

Inopinatum lactosum gen. & comb. nov., the first yeast-like fungus in Leotiomyces

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Abstract

Sporobolomyces lactosus is a pink yeast-like fungus that is not congeneric with other members of *Sporobolomyces* (Basidiomycota, Microbotryomycetes, Sporidiobolales). During our ongoing studies of pink yeasts we determined that *S. lactosus* was most closely related to *Pseudeurotium zonatum* (Ascomycota, Leotiomyces, Thelebolales). A molecular phylogenetic analysis using sequences of the ITS region and the small and large subunit (SSU, LSU) rRNA genes, indicated that four isolates of *S. lactosus*, including three ex-type isolates, were placed in Thelebolales with maximum support. A new genus is proposed to accommodate *S. lactosus*, *Inopinatum*. This is the first pink yeast reported in Leotiomyces.

Pink-pigmented yeasts in the order Sporidiobolales (Basidiomycota, Pucciniomycotina, Microbotryomycetes) produce lipid droplets with carotenoid pigments – mostly β -carotene and torulene – contributing to the pink to orange-reddish colour of colonies [1–3]. These pigments are thought to offer antimicrobial, anticancer, and anti-ageing activities and to protect against radiation [4, 5]. Because of these characteristics, pink-pigmented yeasts have gained interest from pharmaceutical, cosmetics, and biotechnology industries [6–8]. The pink yeasts were historically placed in two anamorphic basidiomycete genera, *Rhodotorula* and *Sporobolomyces*. In their traditional sense, both these asexual genera are polyphyletic, occurring in all three subphyla and several classes and orders of Basidiomycota [9–11].

Following the elimination of the use of dual naming systems for asexual and sexual morphs of fungi, *Rhodotorula* and *Sporobolomyces* are now retained only for those species within Sporidiobolales [12]. Efforts to reassign many of the species once classified into *Rhodotorula* and *Sporobolomyces* into natural genera are ongoing [8, 12, 13]. At present, the order Sporidiobolales is estimated at ca. 260 species of which 42 have been described [3]; *Sporobolomyces* currently includes

ca. 22 species [8, 14, 15]. These are reported from diverse habitats including freshwater and marine ecosystems, fruit must, surfaces of buildings, food, soil, air, and—the most common habitat from which they are isolated—leaf surfaces [3, 15–22].

During our studies of pink yeasts in the genus *Sporobolomyces*, we noted that the internal transcribed spacer (ITS) barcode sequence of *Sporobolomyces lactosus* [23] was not similar to other species in the genus. Moreover, *S. lactosus* is not treated in Kurtzman *et al.* [24]. A general Nucleotide blast search (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) of *S. lactosus* against ex-type sequences of all fungi resulted in *Pseudeurotium zonatum* CBS 329.36^T (Ascomycota, Leotiomyces, Thelebolales) as the closest match with 90.84% shared identity. An ex-type culture of *S. lactosus* was obtained from the Culture Collection of Yeasts (CCY:19-21-1^T) [23] at the Slovak Academy of Sciences (Bratislava, Slovakia). Here we present the results of our phylogenetic analyses of *S. lactosus* and formally describe *Inopinatum* gen. nov. to accommodate it in the Thelebolaceae (Thelebolales), as the first known yeast-like species in the Leotiomyces.

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Abbreviations: ITS, internal transcribed spacer; LSU, large subunit rRNA gene; ML, maximum likelihood; nt, nucleotides; PDA, potato dextrose agar; SSU, small subunit rRNA gene.

The GenBank/EMBL/DDBJ accession numbers of the SSU and ITS sequences of *Inopinatum lactosum* gen. & comb. nov. CCY 19-21-1^T=JCM 8510 are AB021676 and AB038132. The GenBank/EMBL/DDBJ accession number of the ITS sequence of *I. lactosum* C4 is EU551181. The GenBank/EMBL/DDBJ accession numbers of the newly generated SSU, ITS, and LSU sequences of *I. lactosum* CCY 19-21-1^T are MW471137, MW471138 (SSU), MW471139, MW471140 (ITS), MW471141, and MW471142 (LSU). The MycoBank accession numbers are MB835917 for *Inopinatum* gen. nov. and MB835918 for *I. lactosum* comb. nov. The aligned three-locus dataset used for ML phylogenetic inference is available from the figshare online repository with the URL <https://doi.org/10.6084/m9.figshare.12495878>.

