$[Rh_2(O_2CCF_3)_4]$ was obtained using a literature procedure.[14] Sulfur powder, 99.98%, was purchased from Aldrich. The IR spectra were recorded on a Perkin-Elmer 16PC FT-IR spectrophotometer using KBr pellets. Elemental analysis was done by Canadian Microanalytical Services, Ltd.

1: $[Rh_2(O_2CCF_3)_4]$ (0.066 g, 0.10 mmol) was mixed with sulfur powder (0.026 g, 0.10 mmol based on the S_8). The mixture was sealed in an evacuated Pyrex tube and the tube was placed in a furnace at 140 °C. The tube was kept at that temperature for about a week, and then slowly (over 24 h) cooled to room temperature. Several dozen dark violet crystals of **1** deposited in the "cold" zone of the tube, where the temperature was about 5 °C lower. Elemental analysis (%) calcd for $S_8C_8F_{12}O_8Rh_2$: C 10.51, S 28.00; found: C 10.35, S 28.35; IR (KBr): $\tilde{\nu}$ = 1650.0 (s), 1244.6 (m), 1192.5 (s), 1169.5 (s), 861.7 (m), 804.1 (w), 784.4 (w) 739.8 (m) cm^{-1}.

2: $[Rh_2(O_2CCF_3)_4]$ (0.066 g, 0.10 mmol) was mixed with elemental sulfur (0.016 g, 0.063 mmol based on the S_8). The mixture was sealed in an evacuated Pyrex tube and the tube was placed in a furnace at 130 °C. The tube was kept at that temperature for about a week, and then slowly (over 24 h) cooled to room temperature. Several dark violet crystals of **2** deposited not far from the "hot" zone of the tube along with some dark brown polycrystalline solid. Red crystals of free sulfur accompanied the powdered product in the hot zone; these were mechanically separated under the microscope. Elemental analysis (%) calcd for $S_{16}C_{24}F_{36}O_{24}Rh_6$: C 11.59, S 20.63; found: C 11.36, S 20.80; IR (KBr): $\tilde{\nu}$ = 1653.0 (s), 1258.7 (m), 1234.4 (m), 1190.2 (s), 1142.8 (w), 1130.6 (w), 1094.2 (w), 1075.4 (w), 1050.1 (m), 1018.1 (w), 861.3 (m), 823.8 (w), 785.2 (w), 737.7 (m), 688.0 (w) cm^{-1}.

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[8] Crystallographic data for **1** ($S_8C_8F_{12}O_8Rh_2$): M_r = 914.38, dark violet block, 0.25 × 0.08 × 0.05 mm, monoclinic, space group $P2_1/c$, a = 9.1826(5), b = 16.605(1), c = 16.5885(4) Å, β = 92.267(4)°, V = 2527.4(2) Å^3, Z = 4, ρ_{calcd} = 2.403 g cm^{-3}, μ = 2.090 mm^{-1}, $2\theta_{max}$ = 50.18°. Nonius FAST area detector system, $Mo_{K\alpha}$ radiation (λ = 0.71073 Å), T = −60 °C, 14915 reflections measured, data corrected for Lorentz and polarization effects, solution by direct methods (SHELXTL V.5), full-matrix refinement of F^2 (SHELXL-93), R_1 (on F_o) = 0.0585, $wR2$ (on F_o^2) = 0.1125 for 355 parameters and 48 restraints, 4439 independent reflections, R_1 (on F_o) = 0.0496, $wR2$ (on F_o^2) = 0.1009 for 3868 reflections with $I \geq 2\sigma(I)$, highest residual peak 0.96 e Å^{-3}.

