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Alternating $d(G-C)_3$ and $d(C-G)_3$ hexanucleotides containing 7-deaza-2'-deoxyguanosine or 8-aza-7-deaza-2'-deoxyguanosine in place of dG

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ABSTRACT

The synthesis of alternating hexamers (8-13) derived from $d(C-G)_3$ or $d(G-C)_3$ but containing $c^7z^8G_d$ (2) or c^7G_d (3) instead of dG is described employing phosphoramidite-chemistry. Apart from the isobutyryl group the dimethylaminomethylene residue was used for the nucleobase-protection of 3. The methyl- and the cyanoethyl-phosphoramidites of 3 (5a-c) were synthesized. They were employed together with those of c^7G or $c^7z^8G_d$ [12] in automated oligonucleotide synthesis. T_m-values as well as thermodynamic data of the oligomers 9, 10, 12, and 13 indicated that duplexes were destabilized if c^7G_d replaced dG, whereas $c^7z^8G_d$ stabilized the duplex structure. In contrast to $d(C-G)_3$ which underwent salt-dependent B-Z transition, CD-spectra of oligomers containing c^7G_d or $c^7z^8G_d$ in place of dG showed retained B-conformation.

INTRODUCTION

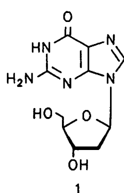
DNA can adopt several conformations depending on the composition, the sequence, or the environmental conditions, e.g. counter ion or humidity [1]. In 1972 spectroscopic studies of Pohl and Jovin suggested a conformational change for poly(dG-dC)·poly(dG-dC) under high salt conditions [2]. The zigzag Z nature, the syn-orientation of the guanine residue, and the left-handed helical sense was definitively established on the basis of single crystal X-ray analysis [3,4]. As hydrogen bonding and stacking interactions are the main forces stabilizing DNA the nucleobase structure plays an important part in the B-Z transition [5,6]. However, structural modification of the phosphodiester moiety can also affect this process [7].

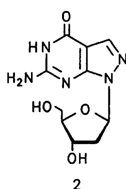
Recently, we have synthesized 7-deaza- 2'-deoxyguanosine (c^7G_d, 3) and 8-aza-7-deaza-2'-deoxyguanosine ($c^7z^8G_d$, 2) two nucleosides being isosteric to dG (1) [8,9]. As incorporation of those nucleosides into oligonucleotides led to unexpected properties [10-12] we considered the synthesis of hexamers derived from $d(C-G)_3$ or $d(G-C)_3$. In the following we report on the synthesis of $d(C-c^7G)_3$ and $d(c^7G-C)_3$ as well as of $d(C-c^7z^8G)_3$ and $d(c^7z^8G-C)_3$. The

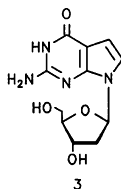
stability of those duplexes and their ability to undergo B-Z transition will be compared with the parent molecules containing dG.

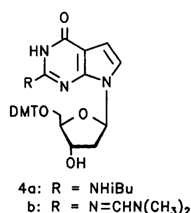
RESULTS AND DISCUSSION

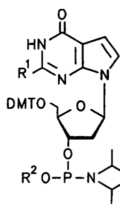
Oligonucleotides with $c^7z^8G_d$ (2) have been prepared by automated synthesis employing regular phosphoramidites together with 6a or 6b [11,13] which have been already described. In case of the oligomers containing c^7G_d (3) the phosphoramidites 5a-c were used. As the protocol of transient protection worked in case of 2 but failed during isobutyrylation of 3 triisobutyrylation followed by selective sugar-deprotection was necessary [14]; later the dimethylaminomethylene residue was selectively introduced as amino protecting group [16]. After dimethoxytritylation (4b) [15] the phosphoramidites 5b,c have been obtained by reaction with methoxy- or cyanoethoxy chlorodiisopropylaminophosphane. Analogously the cyanoethyl phosphoramidite 5a has been obtained from 4a [11, 16].



