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Selective splitting of 3′-adenylated dinucleoside polyphosphates by specific enzymes degrading dinucleoside polyphosphates[✪]

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Several 3′-[^{32}P]adenylated dinucleoside polyphosphates (Np$_n$N′p*As) were synthesized by the use of poly(A) polymerase (Sillero MAG *et al.*, 2001, *Eur J Biochem.*; 268: 3605–11) and three of them, ApppA[^{32}P]A or ApppAp*A, AppppAp*A and GppppGp*A, were tested as potential substrates of different dinucleoside polyphosphate degrading enzymes. Human (*asymmetrical*) dinucleoside tetraphosphatase (EC 3.6.1.17) acted almost randomly on both AppppAp*A, yielding approximately equal amounts of pppA + pAp*A and pA + pppAp*A, and GppppGp*, yielding pppG + pGp*A and pG + pppGp*A. Narrow-leafed lupin (*Lupinus angustifolius*) tetraphosphatase acted preferentially on the dinucleotide unmodified end of both AppppAp*A (yielding 90% of pppA + pAp*A and 10 % of pA + pppAp*A) and GppppGp*A (yielding 89% pppG + pGp*A and 11% of pG + pppGp*A). (*Symmetrical*) dinucleoside tetraphosphatase (EC 3.6.1.41) from *Escherichia coli* hydrolyzed AppppAp*A and GppppGp*A producing equal amounts of ppA + ppAp*A and ppG + ppGp*A, respectively, and, to a lesser extent, ApppAp*A producing pA + ppAp*A. Two dinucleoside triphosphatases (EC 3.6.1.29) (the human Fhit protein and the enzyme from yellow lupin (*Lupinus luteus*)) and dinucleoside tetraphosphate phosphorylase (EC 2.7.7.53) from *Saccharomyces cerevisiae* did not degrade the three 3′-adenylated dinucleoside polyphosphates tested.

Dinucleoside polyphosphates (Np$_n$N′s, where N and N′ are nucleosides and n = 2–7), such as diadenosine 5′,5′′′-P^1,P^3-triphosphate (ApppA or Ap$_3$A) and diadenosine

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5′,5′′′-P^1,P^4-tetraphosphate (AppppA or Ap_4A) are ubiquitous both in prokaryotes and eukaryotes (Garrison & Barnes, 1992; Schlüter *et al.*, 1998). Some data suggest that Np_nN's may be both useful and harmful to organisms (McLennan, 2000). Based on *in vitro* studies it has been assumed that the levels of these dinucleotides result from both their rate of synthesis by ligases and transferases (Guranowski *et al.*, 1990; Plateau & Blanquet, 1992; Ortiz *et al.*, 1993; Sillero & Günther Sillero, 2000) and degradation by specific and nonspecific lytic enzymes (Guranowski & Sillero, 1992; Guranowski, 2000). In addition to metabolizing Np_3N's and Np_4N's, the specific Np_nN'-degrading enzymes exhibit activity towards a number of Np_nN' derivatives including: (a) Np_3N's and Np_4N's with various nucleosides differing both in the base and sugar moieties; (b) mRNA 5′-cap analogues; (c) mono- and di-(N^1,N^6-etheno) derivatives of Ap_3A and Ap_4A; (d) chain-length homologues; (e) nucleoside 5′-tetra- and -pentaphosphates; (f) methylene- and halomethylene analogues; (g) mono- and diphosphorothioate analogues; (h) adenylated derivatives of methanetrisphosphonate; (i) diadenylated polyols (Baraniak *et al.*, 1999; Varnum *et al.*, 2001) and (j) 2′-(deoxy)adenylated Ap_3A and Ap_4A (Guranowski *et al.*, 2000; Maksel *et al.*, 2001). For references concerning the compounds mentioned in (a–h) see reviews (Guranowski & Sillero, 1992; Guranowski, 2000; Blackburn *et al.*, 1992). Here we report that a novel class of compounds, 3′-adenylated Np_nN derivatives, $Np_{3\ \mathrm{or}\ 4}NpA$ (Sillero *et al.*, 2001), are substrates for some, but not other, Np_nN'-degrading enzymes.