[9] Crystallographic data for **2** ($S_{16}C_{24}F_{36}O_{24}Rh_6$): M_r = 2486.66, dark violet plate, 0.25 × 0.08 × 0.03 mm, monoclinic, space group $C2/c$, a = 30.93(2), b = 15.911(4), c = 16.762(8) Å, β = 121.83(4)°, V = 7009(6) Å^3, Z = 4, ρ_{calcd} = 2.357 g cm^{-3}, μ = 2.018 mm^{-1}, $2\theta_{max}$ = 45.02°. Nonius FAST area detector system, $Mo_{K\alpha}$ radiation (λ = 0.71073 Å), T = −60 °C, 15755 reflections measured, data corrected for Lorentz and polarization effects, solution by direct methods (SHELXTL V.5), full-matrix refinement of F^2 (SHELXL-93), R_1 (on F_o) = 0.1430, $wR2$ (on F_o^2) = 0.2462 for 436 parameters and 60 restraints, 4484 independent reflections, R_1 (on F_o) = 0.1047, $wR2$ (on F_o^2) = 0.2165 for 3314 reflections with $I \geq 2\sigma(I)$, highest residual peak 1.36 e Å^{-3}. Crystallographic data (excluding structure factors) for the structures reported in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication nos. CCDC-154796 (**1**) and CCDC-154797 (**2**). Copies of the data can be obtained free of charge on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: (+44) 1223-336-033; e-mail: deposit@ccdc.cam.ac.uk).

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A Ligand-Modulated Padlock Oligonucleotide for Supercoiled Plasmids**

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Supramolecular structures involving a circular oligonucleotide catenated to a double-stranded nucleic acid have been recently described.[1–3] The formation of these structures involves the recognition of a specific region of the DNA by the oligonucleotide through either triple-helix formation[1, 2] or strand displacement.[3] The oligonucleotide can be circularized first and then loaded onto a linear DNA fragment[1] or circularized around the DNA target after triplex formation.[2] In the latter case, the catenated structure can be established on a circular supercoiled DNA. In both cases, the circular oligonucleotide may be able to move along the DNA, whereas in the method developed by Kuhn et al.,[3] sliding does not occur, as the oligonucleotide is threaded between complementary strands of DNA before circularization around the displaced DNA single strand takes place. In the method that we previously described,[2] the linear oligonucleotide is wound around the double-stranded DNA sequence through sequence-specific triple-helix formation, and then circularized by using a template oligonucleotide and T4 DNA ligase. When the target sequence was located on a plasmid, the circular oligonucleotide became catenated to the plasmid. However, it was shown to slide more than 600 bp away from

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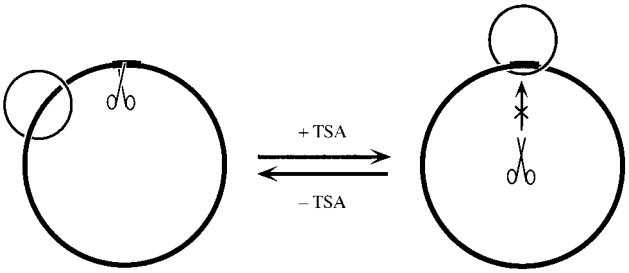
the site of triple-helix formation when the plasmid was linearized under conditions where the triple helix is dissociated.

We have previously shown that some triple helices with a third strand that contains G and T can only be formed in the presence of triplex-stabilizing agents.[4] Herein we investigate if this type of ligand-dependent triple helix could be used for the efficient assembly of padlock oligonucleotides around double-stranded DNA, and if the stability of the triple helix formed by the two catenated molecules could be modulated upon addition or removal of the triplex-stabilizing agent (Scheme 1). We designed a linear oligonucleotide that contains a central triplex-forming sequence linked to two terminal sequences that can hybridize to a 17-mer template oligonucleotide (Figure 1 a). The 5′-phosphorylated linear oligonucleotide was converted into a circular molecule by the addition of the 17-mer template and T4 DNA ligase (not shown). The number of base triplets (12) was chosen so that the oligonucleotide forms one turn around the target double-helical sequence, which is located on the pGA2 plasmid.



Scheme 1. The interactions between the circular oligonucleotide and the plasmid are modulated by the triplex-stabilizing agent (TSA). When the TSA has been removed, the complex can be cleaved by a restriction enzyme (left). Addition of the TSA results in the formation of a structure that is resistant to cleavage (right).

