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Controlling the G-Quadruplex topology: Toward the formation of a Lipid Thrombin Binding Aptamer (TBA) prodrug

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ABSTRACT: Important efforts have been devoted toward the development of modified oligonucleotides capable of controlling the secondary structures of G-quadruplex (G4). Herein, we introduce a photocleavable, lipidated construct of the well-known Thrombin Binding Aptamer (TBA) whose conformation can be dually-controlled by light and/or the ionic strength of the aqueous solution. This novel lipid-modified TBA oligonucleotide spontaneously self-assembles and switches from the conventional antiparallel aptameric fold at low ionic strength to the parallel, inactive, conformation of the TBA oligonucleotide strands in physiologically relevant conditions. The latter parallel conformation can be readily and chemo-selectively switched back to the anti-parallel native aptamer conformation upon light irradiation. Our lipidated construct constitutes an original prodrug of the original TBA with properties prone to improve the pharmacodynamical profile of the unmodified TBA.

In parallel to classical DNA double helices that capitalize on canonical complementary interactions, nucleic acids can adopt multiple three-dimensional supramolecular structures. Among these structures, the guanine-rich regions, which are known for their ability to stabilize G-quadruplex (G4) in the presence of cations, are involved in the regulation of key cellular processes, including the replication, transcription and translation.¹ G4s display many different secondary structures and topologies made of one and up to four DNA (or RNA) strands assembled with different strand directions, loop topologies, length etc.² Cations which are found sandwiched between G-tetrads are necessary to stabilize the G4 structures. The coordination cage that is formed upon folding preferentially binds K⁺ for geometrical as well as thermodynamical reasons. Importantly, the change in the nature of the cation is the primary cause of change in the conformation of G4-prone sequences.³ Moreover, individual G4-forming sequences can usually fold into an equilibrated mixture of different G4 self-assemblies and/or multimers, a property known as polymorphism.^{4,5} If not controlled, the polymorphic nature of G4 self-assemblies clearly impedes the development of G4-based nanotechnologies wherein the exquisite control over the supramolecular folding of the G4 is crucial. Likewise, most therapeutic G4 agents are active under a single topology.⁶ A well-known example is the thrombin-binding aptamer (TBA), which interacts strongly with its target protein only under its antiparallel conformation.⁷⁻⁹ Remarkably, the TBA aptamer has been shown to fold unequivocally into an active antiparallel conformation, in part because of its short (15-mer) oligonucleotide sequence. On the other hand, the control of naturally occurring biological processes that capitalize on a designated G4 fold are mediated mainly through the recruitment of chaperone-like proteins.¹⁰

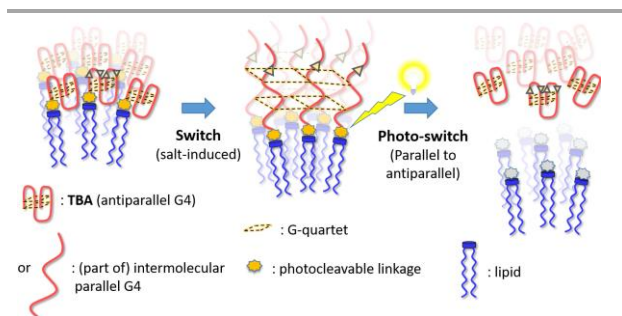
Subtle chemical alterations of oligonucleotide sequences have been shown to elicit drastic G4 conformational changes.¹¹ For instance, a change in conformation from antiparallel to parallel were induced following modifications of the sugar from ribose to deoxyribose in specific guanosine residues.¹² These results were interpreted in terms of the more favorable anti conformation adopted by the guanine-ribose bond that favor parallel G4 folds. Consequently, the conformational landscape accessible to a single G4-prone sequence at equilibrium is often wide and further dependent on the thermodynamic or kinetic control during the annealing process.^{13,14} Changes in the environment of the oligonucleotide were also shown to induce alternative G4 topologies like for instance in the presence of dehydrating agent, the so called crowding effect.¹⁵⁻¹⁷

From the literature, it clearly appears that the artificial control over the morphology of G4 structures is clearly challenging and rely on the profound chemical modification of the original oligonucleotide sequence: addition of a photo-responsive moiety,^{18,19} chemical cross-linking,¹⁵ or by way of a “forced-strand approach”.²⁰