MATERIALS AND METHODS

Chemicals. Unlabeled mono- and dinucleotides were from Sigma, except Gp_4G that was purified from the brine shrimp *Artemia salina* cysts (Vallejo *et al.*, 1974). [α-^{32}P]ATP (3000 Ci/mmole) was from Dupont NEN.

Enzymes. Poly(A) polymerase from *E. coli* was from Amersham Pharmacia Biotech. (code E2180Y), shrimp alkaline phosphatase was from Roche Molecular Biochemicals, (code 1758250). Homogeneous overexpressed human (Thorne *et al.*, 1995) and narrow-leafed lupin (Maksel *et al.*, 2001) (*asymmetrical*) Ap_4A hydrolases (EC 3.6.1.17) were kindly donated by Drs. A. G. McLennan (Liverpool University) and D. Maksel (Melbourne University), respectively. Human Fhit protein, which is a typical dinucleoside triphosphatase (EC 3.6.1.29) (Barnes *et al.*, 1996), overexpressed in *E. coli*, was obtained as described previously (Guranowski *et al.*, 2000). Np_3N' hydrolase from yellow lupin seeds (Guranowski *et al.*, 1996), partially purified (*symmetrical*) Ap_4A hydrolase (EC 3.6.1.41) from *E. coli* (Guranowski *et al.*, 1983) and Ap_4A phosphorylase (EC 2.7.7.53) from *Saccharomyces cerevisiae* (Guranowski & Blanquet, 1985) were obtained as described in the quoted papers.

Synthesis and purification of 3′-[^{32}P]-adenylated Np_nNs. 3′-[^{32}P]Adenylated Ap_3A, Ap_4A, Gp_3G and Gp_4G were synthesized enzymatically by the use of poly(A) polymerase from *E. coli* (Sillero *et al.*, 2001). The incubation mixture (0.05 ml) contained 0.02 mM [α-^{32}P]ATP, 60 μCi/ml), 1 mM of the Np_nN and 3.7 units of poly(A) polymerase. After 3.5 h incubation at 37°C, the mixture was treated with 0.5 μl (0.5 units) of shrimp alkaline phosphatase to degrade, during a 60 min incubation, the remaining [^{32}P]ATP and 3′-adenylated ATP, pppAp*A. Subsequently, the mixture was applied as a 5–6 cm band on a thin-layer silica gel aluminum plate containing fluorescent indicator (Merck), and chromatography was carried out for 120 min in dioxane/ammonium hydroxide/water (6:1:6, by vol.). In this system, each 3′-adenylated derivative migrated slightly faster than its “core” Np_nN (Sillero *et al.*, 2001). The

3′-[^{32}P]adenylated Np$_n$Ns were localized by autoradiography, eluted from the silica gel with water, and used as potential substrates of Np$_n$N′-degrading enzymes. The preparation of AppppAp*A was slightly contaminated with Ap*A.

Enzyme assays. For assaying the asymmetrically acting Ap$_4$A hydrolases and the Ap$_3$A hydrolases, the incubation mixtures (25 μl) contained 50 mM Hepes/KOH (pH 7.6), 0.02 mM dithiothreitol, 5 mM MgCl$_2$, appropriate 3′-[^{32}P]adenylated Np$_n$N and the enzyme under investigation. For assaying yeast Ap$_4$A phosphorylase, the above mixture was supplemented with 5 mM phosphate and, in the assay of (*symmetrical*) Ap$_4$A hydrolase, 5 mM MgCl$_2$ was replaced with 0.1 mM CoCl$_2$ (Guranowski *et al.*, 1983). The reaction mixtures were subjected to thin-layer chromatography. Aliquots of 5 μl were spotted on silica gel plates at the indicated times of incubation at 30°C, the chromatograms developed for 110 min in dioxane/ammonium hydroxide/water (6:1:6, by vol.), and the radioactive compounds detected by autoradiography and quantified with the help of an InstanImager. In pilot experiments, an amount of enzyme completely converting 12 nmoles of "core" Np$_n$N in less than 30 min was used. Based on that, concentrations of the indicated enzymes were appropriately adjusted in order to show progress of substrate degradation on the autoradiograms.

