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Dedicated to Professor Christian Leumann on the occasion of his 65th birthday

Abstract

Chemical modification of aptamers is an important step to improve their performance and stability in biological media. This can be performed either during their identification (mod-SELEX) or after the *in vitro* selection process (post-SELEX). In order to reduce the complexity and workload of the post-SELEX modification of aptamers, we have evaluated the possibility of improving a previously reported, chemically modified aptamer by combining enzymatic synthesis and nucleotides bearing bioisosteres of the parent cubane side-chains or substituted cubane moieties. This method lowers the synthetic burden often associated with post-SELEX approaches and allowed to identify one additional sequence that maintains binding to the PvLDH target protein, albeit with reduced specificity. In addition, while bioisosteres often improve the potency of small molecule drugs, this does not extend to chemically modified aptamers. Overall, this versatile method can be applied for the post-SELEX modification of other aptamers and functional nucleic acids.

Keywords: aptamers; nucleotides;·post-SELEX; cubanes; bioisosteres; polymerases

1. Introduction

Aptamers are a class of nucleic acids that are capable of binding to a broad range of targets with both high affinity and specificity.[1] Aptamers unlike antibodies are generated by application of SELEX (systematic evolution of ligands by exponential enrichment) and related *in vitro* Darwinian selection protocols. During these processes, large pools of individual (i.e. 10^{13}-10^{14}) nucleic acid molecules are subjected to iterative cycles consisting of incubation with target, separation of binders from non-binding species, and PCR amplification.[2] Due to their intrinsic properties, aptamers have found applications as drug delivery agents,[3] diagnostic[4] and imaging tools,[5] and as therapeutics.[6] However, as for other therapeutic oligonucleotides, the nucleosidic scaffold of aptamers needs to be consolidated with chemical modifications to ensure resistance against nuclease degradation and increase circulation times.[7] The introduction of chemical modifications can occur either during (mod-SELEX) or after (post-SELEX) the *in vitro* selection process.[8] In the mod-SELEX approach, canonical nucleotides are replaced by one or multiple modified nucleoside triphosphates analogues during the production of oligonucleotide libraries.[9] These modified libraries are then subjected to SELEX protocols and yield aptamers decorated with additional functional groups. Alternatively, functionalization of randomized libraries can also proceed by click-chemistry[10] or via ligase-based methods which both abrogate the need for synthesizing modified nucleotides and identifying suitable polymerases.[11] In addition, enzymatic synthesis represents a more sustainable alternative to chemical synthesis and is tolerant to a broad range of functional groups.[12] Collectively, mod-SELEX methods permit the direct introduction of a large variety of functional groups without reengineering aptamer sequences using structure activity relationship studies. On the other hand, in the post-SELEX approach, unmodified aptamers identified by *in vitro* selection are subjected to chemical modification campaigns. These mainly include introduction of chemical functional groups at the 3'/5'-termini by phosphoramidite chemistry[13] in order to improve the intrinsic properties of aptamers.[14] Internal nucleotides can also be equipped with functional groups but this approach is more delicate since these alterations might result in important reductions of binding affinity.[15] The post-SELEX approach is also very popular for improving the therapeutic usefulness of other functional nucleic acids, particularly DNAzymes.[16] This chemical approach, while less sustainable, is compatible with larger batch synthesis (up to 10 kg) and permits the site-selective introduction of chemical modifications into functional nucleic acids.[7b, 12c, 16d]

While these methods all produce aptamers decorated with chemical modifications which enhance their properties, alternative approaches that circumvent the limitations of post-SELEX and mod-SELEX are in dire need. Here, we questioned whether chemical modifications of existing aptamers could be substituted with either bioisosteres, i.e. analogues of a parent compound that elicit a similar response from a biological system,[17] or with analogs bearing alternate substituent exit vector patterns.[18] We have previously identified an aptamer modified with cubane moieties (nucleotide **1** in Figure 1) which bound to its protein target with moderate affinity (K_D = 670 nM) but with very high specificity.[19] In order to improve the usefulness of cubamers for the development of aptasensors detecting these malaria biomarkers, we sought to improve the binding efficiency of this aptamer. To do so, we synthesized nucleotides bearing bioisosteres of cubane (nucleotides **2** and **3** in Figure 1) and substituted cubane moieties (nucleotides **4** and **5** in Figure 1). After verifying the suitability of the modified nucleotides with enzymatic DNA synthesis, we prepared analogs of the cubamer and evaluated the binding affinity of the resulting chemical variants.

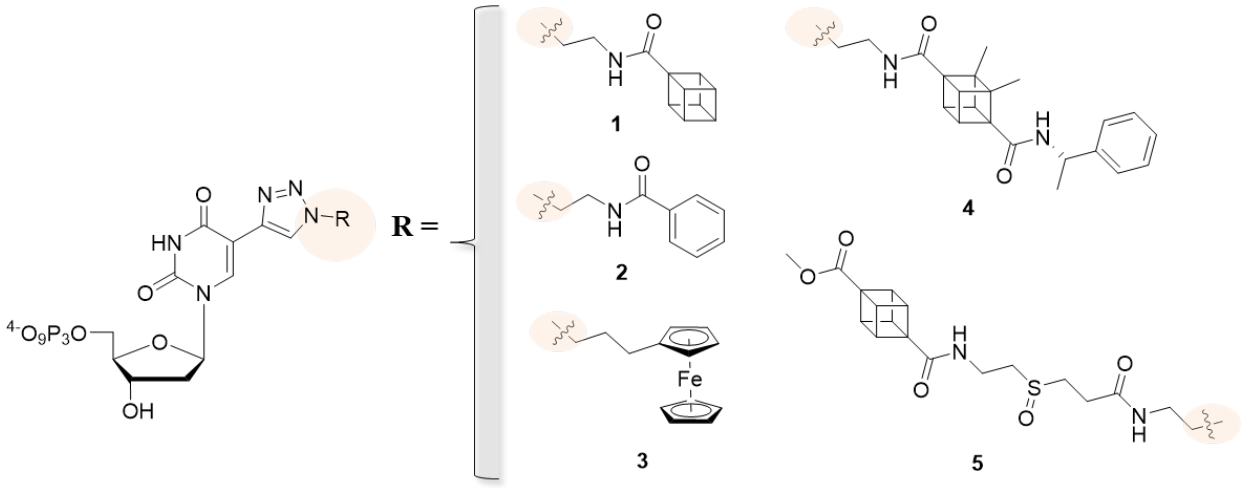


Figure 1. Chemical structures of modified nucleotides dU^{C}TP **1**, dU^{B}TP **2**, dU^{F}TP **3**, dU^{PC}TP **4**, and dU^{SC}TP **5**.

