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Tetrathiafulvalene-Labelled Nucleosides and Nucleoside Triphosphates: Synthesis, Electrochemistry and the Scope of Their Polymerase Incorporation into DNA

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The title 5-substituted pyrimidines (U and C) and 7-substituted 7-deazapurines (7-deazaA and 7-deazaG) bearing tetrathiafulvelene (TTF) attached through an acetylene linker have been prepared by Sonogashira cross-coupling of the corresponding 5- or 7-iodo derivatives of nucleosides with 2-ethynyltetrathiafulvalene. Their subsequent triphosphorylation gave the corresponding nucleoside triphosphates (dNTPs). Square-wave voltammetry of the TTF-labelled nucleosides and nucleotides showed two peaks, one at 0.2–0.3 V and the other at around 0.65 V (vs. Ag|AgCl|3 M KCl), which correspond to two reversible one-electron redox processes in the TTF moiety. Polymerase incorporation of the TTF-labelled dNTPs into DNA has also been studied. Multiple incorporations were rather problematic and only by using **dC^{TTF}TP** was efficient primer extension observed with Vent (*exo*-)polymerase. Single nucleotide extension was successful with labelled A (**dA$^{\star TTF}$TP**) and C (**dC^{TTF}TP**) nucleotides. Inhibition of the polymerase was observed at higher concentrations of **dN^{TTF}TP**s.

Introduction

Base-functionalized nucleic acids are of great current interest due to their applications in chemical biology, bioanalysis, nanotechnology and materials science.[1] Apart from chemical synthesis, base-modified DNA can be prepared enzymatically[2] by polymerase incorporations of base-modified nucleoside triphosphates (dNTPs),[3] which can be further combined with post-synthetic modifications by click chemistry[4] or Staudinger ligation.[5] Particularly efficient is the single-step synthesis of modified dNTPs by aqueous cross-coupling reactions[6] followed by polymerase incorporation. We have recently used this novel approach[7] for the synthesis of DNA bearing amino acids,[8] ferrocenes,[9] amino- and nitrophenyl groups[10] and [Ru/Os(bpy)$_3$] complexes.[11] 5-Substituted pyrimidine dNTPs are usually good substrates for DNA polymerases, whereas 8-substituted purine dNTPs have repeatedly been shown[3c,8,12] to be poor substrates that should be replaced by 7-substituted 7-deazapurine dNTPs.

Tetrathiafulvalene (TTF) is a unique molecule of paramount importance[13] in materials science due to its π-electron-donating and redox properties, and its conjugates and derivatives find numerous applications, for example, in supramolecular chemistry,[14] molecular electronics[15] and organogels.[16] The conjugates of nucleobases[17] with TTF have been prepared and used for self-assembly in crystals. TTF has also been incorporated into oligonucleotides (ONs) as an artificial nucleobase[18] or annulated to the uracil base in phosphorothioate ONs.[19]

Herein we report on our attempts to construct base-modified DNA labelled with TTF. The TTF group could be potentially used in numerous applications. As a redox-active molecule it can be used as an electrochemical marker for electrochemical detection with two distinct redox potentials complementing some of the previously used[9–11] redox markers. Even more attractive could be its use in self-assembly. Donor–acceptor (D–A) complexes of TTF derivatives with π-acceptor macrocycles (e.g., paraquat cyclophanes) are often used[15] in the construction of rotaxanes, molecular shuttles and other supramolecules, and the most important feature of TTF-based D–A complexes is the possibility of reversibly switching them off and on by electrochemical oxidation/reduction. Such reversible switchable complexes with DNA could in principle be used for temporary bioconjugation and for the isolation of modified DNA.

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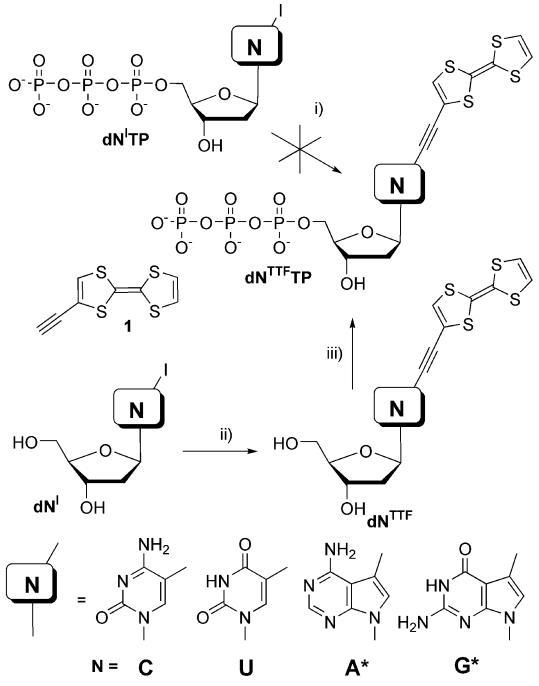
Results and Discussion

To prepare the TTF-modified dNTPs, we first tried to apply our single-step aqueous Sonogashira cross-coupling of halogenated dNTPs, which has been successful in all our previous cases for the attachment of terminal acetylenes bearing ferrocene,[9] amino acids[8] or Ru/Os complexes.[11] The corresponding terminal acetylene of choice was 2-ethynyl-TTF (**1**), which was prepared according to the reported modified procedure.[20] Lithiation of TTF followed by treatment with 1,2-diiodoethane gave 2-iodo-TTF in 84% yield. Its Sonogashira reaction with trimethylsilylacetylene in triethylamine and THF for 3 h catalysed by CuI and $[Pd(PPh_3)_2Cl_2]$ gave 2-(TMS-ethynyl)TTF (77%), which was finally desilylated by treatment with 10 equiv. of KF for 3 h to furnish the desired ethynyl-TTF (**1**) in 74% yield. This compound was quite unstable and darkened within several weeks.

