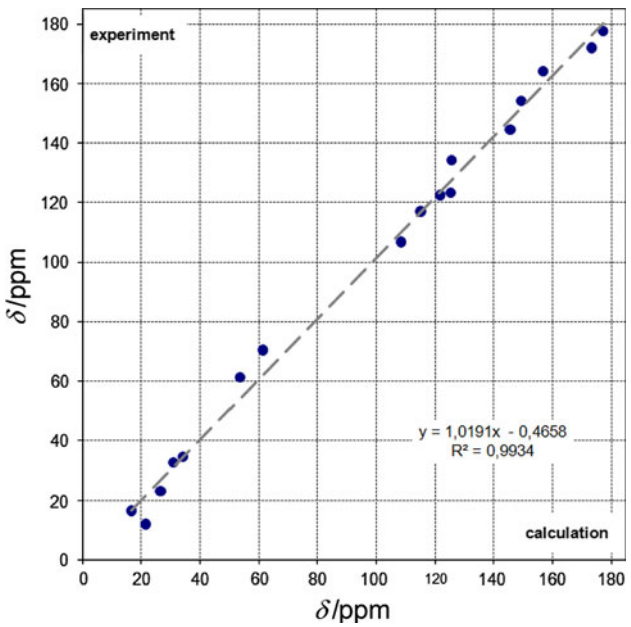

Fig. 4 Comparison of experimental and theoretical ^{13}C NMR data

Table 1 Data from Gaussian 09 calculations

Carbon atom number	Database δ/ppm	Experiment δ/ppm	Calculation δ/ppm	Isotropic
C18	32.60	32.78	31.02	180.4531
C16	16.00	16.53	16.78	197.8628
C7	11.40	11.96	21.56	193.0817
C17	34.10	34.67	34.19	183.6188
C10	22.50	23.01	26.62	188.0192
C14	60.90	61.40	53.59	161.0469
C8	70.00	70.44	61.51	153.1257
C5	122.00	122.48	121.71	106.2380
C13	122.80	123.36	125.27	99.6051
C3	106.20	106.80	108.40	92.9263
C1	116.60	117.11	115.03	89.3739
C15	133.80	134.30	125.59	89.0506
C2	144.00	144.44	145.57	69.0693
C6	163.50	164.08	156.85	65.2542
C4	153.50	154.08	149.39	57.7884
C42	172.80	172.00	173.25	41.3910
C19	179.10	177.63	177.24	37.4010

Reference value of TMS is 214.64 ppm

investigation for various defensin proteins, some of which are very similar to the bubble protein. Among the species under consideration is *P. chrysogenum*, whose PAF-defensin shows similarity to the bubble protein. To our knowledge, we are the first to report the co-production of MPA and the bubble protein.

MPA is known for versatile effects on other microorganisms and hence considered to represent a kind of defense system for *P. brevicompactum*. This fungus is ubiquitous and involved in degrading other biomasses, for example, in compost. In such a habitat, there is high competition for the available nutrients, and it makes sense that any organism has a specific way of prevailing in such a competitive environment. The fact that the fungus develops into different morphologies depending on the used nutrient agar is a hint of the importance of the environment. When grown on a recommended ascomycota fungal media (malt extract agar), *P. brevicompactum* did not develop exudate bubbles. In contrast, when grown on a medium optimised for plants, there was quite strong production of the bubble exudate. This special exudate property may explain why the joint presence of BP and MPA in the bubbles was not described earlier. The plant medium may trigger some kind of stress that the fungus counteracts by producing exudate bubbles. Another medium has been described (Czapek agar) for growing *P. brevicompactum* [2]. In this case, the focus was on observation of brevianamide A and B, which are other metabolites that act as insecticides. In that work it was stated that the brevianamides were only located in the biomass (aerial sporing hyphae) and explicitly not in the exudate (it was not mentioned if the exudate was in the form of bubbles). In another study Bartman [15] followed MPA production by *P. brevicompactum* over time and reported the emergence of significant amounts at about 2 days, actually in line with the emergence of the exudate bubbles. This suggests that MPA is present in the exudate bubbles, further suggesting destructive effects inside cells. In attempts to address possible coordinate action of BP and MPA in the exudate bubbles, both were easy to separate with no indications of covalent, van der Waals, or ionic interactions. Aspects of the potential to generate exudate bubbles could be exploited in MPA purification through new harvesting methods, for example involving a centrifugation and simple column chromatography with silica gel, accentuated by a possible second harvest during one vegetative cycle. Current harvesting involves a troublesome all-biomass organic extraction. The growth phase of the fungus is around 1 week, with exudate bubbles starting to evaporate and leave their content on the surface and—by that time already formed—spores. That residue may form a protective layer around the spores to prevent them from being metabolised by other microorganisms. In this regard, MPA could function by altering cell adhesion properties and thus diminish the effects of phagocytosis by approaching microorganisms [16].