2. Results

2.1. Design and synthesis of bioisosteres and modified cubanes

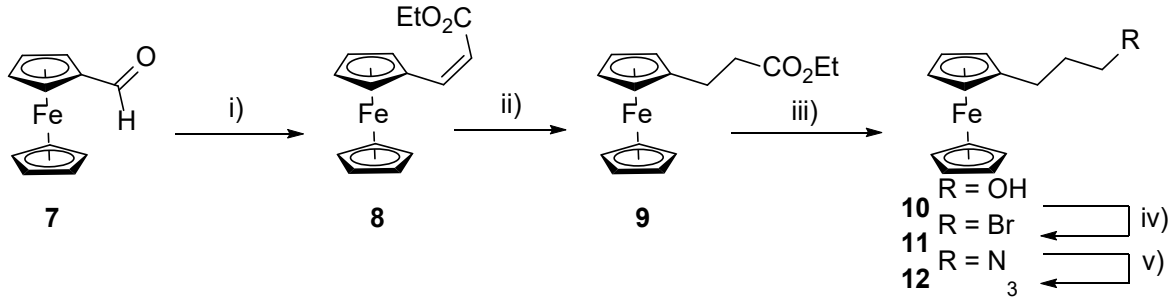
Cubane is a particularly interesting bioisostere of benzene that serves as a substitute of aromatic pharmacophores in medicinal chemistry[20] and has recently been integrated in nucleic acid scaffolds to improve pharmacokinetic and functional properties.[19, 21] Cubamer 1501 was obtained by SELEX using modified nucleotide dU^{C}TP **1** and the biomarker *Plasmodium vivax* lactate dehydrogenase (PvLDH) as target. The resulting sequence contained five modified nucleotides, four of which were found to be directly implicated in the binding mechanism.[19] Cubamer 1501 did not bind to the LDH of *Plasmodium falciparum* (PfLDH) despite a very high sequence homology and this impressive specificity allowed for the detection of PvLDH over PfLDH in a mimetic clinical situation. However, efficient aptasensors rely on both high affinities and specificities to minimize the occurrence of false positives and reach low limits of detection (LOD).[22] Hence, in order to integrate cubamer 1501 into aptasensing devices for the specific detection of PvLDH, binding affinity needs to be improved without affecting the high degree of specificity. Instead of re-selecting aptamers based on partial mutagenesis of 1501 or engaging in an uncertain post-SELEX modification and truncation procedure with phosphoramidite building blocks, we explored the possibility of exchanging dU^{C} **1** with closely, chemically related nucleotides during enzymatic synthesis. Synthetic efforts can be minimized by selecting a common nucleotide precursor (i.e. 5-ethynyl-dUTP) and appending the different functional groups by an orthogonal, high-yielding bioconjugation step. Such a method will then permit the rather rapid screening of modified variants of the parent aptamer. To do so, we deemed that changing cubyls with bioisosteres or substituted analogs might improve the binding capacity of the resulting aptamers. Since cubane is a bioisostere of benzene, we considered that replacing cubyl with phenyl substituents would trigger a similar biological response and potentially even improve binding of modified aptamers to PvLDH. By the same token, metallocenes and particularly ferrocene, are particularly attractive synthons in medicinal chemistry to substitute aromatic moieties of existing drugs and improve their potency.[23] We thus hypothesized that replacing cubane in 1501 with ferrocene surrogates might also induce a positive impact on binding. Lastly, most cubanes inserted in drug candidates display a very limited diversity of functional groups since they are mainly mono-substituted or 1,4-disubstituted.[24] In order to investigate the effect of

extended substitution patterns of cubane moieties of 1501 on the binding affinity, we designed nucleotide **4** which displays an usual 1,2,3,4-tetrasusbtitution motif and nucleotide **5** equipped with an additional moiety at position 4 of the cubyl along with an altered connecting side-chain.

The synthesis of the nucleotides depicted in Figure 1 commenced with the preparation of the corresponding azide building blocks which in turn could be used to decorate the nucleobase of 5-ethynyl-dUTP (EdUTP) via standard Cu(I)-catalyzed azide–alkyne cycloaddition (CuAAC).

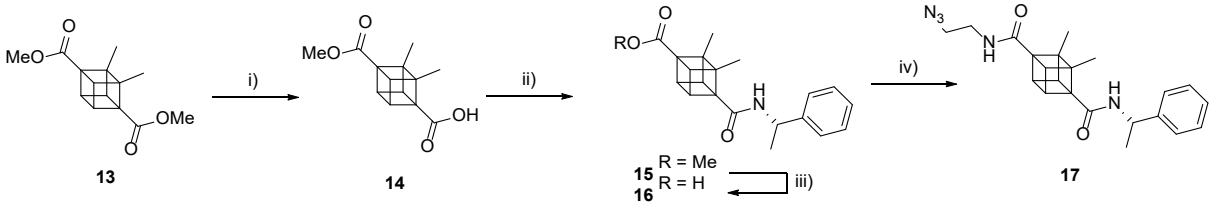
Known *N*-(2-azidoethyl)benzamide **6**[25] required for the synthesis of modified dUTP analog **2** was synthesized by application of standard amide bond coupling conditions using benzoic acid and 2-azidoethylamine as starting materials (see Supporting Information).[26]

Preparation of (2-azidopropyl)ferrocene[27] **12** precursor of triphosphate **3** was prepared according to the synthetic protocol outlined in Scheme 1. Briefly, commercially available ferrocene carboxaldehyde **7** was converted to the corresponding ferrocene unsaturated ester **8** by means of a Wittig-Horner reaction.[28] The resulting ester **8** was then subjected to catalytic hydrogenation yielding saturated ester **9**.[29] Reduction with $LiAlH_4$ produced the corresponding alcohol **10** which was subjected to two consecutive S_N2-type of reactions yielding the desired azide building block **12**.



Scheme 1. Synthesis of azide derivative **12**. Reagents and conditions: i) Triethyl phosphonoacetate, NaH, EtOH, RT, 4 h, 85%; ii) $Pd(OH)_2/C$, H_2, EtOAc, RT, 22 h, 95%; iii) $LiAlH_4$, THF, 0°C, 2 h, 77%; iv) PBr_3, Et_2O, RT, 5 h, 21%; v) NaN_3, DMF, 50°C, 14 h, 98%.

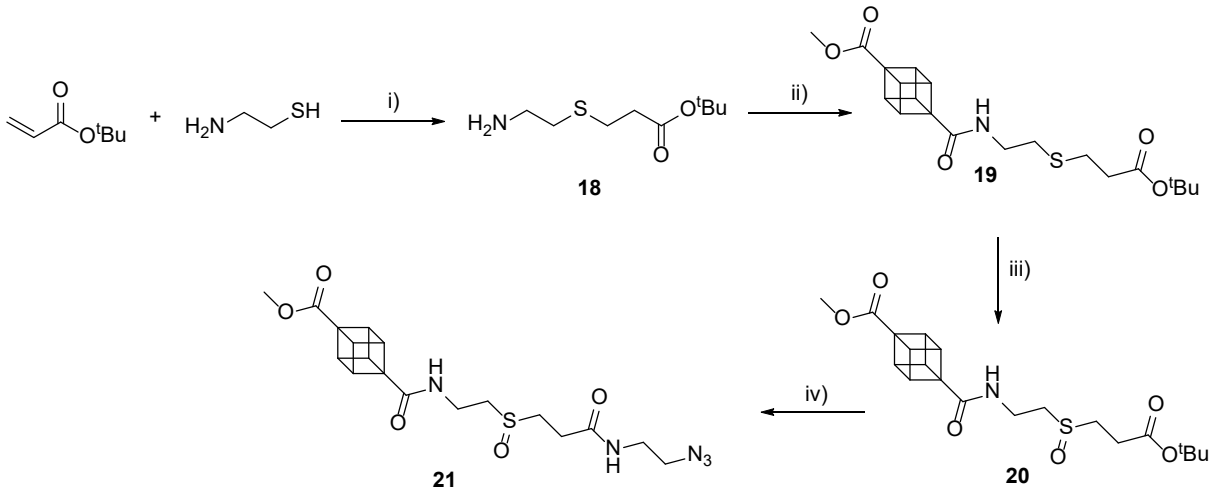
Synthesis of the tetrasubstituted cubane azide derivative **17** started with the mono-saponification of dimethyl (±)-2,3-Dimethylcubane-1,4-dicarboxylate **13** (Scheme 2).[30] This first step introduces a desymmetrization at the level of the cubane moiety but diastereisomers could not be separated.[31] The resulting racemic mixture **14** was then subjected to amide bond coupling conditions with (*S*)-(-)-1-phenylethylamine to yield **15** in good yields (44% from **13**). Hydrolysis of the remaining methyl ester followed by amide bond formation with 2-azidoethylamine led to desired compound **17**.