The reactions of 5-I-dCTP, 5-I-dUTP, and 7-I-7-deaza-dATP with **1** were performed under the previously developed conditions in the presence of $Pd(OAc)_2$, triphenylphosphane 3,3′,3′′-trisulfonate (TPPTS), CuI and Et_3N in water/acetonitrile (2:1) at 70 °C for 1 h. Unfortunately, only partial or complete hydrolysis products of the dNTPs were identified in the reaction mixture and no TTF-modified dNTPs were isolated (Scheme 1).



Scheme 1. Reagents and conditions: i) **1**, $Pd(OAc)_2$, TPPTS, CuI, Et_3N, H_2O/MeCN (2:1), 70 °C, 1 h; ii) **1**, $[Pd(PPh_3)_2Cl_2]$, CuI, Et_3N, DMF, 70 °C, 3 h; iii) 1. $PO(OEt)_3$, $POCl_3$; 2. $(NHBu_3)_2H_2P_2O_7$, DMF; 3. TEAB.

Therefore, the tactics of the synthesis had to be changed to the more classical approach: the synthesis of the modified nucleosides and their triphosphorylation. For the Sonogashira reactions of 5-iodopyrimidine and 7-iodo-7-deazapurine deoxyribonucleosides we used our recent experiences from the synthesis of related nucleoside derivatives bearing acetylene-linked bpy ligands.[21] The reactions of 5-iodocytidine (**dC^{I}**), 5-iodouridine (**dU^{I}**), 7-deaza-7-iodoadenosine (**dA*^{I}**) and 7-deaza-7-iodoguanosine (**dG*^{I}**) with ethynyl-TTF (**1**) were performed in DMF in the presence of $[Pd(PPh_3)_2Cl_2]$ as catalyst (Scheme 1, Table 1). The desired TTF-modified nucleosides **dN^{TTF}s** were obtained in acceptable yields (61–69%). Only the coupling reaction of **dG*^{I}** gave a lower yield (35%) of **dG*^{TTF}**, probably because of stronger complexation of the Pd catalyst to the 7-deazaguanine moiety. The TTF-nucleosides **dN^{TTF}s** were found to be of limited stability and darkened on standing within several weeks.

Table 1. Synthesis of 7-deaza 2′-deoxyadenosine conjugates.

Entry	N	% Yield of **dN^{TTF}**	% Yield of **dN^{TTF}TP**
1	**C**	65	25
2	**U**	61	27
3	**A***	69	39
4	**G***	35	21

Triphosphosphorylation of the **dN^{TTF}s** was performed under classical conditions[22] by treatment with $POCl_3$ in $PO(OEt)_3$ followed by the addition of $(NHBu_3)_2H_2P_2O_7$ and treatment with TEAB (Scheme 1). The desired TTF-modified nucleoside triphosphates (**dN^{TTF}TPs**) were isolated by RP-HPLC in moderate yields (21–39%, Table 1).

The UV spectra (Table 2) of the TTF-modified nucleosides and dNTPs exhibit a strong absorption band at 300 nm and a weaker one at 394–416 nm responsible for yellow/orange colour of the compounds. The electrochemical properties of the modified nucleosides and **dN^{TTF}TPs** were studied by square-wave voltammetry (SWV) at a glassy carbon electrode. All substances produced two reversible peaks corresponding to two consecutive one-electron redox processes in the TTF moiety[20] (Table 2, Figure 1, a). Compared with free TTF, the apparent potentials of both peaks were shifted to more positive values. Such behaviour has been observed previously with base, nucleoside and dNTP conjugates with ferrocene and was attributed to the electron-withdrawing effects of the nucleobase through the unsaturated ethynyl bridge, which makes oxidation of the conjugate electroactive group less feasible.[9] The nucleosides and **dN^{TTF}TPs** of 7-deazapurines produced another peak (Figure 1, a) as a result of the irreversible oxidation of the G* (ca. 0.9 V) or A* (ca. 1.1 V) moieties.[11]

The enzymatic incorporation of the TTF-modified **dN^{TTF}TPs** in primer extension (PEX) experiments was studied by using Klenow, DyNAzyme, Vent (*exo*-) and Pwo polymerases, similarly to our previous work (for sequences of templates and primer, see Table 3). Multiple incorporations into a random sequence containing all four bases (temp^{rnd16}) were tested separately with each of the four **dN^{TTF}TPs** to show that Vent (*exo*-) polymerase was more efficient (Figure 2) compared with Pwo (or other enzymes – not shown). However, only **dC^{TTF}TP** gave a promising (though not fully clean) spot of a fully extended ON, whereas the other **dN^{TTF}TPs** gave mixtures of partly ex-

Table 2. UV/Vis spectral data and redox properties of the TTF-modified nucleosides and dNTPs.