Finally, the present work shows that environmental effects contribute to a large extent to the metabolism of *P. brevicompactum Dierckx*. The change in morphology and fungal development may lead the way for an improvement of production of the important drug mycophenolic acid. Further, the co-production of two defense-related entities raises interesting questions and calls for further inquiry.

Experimental

Plant medium

Contents for 1 dm^3: Murashige and Skoog (MS) powder (Sigma, M-5519) 4.4 g; caseine hydrolysate 1.0 g; phytagel 3.5 g; maltose 30 g; water added up to 1 dm^3; autoclaved at 121°C for 15 min; after cooling to ~50°C it was poured into petri dishes to a final height of about 1 cm. All reagents were from Sigma-Aldrich.

Standard fungal medium: malt extract agar

Contents for 1 dm^3: malt extract 30 g, agar 15 g, mycological peptone 5 g, water added up to 1 dm^3; autoclaved at 121°C for 15 min; after cooling to ~50°C it was poured into petri dishes to a final height of about 1 cm. All reagents were from Sigma-Aldrich.

Standard fungal medium: Czapek agar

Contents for 1 dm^3: $NaNO_3$ 3.0 g, K_2HPO_4 1.0 g, $MgSO_4$ $7H_2O$ 0.5 g, KCl 0.5 g, $FeSO_4$ $7H_2O$ 0.01 g, agar 15.0 g, DI water 900 cm^3; autoclaved at 121°C, cooled, and added the following filter sterilised solution: sucrose (commercial grade) 30.0 g, DI water 100 cm^3. Reference: American Type Culture Collection, ATCC medium Nr. 312 Czapek agar.

Growing of the fungus

For propagation of the fungus, spores from aged cultures were collected by washing the conidia of the cultures with 2–4 cm^3 of water. The resulting suspension was stored at 5°C. For harvesting the MPA-containing liquid exudate bubbles, it was found optimal to propagate the fungus in rows on MS media simply by streaking out spores with standard pipette tips. When grown at room temperature, mycelia can be seen on the second day of propagation, but optimal bubble volumes are obtained at 5 days.

Harvesting of the bubbles

A spoon-like spatula was gently set on the surface of the fungal culture and moved along the colonies. The obtained liquid was passed through a 0.22-μm cellulose acetate filter to remove spores and other particles (Spin-X centrifuge filter unit, Costar Ltd.).

Inhibition studies

Enterococcus faecium M74 and *Lactococcus lactis SR 354* were stored as aliquots at −80°C, after overnight culturing in de Man, Rogosa, Sharpe bouillon (MRS bouillon), inoculation in 10 cm^3 MRS media, growing for 24 h, then plating on MRS plate. Also *E. coli* were tested when growing on Loria Bertani (LB) media and MS media. (1) Influence of BP-containing bubbles: to all three cultures droplets of filtered bubbles were spread on the plate either immediately after inoculation or 24 h later. *E. faecium* and *L. lactis* were cultivated at 37°C, *E. coli* on both media at 20 and 37°C. The growth and development were followed for another 24, 48, and 72 h; plates untreated with bubbles served as controls. (2) Influence of developing fungus: the sterile culture media were inoculated with spores of *P. brevicompactum Dierckx* and 3 days later inoculated with the bacteria, simply to account for the slower growth of the fungus. Culture conditions were the same as in (1); plates untreated with spores served as controls.

Genotyping of the fungus

DNA isolation

Five-day-old fungal colonies were ground in liquid nitrogen; 400 mg of fungal powder was put in a tube. Then 400 mm^3 phenol:water (3.75:1) and 200 mm^3 water were added, and the tube was incubated at 80°C for 15 min with occasional shaking. After centrifugation at 25,000*g* for 5 min and 15°C, the upper phase was taken, and 1 volume of solvent solution (phenol:trichlormethane:isoamylalcohol = 25:24:1) was added. After shaking and centrifugation under the same conditions, the upper phase was taken again, and a solution of phenol and trichlormethane (1:1) was added, repeating shaking and centrifugation as described above. The final extraction of the upper phase was performed with trichlormethane alone. After shaking and spinning, the resulting upper phase contained the genomic DNA. The yield was about 125 μg, at a concentration of 2.5 μg DNA/mm^3.

