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OLIGONUCLEOTIDE PHOSPHORAZOLIDATES — REAGENTS FOR CHEMICAL LIGATION*

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The synthesis has been effected in an aqueous medium of oligonucleotide phosphorazolidates derived from imidazole, 2-methylimidazole, benzimidazole, pyrazole, triazole, and benzotriazole with yields close to quantitative. It has been shown that they are all phosphorylating agents in an aqueous medium and can be used as reagents for the directed complementary template assembly of nucleic acid duplexes (chemical ligation). The most reactive are the imidazole, 2-methylimidazole, and benzotriazole derivatives.

A promising approach to the synthesis of natural compounds is the modeling of processes taking place in their biosynthesis. The use of such an approach, which has acquired the name of biomimetic [1], has led to the creation of a method for the chemical ligation of DNA which permits DNA fragments to be linked within a duplex without the participation of an enzyme [2, 3]. When the method of chemical ligation is used, a number of limitations imposed on the structure of the DNA fragments by the requirements of the substrate specificity of ligases are eliminated. The method permits the solution of a broad spectrum of synthetic problems, such as obtaining preparative amounts of double-stranded oligo(poly)nucleotides and the assembly of modifed DNA duplexes, including those with directedly modified internucleotide bonds [2-5] and of oligonucleotides containing covalently linked ribo- and deoxyribonucleotide frament [2, 6].

As reagent for the synthesis of natural and modified DNA duplexes use has been made previously of oligodeoxyribonucleotide phosphorimidazolidates [4, 6] — compounds imitating the active transition state in a number of enzymatic phosphorylation reactions [1] and possessing phosphorylating properties in an aqueous medium [1, 7].

The development of the technology of recombinant nucleic acids has posed the problem of creating a set of reagents for chemical ligation and, in particular, for solving the problem of the effective ligation of RNA fragments. The necessity has arisen for using the biomimetic

*Abbreviations adopted in this paper: MCC — microcolumn chromatography; OU_{260} — amount of nucleotide material in optical units determined at a wavelength λ = 260 nm; CDI — 1-(3-methylaminopropyl)-3-ethylcarbodiimide; PEG — polyethyleneglycol; PAAG — polyacrylamide gel; MES — 2-morpholinoethanesulfonic acid; HEPS — 2-[4-(2-hydroxyethyl)piperazine-1-yl]ethanesulfonic acid.

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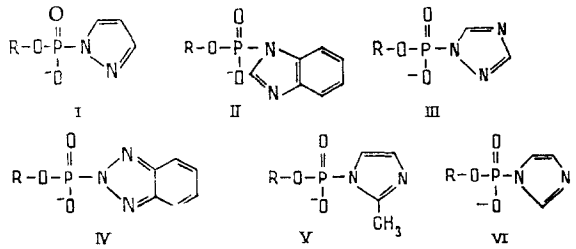
principles upon which chemical ligation is based [3, 6] and of finding reactive groups (including those of nonprotein nature) for the "active site" of a chemical ligase — the unit for the formation of an internucleotide bond. Information of the reactivity of nucleotide phosphorazolidates and phosphorazolidites in organic media [7, 8] gave grounds for assuming that it was possible to use as such groups, together with imidazole derivatives, derivatives of other azoles: triazole, benzotriazole, benzimidazole, 2-methylimidazole, and pyrazole.

The aim of the present work was to choose conditions for the synthesis of derivatives of oligonucleotides with these azoles and to investigate the possibility of using the phosphorazolidates obtained as reagents for chemical ligation.

The carbodiimide method is apparently the most convenient for obtaining phosphorazolidate derivatives of oligonucleotides [9]. It has been shown that the efficacy of the synthesis of a phosphorazolidate under the action of CDI depends on a number of factors such as the pH of the medium and the ratio of the components of the reaction mixture and their concentrations [9]. In view of this, the conditions of synthesis were selected individually for each phosphorazolidate (Table 1). The synthetic procedures were developed initially for the case of the phosphorazolidate derivatives of a mononucleotide — dpA:



where R = dA

The course of the reaction was followed by high-voltage paper electrophoresis for compounds (I-III and V) and by TLC in the case of compound (IV). The optimum conditions ensuring close to quantitative yields of compounds (I-V) are given in Table 1. The electrophoretic mobility of the phosphorazolidates (U_{dpA}), 0.5, indicated the presence in these compounds of a single negative charge. In an acid medium (pH 2, 37°C, 10 min), compounds (I)-(V) were hydrolyzed quantitatively to the initial nucleotide, which, according to [10], confirmed the phosphoramide nature of the bond in the derivatives obtained.