Entry	Compound	λ [nm] (ε [10^4 M^{-1} cm^{-1}])[a]	E^{ox} [V][b]
1	**TTF**	–	0.18, 0.54
2	**dC^{TTF}**	306 (2.1), 413 (0.33)	0.30, 0.61
3	**dU^{TTF}**	305 (2.4), 416 (0.38)	0.29, 0.59
4	**dA*^{TTF}**	301 (2.2), 408 (0.38)	0.33, 0.61
5	**dG*^{TTF}**	314 (2.2), 402 (0.35)	0.27, 0.60
6	**dC^{TTF}TP**	304 (1.5), 406 (0.27)	0.26, 0.64
7	**dU^{TTF}TP**	309 (2.5), 405 (0.45)	0.27, 0.64
8	**dA*^{TTF}TP**	296 (2.3), 402 (0.42)	0.23, 0.64
9	**dG*^{TTF}TP**	315 (2.5), 394 (0.48)	0.25, 0.64

[a] Nucleosides in MeOH, dNTPs in H_2O, all 5×10^{-5} M solutions. [b] Apparent redox potentials (V vs. Ag/AgCl/3 M KCl reference electrode) measured on the net square-wave voltammograms for 20 μM of substrates on the GCE (frequency: 200 Hz, amplitude: 50 mV, supporting electrolyte: 0.2 M sodium acetate, pH 5.0).

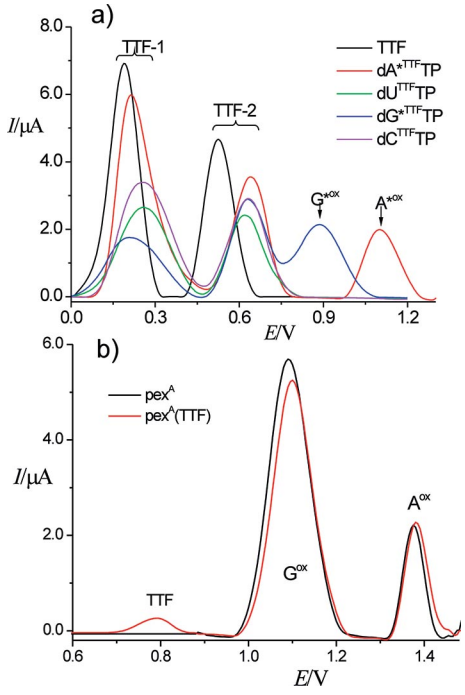


Figure 1. Square-wave voltammetric responses of (a) TTF and TTF-modified dNTPs and (b) PEX products pex^A (unmodified) and pex^A(TTF) bearing a TTF-modified A. The conditions used to record the responses in (a) are given in Table 2; in (b), a pyrolytic graphite electrode (PGE) was used instead of a GCE. The PEX products were synthesized by using a 5′-biotinylated template temp^A and the extended primer strands were separated by using streptavidin-coated magnetic beads.

tended ONs of different lengths (although clearly the **dN^{TTF}TP**s were partly incorporated because the PEX products are longer than negative controls).

To increase the efficiency of the incorporation of the modified **dN^{TTF}TP**s, we tried to increase the concentration of the modified **dN^{TTF}TP**s to 10 times that of the natural dNTPs. Surprisingly, almost complete inhibition of the PEX reaction by **dA*^{TTF}TP**, **dU^{TTF}TP** and **dG*^{TTF}TP** resulted (virtually only the primer is observed in the PAGE analysis, Figure 3) and very limited incorporation of

Table 3. Primers and templates used for primer extension.[a]

prim^{rnd}	5′-CATGGGCGGCATGGG-3′
temp^{rnd16}	5′-**CTAGCAT-GAGCTCAGT***CCCATGCCGCCCATG*-3′
temp^C	5′-**CCCG***CCCATGCCGCCCATG*-3′
temp^U	5′-**CCCA***CCCATGCCGCCCATG*-3′
temp^A	5′-**CCCT***CCCATGCCGCCCATG*-3′
temp^G	5′-**AAAC***CCCATGCCGCCCATG*-3′

[a] In the template (temp) ONs the segments that form a duplex with the primer are printed in *italics*, the replicated segments are printed in **bold**. For magnetic separation of the extended primer strands, the templates were 5′-end biotinylated. The acronyms used in the text for primer extension products are analogous to those introduced for the templates (e.g., the PEX product pex^{rnd16} was synthesized on temp^{rnd16} template).

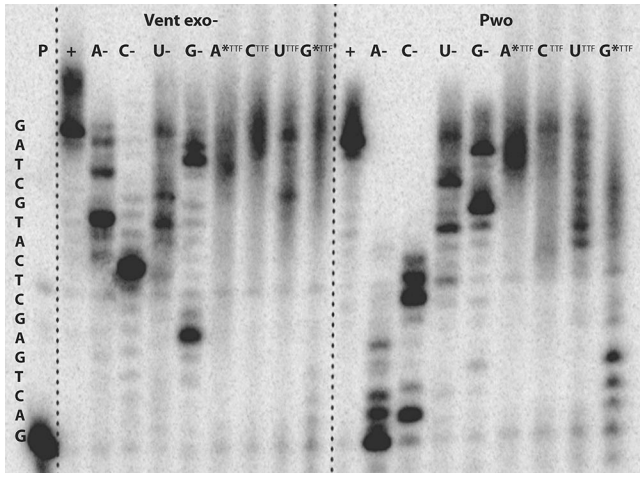


Figure 2. Denaturing PAGE analysis of the PEX experiments using the Vent (*exo*-) and Pwo polymerases and temp^{rnd16}.

dC^{TTF}TP. This inhibition is interesting because it apparently does not proceed by termination of the primer by the modified nucleotide (which is the common mechanism of inhibition of polymerases by triphosphates of nucleoside therapeutics), but even incorporation of unmodified dNTPs into the primer is almost totally prevented. This may indicate a strong (non-covalent or even covalent) binding of the modified dNTPs to the polymerase.