RESULTS AND DISCUSSION

To the best of our knowledge, literature dealing with dinucleoside polyphosphates modified at their 3′-position is confined to two 5′-mRNA cap analogues [chemically synthesized m$_3^{2,2,7}$G$^{5'}$pppAmpUmpA (Sekine *et al.*, 1996) and 7-methyl(3′-*O*-methyl)GpppG (Stępiński *et al.*, 2001)] and to enzymatically synthesized 3′-adenylated diadenosine tri- and tetraphosphates and 3′-adenylated diguanosine tri- and tetraphosphates (Sillero *et al.*, 2001). Degradation of 3′-modified Np$_n$N′s was not the subject of those works.

The approach followed to determine the substrate specificity of the enzymes tested here was similar in all cases, i.e., a radiolabeled substrate was incubated with the specified enzyme and, at different times of incubation, aliquots were taken and subjected to thin-layer chromatography. The nature of the radioactive compounds was deduced mainly from their chromatographic position and coelution with standards. From the radioactive products generated, it could be inferred whether the cleavage of the dinucleotide took place at the phosphoanhydride bond located in position 1, 2 or 3, counting from the unmodified (nonadenylated) nucleotide end. The results obtained are presented in Figs. 1–3 and summarized in Table 1.

When AppppAp*A (Fig. 1A) or GppppGp*A (Fig. 2A) were treated with (*asymmetrical*) dinucleoside tetraphosphatase from narrow-leafed lupin, cleavages at positions 3 (preferential) and 1 were observed in both cases (Table 1). In contrast, random cleavage at positions 1 and 3 was obtained when AppppAp*A (Fig. 1B) or GppppGp*A (Fig. 2B) were treated with dinucleoside tetraphosphatase from human placenta. Exhaustive treatment of GppppG*A (Fig. 2B) with the latter enzyme resulted in complete disappearance of the substrate.

Such a difference in the preference of cleavage of the asymmetrical substrates exerted by the plant and human/animal types of (*asymmetrical*) dinucleoside tetraphosphatases can be explained by the differences in topography of the substrate binding sites between these two subgroups of Ap$_4$A hydrolases whose three-dimensional structures have been revealed only recently (Swarbrick *et al.*, 2000; Bailey *et al.*, 2002; respectively). Although the plant and animal Ap$_4$A hydrolases belong to the same Nudix protein family, they have low sequence similarity outside the Nudix sequence motif. Human and *Caenorhabditis elegans* Ap$_4$A hydrolases are very similar

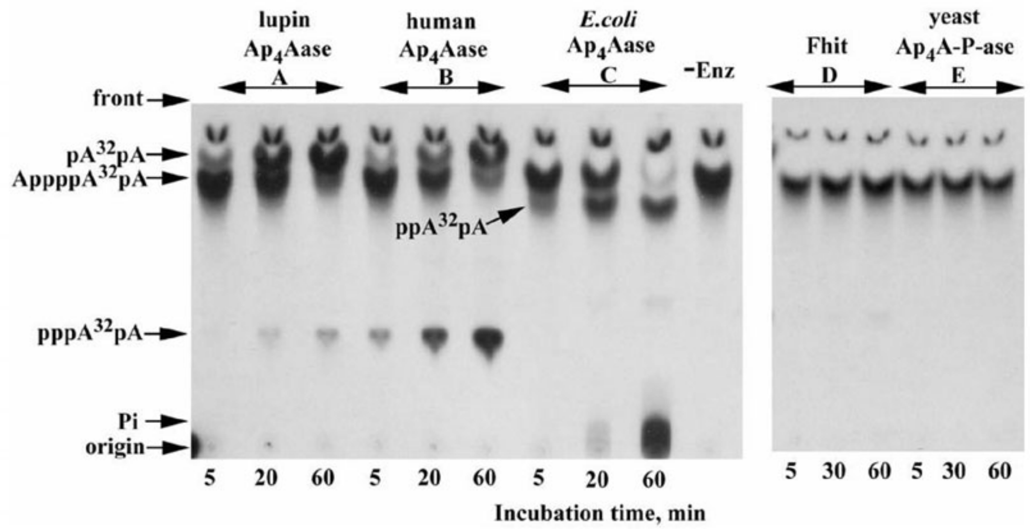


Figure 1. Analysis of enzymatic cleavage of ApppA[^{32}P]A (ApppAp*A) by thin-layer chromatography.