As of the naturally occurring G4s, recent developments show several opportunities for developing new therapeutic strategies targeting these structures.^{21,22} Alternatively, artificial G4-based therapeutic agents have been designed that can elicit different biological mechanisms,^{23,24,25} or used in different biomedical approaches.^{26,27,28}

In this context, the development of G4 structures that can be switched “on demand” between an inactive form and a biologically relevant conformation *via* an external stimulus is highly desirable and still challenging. We previously reported that an otherwise unfolded sequence can be forced to adopt parallel tetramolecular G4 topologies upon lipid modification.²⁹ Lipid modification of oligonucleotide sequences has indeed been shown to provide unique properties to oligonucleotides.³⁰⁻³⁴

Herein, we extend this concept to the conformational switch from an antiparallel to a parallel G4 upon lipidation of the TBA aptamer sequence. Lipid modification of the TBA sequence results in the formation of a prodrug-like TBA that can be further photochemically converted back to its biologically active antiparallel thrombin aptamer provided the lipid is tethered to the TBA sequence via a photocleavable linkage. The active TBA



Scheme 1. General principle of the method. The lipidated TBA sequences switch from their original active antiparallel conformations to the inactive, parallel conformers following an increase in the ionic strength of the medium. In the second step, the TBA prodrug-like parallel conformers fold back to their original antiparallel active conformations upon photolysis. Remark: the exact conformation of the parallel G4s is purely schematic: their actual structure is not known.

anti-parallel G4s can therefore be formed “on demand” after light irradiation (Scheme 1).

To achieve the “on-demand release” of the TBA active form, a photosensitive moiety featuring a *O*-nitrobenzyl linker³⁵ is inserted between the lipid and the oligonucleotide sequence. The synthesis of the new photocleavable phosphoramidite derivative **5** starts from the commercial 1-(5-hydroxy-2-nitrophenyl)ethan-1-one **1** (Scheme S11). In the first step, a Williamson ether synthesis affords the primary alcohol **2**. This aliphatic alcohol will ultimately yield a conventional and stable phosphodiester linkage in the final oligonucleotide structure as opposed to phenyl ester derivatives more prone to hydrolysis. The alcohol **2** is subsequently protected as a dimethoxytrityl ether and the ketone reduced by sodium borohydride. The phosphoramidite **5** is finally obtained using a classical phosphorylation protocol. All these molecules were characterized by ¹H, ¹³C and ³¹P NMR when applicable (see SI).

The oligonucleotide conjugates bearing both the ketal nucleolipid and the photocleavable 2-nitrophenyl linker were synthesized using the conventional phosphoramidite chemistry on a H8 synthesizer (K&A Labs, Germany). The structures of **TBA**, the ketal-lipid modified TBA derivatives featuring either a photocleavable (PC) linker at the 5' end (**K-PC-TBA**) or only a

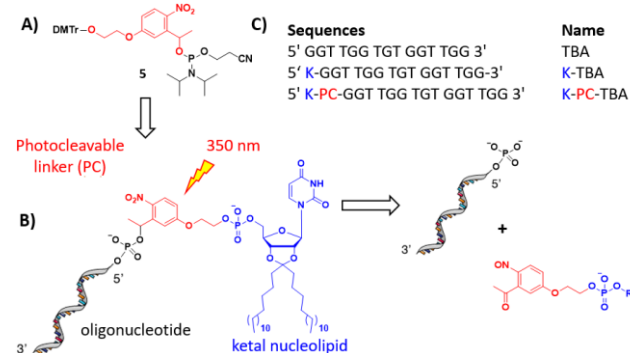


Figure 1. A) Chemical structure of the photocleavable phosphoramidite derivative **5**. B) The photocleavable linker (red) is incorporated between the 5' extremity of the oligonucleotide sequence and the ketal nucleolipid moiety (in blue). Photo irradiation at 350 nm provides the non-lipidic TBA sequence with a phosphate at its 5' end and an *O*-nitrosoacetophenone lipid by-product. C) Modified TBA sequences used in this study).

ketal lipid (**K-TBA**) as a control (Figure 1C) were purified by HPLC.