To obtain clean PEX products for the electrochemical study, we also studied single nucleotide incorporations of **dN^{TTF}TP**s and the incorporation of one modified **dN^{TTF}TP** followed by three natural nucleobases using templates (temp^A, temp^C, temp^U and temp^G). To minimize the misincorporations and erroneous elongations of the synthesized strand we lowered the dNTP concentration to 5 μM and the polymerase concentration to 0.002 U μL^{-1}. Under these conditions we were able to obtain reasonably clean PEX products containing one **C^{TTF}** or **A*^{TTF}** modification (Figure 4), whereas **dU^{TTF}TP** gave a mixture of products and **dG*^{TTF}TP** was not incorporated at all (not shown). Apparently, the polymerase incorporation of TTF-modified dNTPs is rather problematic, which could be a result of the lower stability of the TTF labels, but maybe also a result

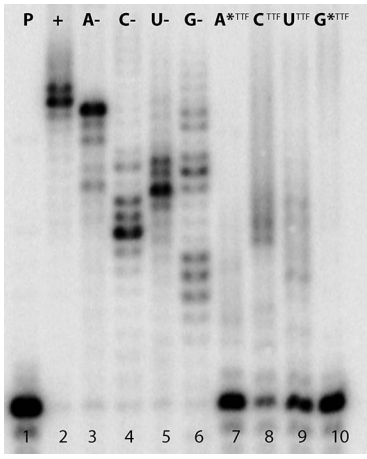


Figure 3. Denaturing PAGE analysis of PEX experiments using Vent (*exo*-) polymerase and the temp^{rnd16} template with a concentration of each **dN^{TTF}TP** 10 times higher than natural dNTPs. Lanes 7, 9 and 10 show full inhibition of PEX.

of the interaction or even chemical reactivity of TTF with polymerase, which causes inhibition of the polymerase at higher concentrations of **dN^{TTF}TP**s.

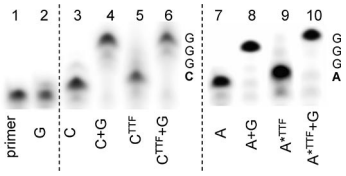


Figure 4. Denaturing PAGE analysis of the incorporation of **dC^{TTF}TP**s or **dA*^{TTF}TP**s using the Vent (*exo*-) polymerase and temp^{C} or temp^{A} templates, respectively. Compositions of the dNTP mixes and nucleotide labelling are given below the lanes; 0.7 μM primer, 1 μM templates, 5 μM dNTPs and 0.002 U μL^{-1} polymerase were used.

By using the 5′-biotinylated templates temp^{A} and temp^{C} we performed the same PEX reactions as shown in Figure 4 and separated the extended primer strands by using streptavidin-coated magnetic beads.[9–11] Ex situ SWV at a pyrolytic graphite electrode showed small reversible signals between 0.75 and 0.8 V, which were produced by the PEX product synthesized in the presence of **dA*^{TTF}TP** (Figure 1, b) or **dC^{TTF}TP** (not shown), but not by unlabelled PEX products. Hence, the additional peak could be attributed to the TTF nucleotide conjugate thus revealing its successful incorporation. A positive shift of the peak potential due to incorporation of the labelled nucleobase into DNA, as compared with the more positive TTF peak of modified nucleosides and dNTPs (Table 1, Figure 4), is in accord with previous observations made with other types of conjugate labels.[9,10]

Conclusions

Ethynyl-TTF derivatives of pyrimidine and 7-deazapurine nucleosides have been prepared by Sonogashira cross-coupling reactions of halogenated nucleosides with ethynyl-TTF and were subsequently converted into TTF-modified dNTPs. Polymerase incorporation of the **dN^{TTF}TP**s was rather problematic and at higher concentrations they inhibited the polymerase. Nevertheless, it is possible to incorporate **A*^{TTF}** and **C^{TTF}** modifications into some sequences. The inhibition (although an unwanted complication of the project) is interesting because it does not proceed by termination of the primer. It is believed[7] that dNTPs bearing small modifications in the 5-position of pyrimidines or the 7-position of 7-deazapurines attached through an acetylene linker should be very well incorporated by DNA polymerases. However, herein we have shown that inhibition of the polymerase can also occur with some modified dNTPs and should be taken into consideration in the design of further modifications of DNA to be introduced by polymerase incorporation.

The modified DNA bearing a single TTF moiety gives a relatively weak redox signal from TTF, which appears not to be very promising for sensitive electrochemical detection. However, other applications of the TTF label (e.g., C-T complexes) could be envisaged in the future if other (more stable) substituted TTF derivatives are prepared and tolerated by the polymerase. Studies in this direction will follow in our laboratory.

