Synthesis of Oligoinosinates with 2′–5′ Internucleotide Linkage in Aqueous Solution Using Pb^{2+} Ion

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Oligoinosinates up to a pentamer were synthesized by the polymerization of inosine-5′-phosphorimidazolide in aqueous solution using Pb^{2+} ion. 2′–5′ Internucleotide linkage was preferentially formed. The yields of the 2′–5′ linked dimer, trimer, and tetramer were 14.8, 4.5, and 1.6%, respectively. A small amount of linkage isomers of oligoinosinates with 3′–5′ linkage was obtained. The products were characterized by sequential enzyme tests.

Recently 2′–5′ linked oligoadenylate ($pppA^{2'}p^{5'}A^{2'}p^{5'}A$) was isolated from interferon-treated cell and has attracted widespread interest because of its strong biological activity and unusual structure.[1–3] The availability of various oligonucleotides with 2′–5′ linkage would facilitate further study of their biological action. As a part of our program in the synthetic approach to oligonucleotides, attention was focused on the selective synthesis of short chained oligonucleotides containing 2′–5′ linkage without using any protecting group.

Reports were given on the synthesis of 2′–5′ linked oligoadenylates from adenosine-5′-phosphorimidazolide in aqueous solution using Pb^{2+} ion.[4,5] The method employs no protecting group, various oligoadenylates being obtained in the one step reaction. We have attempted the synthesis of other homooligonucleotides with 2′–5′ linkage by a similar procedure.[6] This paper describes the oligoinosinates synthesis from inosine-5′-phosphorimidazolide in aqueous solution using Pb^{2+} ion. The products were separated by ion exchange column chromatography and characterized by sequential enzyme and alkaline digestion.

Results and Discussion

Inosine-5′-phosphorimidazolide (ImpI) was prepared from inosine-5′-monophosphate (pI) and imidazole using di-2-pyridyl disulfide and triphenylphosphine as a condensing agent. Phosphorimidazolide bond is labile, hydrolyzing in aqueous solution spontaneously in the absence of divalent metal ion catalyst. The Pb^{2+} ion promoted the internucleotide linkage formation from ImpI. Addition of lead nitrate to a neutral aqueous solution of ImpI caused precipitation, suggesting formation of Pb^{2+}–ImpI complex. The ImpI was allowed to react in a neutral aqueous mixture for 3 d with stirring in the presence of the Pb^{2+} ion for polymerization. Treatment of the reaction mixture with *N*-(2-hydroxyethyl)-ethylenediamine-*N*,*N*′,*N*′-triacetic acid trisodium (Versenol) buffer turned the mixture homogeneous. The Pb^{2+} ion formed a complex with Versenol completely. The formation of the oligoinosinates and Pb^{2+}–Versenol complex was checked by high pressure liquid chromatography (HPLC). The products were separated on a QAE-Sephadex A-25 anion exchange column. The separated products were further purified by paper chromatography when necessary. The elution pattern obtained on column chromatography is shown in Fig. 1. The composition and the yields of the products are given in Table 1 along with the hyperchromicity of the main products.

Oligoinosinates up to a pentamer were formed in addition to the hydrolyzed product pI. The internucleotide linkage formation took place as the following Scheme 1. The 2′–5′ internucleotide linkage was preferentially formed in the reaction.

The oligoinosinates thus obtained were characterized by enzymatic tests, paper chromatography and HPLC. The 2′–5′ internucleotide linkage is not degraded