The reaction mixtures contained about 0.2 μM ApppAp*A, the indicated enzyme and other components as described in Materials and Methods. Accumulation of radioactivity close to the chromatogram origin, observed mostly in lanes C, resulted from [^{32}P]orthophosphate that had been liberated from the ppAp*A product due to the action of phosphodiesterase and nucleotidase/phosphatase which apparently contaminated the partially purified preparation of the *E. coli* hydrolase.

(Abdelghany *et al.*, 2001) and analysis of the three-dimensional structure of the latter reveals much more space in its substrate binding site than one can observe in such a site of the lupin counterpart. This is probably the reason why human Ap$_4$A hydrolase tolerated such a bulky substituent as a nucleotide residue in its potential substrates and bound the investigated asymmetrical substrates randomly, whereas the plant (lupin) enzyme clearly preferred to interact with the asymmetrical substrates from their unmodified end.

The (*symmetrical*) dinucleoside tetraphosphatase from *E. coli* cleaved at position 2 (Table 1) with either ApppAp*A (Fig. 1C) or

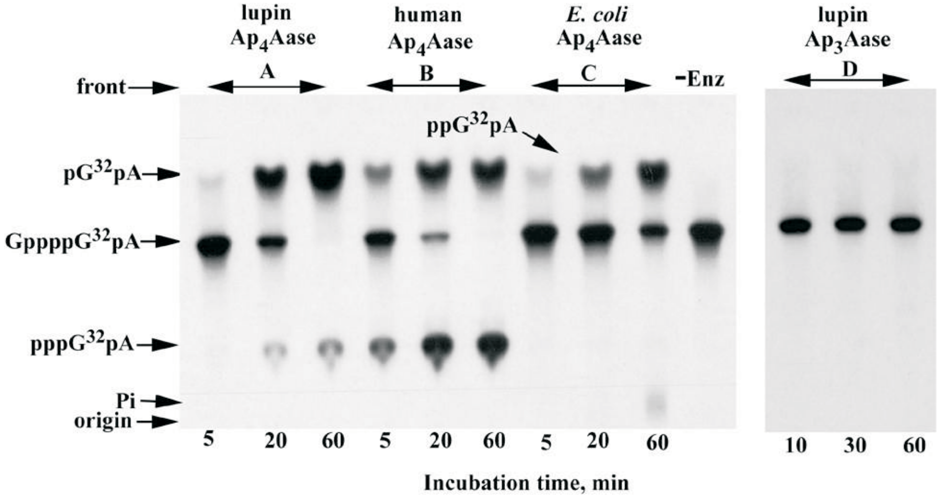


Figure 2. Analysis of enzymatic hydrolysis of GppppG[^{32}P]A (GppppGp*A) by thin-layer chromatography.