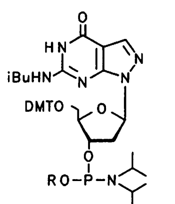




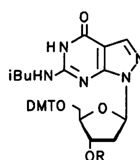


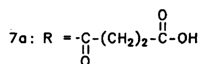


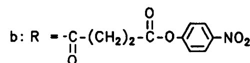
	R^1	R^2
5a	NHiBu	$(CH_2)_2CN$
b	$N{=}CHN(CH_3)_2$	$(CH_2)_2CN$
c	$N{=}CHN(CH_3)_2$	CH_3

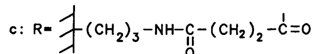


6a: R = $(CH_2)_2CN$
b: R = CH3



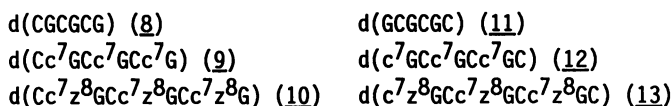






For the synthesis of the oligomers 9 and 10 it was necessary to prepare silica-linked c^7G_d [11] and $c^7z^8G_d$ (7c). The synthesis of the latter was accomplished by succinylation (7a) followed by activation to the p-nitrophenyl ester 7b. Condensation with aminopropyl-functionalized Fractosil

gave 7c. The phosphoramidites 5a-c as well as 6a,b together with those of dC and dG were successfully used in the synthesis of the hexamers 8-13.


d(CGCGCG) (8) d(GCGCGC) (11)
$d(Cc^7GCc^7GCc^7G)$ (9) $d(c^7GCc^7GCc^7GC)$ (12)
$d(Cc^7z^8GCc^7z^8GCc^7z^8G)$ (10) $d(c^7z^8GCc^7z^8GCc^7z^8GC)$ (13)

The synthesis followed a protocol of detritylation, coupling and oxidation according to Caruthers [13]. Purification of the DMT-protected as well as the fully deprotected oligomers was performed by HPLC on RP-18 silica gel. The oligonucleotides were recovered as triethylammonium salts as colorless solids. The nucleoside content was determined by phosphodiester hydrolysis with snake venom phosphodiesterase followed by alkaline phosphatase. Expected amounts of c^7G_d:$c^7z^8G_d$:dG and dC were found from UV-absorbance of HPLC separation. The HPLC pattern also showed that no irreversible side reactions at the lactam moiety of 2 or 3 has taken place during oligonucleotide synthesis.

Next, the T_m-values of the oligomers 8-13 were determined UV-spectrophotometrically at 280 nm in aqueous 1 M NaCl in the presence of 45 mM sodium cacodylate. In all cases cooperative transitions were observed from which T_m values were obtained either from the curve-inflection points or by derivative melting curves. As the table shows oligonucleotides containing c^7G_d exhibited lower T_m values than those of the parent oligomers 8 or 11. In contrast, increased T_m values were observed in case of the oligonucleotides 10 and 13 containing $c^7z^8G_d$ in place of dG. In order to get more information about duplex stability concentration-dependent melting experiments were carried out. The T_m values of 8-13 have been measured at 6 different oligomer concentrations within a range of 1-10 µM (Fig. 1d). This allowed the determination of thermodynamic parameters of helix-coil transition. The ΔG, ΔH and ΔS values were calculated according to [17-20] assuming a two state transition for helix formation (Table). Good agreement was found for the thermodynamic values of the oligomer 8 with data earlier published by Turner and co-workers [21].

From these findings it can be concluded that the ΔH of a dG-dC base pair corresponds to -50 kJ/mol of stack. The value of a dc^7G-dC base pair is definitely lower (-36 kJ/mol of stack). This value is within the range of a dA-dT base pair. On the contrary, the base pair of dc^7z^8G-dC which stabilizes

Table Melting Temperatures (T_m values) and Thermodynamic Parameters for Helix Formation of the Hexamers 8-13.

Compnd.	T_m [°C][a]	ΔH[kJ/mol]	ΔS[J/mol·K]	ΔΔG[b] [kJ/mol]
8	46	-250	-695	-
9	37	-180	-480	-3
10	59	-350	-940	10
11	46	-250	-695	-
12	37	-180	-480	-3
13	62	-345	-940	13

[a] determined at 10 μM oligomer concentration; [b] at 58°C.

the duplex structure exhibits a ΔH = -70 kJ/mol of stack. We assume that the increased stability is due to a better p-electron overlap of the pyrazolo[3,4-d]pyrimidine base probably accompanied by a altered helix geometry.