Polymerase chain reaction (PCR)

For the PCR, 5 mm^3 of purified genomic DNA, 2.5 units Taq DNA polymerase, buffer (1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.0, and 50 mM KCl), 100 pmol of each primer, and 2 μM dNTPs were mixed to a total volume of 50 mm^3 (all reagents from Promega). The PCR was performed with an initial denaturation for 2 min at 95°C, followed by 25 cycles in a Perkin Elmer DNA Thermal Cycler with denaturation for 1 min at 94°C, annealing for 1 min at 60°C, and extension for 1 min at 72°C. The final extension was 10 min at 72°C. PCR products were visualised following electrophoresis on 0.8% agarose gels.

DNA sequencing

The PCR products were purified by electrophoresis on an agarose gel and purified with QIAEX II Gel Extraction

System. The DNA fragments were inserted into pCR 2.1-TOPO vector from Invitrogen. For cloning, TOP 10 One Shot competent *E. coli* (Invitrogen) were used. After propagation of *E. coli* overnight, plasmid DNA was isolated from selected colonies by alkaline lysis (Wizard DNA Purification System Promega). The DNA was automatically sequenced on a MegaBACE 1000 System from General Electric by using the BigDye Terminator v3.1 Cycle Sequencing Kit.

Isolation and crystallisation of MPA

Freshly harvested exudate liquid bubbles (50 cm^3) were filtered through a 0.22-µm cellulose acetate filter and extracted twice for 20 min with 25 cm^3 *t*-butylmethylether at room temperature. After discarding the water phase, that phase with the organic compounds was concentrated to 1 cm^3 and transferred to a fresh tube in which it was dried by application of a nitrogen stream. Next, the residue was dissolved in 250 mm^3 MeOH/H_2O (50%w/50%w) and applied to column chromatography with a UV light-permeable glass cylinder [1 cm diameter, filled with 7 cm^3 silica gel (Merck silica gel 60 silanized 0.063–0.200 µm, art. no. 107719)]. Conditioning (5 column volumes) and elution were performed with the same solution (MeOH/H_2O). Chromatography was done in two cycles, each with applying 125 mm^3 of MPA containing MeOH solution. The UV fluorescence was used to follow the MPA during the chromatography. Under UV light there was one visible band in the column, which contained MPA as deduced by the typical yellow-green fluorescence. After one column volume, the MPA was located at the end of the column; at the beginning of the second column volume, the MPA was eluted within about 5–6 cm^3. After washing the column with five column volumes of elution solution, the second run with the remaining 125 mm^3 was performed. After pooling of the two eluates, these were concentrated to about half of the volume. Finally, the air in the flask was replaced by nitrogen. After 2 days at room temperature, the crystallisation process was completed.

Isolation of BP

BP-containing exudate droplets were harvested on the 5th day of fungal development. The collected droplets were filtered through a 0.22-µm cellulose acetate membrane. Subsequently, a molecular weight cutoff of 5,000 g/mol was performed. The retentate was used and desalted via gel chromatography. A detailed description of the isolation is described elsewhere [10].

MPA NMR analysis

The MPA crystals were isolated and dissolved in $CDCl_3$, and the ^{13}C and ^{1}H NMR spectra were recorded on a Bruker Avance 400 NMR spectrometer at 300K. Chemical shifts are expressed in ppm downfield from tetramethylsilane (TMS). Chemical shifts were checked with the SPECINFO database (Wiley-VCH, Weinheim, Germany) and further analysed manually (Table 1).

Quantum chemical calculations

The result of identification of MPA was verified by quantum chemical calculations with Gaussian 09 software [17]. In a first step the 3D structure of MPA was energy minimised by using B3LYP/6-31G (d, p) basis set. The NMR calculation was also performed with Gaussian 09 using the GIAO method [18].

Fluorescence measurement

The fluorescence measurements were performed on a Perkin Elmer FL Spectrum ASCII PEDS device.

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