Table 1. Oligoinosinates obtained from ImpI

Peak No.	ODU_{248}	h[a]	Structure identification	Yield/%[b]
1	36		I	1.0
2	12		3′,5′-Cyclic IMP	0.3
3	231		ImpI	5.5
4	1558		pI	41
5	72		ImpIpI	1.0
			-pIpI- (2′–5′, 2′–5′)	0.9[c]
6	51		IppI	1.3[c]
7+8	770		-pIpI- (2′–5′, 3′–5′)	1.8[c]
		1.04	-pIpI- (3′–5′, 3′–5′)	5.0
		1.08	$pI^{2'}p^{5'}I$	14.8
9	130	1.06	$pI^{3'}p^{5'}I$	2.6
			$IppI^{2'}p^{5'}I$	0.6[c]
10	146	1.15	$pI^{2'}p^{5'}I^{2'}p^{5'}I$	4.5
11	116	1.18	$pI^{2'}p^{5'}I^{3'}p^{5'}I$	2.6
			Unidentified	0.8[c]
12	66	1.15	$pI^{3'}p^{5'}I^{2'}p^{5'}I$	1.5
			Unidentified	0.5[c]
13	120	1.15	$pI^{3'}p^{5'}I^{3'}p^{5'}I$	1.6
		1.20	$pI^{2'}p^{5'}I^{2'}p^{5'}I^{2'}p^{5'}I$	1.5
			Unidentified	0.4[c]
14	33	1.24	$pI^{2'}p^{5'}I^{3'}p^{5'}I^{2'}p^{5'}I$	0.7
			$pI^{2'}p^{5'}I^{2'}p^{5'}I^{3'}p^{5'}I$	0.4
15	30		$(pI)_4$ (Mixtures of isomers)	0.8[c]
16	72	1.31	$pI^{2'}p^{5'}I^{2'}p^{5'}I^{2'}p^{5'}I^{2'}p^{5'}I$	0.6
			$(pI)_4$ and $(pI)_5$ (Mixture of isomers)	1.3[c]

a) Hyperchromicity calculated from alkaline hydrolysis. b) Yield obtained from UV_{248} after allowing for hyperchromicity of each oligoinosinate. Total ODU_{248} of starting ImpI is 3800 (0.31 mmol). c) Hyperchromicity correction was not carried out.

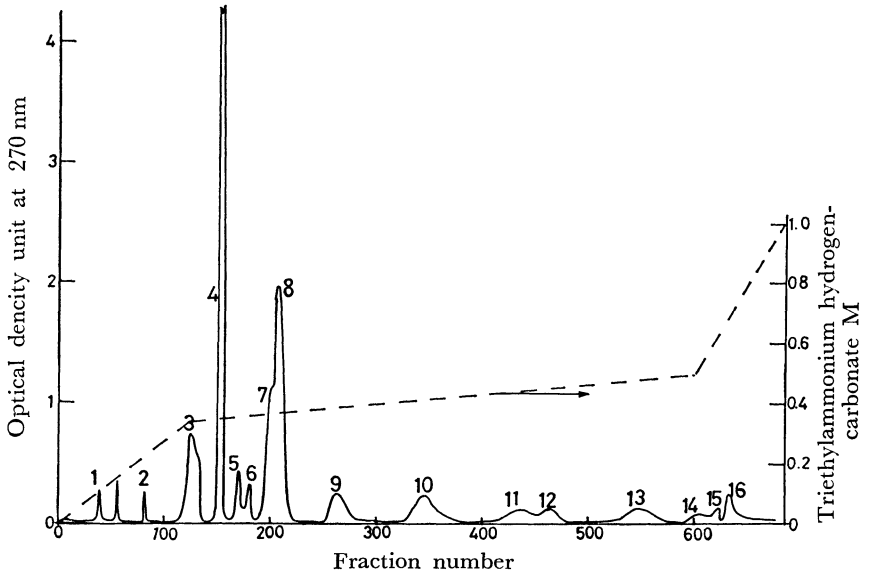
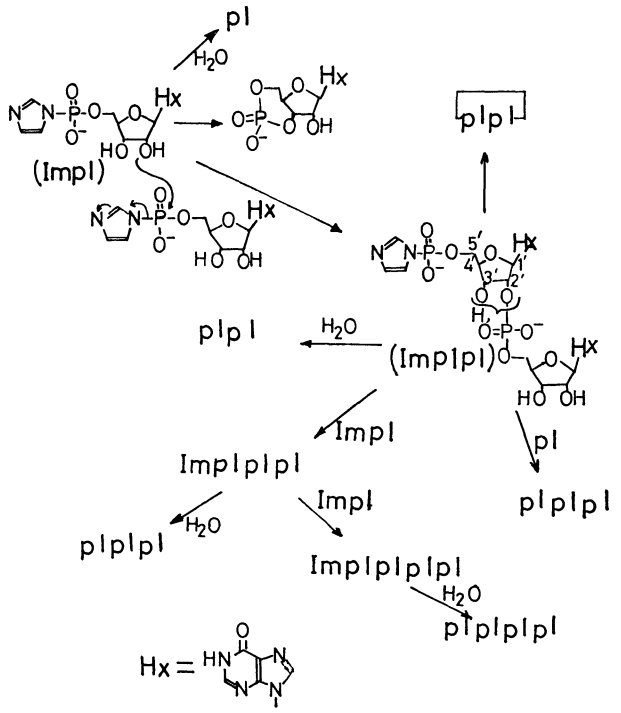