A photolytic cleavage at $\lambda=350$ nm provides a release of the non-lipidic **TBA** and the *O*-nitrosoacetophenone byproduct.^{36,37} As observed by mass spectrometry this photoreaction yields the **TBA** sequence bearing a phosphate at its 5'-end (Figure 1B and SI).

The first conformational switch i.e. the one triggered by salts hereafter simply referred as “switch” (Scheme 1, first antiparallel to parallel switch) was investigated first. Interestingly, the mere lipidation of the TBA sequence is not sufficient to induce any switch as evidenced by the antiparallel signature of **K-TBA** in the CD spectrum at low salt concentrations (Figure 2A). An increase in the concentration of salts is required to observe the switch (Figure 2A and SI1). The switch is observed for concentrations in salts above ca. 20mM, irrespective of the nature of the alkaline-earth cation used (Li^+ , Na^+ or K^+) and is likewise observed with **K-PC-TBA** (Figure SI1). The switch is therefore more likely driven by the ionic strength of the aqueous solution rather than a change in any putative, highly cation dependent, G4 topology. Salting out effects are also unlikely given the low concentration of salts involved in the transition. On the other hand, micellar aggregates of **K-PC-TBA** at low salt concentrations are visible by dynamic light scattering (Figure SI2). Accordingly, we interpret the absence of switch at low ionic strength as the consequence of electrostatic repulsions between the polyanionic antiparallel TBA strands present in the corona of the micelle. The electrostatic repulsion forces are gradually screened upon titration with salts so that the TBA strands of **K-(PC-)TBA** are more likely to interact and switch to the observed parallel conformers. To the best of our knowledge, our results constitute the first example of truly interconverting G4 conformers following titration with the exact same cation present at millimolar, physiologically relevant, concentrations.³⁸ Similar reported conformational switches usually take place following a change in the nature of either the cation,^{39,40} or change in the solvation of the oligonucleotide strands using dehydrating agent such as PEG. PEG was for instance found to favor a G4 parallel conformation for an analog of TBA that contains three (instead of the original two) G-quartets in the central core of the G4.⁴¹ As a control experiment (not shown), we checked that the addition of up to 50% (in volume) of a dehydrating agent (PEG-200) had no effect on the antiparallel conformation of a genuine **TBA** sample in line with previous investigations that showed that a stack of at least 3 G-quartets is required to observe molecular crowding effects.¹⁷

Interestingly, the TBA strands involved in **K-(PC-)TBA** parallel conformers 1) exhibit a higher melting temperature relative to the unmodified antiparallel **TBA** in the same conditions and 2) are not capable of interacting with the target thrombin protein as evidenced by the absence of change in the CD spectrum upon incubation with the protein (Figure SI3). The parallel folds formed from **K-PC-TBA** thus constitute attractive TBA prodrugs that can be further evaluated for their ability to release the aptameric antiparallel active fold of the TBA following a photo-switch hereafter simply referred as “photo-switch” (Scheme 1, right and Figure 2B).

Two different biologically relevant scenarios mimicking either extra- or intracellular fluids were then tested. These conditions primarily differ in the concentration of potassium present in each buffer: KCl is present at 140 and 5 mM inside cells and in extracellular fluids respectively. K^+ constituting one of the best

G4-binder, this difference was expected to have a great impact on the stability of the different G4 folds. This trend was clearly confirmed with the parallel folds of **K-PC-TBA** with an impressive $\Delta T_{1/2}$ (before photocleavage) of ca. 25°C (60 vs 85 °C respectively in extra- and intracellular environments, Figure SI4 and SI5). Moreover, thrombin being an extracellular protein, we primarily focused our investigations toward the photocleavage of **K-PC-TBA** under extracellular conditions.

The original antiparallel fold of TBA is indeed recovered following light exposure at 350 nm at 37°C to further mimic biological conditions. Interestingly, the parallel fold of TBA stands still after completion of the photolysis reaction of **K-PC-TBA** (Figure 2C) at 4°C. The photo-switch is therefore endothermic. The energetic cost is even higher when the photo-switch reaction takes place in the presence of intracellular salts: the photocleaved TBA strands in their parallel conformation remains stable even at 37°C (Figure SI5). Even if not required per se, the partial melting of the parallel folds seems to constitute a prerequisite to observe the switch to the native antiparallel TBA. Hence, $T_{1/2}$ around 43°C (with partial melting at 37°C) and 74°C ($\gg 37^\circ\text{C}$) were observed for the parallel TBA self-assemblies after photocleavage in the presence of the extra- and intracellular buffers respectively (Figure SI5).