Azolides of oligonucleotides, including oligonucleotides ^{32}P-labeled at the terminal phosphate group, were synthesized by the procedures developed. Analysis of the reaction mixtures by the ion-exchange MCC method showed that the time necessary for forming the oligonucleotide derivatives at a total nucleotide concentration of 10^{-3}-10^{-4} M was 2-2.5 h. After incubation for 2.5 h, the reaction mixtures were desalted by gel filtration on Bio-Gel P-2 or by precipitation with ethanol. Conditions were selected that permitted the isolation of the oligonucleotide azolides practically quantitatively, the losses of oligonucleotide material not exceeding 5-10%.

It was interesting to compare the properties of the azolides from (I-V) with the properties of the imidazole derivatives described previously [4, 6, 7]. To characterize the reactivities of the azolides (I-V) and of dpA imidazolide (VI), we studied their hydrolysis on aqueous solutions at pH values of from 5.0 to 8.0. The derivatives of the substituted imidazoles (I), (II), (V), and (VI) and of the triazoles (III) and (IV) differed in their chemical natures, which led to differences in the hydrolytic stability of these phosphorazolidates. The hydrolysis of the phosphorimidazolidates, taking place through the protonation of the azolide residue [11], accelerated with the lowering of the pH of the medium (Table 2). Under these conditions the pyrazolide (I) and the benzimidazolide (II) were more stable than the imidazolides (V) and (VI), which were readily hydrolyzed at pH < 6.0 (Table 2). The triazolide (III) and (IV) behaved differently; they were extremely labile over the whole pH range investigated (Table 2). The weak dependence of the hydrolytic stability of the triazolides on the pH is, to all appearance, explained by the fact that their hydrolysis does not require the preliminary protonation of the azolide residue, as in the case of the imidazolides [11]. This is connected with the fact that in nucleophilic substitution reaction the triazole residue, which is a weak acid, can leave in the form of a monoanion [11].

TABLE 1. Synthesis of Deoxyadenylic Acid Azolides

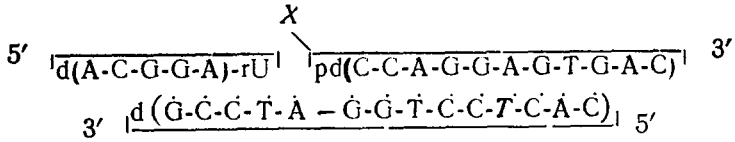
Azole	Concentration, M			pH	Reaction time, h	Yield of product, %
	nucleotide	azole	CDI			
Pyrazole	0,10	3,0	0,5	6,0	0,5	100
Benzimidazole	0,03	0,04*	0,2	6,5	1,0	100
Triazole	0,10	3,0	0,5	6,0	0,25	80—90
Benzotriazole	0,05	0,1*	0,2	7,0	0,5	100
2-Methylimidazole	0,10	3,0	0,5	5,0	0,25	100

*Maximum achieveable concentration of an aqueous solution of the azole.

All the azolides proved to be most stable at a neutral pH, the half-transformation period then exceeding 1.5 h (Table 2). The existence of a range of stability permitted the quantitative isolation of the phosphorazolidates (I-VI) and also their storage in aqueous solutions at a low temperature (<0°C). At the same time, on the addition of nucleophilic catalysts — 1-methylimidazole, 4-dimethylaminopyridine — to the reaction mixtures the half-conversion period of compounds (I-VI) decreased to 5 min.

The capacity of the phosphorazolidates for serving as phosphorylating agents in an aqueous medium was evaluated from their reactions with aliphatic amines — ethylenediamine and butylenediamine. It was found that in aqueous solution at 20°C the azolides (I-VI) reacted with ethylene- and butylenediamines and the phosphorylation of these amides take place quantitatively in 10 min. The ease of preparation of the azolides (I-V) and their high reactivity permit the suggestion that these compounds may find use both for obtaining derivatives of oligo- and polynucleotides at the terminal phosphate group and for the affinity modification of the proteins of the nucleic acid metabolism.