Fig. 1. Chromatography of the oligoinosinates on a QAE-Sephadex A-25 (hydrogen carbonate) column. A linear gradient of triethylammonium hydrogencarbonate was used for elution (dotted line). The characterization of the several peaks is listed in Table 1.



Scheme 1.

with nuclease P_1 (N. P_1) and ribonuclease T_2, while 3′–5′ linkage is degraded. Both 2′–5′ and 3′–5′ internucleotide linkages are hydrolyzed with venom phosphodiesterase (VPD) and by alkaline digestion. Thus, the internucleotide linkage and the chain length of the oligoinosinates were determined by sequential enzyme digestion using bacterial alkaline phosphatase (BAP), N. P_1, and VPD and by alkaline hydrolysis.

The 2′–5′ linked dimer, 5′-phosphoinosinyl-[2′–5′]-inosine ($pI^{2'}p^{5'}I$) and the trimer, 5′-phosphoinosinyl-[2′–5′]-inosinyl-[2′–5′]-inosine ($pI^{2'}p^{5'}I^{2'}p^{5'}I$) were degraded with the enzymes as follows. Digestion of $pI^{2'}p^{5'}I$ with BAP gave inosinyl-[2′–5′]-inosine. $pI^{2'}p^{5'}I$ and $I^{2'}p^{5'}I$ were insensitive to N. P_1. On the other hand, alkaline hydrolysis of $pI^{2'}p^{5'}I$ yielded to inosine (I) and inosine-2′(3′),5′-diphosphate (pIp), and $I^{2'}p^{5'}I$ to I and inosine-2′(3′)-monophosphate (Ip). $I^{2'}p^{5'}I$ was degraded with VPD to I and pI. The tetramer ($pI^{2'}p^{5'}I^{2'}p^{5'}I^{2'}p^{5'}I$) and pentamer ($pI^{2'}p^{5'}$-$I^{2'}p^{5'}I^{2'}p^{5'}I^{2'}p^{5'}I$) were degraded in a similar way. The yields of the 2′–5′ linked dimer, trimer, tetramer, and pentamer were 14.8, 4.5, 1.5, and 0.5%, respectively.

$$pI^{2'}p^{5'}I \xrightarrow{\text{N. } P_1} \not\rightarrow$$

$$pI^{2'}p^{5'}I \xrightarrow{\text{NaOH}} pIp + I \ (1:1)$$

$$pI^{2'}p^{5'}I \xrightarrow{\text{BAP}} I^{2'}p^{5'}I \xrightarrow{\text{N. } P_1} \not\rightarrow$$

$$I^{2'}p^{5'}I \xrightarrow{\text{NaOH}} Ip + I \ (1:1)$$

$$I^{2'}p^{5'}I \xrightarrow{\text{VPD}} I + pI \ (1:1)$$

$$pI^{2'}p^{5'}I^{2'}p^{5'}I \xrightarrow{\text{N. } P_1} \not\rightarrow$$

$$pI^{2'}p^{5'}I^{2'}p^{5'}I \xrightarrow{\text{NaOH}} pIp + Ip + I \ (1:1:1)$$

$$pI^{2'}p^{5'}I^{2'}p^{5'}I \xrightarrow{\text{BAP}} I^{2'}p^{5'}I^{2'}p^{5'}I \xrightarrow{\text{N. } P_1} \not\rightarrow$$

$$I^{2'}p^{5'}I^{2'}p^{5'}I \xrightarrow{\text{NaOH}} Ip + I \ (2:1)$$

$$I^{2'}p^{5'}I^{2'}p^{5'}I \xrightarrow{\text{VPD}} I + pI \ (1:2)$$

The 3′–5′ linked dimer, 5′-phosphoinosinyl-[3′–5′]-inosine ($pI^{3'}p^{5'}I$), and the trimer, 5′-phosphoinosinyl-[3′–5′]-inosinyl-[3′–5′]-inosine ($pI^{3'}p^{5'}I^{3'}p^{5'}I$), were isolated in 2.6 and 1.4% yield, respectively. These compounds were hydrolyzed by the enzymes as illustrated below.