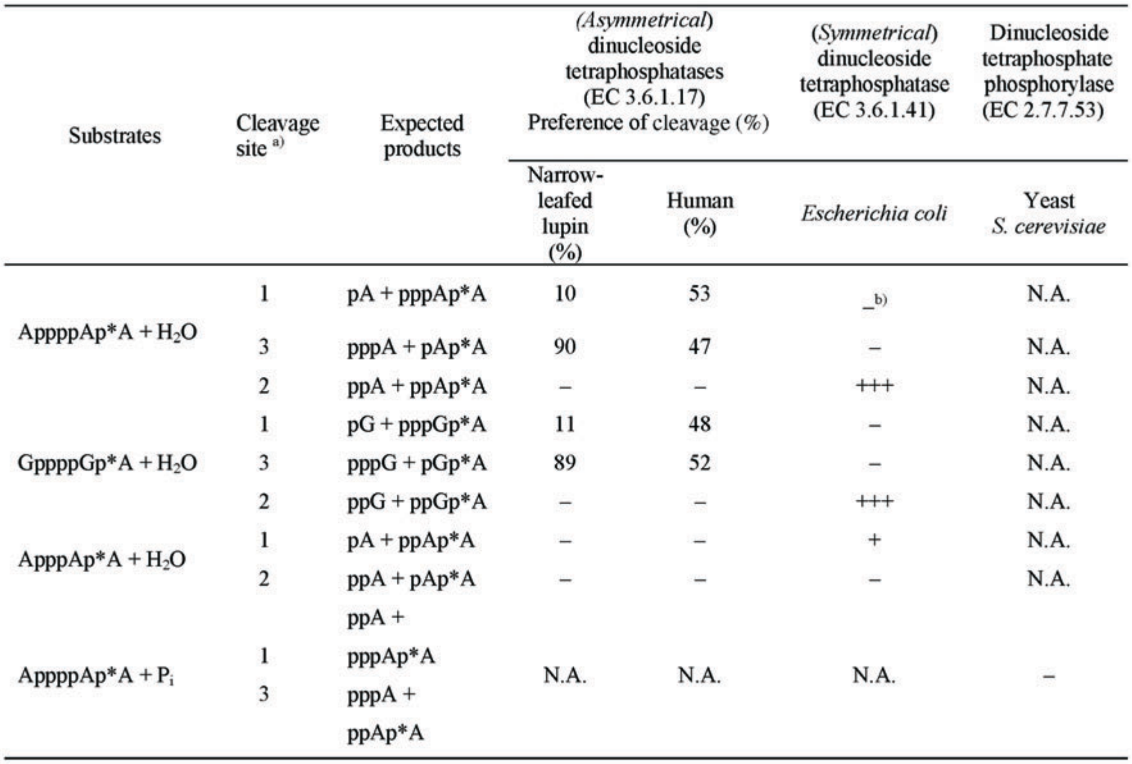
The reaction mixtures contained about 0.4 μM GppppGp*A, the indicated enzyme and other components as described in Materials and Methods.

GppppGp*A (Fig. 2C) as substrates, yielding labeled ppAp*A and ppGp*A, respectively.

Two dinucleoside triphosphatases, which have been demonstrated to hydrolyze also dinucleoside tetraphosphates (Jakubowski & Guranowski, 1983; Barnes *et al.*, 1996), were tested: the enzyme from yellow lupin did not hydrolyze GppppGp*A (Fig. 2D), and the human Fhit enzyme hydrolyzed ApppppAp*A only poorly (Fig. 1D, a faint spot at the position of pppAp*A, after 60 min incubation). ApppppAp*A was also resistant to dinucleoside tetraphosphate phosphorylase from *S. cerevisiae* (Fig. 1E).

ApppAp*A was not a substrate of the reactions catalyzed by either yellow lupin dinucleoside triphosphatase (Fig. 3A) or by the human Fhit protein (not shown), at the same experimental conditions in which unlabeled 0.5 mM ApppA was completely transformed to pA + ppA in less than 30 min. In contrast, ApppAp*A was a substrate for *E. coli* (*symmetrical*) Ap$_4$A hydrolase that can also hydrolyze NppppN to ppN and pN (Guranowski *et al.*, 1983). In the chromatographic system used we observed one of the products, ppAp*A (Fig. 3A). Comigration of ApppApA with pApA does not allow one to determine whether two pairs of products accumulate: the observed ppAp*A + pA and the alternative one, ppA + pAp*A. Both (*asymmetrical*) Ap$_4$A hydrolases and yeast Ap$_4$A phosphorylase did not degrade ApppAp*A, in the same way as the core ApppA was not a substrate of these enzymes (Guranowski & Blanquet, 1985; Jakubowski & Guranowski, 1983; Lażewska *et al.*, 1993).

Table 1. 3′-Adenylated dinucleoside polyphosphates as substrates of enzymes degrading dinucleoside polyphosphates



Substrates	Cleavage site [a]	Expected products	(*Asymmetrical*) dinucleoside tetraphosphatases (EC 3.6.1.17) Preference of cleavage (%)		(*Symmetrical*) dinucleoside tetraphosphatase (EC 3.6.1.41)	Dinucleoside tetraphosphate phosphorylase (EC 2.7.7.53)
			Narrow-leafed lupin (%)	Human (%)	*Escherichia coli*	Yeast *S. cerevisiae*
ApppppAp*A + H_2O	1	pA + pppAp*A	10	53	–[b]	N.A.
	3	pppA + pAp*A	90	47	–	N.A.
	2	ppA + ppAp*A	–	–	+++	N.A.
GppppGp*A + H_2O	1	pG + pppGp*A	11	48	–	N.A.
	3	pppG + pGp*A	89	52	–	N.A.
	2	ppG + ppGp*A	–	–	+++	N.A.
ApppAp*A + H_2O	1	pA + ppAp*A	–	–	+	N.A.
	2	ppA + pAp*A	–	–	–	N.A.
ApppppAp*A + P_i	1 3	ppA + pppAp*A pppA + ppAp*A	N.A.	N.A.	N.A.	–