Scheme 2. Synthesis of azide derivative **17**. Reagents and conditions: i) NaOH, MeOH, THF, RT, 12 h; ii) a) $(COCl)_2$, CH_2Cl_2, DMF (cat.), 0° to RT, 1 h; b) (*S*)-(-)-1-phenylethylamine,

CH_2Cl_2, 0°C, 12 h, 44% over 2 steps; iii) NaOH, MeOH, THF, RT, 3 h, 79%; iv) 2-azidoethylamine, DIC, HOBT, Et_2O, reflux, 12 h, 69%.

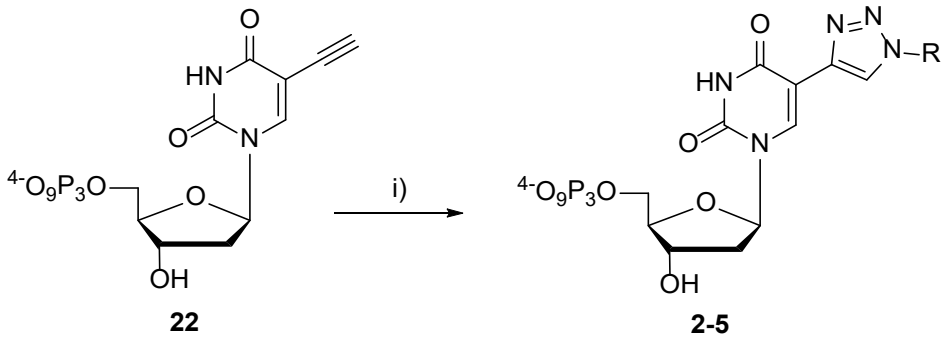
Finally, synthesis of azide precursor **21** proceeded as outlined in Scheme 3. Briefly, Michael addition of cysteamine (2-aminoethanethiol)[32] on *tert*-butyl acrylate produced **18** which was further converted to cubyl derivative **19** by amide bond formation. Further oxidation of the thioether linkage (to increase solubility of the modification and prevent uncontrolled oxidation during enzymatic synthesis) produced sulfoxide **20** in quantitative yields.[33] The desired azide **21** was obtained after removal of the ester masking group and amide bond formation with 2-azidoethylamine.



Scheme 3. Synthesis of azide derivative **21**. Reagents and conditions: i) MeOH, RT, 10 min; ii) EDC, DMAP, 4-(methoxycarbonyl)cubane-1-carboxylic acid, 15 min, RT, 71% over 2 steps; iii) a) $FeCl_3$, CH_3CN, 5 min, b) H_5IO_6, 15 min, c) $Na_2S_2O_3$, quantitative; iv) a) TFA, CH_2Cl_2, RT, 3h, b) 2-azidoethylamine, DIC, HOBT, DMF, RT, 12h, 37% over two steps.

2.2. Synthesis of modified nucleoside triphosphates

With all azide precursors at hand, we next prepared all modified nucleotides by CuAAC reactions with 5-ethynyl-dUTP (**22** in Scheme 4). All nucleotides were obtained in good yields (ranging from 49% to 75%) after RP-HPLC purification.



Scheme 4. Synthesis of modified nucleotides. Reagents and conditions: i) CuI, DIPEA, DMF, CH_3CN, H_2O, azides **6**, **12**, **17**, or **21**, RT, 4 h, 66% ($dU^{B}TP$ **2**), 75% ($dU^{F}TP$ **3**), 64% ($dU^{PC}TP$ **4**), and 49% ($dU^{SC}TP$ **5**).

2.3. Biochemical characterization of modified nucleotides

We next sought to evaluate the possibility of synthesizing modified DNA using nucleotides **2**-**5** under primer extension (PEX) reaction conditions.[12b, 34] To do so, we first considered a system composed of the 71-nucleotide template **T1** and the 18-nucleotide, 5'-FAM-labelled primer **P1** (see Table 1). This primer/template system allows for the incorporation of thirteen modified residues. PEX reactions were performed by substituting natural dTTP with each of the modified dUTPs and in the presence of eleven different DNA polymerases (Phusion, Hemo KlenTaq, Taq, *Bst*, Q5 DNA polymerase, Therminator, Vent (*exo*$^-$), *Sulfolobus* DNA polymerase IV (Dpo4), Deep Vent (*exo*$^-$)), Phi29, and the Klenow fragment of *E. coli* (Kf (*exo*$^-$)).

Table 1. DNA primers and templates used for primer extension reactions and PCR to evaluate the compatibility of modified nucleotides **1**-**5** with enzymatic DNA synthesis

T1	5'-CGT ACG GTC GAC GCT AGC CCC ATA CTC ATC ACC ATT CAC ATC ACT CAC CTA GCC ACG TGG AGC TCG GAT CC-3'
T2	5'-CAC TCA CGT CAG TGA CAT GCG GTA TAG ACC CCT GAG TCC TAC CGA GGG CAC GGC AGT CAG AAA TTT CGC ACC AC-3'
T3	5'-AGA GGC TAT AGT GAG TCG TA-3'
P1	5'-FAM-GGA TCC GAG CTC CAC GTG-3'
P2	5'-GTG GTG CGA AAT TTC TGA C-3'
P3	5'-CAC TCA CGT CAG TGA CAT GC-3'
P4	5'-FAM-TAC GAC TCA CTA TAG CCT C-3'

Analysis of the reaction products by gel electrophoresis unequivocally demonstrates that many polymerases accept the modified nucleotides as substrates and support enzymatic DNA synthesis (see Figure 2 and Figures S1-S4 of the Supporting Information). The four substrates tested are well tolerated by polymerases. Indeed, the observation of high-yield production of fully extended primers to full length products under various conditions confirms this tolerance, with the appearance of only a few truncated side products. It is noteworthy mentioning that six specific polymerases, namely Phusion, *Bst*, Q5, Therminator, Vent (*exo*$^-$), and Deep Vent (*exo*$^-$), favor full elongation of the primer particularly for nucleotides **4** (Figure S3) and **5** (Figure S4) bearing modified cubane moieties. However, nucleotide dU^{F}TP **3** is least tolerated by polymerases and only the reactions catalyzed by Therminator, Vent (*exo*$^-$), and Deep Vent (*exo*$^-$) lead to the formation of the expected product (Figure S2). Nonetheless, as noticed for the parent nucleotide dU^{C}TP **1**,[19] Vent (*exo*$^-$) appears to be the most suitable polymerase for the synthesis of modified DNA with such substrates. After optimization, experimental conditions could be met that led to complete conversion of primer **P1** to modified product for all nucleotides in the presence of Vent (*exo*$^-$) (Figure 2).