A unique property of the oligonucleotides is their capacity for being assembled into duplexes. This property forms the basis of the biomimetic reactions of chemical ligation. To investigate the capacity of the oligonucleotide azolides for taking part in condensation reactions as components of duplexes, we used a DNA duplex constructed of three synthetic oligonucleotides and characterized previously in [12]:



where X = OH in the initial undecanucleotide or an azole residue in its derivative: the pyrazolide (VII), benzimidazolide (VIII), triazolide (IX), benzotriazolide (X), 2-methylimidazolide (XI), or imidazolide (XII) of the undecanucleotide.

The ribo unit present at the 3'-end of the hexanucleotide participates in the formation of an internucleotide bond on condensation in a duplex. Thus, the DNA duplex is the simplext model for the investigation of the chemical synthesis of oligo(poly)nucleotides containing covalently linked ribo and deoxyribo fragments. Such compounds fulfill the role of intermediates in the primary synthesis of DNA ("Okazaki fragments") [13]. The duplexes formed by

TABLE 2. Hydrolytic Stabilities of Azolides of Deoxyadenylic Acid in an Aqueous Medium of 20°C

Azolide	pK_a of the azoles [8]	Half-conversion time in various buffers, h			
		0.1 M MES with pH		0.1 M HEPES with pH	
		5,0	6,0	7,0	8,0
Pyrazolide (I)	2.53	1,5	5,0	50	Stable*
Benzimidazolide (II)	5.48	12—15	Stable	Stable	Stable
Triazolide (III)	1.17	0,5	1,0	1,5	0,5
Benzotriazolide (IV)	1.6	2,0	2,5—3,0	4—5	2,5—3,0
1-Methylimidazolide (V)	6,85	1,5	1,5—2,0	25	Stable
Imidazolide (VI)	6,95	1,0	1,5	25	140

*The period of half-conversion exceeds 150 h.

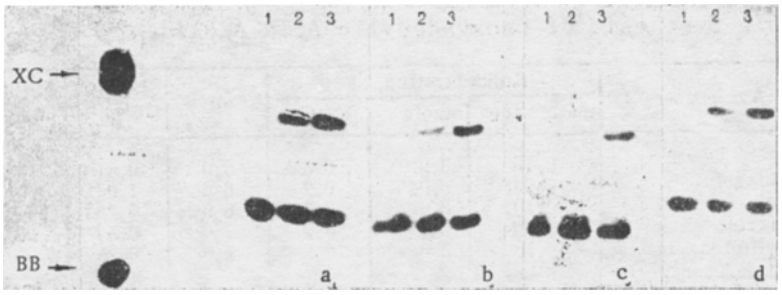


Fig. 1. Autoradioagraph of the gel electrophoresis of reaction mixtures on the condensation of duplexes (scheme 2) of the imidazolide (a), the 2-methylimidazolide (b), the benzimidazolide (c), and the benzotriazolide (d) of the undecanucleotide. Tracks 1, 2, and 3 correspond to reaction for 0.5, 3, and 20 h, respectively. XC and BB are the positions of the marker dyes Xylene Cyanole and Bromphenol Blue. The figures on the right show the numbers of nucleotide units in the oligonucleotides [sic].

oligo(poly)nucleotides containing covalently linked ribo and deoxyribo fragments have an unusual secondary structure combining elements of the A and B forms of DNA [14]. The development of effective methods for synthesizing the compounds will permit performance of a further investigation of the physicochemical properties and biological activity.

All six azolides (VII-XII) of the undecanucleotide were obtained with the aid of CDI and their capacities for condendation within a duplex were investigated. To perform condensation we took an azolide of the ^{32}P-labeled undecanucleotide and the hexa- and tetradecanucleotide. Analysis of the reaction mixtures by electrophoresis in PAAG showed (Fig. 1) that the azolides (VII-XI) were capable of condensing in a duplex in the same way as the imidazolide (XII) studied previously [6]. Information on the efficacy of the formation of the rU–dC bond is summarized in Table 3. The reaction product — the 17-membered oligonucleotide d(ACGGA)rUd-(CCAGGAGTGAC) — was identical with that obtained previously on the condensation of the imidazolide (XII) [6]. The bond formed between the ribo and deoxyribo fragments was to the extent of 70% a (2'–5')-phosphodiester bond, as was confirmed by the cleavage of the heptadecanucleotide by RNase A. The ratio of the (2'–5')- and (3'–5')-isomers obtained in the condensation of the phosphorazolidates (VII-XII) did not depend on the nature of the azolide residue.