Experimental Section

All cross-coupling reactions were performed under argon. Et_3N was degassed in vacuo and stored over molecular sieves under argon. Compounds **1**[20] and **dA*^{I}**[8] were prepared according to literature procedures. **dG*^{I}** was purchased from ChemBiotech, Ltd. (Germany) and **dC^{I}** and **dU^{I}** were supplied by Berry & Associates (USA).

General Procedure for Sonogashira Cross-Coupling Reactions of Nucleoside Analogues dN^{I} with 2-Ethynyltetrathiafulvalene: Dry DMF (2 mL) was added to an argon-purged flask containing 2-ethynyltetrathiafulvalene (68.5 mg, 0.3 mmol, 2 equiv.), nucleoside analogue **dN^{I}** (0.15 mmol, 1 equiv.), CuI (2.9 mg, 0.015 mmol, 10 mol-%) and $[Pd(PPh_3)_2Cl_2]$ (5.2 mg, 0.0075 mmol, 5 mol-%) followed by Et_3N (0.2 mL, 1.5 mmol, 10 equiv.). The reaction mixture was stirred at 70 °C for 3 h until complete consumption of the starting material and then evaporated in vacuo. The products were purified by silica gel column chromatography using chloroform/methanol (0 to 10%) as eluent.

dC^{TTF}: The product was isolated as orange solid (44.1 mg, 65%). ^{1}H NMR (500 MHz, CD_3OD): δ = 2.16 (ddd, J_{gem} = 13.6, $J_{2'b,1'}$ = 6.5, $J_{2'b,3'}$ = 6.1 Hz, 1 H, 2′b-H), 2.40 (ddd, J_{gem} = 13.6, $J_{2'a,1'}$ = 6.1, $J_{2'a,3'}$ = 4.2 Hz, 1 H, 2′a-H), 3.74 (dd, J_{gem} = 12.1, $J_{5'b,4'}$ = 3.6 Hz, 1 H, 5′b-H), 3.85 (dd, J_{gem} = 12.1, $J_{5'a,4'}$ = 3.1 Hz, 1 H, 5′a-H), 3.95 (ddd, $J_{4',3'}$ = 3.8, $J_{4',5'}$ = 3.6, 3.1 Hz, 1 H, 4′-H), 4.37 (ddd, $J_{3',2'}$ = 6.1, 4.2, $J_{3',4'}$ = 3.8 Hz, 1 H, 3′-H), 6.20 (dd, $J_{1',2'}$ = 6.5, 6.1 Hz, 1 H, 1′-H), 6.52 (s, 2 H, SCH=CHS), 6.89 (s, 1 H, SCH=CS), 8.46 (s, 1 H, 6-H) ppm. ^{13}C NMR (125.7 MHz, CD_3OD): δ = 42.50 (CH_2-2′), 62.29 (CH_2-5′), 71.52 (CH-3′), 84.94 (C5-*C*≡C-CS), 86.64 (SC-*C*≡C-C5), 88.08 (CH-1′), 89.09 (CH-4′), 91.50 (C-5), 107.78 (=*C*SC-C≡), 114.65 [=*C*(SCH)$_2$], 115.97 (S*C*-C≡), 120.17 and 120.37 (S-*C*H=*C*H-S), 127.74 (S-*C*H=C-S), 146.91 (CH-6), 156.56 (C-2), 165.79 (C-4) ppm. IR (KBr): $\tilde{\nu}$ = 3441, 3064, 2197, 1636, 1531, 1501, 1089, 1058 cm^{-1}. MS (ESI): *m*/*z* (%) = 475.9 (100) [M$^+$ + Na], 476.9 (27) [M$^+$ + Na + H].

HRMS (ESI$^+$): calcd. for $C_{17}H_{15}O_4N_3S_4Na$ [M$^+$ + Na] 475.9838; found 475.9838.

dU^{TTF}: The product was isolated as an orange solid (41.5 mg, 61%). ^{1}H NMR (500 MHz, CD_3OD): δ = 2.24 (ddd, J_{gem} = 13.7, $J_{2'b,1'}$ = 6.8, $J_{2'b,3'}$ = 6.3 Hz, 1 H, 2′b-H), 2.33 (ddd, J_{gem} = 13.7, $J_{2'a,1'}$ = 6.1, $J_{2'a,3'}$ = 3.8 Hz, 1 H, 2′a-H), 3.74 (dd, J_{gem} = 12.0, $J_{5'b,4'}$ = 3.4 Hz, 1 H, 5′b-H), 3.84 (dd, J_{gem} = 12.0, $J_{5'a,4'}$ = 3.0 Hz, 1 H, 5′a-H), 3.94 (ddd, $J_{4',3'}$ = 3.5, $J_{4',5'}$ = 3.4, 3.0 Hz, 1 H, 4′-H), 4.37 (ddd, $J_{3',2'}$ = 6.3, 3.8, $J_{3',4'}$ = 3.5 Hz, 1 H, 3′-H), 6.24 (dd, $J_{1',2'}$ = 6.8, 6.1 Hz, 1 H, 1′-H), 6.51 (s, 2 H, SCH=CHS), 6.81 (s, 1 H, SCH=CS), 8.44 (s, 1 H, 6-H) ppm. ^{13}C NMR (125.7 MHz, CD_3OD): δ = 41.86 (CH_2-2′), 62.41 (CH_2-5′), 71.83 (CH-3′), 84.76 (SC-*C*≡C-C5), 85.99 (C5-*C*≡C-CS), 87.19 (CH-1′), 89.17 (CH-4′), 99.29 (C-5), 107.89 (=*C*SC-C≡), 114.36 [=*C*(SCH)$_2$], 116.27 (S*C*-C≡), 120.15 and 120.36 (S-*C*H=*C*H-S), 127.09 (S-*C*H=C-S), 145.99 (CH-6), 151.06 (C-2), 164.07 (C-4) ppm. IR (KBr): $\tilde{\nu}$ = 3429, 3065, 2203, 1691, 1574, 1532, 1457 1090, 1058 cm^{-1}. MS (ESI$^+$): *m*/*z* (%) = 453.8 (54) [M$^+$ + H], 476.8 (91) [M$^+$ + H + Na], 930.5 (100) [2 M$^+$ + Na]. HRMS (ESI): calcd. for $C_{17}H_{14}O_5N_3S_4$ [M$^+$ + H] 453.9780; found 453.9781.