As the left-handed double helical form of the oligomer 8 (Z-DNA) has been determined by single crystal X-ray analysis [3,4] and a salt-dependent B-Z transition was detected by the CD-spectrum [22], we have measured the CD-spectra of the oligomers 8-10 in 1 M and 4 M NaCl (Figure 1a-c). At 1 M NaCl each oligomer showed the CD-spectrum of a B-DNA. As expected, at 4 M NaCl the CD-spectrum of the guanine-containing 8 (Figure 1a) is different due to Z-DNA formation [21, 22]. Only small changes are observed in case of $d(C\text{-}c^7G)_3$ (Figure 1b) between 1 M and 4 M NaCl concentration. The observed decrease of the CD-magnitude at high salt may be due to base-destacking, as it is usually observed in single-stranded oligomers. From this finding it is concluded that the oligomer 9 is not able to perform a B-Z transition. A similar situation is found in case of $d(C\text{-}c^7z^8G)_3$ (figure 1c); here the CD-spectra at low and high salt concentration are almost identical.

There are several explanations for the behaviour of the oligomers 9 and 10 compared to 8. From Z-DNA structure it can be seen that the d(G-C) base pair occupies a position at the periphery of the molecule instead of the center like in right-handed B-DNA. The N-7 and C-8 atoms of guanine are near the outer wall of the molecule, an arrangement in wich N-7 can interact with counter ions. As c^7G_d lacks this particular nitrogen and its nucleobase is more hydrophobic it shows only a small tendency to leave the hydrophobic environment of the stacked bases in the center of the B-duplex. Moreover, cations such as Mg^{2+} or Na^+ cannot interact with the five-membered ring thus

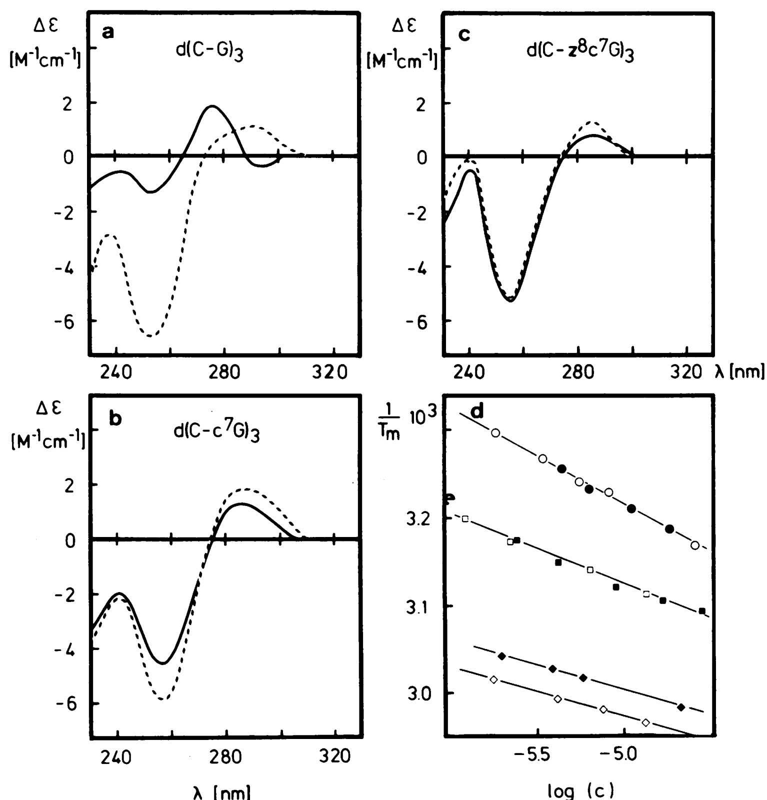


Figure 1a-d. CD-Spectra of the hexamers 8 (a), 9 (b) and 10 (c) in 1 M NaCl (--) and 4 M NaCl (—) containing 45 mM Na-cacodylate, pH 7.0. The oligomer concentration was 10 mM; Plots of $1/T_m$ vs. log(c) of the oligomers 8 (□), 9 (●), 10 (◆), 11 (■), 12 (○) and 13 (◇) (d). All solutions contained 1 M NaCl and 45 mM Na-cacodylate, pH 7.0.