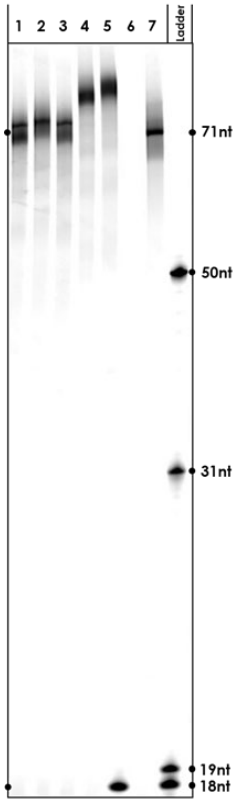


Figure 2. Gel (PAGE 20%) analysis of the PEX reactions with primer **P1** (10 pmol), template **T1** (15 pmol), natural nucleotides dACG (200 μM), **dU*TP** (200 μM), and Vent (exo-) (1 μL) for a total volume of 10 μL. The following nucleotides were used: dU^{C}TP **1** (lane 1), dU^{F}TP **3** (lane 2), dU^{B}TP **2** (lane 3), dU^{PC}TP **4** (lane 4), dU^{SC}TP **5** (lane 5). Negative controls (T-): (lane 6) No polymerase. Positive control (T+): (lane 7) with all natural dNTPs and Vent (exo-) polymerase. All reactions were incubated at adequate reaction temperatures for 4 h. 18 nucleotides (nt) corresponds to the length of primer **P1** and 71 to the one of the full-length product.

With the aim of further demonstrating the compatibility of modified nucleotides with polymerase-mediated DNA synthesis, we carried out digestion-LC-MS analysis experiments.[26b] To this end, we designed a system comprising a 20-nucleotide long template **T3** and a complementary, 19-nucleotide primer **P4** (see Table 1). The 5'-terminal region of **T3** consists of a unique deoxyadenosine (dA) which leads to single incorporation events of modified dU nucleotides and ease of detection during the LC-MS analysis. We thus first carried out PEX reactions with the conditions devised previously with Vent (*exo*$^{-}$) and keeping relatively short reaction times (10 min) to avoid untemplated addition events from occurring. All nucleotides were incorporated efficiently under these conditions as shown by gel electrophoresis (Figure S5). PEX reaction products were then purified using Monarch DNA clean-up columns and quantified by UV spectroscopy. After purification, the resulting dsDNA were digested down to single nucleosides by nucleases (see Experimental Section for details). In parallel, standards of the modified nucleosides were derived from the corresponding nucleoside triphosphates by hydrolysis with the shrimp alkaline phosphatase (rSAP). As expected, the LC-MS chromatograms of the digested dsDNA reveal four dominant peaks corresponding to the natural nucleosides (Supporting Information, Figures S8-12). Additional peaks corresponding to the modified nucleosides can be detected in all reactions and these peaks align perfectly with the nucleoside standards. Hence, this analysis confirms the incorporation of all modified nucleotides into DNA by Vent (*exo*$^{-}$).

After identifying conditions permitting enzymatic synthesis under PEX reaction conditions, we next evaluated the possibility of using the modified nucleotides in PCR. PCR experiments were conducted by using a 74-mer template **T2** flanked by 19 and 20-nt primers **P2** and **P3**, respectively along with seven different thermophilic DNA polymerases (Figure S6). We have previously used this system to evaluate the compatibility of modified nucleotides with PCR.[35] This analysis reveals that nucleotides **1**, **2**, **4**, and **5** are well-tolerated particularly by the Vent (*exo*$^-$) and Deep Vent (*exo*$^-$) polymerases and faithfully amplified template **T3** producing the expected amplicons. Nucleotide **3**, as already observed in PEX reactions, did not act as a substrate for polymerases and cannot be used in PCR to produce modified DNA. Overall, the best conditions leading to good yields of modified DNA are met when Vent (*exo*$^-$) serves as polymerase (Figure 4).

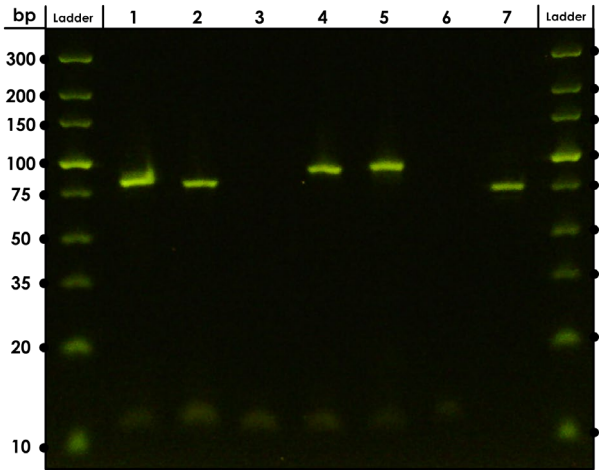


Figure 4. Gel image (agarose 4%) shows the outcome of the PCR reactions with primers **P2** and **P3** (5 μM), template **T2** (0.01 μM), natural nucleotides dACG (200 μM), modified nucleotides (200 μM), Mg^{2+} (2 mM), and Vent (*exo*$^-$) (1 μL) for a total volume of 20 μL. The following nucleotides were used: (lane 1) dU^{C}TP **1**, (lane 2) dU^{B}TP **2**, (lane 3) dU^{F}TP **3**, (lane 4) dU^{PC}TP **4**, (lane 5) dU^{SC}TP **5**, Negative control (T-): (lane 6) no polymerase added to the reaction mixture; positive control (T+): (lane 7) 10 cycles with all natural dNTPs and Vent (*exo*$^-$) polymerase. PCR program: 95°C for 5 min, (95°C 1 min, 52°C 1 min, 72°C 2 min) X 25 cycles, 72°C 5 min.