Table 1. Sequences of Thelebolales used in phylogenetic analysis

Species	Isolate/strain	SSU	ITS	LSU	Reference(s)
<i>Antarctomyces pellizariae</i>	UFMG 12416 ^T	NA	NR_164245	NA	[62]
<i>Antarctomyces psychrotrophicus</i> *	IMI 378528 ^T	NA	AJ133431	NA	[69]
<i>Inopinatum lactosum</i> *	JCM 8510	AB021676	AB038132	NA	–
<i>Inopinatum lactosum</i> *	C4	NA	EU551181	NA	[64]
<i>Inopinatum lactosum</i> *	D. Haelew. F-3088a (ex-CCY 19-21-1 ^T)	MW471137	MW471139	MW471141	This study
<i>Inopinatum lactosum</i> *	D. Haelew. F-3088b (ex-CCY 19-21-1 ^T)	MW471138	MW471140	MW471142	This study
<i>Cleistothelobolus nipigonensis</i> *	CBS 778.70 ^T	NA	NR_164284	MH871738	[70]
<i>Connersia rilstonii</i> *	CBS 537.74	AF096174	KJ755499	FJ176866	[71–73]
<i>Crinula caliciiformis</i> *	AFTOL-ID 272	AY544729	KT225524	AY544680	–
<i>Geomyces auratus</i>	CBS 108.14 ^T	AB015785	NR_111872	NG_042776	[42, 74, 75]
<i>Gymnostellatospora alpina</i>	CBS 620.81 ^T	NA	MH861383	MH873132	[70]
<i>Gymnostellatospora japonica</i> *	UAMH 9239	NA	DQ117454	NA	[76]
<i>Holwaya mucida</i> *	TU 112863	KX090898	MH752062	KX090844	[44, 77]
<i>Leuconeurospora pulcherrima</i> *	AFTOL-ID 1397	FJ176828	KF049206	FJ176884	[73]
<i>Pleuroascus nicholsonii</i>	CBS 345.73 ^T	AF096182	NR_156627	AF096196	[71]
<i>Pleuroascus rectipilus</i>	CBS 120411 ^T	NG_067690	NR_165899	NA	[78]
<i>Pseudeurotium hygrophilum</i> [as <i>Teberdinia hygrophila</i>]	CBS 102670 ^T	AY129282	AY129291	NA	[79]
<i>Pseudeurotium</i> sp.	01NH01	NA	JX270336	NA	[80]
<i>Pseudeurotium zonatum</i>	AFTOL-ID 1912 ^T	DQ471040	NR_111127	DQ470988	[75, 81]
<i>Pseudogymnoascus destructans</i>	JGI Genome	NA	JGI genome	NA	–
<i>Pseudogymnoascus roseus</i> *	CBS 395.65 ^T	AB015778	NR_165894	MH870271	[70, 74]
<i>Ramgea ozimecii</i>	CNF 2/9997 ^T	NA	NR_164248	KY368753	[82]
<i>Thelebolus balaustiformis</i>	MUT 2357 ^T	NA	NR_159056	NG_067559	[83]
<i>Thelebolus globosus</i>	CBS 113940 ^T	NG_062682	NR_138367	NG_067263	[60, 73]
<i>Thelebolus stercoreus</i>	CBS 718.69 ^T	NA	MH859396	MH871167	[70]
<i>Thelebolus stercoreus</i> *	JGI Genome	NA	JGI genome	NA	–

*, Type species; ^T, ex-type; NA, Not available.