Overall, our experimental results suggest that our lipidated TBA platform meets all the specifications of a prodrug i.e. 1) the parallel prodrug spontaneously self-assembles under physiologically relevant conditions ($>50\text{mM}$ salt concentrations) and 2) following photocleavage, it folds back to the active TBA drug at 37°C only in extracellular environments where the targeted thrombin protein is present.

Finally, we investigated the effect the target thrombin protein has on the photo-switch. Thrombin has indeed been shown to assist the folding of the antiparallel TBA.⁸ Consequently, thrombin was expected to assist and ease the photo-switch. On one hand, thrombin effectively stabilizes the native antiparallel form of the photocleaved 5'PO₃²⁻-TBA as evidenced by a higher intensity signal in CD for the antiparallel signal in the presence in comparison to the absence of the protein (Figure SI3). On the other hand, no photo-switch (in the presence of the intracellular buffer at 37°C) or acceleration of the photo-switch of any sort (in the presence of the extracellular buffer at 20°C where a slow kinetic of switch is observable) are noticed in the presence of the protein. These results suggest that following photolysis, the parallel folds of TBA are kinetically trapped and exhibit a high energetic barrier to overcome to fold back to the original antiparallel aptamer and that thrombin does not ease the conformational switch even though it binds the final aptameric structure.

Salt induced antiparallel to parallel switch of the TBA topology (Figure 2A).

The TBA aptamer truly constitutes a case study of an antiparallel G4 conformation in the realm of G4 supramolecular structures, even when present at high concentrations where dimeric structures have been observed.⁴² Drastic changes in its structure (specific loop mutations,^{43,44} addition of flanking oligonucleotide sequences,^{39,45} or chemical modification⁴⁶) are thus required to drive the TBA strands into a parallel topology. These TBA-derived oligonucleotide sequences are yet not amenable to any conformational switch as no alternative antiparallel G4 conformations have been observed to coexist in solution. In this contribution, we demonstrate that the conformation of TBA in its original antiparallel G4 conformation can be switched to parallel conformers (Figure 2A) following lipidation of the TBA

oligonucleotide sequence. The switch is controlled by the concentration of salts present in solution. While antiparallel G4s usually exhibit lateral and/or diagonal loops (the TBA aptamer is a typical chair-type, antiparallel G4 with lateral loops), parallel G4s usually rely on short propeller- or snapback-type loops. In other words, the switch observed here upon salt titration with **K-(PC-)TBA** involves a profound reorganization of the original, already stable, TBA antiparallel conformation. The thermal stabilities of G4 are in general tightly associated with the number of G-tetrads stacks. Given the restricted number of guanines in the TBA sequence (eight), no more than 2 G-tetrads can stack in any putative monomeric parallel self-assembly. In our case, the high thermal stability observed for the parallel G4 formed from **K-(PC-)TBA** would suggest the formation of a multimeric structure. Indeed, intramolecular two tetrads parallel G4s are not thermodynamically stable as demonstrated lately by Mergny et al.^{47,48} Formation of dimers, or alternative multimeric species are therefore necessary to expand the number of G-tetrad stacks that ultimately provide sufficient stabilization energy to the overall self-assembly.^{49,50} As a consequence, two TBA strands at the very least are poised to interact in the final parallel folds at high ionic strength in our case. The exact structure of the parallel folds of **K-(PC-)TBA** is yet not known and most probably complex. Nevertheless, these results shed some light onto the role the lipid plays during the self-assembly of **K-(PC-)TBA**. The micellization of the lipid-oligonucleotide conjugates results in a huge increase in local TBA strands concentration that ultimately favors multimerization of the TBA strands in a parallel topology (the lipid modifications at the 5'-end of the DNA sequence forces the orientation of the neighboring DNA strands in the same strand direction). Furthermore, the salt-induced conformational switch in **K-(PC-)TBA** turned out to be spontaneous in contrast to conventional, unmodified G4 tetrameric structures for instance.⁵¹ Again, the velocity in the self-assembling process is a consequence of the micellization as already observed with another lipid-oligonucleotide tetrameric G4.²⁹ Interestingly, while in this latter example, the lipidation was shown to drive the folding process from poorly structured oligonucleotide sequences, the lipidation of the TBA sequence triggers a conformational switch from a pre-existing, stable, antiparallel G4 conformation. Our results suggest that in addition to the nature of the cations,⁴⁰ or the presence of dehydrating agents, proximity effects may channel G4-prone sequences in an otherwise entropically disfavored energetic path when the oligonucleotide are free in solution.