dA*^{TTF}: The product was isolated as an orange solid (49 mg, 69%). ^{1}H NMR (500 MHz, CD_3OD): δ = 2.35 (ddd, J_{gem} = 13.5, $J_{2'b,1'}$ = 6.1, $J_{2'b,3'}$ = 2.9 Hz, 1 H, 2′b-H), 2.63 (ddd, J_{gem} = 13.5, $J_{2'a,1'}$ = 8.0, $J_{2'a,3'}$ = 6.0 Hz, 1 H, 2′a-H), 3.73 (dd, J_{gem} = 12.2, $J_{5'b,4'}$ = 3.7 Hz, 1 H, 5′b-H), 3.80 (dd, J_{gem} = 12.2, $J_{5'a,4'}$ = 3.3 Hz, 1 H, 5′a-H), 4.01 (ddd, $J_{4',5'}$ = 3.7, 3.3, $J_{4',3'}$ = 2.7 Hz, 1 H, 4′-H), 4.52 (m, $J_{3',2'}$ = 6.0, 2.9, $J_{3',4'}$ = 2.7, $J_{3',1'}$ = 0.6 Hz, 1 H, 3′-H), 6.51 (dd, $J_{1',2'}$ = 8.0, 6.1 Hz, 1 H, 1′-H), 6.52 (s, 2 H, SCH=CHS), 6.87 (s, 1 H, SCH=CS), 7.77 (s, 1 H, 6-H), 8.13 (s, 1 H, 2-H) ppm. ^{13}C NMR (125.7 MHz, CD_3OD): δ = 41.70 (CH_2-2′), 63.56 (CH_2-5′), 72.92 (CH-3′), 83.67 (SC-*C*≡C-C5), 86.71 (CH-1′), 87.13 (C5-*C*≡C-CS), 89.30 (CH-4′), 95.95 (C-5), 104.30 (C-4a), 107.72 (=*C*SC-C≡), 114.76 [=*C*(SCH)$_2$], 116.16 (S*C*-C≡), 120.18 and 120.35 (S-*C*H=*C*H-S), 126.76 (S-*C*H=C-S), 129.41 (CH-6), 150.35 (C-7a), 153.53 (CH-2), 159.16 (C-4) ppm. IR (KBr): $\tilde{\nu}$ = 3446, 3068, 2193, 1622, 1591, 1564, 1543, 1469, 1300, 1093, 1055 cm^{-1}. MS (ESI$^+$): *m*/*z* (%) = 475.9 (23) [M$^+$], 477.0 (18) [M$^+$ + H], 974.5 (100) [2 M$^+$ + Na], 975.5 (49) [2 M$^+$ + H + Na]. HRMS (ESI$^+$): calcd. for $C_{19}H_{17}O_3N_4S_4$ [M$^+$ + H] 477.0178; found 477.0178.