Sporobolomyces lactosus, CCY:19-21-1^T, (Poland: Warsaw, Plock Refinery sewage treatment plant) [23], was grown on potato dextrose agar (PDA) with 2% agar, supplemented with 50 µg ml⁻¹ chloramphenicol and 100 µg ml⁻¹ ampicillin (BD, Franklin Lakes, New Jersey) to inhibit bacterial growth. Samples were removed for DNA isolation by using a J-hook to superficially scrape off pieces of fungal tissue. DNA was extracted using the Wizard Genomic DNA Purification kit (Promega Co., Madison, Wisconsin), following the

manufacturer's instructions. Next, we amplified the internal transcribed spacer, and nuclear small and large subunits of the ribosomal DNA repeat (ITS, SSU, and LSU, respectively). Primer combinations used were NS1/NS4 for SSU [25], ITS1f/ITS4 for ITS [25, 26], and LR0R/LR5 and LR0R/LR7 for LSU [27, 28]. PCR reactions consisted of 12.5 µl of Promega 2×PCR Master Mix, 1.25 µl of each 10 µM primer, 9.0 µl of H₂O, and 1.0 µl of template DNA. All amplifications were done in an Eppendorf Mastercycler ep Thermal Cycler (Hauppauge, New

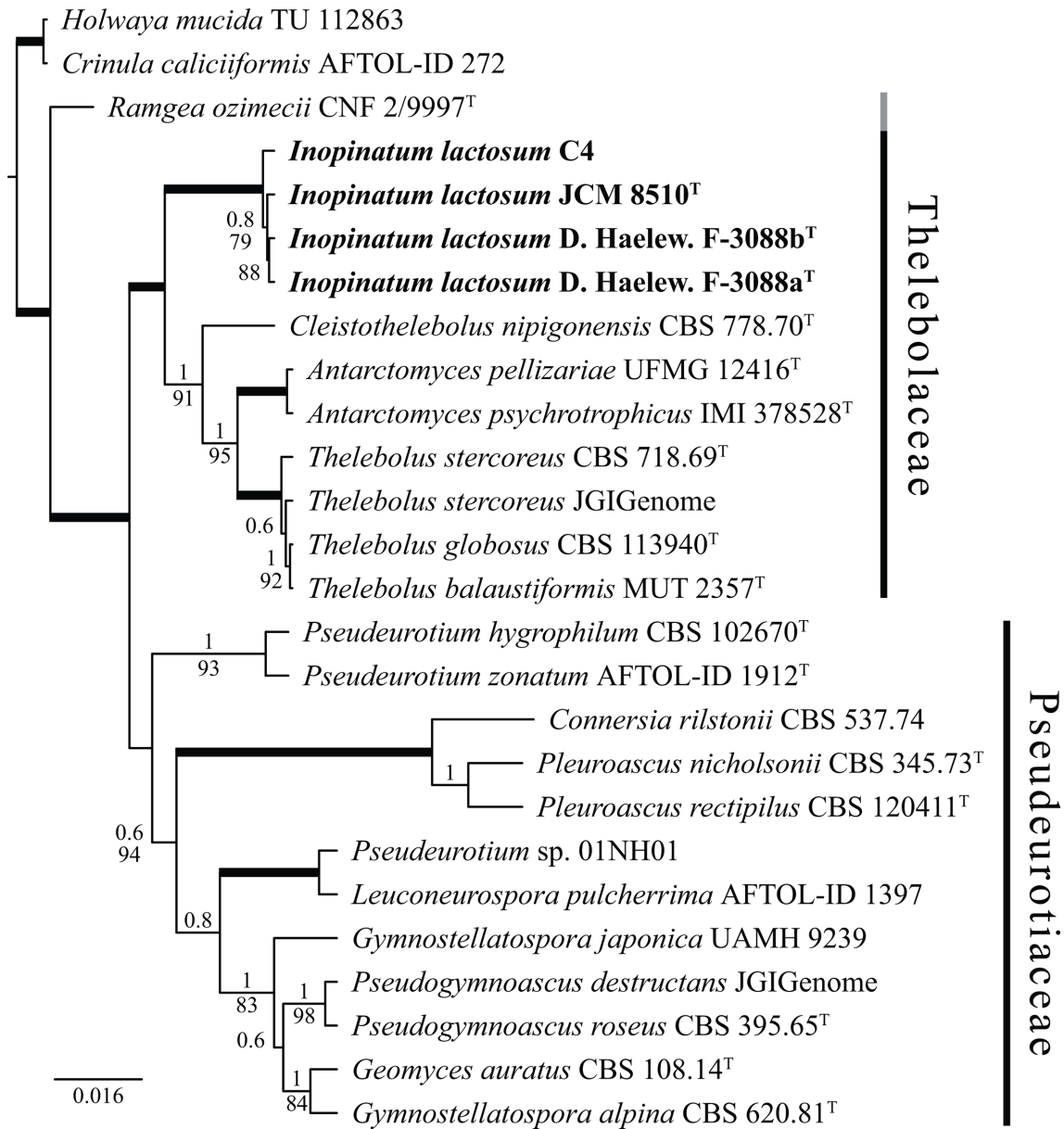


Fig. 1. Phylogenetic placement of *Inopinatum lactosum* gen. and comb. nov. within Thelebolales, reconstructed from a combined dataset of SSU, ITS, and LSU sequences (26 isolates, 2496 characters). The topology is the result of Bayesian inference performed with BEAST. *Crinula caliciiformis* AFTOL-ID 272 and *Holwaya mucida* TU 112863 were used as outgroups. For each node, pp ≥ 0.6 and ML bootstrap ≥ 60 are presented above/below the branch leading to that node. Thick branches, maximum support from both Bayesian and ML inference; ^T, ex-type; bar, number of substitutions per site.