Photo-switch: recovery of the original antiparallel topology of the TBA after photocleavage (Figure 2B).

While the photocleavage reaction readily takes place at low temperatures (Figure 2C), the TBA oligonucleotide strands are kinetically trapped as parallel conformers. Not surprisingly, the thermal stability of the parallel conformers of the photocleaved 5'PO₃²⁻-TBA increases in the presence of K⁺ in comparison to Na⁺. Accordingly, the photo-switch readily takes place at 37°C using a buffer mimicking extracellular conditions whereas no photo-switch is observed in the presence of a higher concentration of K⁺, i.e. under intracellular conditions. The kinetic stability of the parallel folds for the photocleaved 5'PO₃²⁻-TBA also allowed us to assess the contribution the lipid plays in the stabilization of the G4 parallel assemblies. The $\Delta T_{1/2}$ of around 16°C obtained in the presence and absence of the lipid (i.e. for **K-PC-TBA** and 5'PO₃²⁻-TBA respectively) reflects the contribution the hydrophobic effect has in the stabilization of the parallel G4 conformers of **K-PC-TBA**. This result compares well

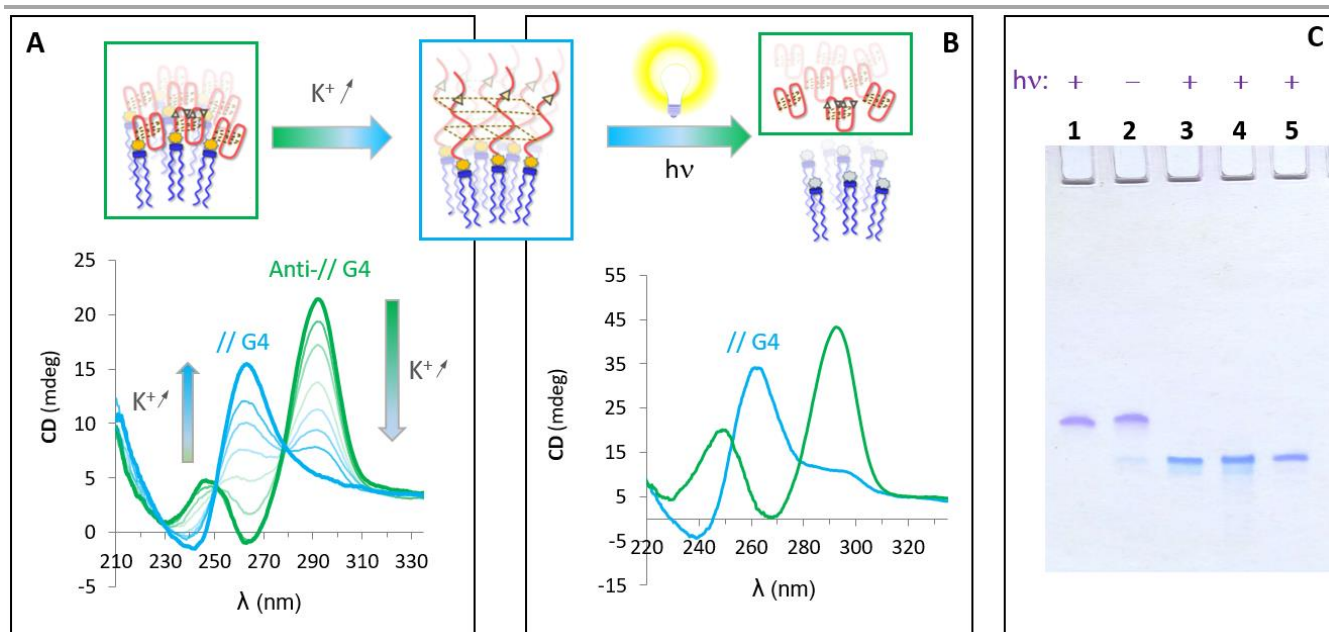


Figure 2. A: CD of the anti-parallel to parallel G4 conformational switch in **K-TBA** at 40°C triggered by KCl titration (5 (green) → 75 mM (blue), last curve (75mM) at 60°C). B: Photo-induced conformational switch at 37°C from parallel (blue) to the original antiparallel (green) TBA conformation. C: Denaturing PAGE showing the end products of the photocleavage reaction with: 1: **K-TBA** (negative control), 2 & 3: **K-PC-TBA**, 4: **PC-TBA**, 5: **TBA**.

with other previous reports obtained with lipophilic DNA duplexes.⁵²

Satisfyingly, the photocleaved 5'**PO₃²⁻-TBA** parallel conformers reverse back to the desired aptameric folds at physiological temperatures only when the photolysis reaction is carried out in conditions mimicking extracellular fluids i.e. in the environment of its target thrombin protein. In contrast, the photocleaved 5'**PO₃²⁻-TBA** remains in a prodrug-like inactive parallel conformation in buffers mimicking intracellular conditions, providing an extra level of selectivity for the prodrug.

Conclusion

Lipidation of the thrombin binding aptamer (TBA) was shown to provide unique properties to this emblematic G4 structure. First, the lipid oligonucleotide conjugates (**K-(PC-)TBA**) can switch from the original antiparallel to the parallel G4 conformers upon titration with salts. This result was interpreted in terms of an interplay between the supramolecular oligomerization of the aptamer brought about by the lipid and the screening of electrostatic repulsion forces. While such conformational changes are common when the G4 structures are annealed in the presence of cations of different types, the switch is observed in our case following