not inducing a positive charge at the 7-position. That the generation of a positive charge is important for the B-Z transition was already supported by methylation studies. Oligonucleotides with m^7G_d markedly accelerate the ease of the conversion from B- to Z-DNA. At 100% N-7 methylation of the guanine residues the DNA is fully converted to Z-DNA in a physiolocical salt solution [5]. As the oligomer 9 lacks the N-7 acceptor site and N-8 within the oligomer 10 may not have an acceptor ability the unwillingness of the oligomers 9 and 10 to undergo B-Z transition is in aggreement with this interpretation. The increased stability of the oligomer 10 (Table) compared to the parent $d(C\text{-}G)_3$ may additionally hinder the rotation of the nucleobase

from the anti- into the syn-orientation. Further experiments which verify these findings are in progress.

EXPERIMENTAL SECTION

NMR spectra were recorded on a Bruker AC 250 spectrometer; δ values are in ppm relative to external 85% H_3PO_4 (^{31}P) or tetramethylsilane (^{1}H,^{13}C). Chemical shifts are downfield from the appropriate standard. CD-spectra were measured with a Jasco J-600 spectropolarimeter. Thin-layer chromatography (TLC) was performed on silica gel SIL G-25 UV_{254} plates (Macherey-Nagel, West Germany), and flash chromatography on silica gel 60H (Merck, West Germany) at 0.5 bar N_2. Snake venom phosphodiesterase (EC 3.1.16.1, Crotallus durissus) and alkaline phosphatase (EC 3.1.3.1, Escherichia coli) are products of Boehringer Mannheim (West Germany).

Melting Curves.

The melting curves were measured in Teflon-stoppered cuvettes with a 1 cm light path length in a thermostatically controlled cell holder with a Shimadzu 210A recording spectrophotometer connected with a Kipp & Zonen BD-90 recorder. The increase of absorbance (Lauda PM-351 programmer and a Lauda RCS 6 bath equipped with an R 22 unit; MWG Lauda, West Germany) at a certain wavelengths was recorded while the temperature of the solution was increased linearly (20°C/h). The actual temperature was measured in the probe cell with a Pt-100 resistor.

HPLC-Separation.

HPLC (Merck-Hitachi, model 655-12 with proportioning valve, model 655A variable-wavelength monitor, model L-5000 controller, and D-2000 integrator) was carried out on a 250 x 4 mm (7 µm) RP-18 LiChrosorb column with a RP-18 25 x 4 mm precolumn (Merck, West Germany). Solvents: (A) 0.1 M aq. $(C_2H_5)_3N^+$ OAc^- -MeCN (95:5), pH 7.0; (B) MeCN; (C) H_2O and (D) $MeOH/H_2O$ (3:2). System I, 100 % A; system II, 15 min (0 - 20% B) in (A); system III, 15 min (15 - 60 % B) in (A); system IV, 10 min C followed by 15 min D.

Enzymatic Analysis of the Hexanucleotides 8-13.

The oligomers (0.3 A_{260} units) were dissolved in 0.1 M TRIS-HCl buffer, pH 8.5 (100 µl), and incubated with snake venom phosphodiesterase (2 µg) for 10 min at 37°C followed by alkaline phosphatase (2 µg) at 25°C for 15 min. The reaction mixture was analyzed by HPLC with system I as eluent. Quantification was made on the bases of the peak areas and the extinction coefficients of the nucleosides (ϵ_{260}: dC = 7300; dG = 11400; c^7G_d = 13400; $c^7z^8G_d$ = 11400).

<u>7-[2'-Deoxy-5'-O-(4,4'-dimethoxytrityl)-β-D-erythro-pentofuranosyl]-2-[(2-methylpropionyl)amino]-7H-pyrrolo[2,3-d]pyrimidin-4(3H)-one 3'-[(2-Cyanoethyl) N,N-Diisopropylphosphoramidite] (5a)</u>.