2.4. Enzymatic synthesis and evaluation of binding affinity of modified aptamers

NGS analysis of the SELEX conducted with modified nucleotide dU^{C}TP **1** clearly highlighted that mainly two sequences evolved and dominated the enriched library of round 15. Indeed, sequences 1501 with five modified dU^{C} units and 1506 with six cubane-modified nucleotides covered 23.1% and 22.4% of pool sequences, respectively.[19] While 1501 displayed a moderate K_D and high specificity, 1506 was very reluctant at binding to target PvLDH. Hence, we questioned whether the introduction of modified nucleotides **2**-**5** instead of parent cubane analog **1** in sequences 1501 and 1506 would either enhance or permit binding, respectively. To do so, we carried out large scale PEX reactions using templates corresponding to the complementary sequences of 1501 and 1506 equipped with a primer **P3** binding sequence (see Tables S1 and S2, Supporting Information). Using the optimized conditions established with Vent (*exo*$^-$), all modified sequences could be obtained in good to excellent yields (see Figure S7). Conversion of dsDNA to ssDNA is not an obvious undertaking since most methods lead to poor recovery yields or partial template contamination, particularly for sequences containing hydrophobic moieties.[34] In order to avoid presence of unmodified DNA template, we purified the products of the PEX reactions by denaturing gel electrophoresis followed by excision of the bands corresponding to modified ssDNA. The oligonucleotides were then

extracted into an elution buffer containing 0.5 M ammonium acetate and 10 mM magnesium acetate. After ethanol precipitation, modified ssDNA sequences were recovered in 10-20% yields from starting primer. The integrity of the sequences was verified by PAGE (Figure 5).

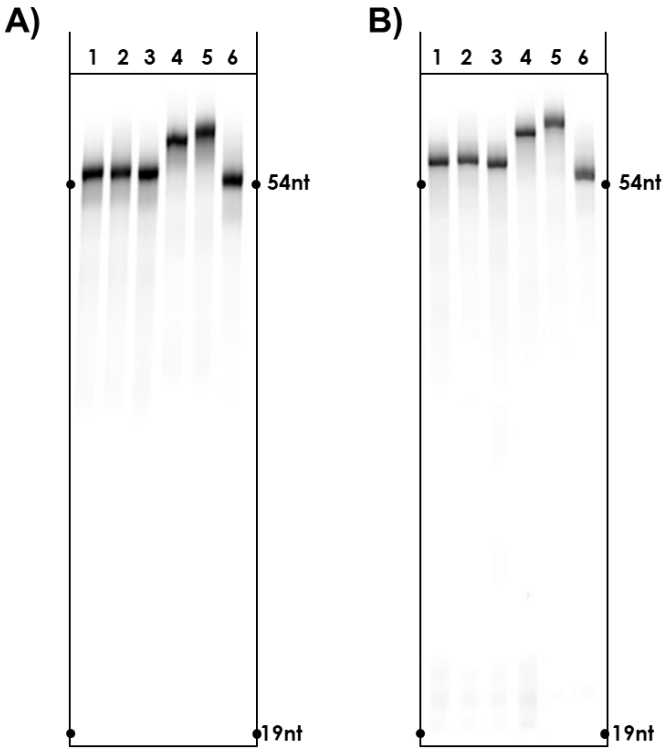


Figure 5. Gel image (20% PAGE) of gel-purified ssDNA. PAGE analysis of purified single-stranded DNA (ssDNA) obtained from **A)** cubamer 1501 and **B)** 1506. **A)** lane1: 1501-C, lane 2: 1501-F, lane 3: 1501-B, lane 4: 1501-PC, and lane 5: 1501-SC; **B)** lane1: 1506-C, lane 2: 1506-F, lane 3: 1506-B, lane 4: 1506-PC, and lane 5: 1506-SC. Positive controls (T+): lanes 6 with natural dTTP.

After producing sequences 1501 and 1506 equipped with nucleotides **1**-**5**, we next sought to evaluate their propensity at binding to the malaria biomarker PvLDH. In terms of nomenclature, we associated each evaluated sequence to the chemistry that was used during PEX reactions (e.g. 1501-C corresponds to sequence 1501 prepared with cubane-modified nucleotide **1** while 1506-B is the sequence 1506 containing modified nucleotide **2**). To do so, we performed biolayer interferometry (BLI) experiments using His_6-tagged PvLDH protein. As negative controls, sequences 1501 and 1506 containing only natural nucleotides were also included in these binding experiments.

In a first screening step, we verified oligonucleotide binding to PvLDH by using a single, set concentration of 670 nM, which corresponds to the K_D of 1501-C for PvLDH protein reported previously by SPR.[19] We surmised that by setting the oligonucleotide concentration at this value significant binding would be observed for 1501-C and would allow evaluating whether all other modified sequences displayed similar, higher, or slightly lower affinities. Binding was assessed in two different buffers: PvLDH-buffer only and PvLDH-buffer supplemented with 0.5 $mg \cdot mL^{-1}$ BSA and 0.005% Tween which had been used for SPR binding studies. Under these conditions, we observed a clear binding signal for 1501-C (Supporting Information Figure S13A) in conditions containing BSA and tween. In PvLDH-buffer only, we observed a clear binding signal for 1501-PC, whereas surprisingly, no binding signal could be observed when

BSA and Tween are present inside the buffer (Supporting Information Figure S13B). On the other hand, all other modified sequences (as well as the unmodified controls) failed to bind to PvLDH under either of the two conditions tested. Similarly, none of the modified sequences 1506 displayed any propensity at binding to the target protein. In addition, in order to rationalize the absence of binding of 1501-PC in presence of BSA and Tween, we surmised that due to the presence of additional hydrophobic moieties on nucleotide **4** compared to parent cubane **1**, the presence of Tween could interfere with the binding to PvLDH protein. Finally, increasing the oligonucleotide concentration to 1 µM, confirmed that none of the other modified sequences except for 1501-C and 1501-PC could bind to PvLDH (Supporting Information Figure S14)

We next determined precise affinity constants for the 1501-C and 1501-PC sequences for the PvLDH target protein (Figure 6). For each oligonucleotide, two independent experiments were performed, using the most adapted buffers determined during the first screening step, namely PvLDH-buffer supplemented with BSA 0.5 $mg \cdot mL^{-1}$ and Tween 0.005% for 1501-C and PvLDH-buffer only for 1501-PC. The K_D value measured for 1501-C was 389 ± 61 nM (Figure 6A), which is very close to that determined by SPR (i.e. 670 ± 9 nM).[19] In the case of modified aptamer 1501-PC, the K_D value was estimated at 524 ± 61 nM (Figure 6B). However, accurate K_D determination was complicated as no binding signal could be observed for the smallest concentrations (31.25, and 62.5 nM). We hypothesized that at lower concentrations, 1501-PC is highly retained on surfaces, a phenomenon amplified due to the absence of BSA and Tween, preventing any binding to PvLDH protein.

To assess the preservation of the binding specificity of parent 1501, we tested the binding of 1501-C and 1501-PC to streptavidin. We opted for streptavidin since both proteins display similar isoelectric points (pI ~ 6.2)[36] but differ in size and sequence composition. No binding to streptavidin could be observed for 1501-C, whereas a weak interaction could be observed for 1501-PC (Supporting Information Figure S15). Hence, swapping of cubane side-chains with tetra-substituted, chiral analogs in 1501 does not suppress binding capacity to PvLDH although the K_D value slightly increased, but the high degree of specificity of the parent aptamer was lost.