We previously reported the design and synthesis of a very efficient triplex-stabilizing agent, 6-[3-(dimethylamino)propyl]amino-11-methoxybenzo[*f*]quino-[3,4-*b*]quinoxaline (BQQ) (Figure 1 b).[5] Preliminary circularization experiments were carried out with a radiolabeled oligonucleotide, and the results were analyzed by using agarose gel electrophoresis (Figure 2). Under the conditions used for electrophoresis, the triple helix formed by the linear oligonucleotide and pGA2 in the presence of BQQ was dissociated (Figure 2, lane 2). In contrast, the radiolabeled oligonucleotide was shown to comigrate with the plasmid when it had been circularized in the presence of pGA2 and BQQ (Figure 2, lane 3). This comigration was not observed when the oligonucleotide was circularized before the addition of the plasmid (Figure 2, lane 5), when the triplex-stabilizing agent was omitted (Figure 2, lane 4), or in the presence of the pBl plasmid, which does not contain the target sequence (not shown). Finally, comigration was excluded upon digestion of the plasmid by *Hin*d III, which linearizes the plasmid at a site located outside the oligonucleotide-binding region (Figure 2, lane 6). The circularization reaction did not alter the topology of the plasmid, which remains mostly in the supercoiled form, as observed in the case of the untreated plasmid (Figure 2,

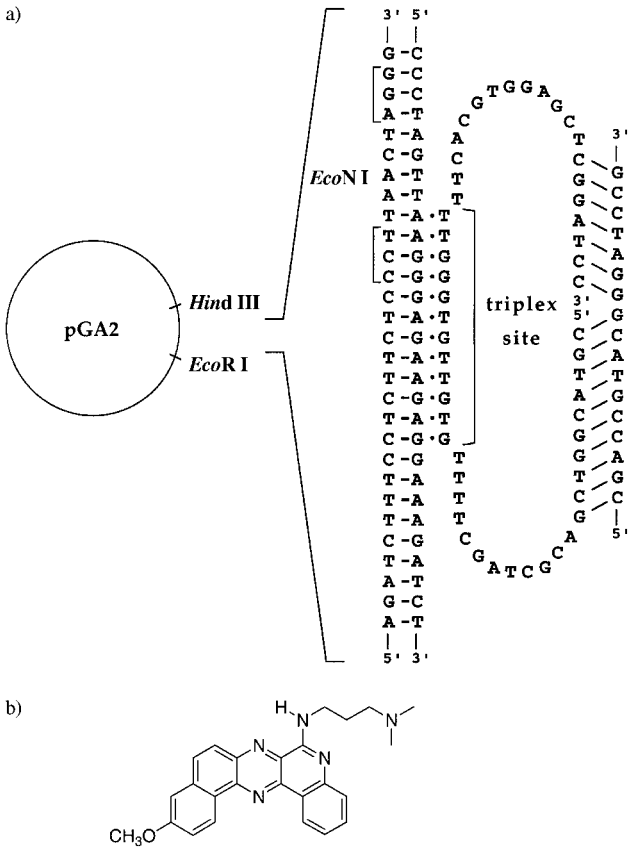


Figure 1. a) Description of the plasmid and oligonucleotides used in this study. Sequences of the 54-mer TFO and the 17-mer template are shown. The 54-mer can form a triple helix by binding to a 12 bp sequence target in the pGA2 plasmid, and its extremities can hybridize to the 17-mer template. The position of the restriction site for *Eco*N I is also indicated. b) Structure of BQQ.