York) under the same cycling conditions as in Haelewaters et al. [13].

Maximum likelihood (ML) analyses were done using IQ-TREE [29], on a multi-locus dataset of all three amplified loci. Representative sequences for all genera in Thelebolales were downloaded from NCBI GenBank (Table 1). Sequences for each locus were aligned using MUSCLE [30] available from the Cipres Science Gateway [31], and then trimmed using the command-line version of TrimAl 1.3 [32] with gap threshold of 0.6 and minimal coverage of 0.5. Substitution models were

selected using ModelFinder [33] by considering the Akaike Information Criterion corrected for small sample size (AICc): TN+F+G4 for SSU (-lnL=2007.697), TIM2e+R2 for ITS (-lnL=2411.356), and TIM3 +F+R2 for LSU (-lnL=2300.016). ML was inferred for the concatenated SSU-ITS-LSU dataset under partitioned models, with rapid bootstrapping under 1000 replicates [34, 35].

Bayesian analyses were done using a Markov chain Monte Carlo (MCMC) approach implemented in the BEAST package [36], with a strict clock assuming a constant rate of evolution

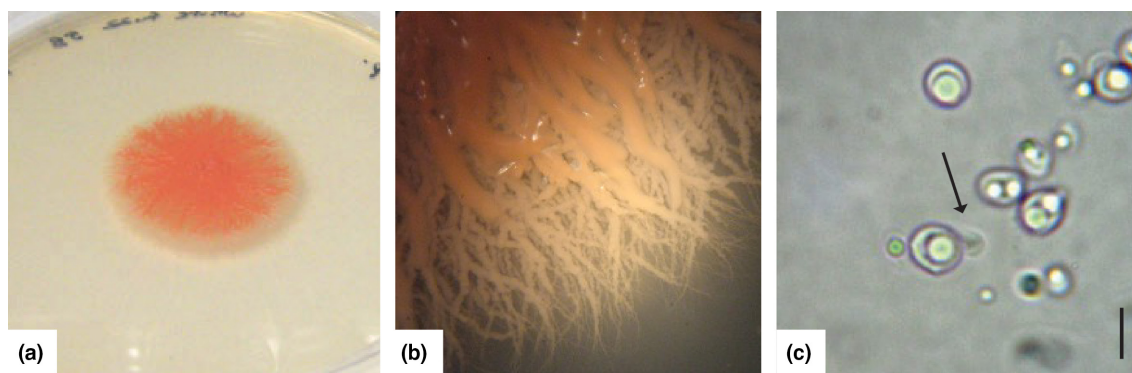


Fig. 2. *Inopinatum lactosum* gen. and comb. nov. Strain C4 from [64], growth on PDA supplemented with chloramphenicol (100 mg l⁻¹) and ampicillin (100 mg l⁻¹), after incubation of 5–7 days. (a) Colony, with (b) detail of colony with thick and 'veiny' undulating margin. (c) Vegetative cells, arrow pointing at daughter blastoconidium connected to its mother cell. Bar, 10 µm.