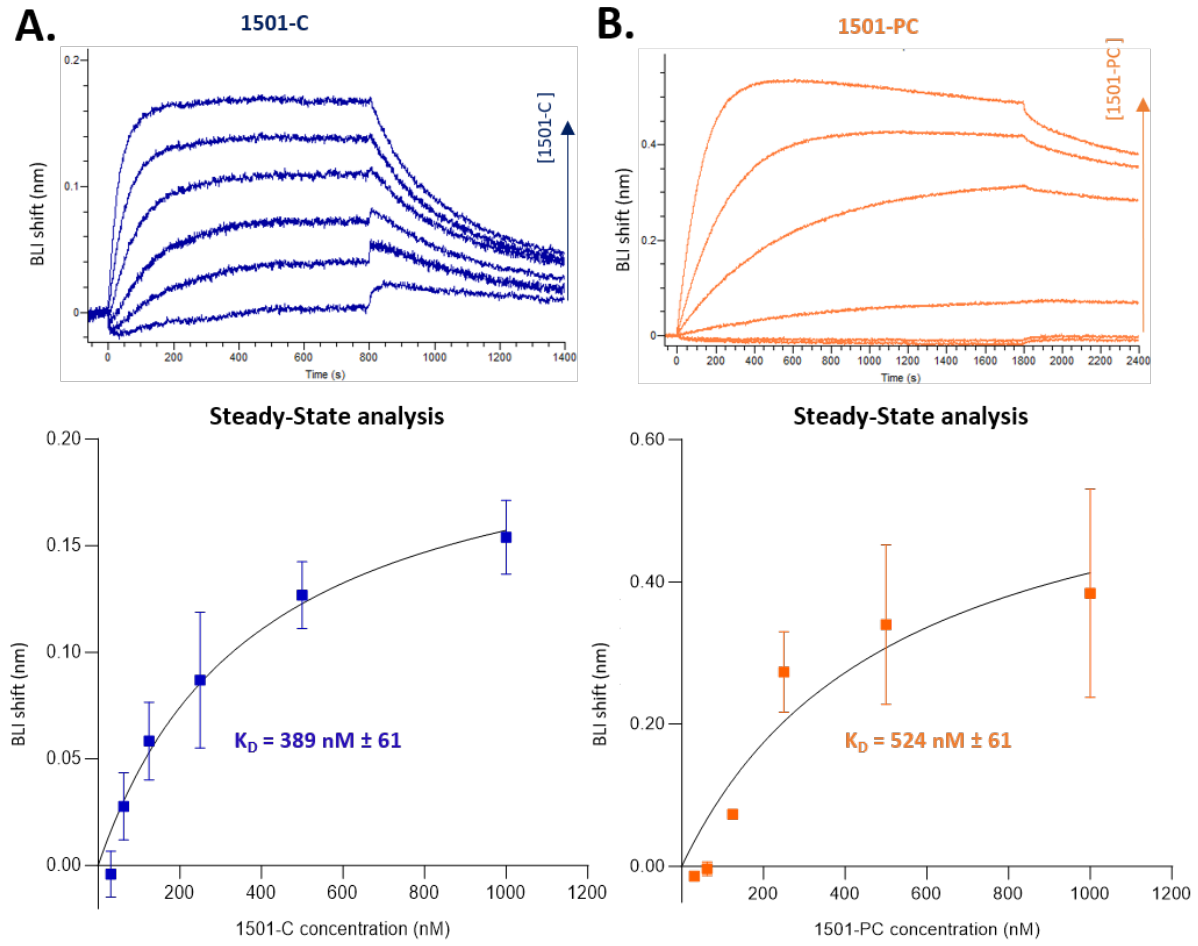


Figure 6. Affinity measurement of 1501-C and 1501-PC for PvLDH protein measured by BLI. We measured, in two independent duplicates, binding for target PvLDH protein at different concentrations of modified 1501 sequences (from 1 µM to 31.25 nM). Top portion of the figure represents binding sensograms of one of the two duplicates. Bottom part displays nonlinear curve fitting of steady-state analysis calculated for the two duplicates using signals at the end of the association step. **A)** 1501-C affinity measurements were performed in PvLDH buffer with 0.5 mg·mL^{-1} BSA and 0.005% tween, association step was lengthened to 800 s to reach equilibria. **B)** 1501-PC affinity measurements were performed in PvLDH buffer, association step was lengthened to 1800 s to reach equilibria.

3. Discussion

Post-SELEX engineering can improve the overall performance of functional nucleic acids and concomitantly reduce their size. However, whether truncation, chemical modification or partial mutagenesis are applied, most post-SELEX approaches are labor-intensive and have an uncertain outcome.[37] Here, we sought to accelerate post-SELEX improvement of a cubane-modified aptamer capable of binding to a malaria biomarker and high discrimination of a related protein target by merging medicinal chemistry and enzymatic synthesis. An important step in accelerating post-SELEX protocols is the reduction of the synthetic burden. To do so, we

introduced a common synthetic intermediate for the production of modified nucleotides and we have opted for enzymatic rather than solid-phase synthesis. By this approach we have introduced functionally similar modification patterns, either by direct bioisosteric replacement or by alteration of the substitution pattern of cubanes, directly into aptamers. Interestingly, substitution of cubanes with a benzene completely rescinds the binding capacity of 1501. This finding is not totally surprising by inspecting the crystal structure of the 1501-PvLDH complex since replacing bulky cubanes with flat, aromatic benzene moieties is expected to have severe implications in the binding mode and capacity. On the other hand, this finding has an important implication. Indeed, while cubane-modified drugs often trigger a similar or better response from a biological system than the corresponding parent, aromatic compounds, this does not hold for aptamer binding. Similarly, ferrocene often acts as a good surrogate of aromatic derivatives for the production of drugs with enhanced properties,[23a] but this does not seem to be the case for binding affinity of aptamers. The only modified sequence that maintained a similar binding affinity to that of the parent 1501 was 1501-PC which is equipped with a tetrasubstituted, chiral cubane moiety. This is somewhat surprising since this extensive substitution modification pattern was expected to abrogate rather than maintain binding of the resulting aptamer (which is the case for 1501-SC). However, maintenance of binding activity comes at the expense of loss of specificity since 1501-PC displayed a weak, yet non-negligible interaction with streptavidin. These properties might be improved by diastereoselective synthesis of the chiral cubane residues followed by incorporation onto the nucleobase of nucleotide analogs.[38] On the other hand, all modified 1506 sequences, including with unsubstituted cubane, are not capable of interacting with PvLDH despite being over-represented in the enriched pool of the 15th round of the SELEX experiment.

4. Conclusions

In the present study, we have evaluated the possibility of post-SELEX modification of aptamers by combining enzymatic synthesis with nucleotides modified with similar functional side-chains to that of the parent sequence. This process allows a reduction of the synthetic burden often associated with post-SELEX strategies. In addition, one specific sequence, 1501-PC, was identified by this process and shown to maintain binding to target PvLDH despite bearing a more complex substitution pattern than the parent cubane moiety. On the other hand, while bioisosteric substitution is a common and efficient method for the identification of more potent small molecule drugs, bioisosteres do not appear to trigger the same response, i.e. binding, in the context of aptamers.

This enzymatic, post-SELEX approach might be further improved by controlled enzymatic synthesis[39] or ligation[40] which would permit the introduction of modified nucleotides at site-specific locations or multiple nucleotides with different substation patterns. Finally, cubanes offer the possibility of direct conversion to other scaffolds such as cuneanes[41] or 1-azahomocubanes[42] either at the level of the nucleotides or once installed within the sequences which augments the number of modified residues to be processed by this post-SELEX approach without the need of long synthetic campaigns. Such a protocol could be amenable to other functional nucleic acids such as DNAzymes where intensive post-SELEX protocols are required to improve their performance.