The condensation of the azolides (VII-XII) took place most effectively in the presence of 1-methylimidazole (Table 3). The existence of 1-methylimidazole catalysis shows that the condensation of the azolide (VII-XII) took place through the formation of a highly reactive phospho-1-methylimidazolium intermediate the existence of which in aqueous solution has been demonstrated previously [15]. An attempt was made to increase the efficiency of condensation by the addition of PEG-6000 to the reaction mixture. It is known that PEG catalyzes the enzymatic ligation reaction [16] by raising the local concentration of nucleic acids through the excluded-volume effect [17]. However, the addition of PEG to the reaction mixture in a concentration of 5%, which is the optimum for enzymatic ligation [16], caused a fall in the efficiency of the reaction (Table 3). To all appearances, the compactization of the DNA in the presence of PEG had an adverse effect on the accessibility of the internucleotide-bond forming unit for the approach of the catalyst from outside.

For all the reactions performed in 1-methylimidazole buffer with pH 8.0 we detected a fall in the yield of the desired product when the reaction mixture was incubated for more than a day (Table 3). The ease of the breakdown of the reaction product is connected with the fact that it contains an alkali-labile unit — a internucleotide phosphodiester bond formed by the participation of a pyridine ribonucleotide [18]. 1-Methylimidazole may also make its own contribution to the degradation of the heptadecanucleotide as a general-base catalyst in a similiar manner to what is described in [19].

The results obtained enable us to compare the efficacies of template-directed condensation with the use of the azolides (VII-XI) and the imidazolide (XII) (Table 3). The comparison shows that the least reactive was the pyrazolide (VII). The initial rates of condensation of the azolides (VIII-XI) were equal to the initial rate of phosphorimidazolidate con-

TABLE 3. Formation of a Phosphodiester Bond on Phosphorazolidate Condensation within a DNA Duplex (Scheme 2) at 4°C

Phosphorazolidate	Conditions of condensation		Yield of the heptadecanucleotide (%) after time, h			
	buffer	pH	0,5	3,0	20	160
Imidazolidate (XII)	1-Melm[a]	8,0	14	20	48—50	46—48
Imidazolidate	1-Melm, PEG[b]	8.0	8	13	17—20	15
Imidazolidate	2,6-Lut[c]	7,6	0	Tr.	3—5	10
Imidazolidate	Tetr[d]	7,0	0	0	1	3
2-Methylimidazolidate (XI)	1-Melm	8,0	15	22	50—55	52—53
2-Methylimidazolidate	2,6-Lut	7,6	0	Tr.	2—3	5—8
Benzimidazolidate (VIII)	1-Melm	8,0	13	19	35—37	32
Triazolidate (IX)	1-Melm	8,0	13	17	27—30	24—26
Triazolidate	2,6-Lut	7,6	0	Tr.	2—3	5—8
Benzotriazolidate (X)	1-Melm	8,0	10	15	45—47	42—43
Pyrazolidate (VII)	1-Melm	8,0	7	12	18—20	16—18

[a]0.2 M 1-methylimidazole, 0.2 M NaCl, 0.12 M MgCl.
[b]1-Methylimidazole buffer (see a) containing 5% of PEG-6000.
[c]0.2 M 2,6-lutidene, 0.2 M NaCl, 0.12 M MgCl.
[d]0.2 M tetrazole, 0.2 M NaCl, 0.12 M MgCl.

densation (see Table 3). The highest efficacy of condensation was achieved on the activation of the terminal phosphate with the aid of 2-methylimidazole, imidazole, and benzotriazole. The yield of the desired heptadecanucleotide then amounted to from 45 to 55% after 20 h. Condensation of the benzimidazolide (VIII) took place somewhat less effectively (see Table 3). On the condensation of the triazolide (IX), the yield of product did not exceed 30% because of the high lability of the (IX) under the conditions of condensation. Thus, as reagents for the chemical ligation of fragments of nucleic acids it is possible to recommend, in addition to oligonucleotide imidazolides, the corresponding derivatives of 2-methylimidazole and of benzotriazole.