dG*^{TTF}: The product was isolated as an orange solid (25.8 mg, 35%). ^{1}H NMR (500.0 MHz, CD_3OD): δ = 2.27 (ddd, J_{gem} = 13.5, $J_{2'b,1'}$ = 6.1, $J_{2'b,3'}$ = 3.0 Hz, 1 H, 2′b-H), 2.49 (ddd, J_{gem} = 13.5, $J_{2'a,1'}$ = 8.0, $J_{2'a,3'}$ = 6.0 Hz, 1 H, 2′a-H), 3.70 (dd, J_{gem} = 12.0, $J_{5'b,4'}$ = 4.2 Hz, 1 H, 5′b-H), 3.75 (dd, J_{gem} = 12.0, $J_{5'a,4'}$ = 3.8 Hz, 1 H, 5′a-H), 3.94 (ddd, $J_{4',5'}$ = 4.2, 3.8, $J_{4',3'}$ = 2.8 Hz, 1 H, 4′-H), 4.46 (ddd, $J_{3',2'}$ = 6.0, 3.0, $J_{3',4'}$ = 2.8 Hz, 1 H, 3′-H), 6.38 (dd, $J_{1',2'}$ = 8.0, 6.1 Hz, 1 H, 1′-H), 6.50 (s, 2 H, SCH=CHS), 6.73 (s, 1 H, SCH=CS), 7.33 (s, 1 H, 6-H) ppm. ^{13}C NMR (125.7 MHz, CD_3OD): δ = 41.45 (CH_2-2′), 63.50 (CH_2-5′), 72.83 (CH-3′), 82.26 (SC-*C*≡C-C5), 85.40 (CH-1′), 88.29 (C5-*C*≡C-CS), 88.79 (CH-4′), 99.56 (C-5), 101.22 (C-4a), 108.59 (=*C*SC-C≡), 113.67 [=*C*(SCH)$_2$], 117.35 (S*C*-C≡), 120.12 and 120.33 (S-*C*H=*C*H-S), 124.90 (S-*C*H=C-S), 125.18 (CH-6), 152.40 (C-7a), 154.68 (C-4), 161.08 (C-2) ppm. IR (KBr): $\tilde{\nu}$ = 3293, 3198, 3065, 2196, 1670, 1625, 1589, 1542, 1528, 1087, 1049 cm^{-1}. MS (ESI$^+$): *m*/*z* (%) = 492 (31) [M$^+$ + H], 514.9 (100) [M$^+$ + H + Na], 1006.6 (75) [2 M$^+$ + Na]. HRMS (ESI$^+$): calcd. for $C_{19}H_{16}O_4N_4S_4$ [M$^+$ + H] 492.0049; found 492.0050.

General Procedure for the Phosphorylation of Tetrathiafulvalene-Functionalized Nucleosides (dN^{TTF}s): Dry trimethyl phosphate (0.2 mL) was added to an argon-purged flask containing nucleoside analogue **dN^{TTF}** (0.02 mmol, 1 equiv.) cooled to 0 °C on ice followed by the addition of $POCl_3$ (3.7 µl, 0.04 mmol, 2 equiv.). After 16 h, a solution of $(NHBu_3)_2H_2P_2O_7$ (38 mg, 0.1 mmol, 5 equiv., 1 mL) in dry DMF was added to the reaction mixture and stirred for another 1 h and quenched by 2 M TEAB buffer (1 mL). The products were purified by C18 reversed-phase column chromatography using water/methanol (5–100%) as eluent.

dC^{TTF}TP: The product was isolated as an orange solid (3.9 mg, 25%). ^{1}H NMR (500 MHz, D_2O, ref.$_{dioxane}$ = 3.75 ppm, pD = 7.1): δ = 2.31 (dt, J_{gem} = 14.0, $J_{2'b,1'}$ = $J_{2'b,3'}$ = 6.8 Hz, 1 H, 2′b-H), 2.46 (ddd, J_{gem} = 14.0, $J_{2'a,1'}$ = 6.1, $J_{2'a,3'}$ = 4.3 Hz, 1 H, 2′a-H), 4.12–4.25 (br. m, 3 H, 4′,5′-H), 4.59 (br. m, 1 H, 3′-H), 6.19 (dd, $J_{1',2'}$ = 6.8, 6.1 Hz, 1 H, 1′-H), 6.47 (br. s, 2 H, SCH=CHS), 6.93 (br. s, 1 H, SCH=CS), 8.14 (br. s, 1 H, 6-H) ppm. ^{31}P NMR (202.3 MHz, D_2O, ref.$_{phosphate\ buffer}$ = 2.35 ppm, pD = 7.1): δ = –21.04 (br. dd, *J* = 19.9, 18.8 Hz, P_β), –10.37 (br. d, *J* = 18.8 Hz, P_α), –6.35 (br. d, *J* = 19.9 Hz, P_γ) ppm. MS (ESI$^-$): *m*/*z* (%) = 232.5 (31) [M^{3-}], 345.2 (100) [M^{2-} + H], 691.8 (8) [M$^-$ + 2 H]. HRMS (ESI$^-$): calcd. for $C_{17}H_{17}O_{13}N_3P_3S_4$ [M + 2 H]$^-$ 691.8863; found 691.8848.

dU^{TTF}TP: The product was isolated as an orange solid (4.2 mg, 27%). ^{1}H NMR (500 MHz, D_2O, ref.$_{dioxane}$ = 3.75 ppm, pD = 7.1): δ = 2.36–2.47 (br. m, 2 H, 2′-H), 4.07–4.30 (br. m, 3 H, 4′,5′-H), 4.62 (br. m, 1 H, 3′-H), 6.22 (t, $J_{1',2'}$ = 6.6 Hz, 1 H, 1′-H), 6.49 (br. s, 2 H, SCH=CHS), 6.90 (br. s, 1 H, SCH=CS), 8.14 (br. s, 1 H, 6-H) ppm. ^{31}P NMR (202.3 MHz, D_2O, ref.$_{phosphate\ buffer}$ = 2.35 ppm, pD = 7.1): δ = –21.07 (br. dd, *J* = 18.5, 17.5 Hz, P_β), –10.48 (br. d, *J* = 18.5 Hz, P_α), –6.29 (br. d, *J* = 17.5 Hz, P_γ) ppm. MS (ESI$^-$): *m*/*z* (%) = 305.6 (100) [M^{2-} + H – PO_3^-], 345.7 (82) [M^{2-} + H], 612.7 (34) [M$^-$ + H – PO_3^-]. HRMS (ESI$^-$): *m*/*z* for $C_{17}H_{15}O_{14}N_2P_3S_4$ [M^{2-} + H] 345.9304; found 345.9311.