5. Experimental Section

General protocol for the synthesis of modified nucleotides:

The suitable azide and 5-ethynyl-dUTP[43] are dissolved in 100 µL of DMF and 100 µL H_2O in a 1.5 mL Eppendorf tube and degassed under Argon. In a separated Eppendorf tube, CuI (2 eq.) are suspended in *N*,*N*-diisopropylethylamine (DIPEA, 6 eq.), 20 µL H_2O and 100 µL MeCN and degassed with Argon. The two fractions were merged together and 200 µL DMF were added. The reaction mixture was degassed with Argon and left to shake at 1000 rpm, at RT for 4 hours. The reaction mixture was evaporated *in vacuo* and resuspended in 300 µL H_2O. The mixture was poured in 12 mL $NaClO_4$ 2% in acetone to precipitate the crude product, centrifuged at 4000 rpm for 15 minutes, and the supernatant was discarded. The crude was dissolved in 3 mL H_2O and purified by semi-preparative reverse phase HPLC (50% B in 25 minutes; Buffer A, 20 mM TEAA in H_2O; Buffer B, 30% 20 mM TEAA in H_2O:CH_3CN = 3/7) with a Kinetex 5 µm C18 100 Å LC column. Prior to each HPLC injection, 50 µL 0.5 M EDTA in H_2O (pH=8) are added to the crude triphosphate to remove excess copper.

Nucleotide $dU^{B}TP$ ***2***

This modified nucleotide was obtained by reacting azide **6** (3.2 mg, 5.3 µmol, 1.3 eq.) with 5-ethynyl-dUTP (2 mg, 4 µmol, 1 eq.) by application of the general protocol and was obtained as a white solid (1.9 mg, 66%).

^{1}H NMR (500 MHz,D_2O) δ 8.24 (s, 1H), 8.12 (s, 1H), 7.53-7.35 (m, 5H), 6.21-6.18 (t, 1H, *J* = 6.2 Hz), 4.61-4.59 (m, 2H), 4.54-4.51 (m, 1H), 4.10-4.04 (m, 3H), 3.79-3.76 (m, 2H), 2.38-2.27 (m, 2H).

^{31}P NMR (202 MHz, D_2O) δ -10.41 (d, *J* = 19.9 Hz), -11.49 (d, *J* = 20.0 Hz), -23.26 (t, *J* = 20.2 Hz).

HRMS (ESI) calcd for $C_{20}H_{25}N_6O_{15}P_3$, $[M-H]^-$ = 681.0518, found $[M-H]^-$ = 681.0517.

Nucleotide $dU^{F}TP$ ***3***

This modified nucleotide was obtained by reacting azide **12** (10.2 mg, 0.04 mmol, 4 eq.) with 5-ethynyl-dUTP (2 mg, 4 µmol, 1 eq.) by application of the general protocol and was obtained as a white solid (5.7 mg, 75%). ^{1}H and ^{31}P NMR spectra could not be analyzed due to partial oxidation of the ferrocene moiety.[44]

HRMS (ESI) calcd for $C_{24}H_{30}FeN_5O_{14}P_3$, $[M]^-$ = 761.04, found $[M-H]^-$ = 760.0282

Nucleotide $dU^{PC}TP$ ***4***

This modified nucleotide was obtained by reacting azide **17** (4.5 mg, 0.014 mmol, 1.8 eq.) with 5-ethynyl-dUTP (3.9 mg, 0.0079 mmol, 1 eq.) by application of the general protocol and was obtained as a white solid (4.5 mg, 64%).

^{1}H NMR (500 MHz, D_2O) δ 8.37 (s, 1H), 8.31 (s, 1H), 7.49 – 7.27 (m, 5H), 6.36 (t, *J* = 6.9 Hz, 1H), 5.04 – 4.98 (m, 1H), 4.68 (s, 1H), 4.63 (t, *J* = 5.8 Hz, 2H), 4.27 – 4.19 (m, 3H), 4.09 – 4.02 (m, 1H), 4.02 – 3.93 (m, 1H), 3.86 – 3.71 (m, 4H), 3.21 (q, *J* = 7.3 Hz, 24H), 2.54 – 2.42 (m, 2H), 1.48 (d, *J* = 7.0 Hz, 3H), 1.29 (t, *J* = 7.2 Hz, 35H), 1.10 (d, *J* = 1.6 Hz, 3H), 1.03 (d, *J* = 4.8 Hz, 3H).

^{31}P NMR (202 MHz, D_2O) δ -10.63 (d, *J* = 19.9 Hz), -11.57 (d, *J* = 21.1 Hz), -23.42 (t, *J* = 20.5 Hz).

HRMS (ESI) calcd for $C_{33}H_{40}N_7O_{16}P_3$, $[M-H]^-$=882.1672, found $[M-H]^-$= 892.1672.

Nucleotide $dU^{SC}TP$ ***5***

This modified nucleotide was obtained by reacting azide **21** (4.5 mg, 0.0107 mmol, 1.5 eq.) with 5-ethynyl-dUTP (3.5 mg, 0.007 mmol, 1 eq.) by application of the general protocol and was obtained as a white solid (3.2 mg, 49%).

^{1}H NMR (500 MHz, D_2O) δ 8.37 (s, 1H), 8.31 (s, 1H), 6.35 (t, *J* = 6.8 Hz, 1H), 4.69 – 4.65 (m, 1H), 4.62 (t, *J* = 5.5 Hz, 2H), 4.23 (d, *J* = 4.6 Hz, 3H), 4.19 (q, *J* = 4.1 Hz, 4H), 3.74 (d, *J* = 3.8 Hz, 1H), 3.73 (s, 3H), 3.68 – 3.63 (m, 3H), 3.20 (q, *J* = 7.3 Hz, 33H), 3.16 – 2.97 (m, 6H), 2.68 (t, *J* = 7.3 Hz, 2H), 2.54 – 2.40 (m, 2H), 1.28 (t, *J* = 7.3 Hz, 48H).

^{31}P NMR (202 MHz, D_2O) δ -10.79 (d, *J* = 19.9 Hz), -11.62 (d, *J* = 19.9 Hz), -23.46 (t, *J* = 19.9 Hz).

HRMS (ESI) calcd for $C_{29}H_{38}N_7O_{19}P_3S$, $[M-H]^-$ = 912.1083, found $[M-H]^-$ = 912.1086.