across the tree and a Yule Speciation tree prior [37, 38]. The nucleotide substitution models, as determined as by jModelTest 2.1.6 [39] under AICc, were as follows: HKY+G for SSU (-lnL=2008.6823), TIM2ef+G for ITS (-lnL=2406.8675), and TrN +I+G for LSU (-lnL=2300.4287). Two runs were undertaken from a random starting tree for 40 million generations, with a sampling frequency of 4000. Tracer 1.6 [40] was used to check MCMC trace plots. After removed of 10% as burn-in, trees files were combined, consensus trees were generated (with 0% burn-in), and the Maximum Clade Credibility (MCC) tree was inferred with the higher product of individual clade posterior probabilities (pp).

Intra- and interspecific divergence in the ITS and LSU regions was calculated using the Compute Pairwise Distances function in MEGA7 [41] with model/method set at 'No. of differences', gaps/missing data treatment set at 'pairwise deletion', and default settings for other parameters. The aligned, trimmed ITS sequences of our two ex-CCY 19-21-1^T isolates and of ex-type strain JCM 8510 were 100% identical. Isolate C4 differed in its ITS in four nucleotides (nt), followed by *Pseudeurotium zonatum* CBS 329.36^T=AFTOL-ID 1912^T with 42 nt differences in the ITS. The two ex-CCY 19-21-1^T isolates also shared 100% identity in their LSU sequences. The isolate from our dataset with least nt differences in the LSU region was *Thelebolus balaustiformis* MUT 2357^T (28 nt), followed by *Leuconeurospora pulcherrima* AFTOL-ID 1397 (29 nt), and *Cleistothelobolus nipigonensis* CBS 778.70^T and *Pseudeurotium zonatum* CBS 329.36^T (both 30 nt).

The phylogenetic reconstruction of Thelebolales based on the concatenated three-locus dataset is shown in Fig. 1. *Crinula caliciiiformis* and *Holwaya mucida* (Leotiomycetes incertae sedis) were chosen as outgroup taxa. All included genera except *Ramgea* were placed in either Pseudeurotiaceae or Thelebolaceae as currently accepted [42–44]. In our three-locus phylogenetic reconstruction, *Ramgea ozimecii* CNF 2/9997^T was retrieved as the earliest diverging clade in the order with maximum support, resulting in a paraphyletic family Thelebolaceae. *Inopinatum lactosum* gen. and comb.

nov. was maximally supported as sister to other members of Thelebolaceae.

Leotiomycetes are a diverse class within subphylum Pezizomycotina [44, 45] comprising ca. 6500 described species in 630 genera. These fungi are often found as major components of environmental samples. Nonetheless, many taxa remain unnamed or *incertae sedis* within the class. Leotiomycetes species appear to be predominantly saprotrophic and parasitic, including economically and ecologically important pathogens such as the powdery mildews (Erysiphaceae) and the causal agent of white-nose syndrome in bats (*Pseudogymnoascus destructans*, only known from its asexual morph) [43, 44]. Other species, however, are mycorrhizal mutualists (ectomycorrhizae and ericoid mycorrhizae) and plant endophytes [46–49].

Ascomycetous yeasts and yeast-like taxa are primarily found in the subphyla Saccharomycotina (Saccharomycetes) and Taphrinomycotina (Neoelectromycetes, Pneumocystomycetes, Schizosaccharomycetes, Taphrinomycetes) [24], but have also been revealed in other lineages: Arthoniomycetes, Dothideomycetes, Eurotiomycetes, Xylonomycetes (subphylum Pezizomycotina), and *Gemmulina* (Ascomycota *incertae sedis*) [50–56]. Only recently, the black yeast genus *Phaeococcomyces* was placed in a newly erected order Lichenostigmatales (Arthoniomycetes) along with taxa forming colonies of stromatic ascomata or conidiomata (*Etayoa*, *Lichenostigma*)—a lineage that is unique within this class, which is otherwise composed primarily of lichenized species [51].