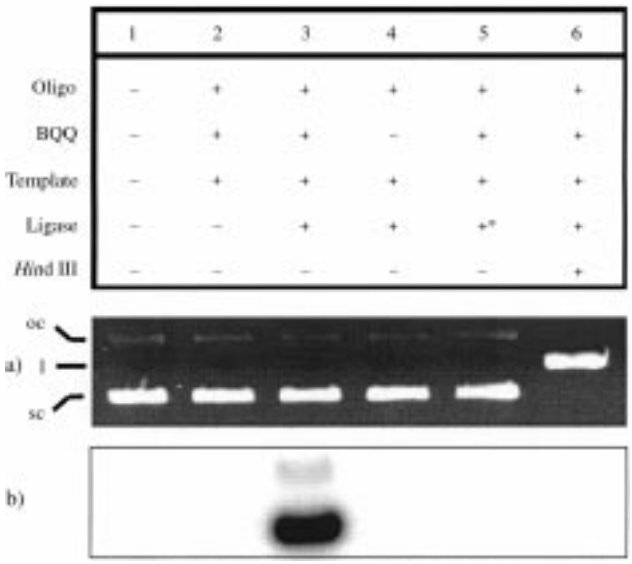


Figure 2. Ligand-induced formation of a triple-helix to give a catenated complex, as demonstrated by using agarose gel electrophoresis. The 54-mer oligonucleotide is radiolabeled and treated as indicated, before analysis on agarose gel (1 %). The pGA2 plasmid was present during the ligation reaction except in lane 5 (* pGA2 was added after the circularization reaction, followed by heat denaturation of DNA ligase). In lane 6, the complex of lane 3 was digested with *Hin*d III. a) Ethidium bromide staining of the gel. b) Autoradiogram of the gel. sc = supercoiled plasmid, oc = open circular form, l = linearized plasmid.

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lane 1). All these data are in agreement with the proposed formation of a catenated structure that yields a sequence-specific oligonucleotide label linked to a plasmid in an irreversible (but not covalent) way owing to ligand-induced triplex formation.

We undertook further experiments to probe the efficiency of the labeling reaction and to determine whether the circular oligonucleotide could be displaced from the sequence to which it was bound during the circularization reaction. Triple-helix formation can inhibit DNA cleavage by a restriction enzyme.[6] On the pGA2 plasmid, the *Eco*NI unique site partially overlaps the oligonucleotide-binding region (Figure 1a), and triple-helix formation inhibits the cleavage by the restriction enzyme (not shown). The circularization reaction was carried out in the presence of excess 5′-phosphorylated triplex-forming oligonucleotide (TFO). We devised a purification protocol based on the specific precipitation of plasmids in the presence of spermidine (see *Experimental Section*). This procedure allowed us to recover more than 95% of the plasmids and to get rid of all the unbound oligonucleotides. When submitted to this treatment, the pGA2 plasmid was only partially linearized by *Eco*NI, thus indicating that the triple-helical complex was not fully dissociated. Therefore, we tried to remove BQQ by heating in the presence of excess so-called "trap" oligonucleotide, which is folded into an intramolecular triple-helical structure. After this treatment, the plasmid was totally digested by *Eco*NI, as shown by agarose gel electrophoresis (Figure 3, lane 2). When BQQ (2 μM) was

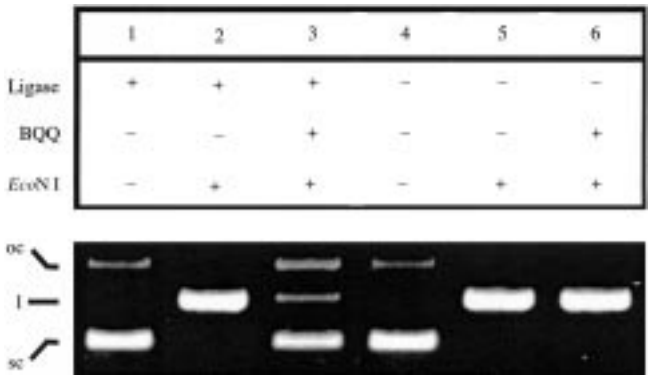

Figure 3. Restriction endonuclease probing of the padlock-modified plasmid, as revealed by agarose gel electrophoresis. Samples were prepared as described in the Experimental Section. The plasmid preparation contains mostly the supercoiled form (sc) of the plasmid and some open circular DNA (oc). Plasmid cleavage by *Eco*NI leads to the formation of linear plasmid (l).

again added to this plasmid, inhibition of *Eco*NI was restored (Figure 3, lane 3). When the plasmid was treated in the same way, but the ligation reaction was omitted, complete cleavage by *Eco*NI occurred (Figure 3, lane 6). Also, no inhibition could be observed when the oligonucleotide was circularized before the addition of the plasmid, or when the ligase was added, but the template was omitted (not shown). These experiments demonstrate that the oligonucleotide, which has been circularized around the target, inhibits the cleavage of the restriction enzyme in the presence of the triplex-stabilizing agent BQQ, and that removal of BQQ by the trap oligonucleotide is sufficient to disrupt triple-helix formation, therefore allowing digestion of the restriction enzyme.