General protocol for PEX reactions:

The 5'-FAM-labelled primer (10 pmol) was annealed to the appropriate template (15 pmol) in DNase/RNase-free ultrapure water by heating to 95°C and gradually cooling down to room temperature over one hour. The appropriate DNA polymerase, the adequate reaction buffer (1 μL of 10X or 2 μL of 5X), and the dNTP(s) were then added to the reaction mixture for a total reaction volume of 10 μL. The reaction mixture was incubated for the appropriate time at the optimal temperature for the polymerase. The incubation temperatures associated to each polymerase were respectively: Phusion (60°C), Hemo KlenTaq (60°C), Taq (60°C), *Bst* (60°C), Q5 DNA polymerase (60°C), Therminator (60°C), Vent (*exo*$^-$) (60°C), Dpo4 (55°C), Deep Vent (55°C), Phi29 (37°C), Kf (*exo*$^-$) (37°C). The reaction was stopped by adding 10 μL of the quenching solution (formamide (70%), ethylenediaminetetraacetic acid (EDTA, 50 mM), bromophenol (0.1 %), xylene cyanol (0.1 %)). The reaction mixtures were subjected to gel electrophoresis in denaturing polyacrylamide gel (20 %) containing trisborate– EDTA (TBE) 1× buffer (pH 8) and urea (7 M).

General protocol for PCR

The primers (5 μM each), the template (0.01 μM), the dNTP(s) (200 μM), Mg^{2+} (2 mM), the polymerase (0.4 μL) and the appropriate polymerase buffer (2 μL of 10X, 4 μL of 5X) were mixed together in a total volume of 20 μL. Each PCR reaction consisted of an initial denaturation of 5 min at 95°C, followed by 25 cycles composed of 1 min at 95°C, 1 min at 52°C, 2 min at 72°C, and a final extension step of 5 min at 72°C. The PCR products were analyzed by agarose gels (4%) with E-GEL sample loading buffer (1X).

Synthesis of modified nucleosides from the corresponding triphosphates

100 pmoles of modified nucleotide were combined with 10 μL rSAP and 2.2 μL of 10X rCutSmart buffer, incubated at 37 °C for 1 hour. rSAP was subsequently inactivated by a 10-minute exposure to 65°C. The final products (modified nucleosides) were purified and subjected to LC-MS for characterization.

General Protocol for digestion-LC-MS analysis of modified DNA

Primer **P4** (100 pmol) was annealed with the template **T3** (150 pmol) in DNase/RNase-free ultrapure water. This was carried out by first raising the temperature to 95°C, then gradually cooling down to room temperature over a period of one hour. The resulting solution was mixed with 1 μL of Vent (*exo*$^-$) polymerase, 1 μL of Thermopol buffer (supplied by the DNA

polymerase supplier) and 200 µM of modified dUTP or dTTP for the positive control, giving a total reaction volume of 10 µL. Next, the combined mixture was left to incubate for a period of four hours at a temperature of 60°C. Next, 1 µL of the reactions in 9 µL H_2O were quenched by adding the stop solution. The reaction mixtures were subjected to gel electrophoresis in denaturing polyacrylamide gel (20%) containing trisborate- EDTA (TBE) 1× buffer (pH 8) and urea (7 M).

After gel confirmation, products were purified using Monarch DNA Cleanup columns (5 µg), each column processing a maximum of 250 pmol of product. The purified products (around 100 pmol in 10 µL) was then combined with Nucleoside Digestion Mix buffer (2 µL of 10X) and Nucleoside Digestion Mix (1 µL) in a final volume of 20 µL. The solution was left to incubate at 37°C for one hour. Finally, the resulting products were subjected to LC-MS analysis (see below) without further purification.

A solution of digested dsDNA was introduced into a ThermoFisher Hypersil Gold aQ chromatography column (100 X 2.1 mm, with a particle size of 1.9 µm), maintained at a temperature of 30°C. Flow rate was set at 0.3 ml/min, and isocratic elution was performed at 1% MeCN in H2O with 0.1% formic acid for 8 minutes, then at 100% CH_3CN from the 9th to the 11th minute. In positive ion mode, parent ions were fragmented using a normalized collision energy of 10% in PRM (Parallel Reaction Monitoring) mode. MS2 resolution was set at 17,500 with an AGC target of 2e5, a maximum injection time of 50 ms and an isolation window of 1.0 *m/z*. The inclusion list contained the following masses: dC (228.1), dA (252.1), dG (268.1), dT dT (243.1), dU^C (469.18), dU^B (443.17), dU^F (520.14), dU^{PC} (644.28), dU^{SC} (674.22). For detection, chromatograms of ions extracted from the base fragments (± 5 ppm) were used (112.0506 Da for dC; 136.0616 for dA; 152.0565 Da for dG; 127.0501 Da for dT, dU^C (353.13), dU^B (327.12), dU^F (404.1), dU^{PC} (528.23), and dU^{SC} (558.17). To confirm assignment (fragment ion and retention time), synthetic standards were injected beforehand.

Bio-layer interferometry (BLI) binding studies

BLI experiments were performed on an Octet RED384 instrument (ForteBio). All measurements were performed on 96-well plates agitated at 1000 rpm at 25°C. The PvLDH-buffer used was, as described previously,[19] composed of 25 mM Tris-HCl, 100 mM NaCl and 20 mM imidazole, the final solution filtrated over 0.22 µm filters.

For the first screening study, His-PvLDH protein (produced as described previously[19]) was diluted in PvLDH-buffer supplemented with 0.5 $mg.mL^{-1}$ bovine serum albumin (BSA) and 0.005 % tween, was captured at 20 $\mu g.mL^{-1}$ on Ni-NTA biosensors during 800 s, to reach a signal of 3.5 nm. Association and dissociation phases were monitored during 600 s each. When oligonucleotide binding measurement was performed in buffer containing no BSA or tween, a new baseline was established during 10 minutes in the new buffer. At the end of each cycle (PvLDH loading, oligonucleotides association/dissociation steps) sensors were regenerated with glycine solution (pH 1.5) and EDTA 0.5 M, for 5 s each. This regeneration process was repeated 3 times.

For the next experiments, some parameters were optimized: His-PvLDH protein was captured at 40 $\mu g.mL^{-1}$ on Ni-NTA biosensors during 450 s to reach a signal of 4. The regeneration process was repeated 4 times, with 30 s for each step.

Precise affinity measurements were performed for 1501-C and 1501-PC only. Binding was monitored in PvLDH-buffer supplemented with BSA 0.5 $mg.mL^{-1}$ and 0.005 % tween for 1501-C, whereas binding to 1501-PC was performed in PvLDH-buffer only. Association phase was lengthened to reach equilibria: 1501-C was monitored for 800 s, and 1501-PC for 1800 s. Six serial oligonucleotide concentrations were assayed for each oligonucleotide: 31.25, 62.5, 125,

250, 500 and 1000 nM. Buffer-only reference was subtracted from all curves. Affinities were determined by fitting the concentration dependence of the experimental steady-state signals with the GraphPad Prism software (version 10.0.0), using the non-linear regression model "Binding-Saturation, One-site specific binding".

For binding specificity tests, the experimental layout was the same as described above except that the loading concentration of Streptavidin His-tag was 5µg·mL^{-1}.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Post-SELEX modification of aptamers allows to improve their performance but comes at the cost of uncertain and labor-intensive structure activity relationship studies. Here, we have evaluated the possibility of improving a previously reported, chemically modified aptamer by combining enzymatic synthesis and nucleotides bearing bioisosteres of the parent cubane side-chains or substituted cubane moieties. This method lowers the synthetic burden associated with post-SELEX approaches and allowed to identify one additional sequence that maintains binding to the PvLDH target protein, albeit with reduced specificity.