EXPERIMENTAL

UV spectra were taken on a Specord UV-VIS spectrophotometer, and high-voltage electrophoresis was performed in a Labor instrument on Filtrak No. 1 chromatographic paper. Silufol-254 plates (Chemapol) were used for thin-layer chromatography. The electrophoretograms and chromatograms were scanned on an ultrachemiscope. Microcolumn ion-exchange chromatography was performed on an Ob'-4 chromatograph using columns with dimensions of 2 × 50 mm with the support LiChromosorb NH_2 (Merck) and a linear gradient of Na phosphate buffer of from 0.02 to 0.3 M as eluent. The radioactivities of the samples were measured in a Delta-300 liquid scintillation counter (Tracor).

We used imidazole, 2-imidazole, triazole, tetrazole, and 1-(3'-dimethylaminopropyl)-3-ethylcarbodiimide from Merck; polyethyleneglycol-6000 and ATP from Serva; acylamide, N,N'-methylenebisacrylamide, and Bio-Gel P-2 from Bio-Rad; deoxyadenylic acid, 1-methylimidazole, 2,6-lutidine, and T4 polynucleotide kinase of domestic production; and [γ-^{32}P]ATP from Izotop. The benzimidazole and benzotriazole were kindly supplied by P. O. Purygin (Kubyshev State University). The oligonucleotides were synthesized by the phosphotriester method [12].

Synthesis and Isolation of the Mononucleotide Azolides. A weighed sample of a mononucleotide or a solution of it that has been evaporated to dryness was dissolved in 100 µl of an aqueous solution of an azole which was then titrated to the pH value shown in Table 2, after which CDI was added and incubation was carried out at room temperature. The optimum ratios of the molar concentrations of nucleotide, azole, and CDI are shown in Table 1. For the synthesis of the triazolide, pyrazolide, and 2-methylimidazolide, 100 µl of a 3 M solution of the corresponding azole and 120 mg of CDI were taken per 100 OU_{260} of deoxyadenylic acid. After the end of the reaction, the mixture was deposited on chromatographic paper and was separated by high-voltage electrophoresis in triethylammonium bicarbonate buffer with pH 7.5-8.0 at a voltage of 900 V. The U_{dpA} value of the nucleotide azolides was 0.5. To isolate the nucleotide benzotriazolide we used thin-layer chromatography on Silufol 254 in the chloroform—methanol—1 M ammonium acetate (10:10:3 by volume) system. The nucleotide azolides were eluted from the paper or the plates with a 0.05 M solution of triethylammonium bicarbonate buffer having pH 7.5 (compounds (I), (II), and (V)) or with water (compounds (III) and (IV)).

Synthesis and Isolation of the Oligonucleotide Azolides. A solution of 0.1 OU_{260} of an oligonucleotide in 50 µl of an aqueous solution of an azole (the polarity and pH of the solution are given in Table 1) was treated with 2.5 mg of CDI and, after incubation for 1.5 h, with another 2.5 mg of CDI. After 1 h, the reaction mixture was desalted by gel filtration or by precipitation with ethanol. The resin Bio-Gel P2 (200-400 mesh) was used for gel filtration, with a 0.6 × 15 cm column and elution by means of 0.05 M solution of triethylammonium bicarbonate pH 7.5, in the case of compounds (VII), (VIII), (XI), and (XII) or water in the case of compounds (IX) and (X). When the precipitation method was used, a fivefold excess, by volume, of a 1 M solution of potassium acetate was added to the reaction mixture that had been titrated to pH 7.0, together with a 50-fold excess, by volume, of ethanol. The mixture was cooled to −70°C (20 min) and was then centrifuged (7 min) at 15 thousand rpm, and the supernatant was taken off. The residue was washed twice with 200 µl of ethanol, the time of cooling in this process being reduced to 10 min and that of centrifugation to 5 min. The ethanol residue was eliminated by evaporation in a vacuum desiccator.