[a] Cleavage site 1, 2 or 3 refers to the first, second or third (where applicable) phosphoanhydride bond, counting from the unmodified end of the dinucleotide; [b] No cleavage was observed; N.A., not assayed; not applicable; p* corresponds to ^{32}P-labeled products. The investigated compounds were substrates neither for lupin nor human (Fhit protein) dinucleoside triphosphatase (EC 3.6.1.29).

This study shows that both Ap_4A phosphorylase, and the human (Fhit protein) and yellow lupin Ap_3A hydrolases are rather strict concerning the 3′-adenylation of their respective substrates, Ap_4A and Ap_3A. Similarly, previous studies showed that 2′-(deoxy)adenylated Ap_3A and Ap_4A were not substrates of the human Ap_3A hydrolase (Guranowski *et al.*, 2000). In contrast, both the *symmetrical* and *asymmetrical* Np_4N' hydrolases are able to cleave their 3′-adenylated substrates Ap_4A or Gp_4G, in line with previous findings showing the susceptibility of 2′-deoxyadenylated Ap_4A to the hydrolysis catalyzed by (*asymmetrical*) dinucleoside tetraphosphatases from human (Guranowski *et al.*, 2000) or lupin (Maksel *et al.*, 2001). Altogether, these results show that both types of Ap_4A-degrading enzymes tolerate such a bulky substituent as adenylate at the 3′ or 2′ position of their substrates. The substrate specificity of rat liver (*asymmetrical*) dinucleoside tetraphosphatase was previously tested using AppppA, AppppddA and ddAppppddA as substrates (Sillero *et al.*, 1997). The main conclusion was that lack of only one of their 3′-OH residues greatly diminishes the rate of catalysis of the enzyme, as reflected by the similarity between the actual velocities observed with equal concentrations of AppppddA and ddAppppddA. With AppppddA, the products of the reaction were preferentially pA and pppddA, i.e. the enzyme cleaved the substrate at position 1. It is clear that more investigation is needed to elucidate the mechanism of catalysis of the enzymes cleaving specifically this type of dinucleotides. Unfortunately, 3′-adenylated substrates were available in limited amounts which precluded detailed kinetic studies. The work presented here, although largely qualitative, widens nevertheless the spectra of substrate specificities of the dinucleoside polyphosphate-cleaving enzymes. Since none of the eukaryotic Np_nN'-degrading enzymes investigated here was able to degrade 3′-adenylated dinucleoside triphosphates, Np_3NpAs, involvement of those enzymes in mRNA decapping is unlikely. The decapping is controlled by different, very specific enzymes (Liu *et al.*, 2002; Milone *et al.*, 2002).

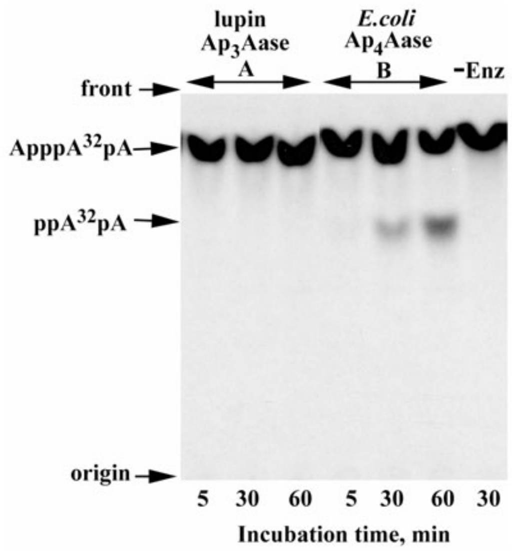


Figure 3. Thin-layer chromatography analysis of hydrolysis of ApppA[^{32}P]A (ApppAp*A).

The reaction mixtures contained about 0.5 μM ApppAp*A, the indicated enzyme and other components as described in Materials and Methods.

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