$$pI^{3'}p^{5'}I \xrightarrow{N.P_1} pI$$

$$pI^{3'}p^{5'}I \xrightarrow{BAP} I^{3'}p^{5'}I \xrightarrow{N.P_1} I + pI \ (1:1)$$

$$I^{3'}p^{5'}I \xrightarrow{NaOH} Ip + I \ (1:1)$$

$$pI^{3'}p^{5'}I^{3'}p^{5'}I \xrightarrow{N.P_1} pI$$

$$pI^{3'}p^{5'}I^{3'}p^{5'}I \xrightarrow{BAP} I^{3'}p^{5'}I^{3'}p^{5'}I \xrightarrow{N.P_1} I + pI \ (1:2)$$

$$I^{3'}p^{5'}I^{3'}p^{5'}I \xrightarrow{NaOH} Ip + I \ (2:1)$$

Two linkage isomers, $pI^{3'}p^{5'}I^{2'}p^{5'}I$ and $pI^{2'}p^{5'}I^{3'}p^{5'}I$, were present in peaks 11 and 12, respectively. Their characterization was accomplished by the following enzyme digestion.

$$pI^{3'}p^{5'}I^{2'}p^{5'}I \xrightarrow{N.P_1} pI + pI^{2'}p^{5'}I \ (1:1)$$

$$pI^{3'}p^{5'}I^{2'}p^{5'}I \xrightarrow{BAP} I^{3'}p^{5'}I^{2'}p^{5'}I \xrightarrow{N.P_1} I + pI^{2'}p^{5'}I \ (1:1)$$

$$\downarrow NaOH$$

$$pIp + I \ (1:1)$$

$$pI^{2'}p^{5'}I^{3'}p^{5'}I \xrightarrow{N.P_1} pI^{2'}p^{5'}I + pI \ (1:1)$$

$$pI^{2'}p^{5'}I^{3'}p^{5'}I \xrightarrow{BAP} I^{2'}p^{5'}I^{3'}p^{5'}I \xrightarrow{N.P_1} I^{2'}p^{5'}I + pI \ (1:1)$$

$$\downarrow NaOH$$

$$Ip + I \ (1:1)$$

Two tetramers containing one 3′–5′ and two 2′–5′ internucleotide linkages in a different position, $pI^{2'}p^{5'}I^{3'}p^{5'}I^{2'}p^{5'}I$ and $pI^{2'}p^{5'}I^{2'}p^{5'}I^{3'}p^{5'}I$, were isolated in a small amount. They were degraded with the enzymes as follows.

$$pI^{2'}p^{5'}I^{3'}p^{5'}I^{2'}p^{5'}I \xrightarrow{N.P_1} pI^{2'}p^{5'}I$$

$$pI^{2'}p^{5'}I^{3'}p^{5'}I^{2'}p^{5'}I \xrightarrow{BAP} I^{2'}p^{5'}I^{3'}p^{5'}I^{2'}p^{5'}I \xrightarrow{N.P_1} I^{2'}p^{5'}I + pI^{2'}p^{5'}I \ (1:1)$$

$$pI^{2'}p^{5'}I^{2'}p^{5'}I^{3'}p^{5'}I \xrightarrow{N.P_1} pI + pI^{2'}p^{5'}I^{2'}p^{5'}I$$

$$pI^{2'}p^{5'}I^{2'}p^{5'}I^{3'}p^{5'}I \xrightarrow{BAP} I^{2'}p^{5'}I^{2'}p^{5'}I^{3'}p^{5'}I \xrightarrow{N.P_1} I^{2'}p^{5'}I^{2'}p^{5'}I + pI \ (1:1)$$

$$\downarrow NaOH$$

$$Ip + I \ (2:1)$$

Linkage isomers of the tetramer and pentamer were present in peaks 15 and 16 in a small amount. They remain unidentified.