Hydrolysis of the Mononucleotide Azolides. A mononucleotide azolide (30 OU_{260} evaporated to dryness) was dissolved in 300 µl of buffer with a given pH (see Table 2) at 20°C. Samples with a volume of 30 µl were taken from the reaction mixture, deposited on chromatographic paper, and analyzed by high-voltage electrophoresis. The zones corresponding to the initial substances (U_{dpA} = 0.5) and the products of their transformation — deoxyadenylic acid (U taken as unity) and the symmetrical 5',5'-pyrophosphate (U_{dpA} = 0.7-0.75) were cut out from the paper and were eluted with water, and the hydrolytic stability of each nucleotide azolide was determined from the ratio of the UV absorption of the initial nucleotide azolide to the total UV absorption.

Interaction of the Nucleotide Azolides with Aliphatic Amines. A mononucleotide azolide (30 OU_{260} evaporated to dryness) was dissolved in 300 µl of a 1 M aqueous solution of ethylenediamine or butylenediamine. To obtain amides of dpA, amine solutions with pH 5.0 were used in the reaction with the pyrazolide (I) and the benzimidazolide (II), with pH 8.0 in the reactions with the triazolides (III) and (IV), and with pH 6.0 in the reactions with the imidazolides (V) and (VI). The mononucleotide phosphoramides obtained were identified from their electrophoretic mobilities (total charge equal to zero, U_{dpA} = 0-0.1) and by their coloration with ninhydrin. The degree of conversion was determined in the same way as in the case of the hydrolysis of the phosphorazolidates.

Condensation of the Oligonucleotide Azolides within a DNA Duplex. A solution of 0.01 OU_{260} of the hexanucleotide and 0.02 OU_{260} of the tetradecanucleotide in 5 µl of buffer containing 0.12 $MgCl_2$ and 0.2 M NaCl was cooled to 0°C and was added to a 5'-^{32}P-labeled undecanucleotide azolide that had been evaporated to dryness (0.01 OU_{260}, 10^5 pulses/min). Then 5 µl of a 0.4 M 1-methylimidazole buffer that had been titrated with 1 N hydrochloric acid to pH 8.0 and contained 0.12 M MgCl, and 0.2 M NaCl was added to the cooled mixture, and it was incubated at 4°C. Samples (2 µl) were taken from the reaction mixture and were desalted by precipitation with ethanol as described above. A sample after evaporation to dryness was dissolved in 80% aqueous formamide containing Xylene Cyanole and Bromophenol Blue in a concentration of 1 mg/ml. The samples were analyzed by electrophoresis in 20% PAAG in 7 M urea in 0.05 M Tris-borate buffer, pH 8.3, the size of the gel plates being 0.03 × 20 × 30 cm. After autoradiography, the zones corresponding to the initial 5'-^{32}P-labeled undecanucleotide and the condensation product — the heptadecanucleotide — were cut out from the gel, placed in bottles containing water, and counted in the liquid scintillation counter. The yields of products were determined as percentages of the total radioactivity.

CONCLUSIONS

1. Procecures have been developed which permit azolides of mono- and oligonucleotides to be obtained in aqueous medium with 80-100% yield. The procedures are suitable for obtaining azolides of radioactively labeled oligonucleotides.

2. The reactivities of the mono- and oligonucleotide azolides have been characterized. It has been shown that all the phosphorazolidates are good phosphorylating agents in an aqueous medium and can be used for obtaining amide derivatives of oligonucleotides at the terminal phosphate group.

3. An investigation of the reactivities of the oligonucleotide azolides within a DNA duplex has shown that they all possess the capacity for taking part in a chemical ligation

reaction. This opens up the possibility of the use in the "active site" of a chemical ligase — the unit for the formation of an internucleotide bond — of functional groups of nonprotein nature.

4. Phosphorazolidate condensation within nucleic acid duplexes enables oligo(poly)nucleotides containing covalently linked ribo and deoxyribo fragments to be obtained and also provides the possibility of the elimination of breaks in the sugar phosphate backbone of an NA, but the field of use of the method is limited because of the formation of condensation of two types of phosphodiester bond (3'-5') and (2'-5'). The greatest efficacy of condensation is achieved with the use of oligonucleotide derivatives of 2-methylimidazole, imidazole, and benzotriazole.

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