titration of the aptameric sequences with the exact same cation. Our results clearly show that the proximity effect provided by the micellization of **K-(PC-)TBA** can force an already stable antiparallel G4 structure to irreversibly switch to a parallel G4 conformation not present in solution at first. It is therefore tempting to speculate how these observations pertain in the context of gene regulation. Our fluid micellar platform may well mimic the natural environment of a gene with high local concentration in DNA segments. Depending on trans-interacting elements, original G4s present in promoter regions could well switch to other topologies when forced to interact with other DNA sequences giving rise to inhibition or promotion of gene transcription.

An additional control over the G4 morphologies was provided by the insertion of a photocleavable linkage between the lipid and the TBA oligonucleotide sequence of **K-PC-TBA**. The original parallel folding of the TBA strands can readily release the TBA drug in its active antiparallel form upon light irradiation at 350 nm.

Ongoing phase II clinical trials and in vivo experiments confirmed the potential for the TBA aptamer (also referred as BC 007 in the literature) in the clinic.^{6,53–55} Originally selected for its ability to inhibit thrombin during coronary bypass graft surgery, the use of TBA was abandoned because of a suboptimal dosing profile.^{8,9} Our **K-PC-TBA** platform offer a clear advantage over the unmodified **TBA** in this regard. In fact, the **K-PC-TBA** prodrug in its parallel conformation is thermally stable in both intra- and extracellular fluids and folds back into the active antiparallel TBA aptamer once photo-cleaved only in extracellular environments where the targeted thrombin protein is located. Fewer intracellular off-target effects are then to be expected with **K-PC-TBA** compared to the unmodified **TBA**. The development of stimuli responsive modified oligonucleotides is of high interest for several medical treatments. As reported recently lipid-oligonucleotide can be subcutaneously (SC) administered.⁵⁶ Likewise, **K-PC-TBA** could be SC injected to form a reservoir of non-active prodrug of TBA. Upon light stimulation such a reservoir could release “on demand” the active antiparallel form of TBA.

ASSOCIATED CONTENT

Supporting Information

Experimental procedures and characterization data (NMR, ESI MS), CD, PAGE. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/XXXX.

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Author Contributions

The manuscript was written through contributions of all authors. / All authors have given approval to the final version of the manuscript. / #These authors contributed equally

Notes

The authors declare no competing financial interests.

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