Inopinatum lactosum is the first described yeast-like fungus in Leotiomycetes, adding to the body of work that is expanding the known morphological and ecological diversity in the class. For example, the perithecioid apothecial *Annabella australiensis* (Leotiomycetes, Cordieritidaceae) was recently discovered from mangrove wood [57], a habitat that is otherwise mostly populated by Dothideomycetes and Sordariomycetes [58]. *Inopinatum* is placed in Thelebolaceae. This family includes many coprophilic and psychrophilic species [59–62] and, based on

genomic-scale data, was recently determined to be the sister to Pseudeurotiaceae—the order that contains the asexual fungus *P. destructans* [44, 63]. Although little is known of the ecology of *I. lactosum*, a coprophilous habit is likely given its isolation from animal faeces [64] and petrochemical wastewater [23].

Inopinatum lactosum is represented in NCBI GenBank by SSU, ITS, and LSU sequences of two ex-CCY 19-21-1^T isolates (this study); SSU, ITS, LSU, and cytochrome b (*cytb*) sequences of strain JCM 8510 (unpublished data); and an ITS sequence of isolate C4 [64], which shares 99.01% identity with the ex-type sequences. The C4 isolate was screened for production of enzymes on agar plates containing different substrates. Protease, amylase, mannanase, and variable xylanase activity was observed at 25°C, while at 15°C and 39°C all enzymatic activity was either variable or absent [64].

DESCRIPTION OF *INOPINATUM* HAELEW. & AIME, GEN. NOV.

Inopinatum (Latin, meaning ‘unexpected’ and referring to the unexpected placement of this pink yeast genus in Leotiomyces) MycoBank number: MB835917.

Type species: *Inopinatum lactosum* (E. Sláviková and Grab.-Łon.) Haelew. and Aime

Description: Yeast-like fungi belonging to Theobolaceae (Theobolales, Leotiomyces). Teleomorph unknown. Anamorph pink-pigmented, forming pseudohyphae and hyphae; blastoconidia bilaterally symmetrical; no known fermentation. Isolated from animal faeces and wastewater.

Inopinatum lactosum (E. Sláviková and Grab.-Łon.) Haelew. and Aime, comb. nov. MycoBank number: MB835918. Fig. 2.

Basionym: *Sporobolomyces lactosus* E. Sláviková and Grab.-Łon., Anton Leeuw 61 (3): 246 (1992).

Inopinatum lactosum forms pink, glistening, ropey colonies on PDA (Fig. 2a). The colony margin is coarsely fimbriate, with a ‘veiny’ appearance (Fig. 2b) reminiscent of growth of some *Aureobasidium* Viala and G. Boyer and *Kabatiella* Bubák species in culture [65, 66]. Whereas *Aureobasidium* cultures become black with time, *I. lactosum* retains its pink pigmentation (Fig. 2a). Growth is dimorphic, producing short chains of pseudohyphae and a few true hyphae near margins, and blastoconidia from older growth in the center (Fig. 2c). CCY 19-21-1^T, the holotype strain of *I. lactosum*, was isolated from an activated sludge in Poland [23]. The conidia were described as ballistoconidia in the protologue but are blastoconidia [67], analogous to *Aureobasidium* [68]. The C4 strain was isolated from koala faeces [64], a habitat that is consistent with that of other members of Theobolaceae that are mainly known from dung [59].

The holotype is CCY 19-21-1, from petrochemical wastewater in Warsaw, Poland, and is permanently preserved in a metabolically inactive state in the Culture Collection of Yeasts, Bratislava, Slovakia. Ex-type cultures are preserved as JCM 8510 and JCM 10082 in the Japan Collection of

Microorganisms, Tsukuba, Japan; and as NCYC 2618 in the National Collection of Yeast Cultures, Norwich, UK.

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Author contributions

Conceptualization, Formal Analysis, Visualization, Writing – original draft: D.H., Methodology: D.H. and M.C.A., Writing – review and editing: D.H., R.A.P., K.M.H.N., and M.C.A.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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