We have shown herein that the triplex-mediated assembly of a padlock oligonucleotide around plasmid DNA can be carried out by using a triple helix that is made up of C–G·G and T–A·T base triplets (see Figure 1a), whose formation is induced in the presence of the triplex-specific ligand BQQ. The low fraction of guanines in the oligonucleotide reduces the possibility of it forming self-associated structures that may compete with triple-helix formation.[7] The plasmid was very efficiently modified, as shown by an inhibition of more than 90% in the cleavage by the restriction enzyme. Furthermore, the structural integrity of the plasmid was not affected, as no covalent bond was formed between the oligonucleotide and the supercoiled plasmid. In addition, the noncovalent interaction that involves the formation of hydrogen bonds between the bases of the circular oligonucleotide and the plasmid can be disrupted by removing the BQQ ligand. Finally, further addition of BQQ locks the circular oligonucleotide around its target sequence on the plasmid.

Padlock oligonucleotides for single-stranded DNA have been used as tools for the specific detection of DNA sequences, using fluorescent oligonucleotides or rolling circle amplification.[8] By combining such approaches with the method described herein, the detection of double-stranded DNA sequences should be possible without the need for a DNA denaturation step. This method also opens the route to a new type of noninvading DNA vector chemistry, as the attached circular oligonucleotide may be used as a substrate for linking any chemical moiety to a supercoiled plasmid in an irreversible, but noncovalent way. Such a strategy has potential applications in the development of nonviral vectors for gene therapy.[9] The biological properties of the plasmid, such as its ability to be transcribed, should be intact. In its unbound state, the circular oligonucleotide may be displaced by proteins that translocate on double-stranded DNA[10] and therefore could be used as a marker for observing their one-dimensional motion. On the other hand, locking the circular oligonucleotide around its target by the addition of a triplex-stabilizing agent may interfere with DNA–protein interactions. Furthermore, the complex described herein represents a new type of supramolecular structure in which interactions between two catenated circular molecules can be modulated by the use of a low molecular weight compound.

Experimental Section

Plasmid pGA2 was constructed by cloning the appropriate oligonucleotide pair between the *Eco*RI and *Hin*dIII sites of pBluescript (Stratagene). The synthesis of the triplex-stabilizing agent BQQ has been previously described.[5] A 5′-phosphorylated 54-mer TFO and a 17-mer template were used in the circularization experiments (see Figure 1 for sequences). A "trap" oligonucleotide that can form an intramolecular triplex (5′CTTTCCTTCTCTCCTTTTTGGAGAGAAGGAAAGTTTTTGTTT-GGTTGTGTGG3′) was used to remove BQQ from the samples. All these oligonucleotides, obtained from Eurogentec (Seraing, Belgium), were precipitated with ethanol, and their concentration was calculated by using a nearest neighbor model for the absorption coefficients.

For radioactive phosphorylation, TFO (10 pmol) was incubated in T4 Polynucleotide Kinase (PNK) buffer (20 μL; New England Biolabs, NEB) with [γ-^{32}P]ATP (10 μCi, >5000 Ci mmol^{-1}, Amersham) and T4 PNK

(5 units, NEB) for 1 h at 37 °C. Unincorporated ATP was removed by using Micro Bio-Spin columns (Bio-Rad). For nonradioactive phosphorylation, TFO (300 pmol) was incubated in T4 DNA ligase buffer (50 μL—50 mM Tris–HCl, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, and 25 μg mL^{-1} BSA, pH 7.8 at 25 °C) with T4 PNK (10 units), for 1.5 h at 37 °C. The oligonucleotide was used without any further purification.