To a solution of 4a (500 mg, 0.78 mmol) [14] and N-ethyldiisopropyl amine (280 µl, 1.6 mmol) in dry THF chloro-β-cyanoethoxy-(N,N-diisopropyl-amino)phosphane (190 mg, 0.8 mmol) [16] was added within 2 min at room temperature under argon. After 1 h insoluble salt was filtered off and the filtrate was evaporated to dryness. The resulting oil was dissolved in EtOAc (saturated with argon) and chromatographed on silica gel 60 H (column: 2.5 x 20 cm, EtOAc). Isolation of the main zone furnished colorless amorphous 5a (573 mg, 88%) upon evaporation. TLC (silica gel, CH_2Cl_2-acetone 9:1): R_f 0.6. - ^{31}P-NMR ($CDCl_3$): 148.42, 148.82.

<u>7-[2'-Deoxy-5'-O-(4,4'-dimethoxytrityl)-β-D-erythro-pentofuranosyl]-2-[(dimethylamino)methylidenamino]-7H-pyrrolo[2,3-d]pyrimidin-4-(3H)-one 3'-[Methyl N,N-Diisopropylphosphoramidite] (5c)</u>.

To a solution of 4b (500 mg, 0.8 mmol) [15] in CH_2Cl_2 (2.5 ml), bis-(diisopropylamino)methoxyphosphane (262 mg, 1.0 mmol) [23] and diisopropyl-ammonium tetrazolide (70 mg, 0.4 mmol) were added (argon, r.t.). The solution was stirred for 1 h, diluted with CH_2Cl_2 (50 ml) and washed with 5% aq. $NaHCO_3$. The organic layer was dried over Na_2SO_4, filtered and the volume was reduced to 2 ml. The resultant was chromatographed on silica gel (column: 2.5 x 20 cm, solvent CH_2Cl_2-EtOAc-$(Et)_3N$, 45:45:10). Isolation of the main zone furnished a colorless amorphous foam (604 mg, 91%). - TLC (silica gel, CH_2Cl_2-EtOAc-$(Et)_3N$, 45:45:10): R_f 0.4. - ^{31}P NMR ($CDCl_3$): δ = 147.04.

<u>7-[2'-Deoxy-5'-O-(4,4'-dimethoxytrityl)-β-D-erythro-pentofuranosyl]-2-[(dimethylamino)methylidenamino]-7H-pyrrolo[2,3-d]pyrimidine-4-(3H)-one 3'-[(2-Cyanoethyl) N,N-Diisopropylphosphoramidite] (5b)</u>.

Compound 5b was synthesized as described for 5a except that compound 4b (500 mg, 0.8 mmol) [15] was used. After work-up 5b was obtained as a colorless amorphous foam (499 mg, 76%). TLC (silica gel, CH_2Cl_2-EtOAc-$(Et)_3N$, 45:45:10): R_f 0.8. - ^{31}P-NMR ($CDCl_3$): 147.9, 147.7.

<u>1-[2'-Deoxy-5'-O-(4,4'-dimethoxytrityl)-3'-O-succinyl-β-D-erythro-pentofuranosyl]-6-[(2-methylpropionyl)amino]-1H-pyrazolo[3,4-d]pyrimidin-4(5H)one(7b).</u>

To a solution of 1-[2'-deoxy-5'-O-(4,4'-dimethoxytrityl)-β-D-erythro-pentofuranosyl]-6-[(2-methylpropionyl)amino]-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-one [11] (250 mg, 0.4 mmol) in pyridine (10 ml), 4-dimethylamino-pyridine (60 mg, 0.47 mmol) and succinic anhydride (100 mg, 1.0 mmol) were

added. The mixture was stirred for 48 h at room temperature. Water (3 ml) was added and the resultant was evaporated to dryness. Coevaporation with toluene removed pyridine. The resulting oil was dissolved in CH_2Cl_2 and washed with 10% aq. citric acid and water. The organic layer was dried over Na_2SO_4 and evaporated. The residue was dissolved in CH_2Cl_2-pyridine (95:5, 2 ml) and added slowly to n-pentane-ether (1:1, 50 ml). The precipitate was filtered off yielding colorless powder (258 mg, 94%). - TLC (silica gel, MeCN-H_2O, 9:1): R_f 0.7. - ^{1}H-NMR ($CDCl_3$): 1.23 (d, 6H, 2 CH_3, J 5.4 Hz), 2.43, 3.03 (2m, 2H, 2'-$H_{a,b}$), 2.69 (m, 5H, CH_2 und CH), 3.77 (m, 8H, 2 OCH_3 und 5'-H), 4.20 (m, 1H, 4'-H), 5.49 (m, 1H, 3'-H), 6.43 (m, 1H, 1'-H), 6.80, 7.15, 7.25 (m, 13 aromat. H), 8.02 (s, 1H, 3-H), 10.03 (s, 1H, NH), 12.09 (s, 1H, NH); ^{13}C-NMR ($CDCl_3$): 18.84, 18.90 (CH_3), 28.81, 28.91 (CH_2), 34.99 (CH), 55.05 (OCH_3), 64.06 (C-5'), 74.86 (C-3'), 83.24 (C-1'), 84.03 (C-4'), 103.15 (C-3a), 135.70 (C-3), 150.27 (C-6), 153.12 (C-7a), 155.85 (C-4), 180.67 (C=O).