Contrary to the oligoadenylate formation,[5] appreciable amounts of cyclic diinosinates were obtained in peaks 6 and 7. They were characterized by resistance to BAP and by their chromatographic mobilities. The internucleotide linkage of the cyclic diinosinates was determined by susceptibility to N. P_1. N. P_1 degraded 3′–5′, 3′–5′, and 3′–5′, 2′–5′ linked isomers to pI and $pI^{2'}p^{5'}I$, respectively. 2′–5′,2′–5′ Linked cyclic diinosinate was insensitive to N. P_1, being degraded to pI with VPD. The degradation rate of the cyclic diinosinates with the enzymes was lower than that of the linear dimer. Of the three linkage isomers, the 3′–5′, 3′–5′ linked cyclic diinosinate was predominant. A molecular model indicates that the cyclic dinucleotide prefers 3′–5′ linkage to 2′–5′ linkage conformationally.

The side products containing pyrophosphate bond were characterized as 5,5′-diinosine diphosphate (IppI) and 5′-inosine-5′-inosinyl-[2′–5′]-inosine diphosphate ($IppI^{2'}p^{5'}I$) by resistance to BAP and mobility on paper chromatograms. Both IppI and $IppI^{2'}p^{5'}I$ were degraded with VPD giving pI. Alkaline hydrolysis of $IppI^{2'}p^{5'}I$ followed by incubation with BAP gave IppI.

Peaks 1, 2, 3, and 4 were identified as I, 3′,5′-cyclic IMP, ImpI, and pI, respectively, by comparison of paper chromatogram mobility and HPLC with those of authentic samples.

The selective formation of 2′–5′ linked oligoinosinates was established in this polymerization reaction. Yield and regioselectivity of internucleotide linkage were lower than those for the oligoadenylates.[5] This procedure employs no protecting group, condensation reaction taking place in an aqueous solution. It provides a simple preparative method for the 2′–5′ linked oligoinosinates, though the yield is not satisfactory.

Experimental

Materials. Inosine-5′-monophosphate sodium salt (Yamasa) and commercial imidazole, triethylamine, triphenylphosphine, Versenol trisodium salt, and lead nitrate were used. Di-2-pyridyl disulfide was prepared by a modification of the procedure of Jones and Katrizky.[7] Inosine-5′-phosphorimidazolide was prepared from pI and imidazole using triphenylphosphine and di-2-pyridyl disulfide as a condensing agent in a similar way to that of Lohrmann and Orgel.[8] Bacterial alkaline phosphatase, (BAPF) and venom phosphodiesterase (VPD) from Worthington, nuclease P_1 (N. P_1) from Yamasa and QAE-Sephadex A-25 from Pharmacia Fine Chemicals were used.

Paper Chromatography and HPLC. Paper chromatography was carried out by a descending technique on Whatman 3MM paper. The solvent systems were (**1**), 1-propanol–concentrated ammonia–water (55:10:35 v/v), and (**2**), saturated ammonium sulfate–0.1 M sodium acetate (pH 6.5)–2-propanol (79:19:2 v/v). R_f values of various oligoinosinates are given in Table 2.

HPLC was performed with a Hitachi 538 apparatus using a RPC-5 column (4 mmϕ × 25 cm). RPC-5 was prepared according to the method of Pearson *et al.*[9] The elution of the HPLC column was carried out with a linear gradient of aqueous $NaClO_4$ solution (H_2O–0.025 M $NaClO_4$) containing 0.0025 M Tris-acetate buffer (pH 7.5). The compounds were monitored by UV absorption at 260 nm.

Preparation of Oligoinosinate. Lead nitrate solution (0.25 M 0.3 ml) was added to a 5.7 ml aqueous solution of ImpI (150 mg, 3800 optical density unit at 248 nm, 0.3 mmol) containing 0.2 M imidazole buffer at pH 7.0 with stirring. White precipitates were formed. The solution was kept at 20 °C for 3 d with stirring. The reaction mixture was treated with 0.5 ml of 0.25 M Versenol buffer to complex the Pb^{2+} ion. A small portion (10 μl)