To assemble the radiolabeled oligonucleotide and the plasmid, the 5′-labeled TFO (20 nM) was incubated with plasmid (1 μg, 50 nM final) and BQQ (20 μM) in T4 DNA ligase buffer. The sample was heated to 75 °C and slowly cooled down to 37 °C. The 17-mer oligonucleotide template (100 nM) and T4 DNA ligase (40 units) were added to achieve circularization of the oligonucleotide. The reaction products were analyzed by using a 1% agarose gel containing ethidium bromide. The gels were visualized on a UV lamp, then dried and autoradiographed by using a phosphorimager system (Molecular Dynamics).

For the unlabeled oligonucleotides, the process was the same, except that different amounts of plasmid (100 nM, 4 μg in 20 μL), TFO (1 μM), template (2 μM), and DNA ligase (400 units) were used. To remove BQQ from the triplex, the sample was diluted to 50 μL so that final buffer concentration was 50 mM Tris–HCl pH 8.0, 100 mM NaCl, 20 mM $MgCl_2$, and 20 μM trap oligonucleotide. The sample was heated to 80 °C and then slowly cooled to 37 °C. Spermidine (50 μL, 40 mM) was then added, and the samples were left at room temperature for 30 min with frequent vortex-mixing in order to allow plasmid compaction. After centrifugation, the pellet was washed with a solution (200 μL) containing isopropanol (50%), $MgCl_2$ (10 mM), NaCl (300 mM), and EDTA (25 mM), as described by Murphy et al.[11] The plasmid was resuspended in TE buffer (40 μL, 10 mM Tris pH 8.0, 0.1 mM EDTA).

The restriction enzyme inhibition assay was carried out in the following way: the plasmid (0.2 μg) was incubated in NEB 3 buffer (40 μL—50 mM tris–HCl, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT, pH 7.9 at 25 °C) containing BQQ (2 μM). The samples were heated to 75 °C and slowly cooled to 37 °C. The cleavage reaction was performed at 37 °C for 60 min with *Eco*NI (30 units), then stopped by the addition of SDS 2%/EDTA 0.2 M (5 μL). The samples were precipitated with ethanol (200 μL), resuspended in TE buffer (10 μL), and analyzed by agarose gel electrophoresis.

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Selective Inclusion of a Hetero-Guest Pair in a Molecular Host: Formation of Stable Charge-Transfer Complexes in Cucurbit[8]uril**

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The inclusion of two or more guest molecules in a molecular host is attractive because it provides unique opportunities to study new forms of stereoisomerism,[1] bimolecular reactions,[2] and molecular recognition[3] in microenvironments. Although the inclusion of two different types of guest molecules in a host has been reported,[4–6] the resulting complexes have been characterized only by spectroscopy. For example, the formation of pyrene-cyclodextrin-alcohol ternary complexes has been studied extensively by emission spectroscopy, but they are not stable enough to be isolated or structurally characterized.[5] Thus, the selective inclusion of two different guests in a molecular host which leads to an isolable ternary complex is still difficult to achieve.

Cucurbituril (CB[6]), a macrocycle comprising six glycoluril units, has a cavity that is accessible through two identical carbonyl-fringed portals.[7] The polar carbonyl groups at the portals and the hydrophobic cavity allow the cavitand to form stable host–guest complexes with small molecules such as protonated aliphatic and aromatic amines.[7, 8] We recently reported new cucurbituril homologues, namely cucurbit[*n*]uril (CB[*n*]; $n = 5$, 7, and 8), that contain five, seven, and eight glycoluril units.[9] The largest member of the cucurbituril family, CB[8], which has a cavity comparable to that of γ-cyclodextrin, can accommodate two molecules of a naphthalene derivative to form a 1:2 host–guest complex.[9] The capability of CB[8] to form 1:2 host–guest complexes prompted us to study the inclusion of two different guests within CB[8]. Here we report the selective inclusion of a hetero-guest pair in CB[8] that is driven by a charge-transfer interaction between the guests. The resulting ternary complexes are highly stable, which enables them to be isolated and characterized by X-ray crystallography.

Host–guest interactions between redox-active viologens and cyclodextrins has been extensively studied.[10] While methylviologen ($\mathbf{MV}^{2+}$) shows little interaction with β- or γ-cyclodextrin,[10a] it readily forms a 1:1 host–guest complex with CB[8], as evidenced by ^{1}H NMR spectroscopy and mass

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