Fractosil-linked 8-aza-7-deaza-2'-deoxyguanosine (7c).

To a solution of 7a (100 mg, 0.07 mmol) in p-dioxane-5% pyridine (1 ml), p-nitrophenol (19 mg, 0.14 mmol), and N,N-dicyclohexylcarbodiimide (29 mg, 0.14 mmol) were added under stirring at room temperature. The reaction was monitored by TLC (silica gel, benzene-p-dioxane, 3:1). After 2 h dicyclohexyl urea was removed by centrifugation. The supernatant containing the compound 7b was added to a suspension of Fractosil 200 (200 mg, 450 µmol NH_2/g; Merck) in DMF (1 ml). Triethylamine (200 µl) was added and the mixture was shaken for 4 h at room temperature. After addition of acetic anhydride (60 µl) shaking was continued for another 30 min. The silica was filtered off, washed with DMF, EtOH, and ether and dried in vacuo. The amount of silica gel bound nucleoside was determined by treatment of 7c with 0.1 M p-toluene sulfonic acid (5 ml) in MeCN. From the absorbance at 498 nm of the supernatant obtained after centrifugation 94 µmol linked 2/g Fractosil were calculated (ϵ_{DMT} = 70000).

Synthesis of the Oligomers 8-13.

The oligomers 8-13 were synthesized with an automated DNA synthesizer (Applied Biosystems, model 680 B) employing the phosphoramidites 5a-c or 6a,b [11] together with phosphoramidites of dG and dC [13, 16]. The synthesis was carried out on solid support in 1 µmol scale. It followed a reaction cycle of detritylation, coupling, oxidation, and capping according to [13]. Thiophenol reaction was carried out on solid support. The cleavage of the oligomer from the solid support was performed with conc. aq. ammonia at room temperature on the stage of the 5'-protected oligomers which also removed the cyanoethyl

group. Nucleobase deprotection occurred in conc. aq. ammonia at 60°C within 24 h. The 5'-protected oligomers were purified by HPLC (system III; see following table), and detritylated with 80% acetic acid within 5 min. After evaporation of the acid the oligomers were dissolved in water (5 ml), triphenylcarbinol was extracted with ether and the aqueous solution was evaporated. The oligomers 8-13 were purified by HPLC (system II; see following table. The contents of the main zone were isolated and lyophilized. Desalting was accomplished on a 25 x 4 mm HPLC column (silica gel RP-18) by elution of inorganic material with water. The oligomers were recovered from the MeOH-eluant as colorless solid after lyophilization. Yield: 0.20 - 0.35 µmol; 15 - 25%. The oligomer 8-13 were characterized by HPLC-retention times; data of the 5' DMT-protected oligonucleotides are given in parenthesis.

Oligomer	Retention Time	Solvent System
d(CGCGCG) (8)	11.0 (13.0)	II (III)
d(Cc^{7}GCc^{7}GCc^{7}G) (9)	11.7 (11.0)	II (III)
d(Cc^{7}z^{8}GCc^{7}z^{8}GCc^{7}z^{8}G) (10)	12.9 (12.0)	II (III)
d(GCGCGC) (11)	11.1 (13.1)	II (III)
d(c^{7}GCc^{7}GCc^{7}GC) [14] (12)	11.7 (12.4)	II (III)
d(c^{7}z^{8}GCc^{7}z^{8}GCc^{7}z^{8}GC) (13)	13.0 (12.1)	II (III)

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