TABLE 2. R_f VALUES OF THE COMPOUNDS

Compound	R_f values relative to pI	
	Solvent 1	Solvent 2
I	1.57	0.80
3′,5′-Cyclic IMP	1.43	0.87
ImpI	1.56	
pI	1.00	1.00
IppI	0.91	0.86
ImpIpI	1.12	
$\overline{\text{pIpI}}$ - [3′-5′, 3′-5′ / 3′-5′, 2′-5′ / 2′-5′, 2′-5′]	1.05	0.30—0.50
$pI^{2'}p^{5'}I$	0.78	0.92
$pI^{3'}p^{5'}I$	0.76	0.81
$I^{2'}p^{5'}I$	1.17	
$I^{3'}p^{5'}I$	1.14	
$IppI^{2'}p^{5'}I$	0.60	0.80
$pI^{2'}p^{5'}I^{2'}p^{5'}I$	0.53	0.83
$pI^{2'}p^{5'}I^{3'}p^{5'}I$	0.48	0.77
$pI^{3'}p^{5'}I^{2'}p^{5'}I$	0.50	0.75
$pI^{3'}p^{5'}I^{3'}p^{5'}I$	0.49	0.64
$pI^{2'}p^{5'}I^{2'}p^{5'}I^{2'}p^{5'}I$	0.37	0.79
$pI^{2'}p^{5'}I^{3'}p^{5'}I^{2'}p^{5'}I$	0.35	0.67
$pI^{2'}p^{5'}I^{2'}p^{5'}I^{2'}p^{5'}I^{2'}p^{5'}I$	0.23	0.64

of the resulting homogeneous solution was analyzed with HPLC. The bulk of the solution was applied to the top of a QAE-Sephadex A-25 (hydrogencarbonate form) column, 25 mmϕ × 36 cm. After washing with water, elution was carried out by a stepwise linear gradient of triethylammonium hydrogencarbonate buffer (pH 7.5); (1) H_2O (0.75 l)–1/3 M (0.75 l); (2) 1/3 M (3 l)–1/2 M (3 l); 1/2 M (1 l)–1 M (1 l). *Ca.* 14 ml fractions were collected every 10 min. UV absorption of each fraction was measured at 270 nm. The fractions containing the UV absorbing compound were pooled and evaporated *in vacuo* below 30 °C. The excess of triethylammonium hydrogencarbonate was removed by repeated evaporation after addition of water. UV absorption of the oligoinosinates was measured at 248 nm. The yield was calculated from ODU_{248} after allowing for the hyperchromicity of each oligoinosinate. The hyperchromicity was obtained by the ratio of UV absorption at 248 nm after and before alkaline hydrolysis of the oligoinosinate.

Characterization of Products. Characterization of the products was carried out by means of sequential enzymes and alkaline digestion. The structure was confirmed by comparing the paper chromatograms and HPLC with those of authentic samples. The separated products were subjected to paper chromatography in solvent systems **1** and **2**. The UV active spot on the paper developed with system **1** was eluted with water, and then incubated with BAP. The incubated mixture was chromatographed on the paper using system **1**. The spot on the paper was elute with water. The eluate was evaporated *in vacuo* and incubated with N. P_1. The mixture was analyzed with HPLC, and applied to paper chromatography in system **1**. The UV absorbing spot was eluted with water and divided into two portions. One was digested with VPD and the other was degraded in a 0.5 M alkaline solution. The digested solution was analyzed with HPLC. The molar ratio of inosine and inosine nucleotides was determined with HPLC.

Digestion of the Products with Enzymes. Digestion with BAP was carried out by incubating a mixture of 0.5—1.5 μmol of nucleotidic material in a 50 μl solution containing 0.1 M Tris-HCl (pH 8.05), 0.001 M $MgCl_2$ and 0.1 unit of enzyme, at 37 °C for 2.5 h. The mixture was spotted directly on a Whatman 3MM paper and developed in system **1**.

Degradation with N. P_1 was performed at 37 °C for 2.5 h in a mixture (50 μl) containing the nucleotidic material (0.5—1.5 μmol), 0.006 M Veronal-acetate buffer (pH 5.75) and an enzyme solution (5 μg in 5 μl).

Degradation with VPD was carried out at 37 °C for 2.5 h in a mixture (50 μl) containing the substrate (0.2—0.4 μmol), 0.01 M Tris–acetate (pH 8.8), 0.01 M $MgCl_2$ and enzyme solution (0.01 unit).

Alkaline hydrolysis was carried out at room temperature for 1 d in a mixture (50 μl) containing the substrate (0.2—0.5 μmol) in a 0.5 M NaOH solution.

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