dA*^{TTF}TP: The product was isolated as an orange solid (6.1 mg, 39%). ^{1}H NMR (499.8 MHz, D_2O, ref.$_{dioxane}$ = 3.75 ppm): δ = 2.51 and 2.67 (2 × m, 2 × 1 H, 2′-H), 4.14 (br. m, 2 H, 5′-H), 4.23 (br. m, 1 H, 4′-H), 4.72 (br. m, 1 H, 3′-H), 6.50 (br. s, 2 H, SCH=CHS), 6.47 (br. t, $J_{1',2'}$ = 6.6 Hz, 1 H, 1′-H), 6.77 (br. s, 1 H, SCH=CS), 7.67 (br. s, 1 H, 6-H), 8.03 (s, 1 H, 2-H) ppm. ^{31}P NMR (202.3 MHz, D_2O): δ = –21.37 (br., P_β), –10.31 (br. d, *J* = 19.3 Hz, P_α), –7.41 (br., P_γ) ppm. ^{1}H NMR (499.8 MHz, D_2O, ref.$_{dioxane}$ = 3.75 ppm, pD = 7.1): δ = 2.49 and 2.63 (2 × m, 2 × 1 H, 2′-H), 4.13 (br. m, 2 H, 5′-H), 4.22 (br. m, 1 H, 4′-H), 4.67 (br. m, 1 H, 3′-H), 6.20–6.50 (br. m, 3 H, 1′-H and SCH=CHS), 6.70 (br. s, 1 H, SCH=CS), 7.57 (br. s, 1 H, 6-H), 7.99 (br. s, 1 H, 2-H) ppm. ^{31}P NMR (202.3 MHz, D_2O, ref.$_{phosphate\ buffer}$ = 2.35 ppm, pD = 7.1): δ = –20.54 (br., P_β), –9.80 (br. d, *J* = 18.1 Hz, P_α), –6.79 (br., P_γ) ppm. MS (ESI$^-$): *m*/*z* (%) = 237.4 (58) [M^{3-}], 356.7 (100) [M^{2-} + H], 634.7 (20) [M$^-$ + H – PO_3^-], 714.6 (9) [M$^-$ + 2 H]. HRMS (ESI$^-$): calcd. for $C_{19}H_{18}O_{12}N_4P_3S_4$ [M$^-$ + 2 H] 714.9022; found 714.9004.

dG*^{TTF}TP: The product was isolated as an orange solid (3.4 mg, 21%). ^{1}H NMR (499.8 MHz, D_2O, ref.$_{dioxane}$ = 3.75 ppm): δ = 2.42 and 2.65 (2 × m, 2 × 1 H, 2′-H), 4.16 (br. m, 2 H, 5′-H), 4.20 (br. m, 1 H, 4′-H), 4.75 (br. m, 1 H, 3′-H, overlapped with HDO signal), 6.37 (br. t, $J_{1',2'}$ = 6.5 Hz, 1 H, 1′-H), 6.50 (br. s, 2 H, SCH=CHS), 6.76 (br. s, 1 H, SCH=CS), 7.38 (br. s, 1 H, 6-H) ppm. ^{31}P NMR (202.3 MHz, D_2O): δ = –21.12 (br. , P_β), –10.22 (br. d, *J* = 19.0 Hz, P_α), –5.55 (br., P_γ) ppm. ^{1}H NMR (499.8 MHz, D_2O, ref.$_{dioxane}$ = 3.75 ppm, pD = 7.1): δ = 2.42 and 2.64 (2 × m, 2 × 1 H, 2′-H), 4.14 (br. m, 2 H, 5′-H), 4.20 (br. m, 1 H, 4′-H), 4.78 (br. m, 1 H, 3′-H, overlapped with HDO signal), 6.00–6.80 (br. m, 4 H, 1′-H, SCH=CHS and SCH=CS), 7.30 (br. s, 1 H, 6-H) ppm. ^{31}P NMR (202.3 MHz, D_2O, ref.$_{phosphate\ buffer}$ = 2.35 ppm, pD = 7.1): δ = –21.06 (br., P_β), –9.97 (br. d, *J* = 17.6 Hz, P_α), –6.35 (br., P_γ) ppm. MS (ESI$^-$): *m*/*z* (%) = 375.9 (20) [M^{2-} + H], 650.7 (70)

$[M^- + H - PO_3^-]$, 672.6 (100) $[M^- + Na - PO_3^-]$, 730.8 (31) $[M^- + 2\ H]$. HRMS (ESI^-): calcd. for $C_{19}H_{18}O_{13}N_4P_3S_4$ $[M^- + 2\ H]$ 730.8972; found 730.8976.

UV/Vis Spectra: The UV/Vis spectra were measured with a Varian CARY 100 Bio spectrophotometer at room temperature. TTF-modified nucleosides were measured as 5×10^{-5} M solutions in methanol. TTF-modified nucleoside triphosphates were measured as 5×10^{-5} M solutions in water.

Electrochemistry: Voltammetric measurements were performed with an Autolab analyzer (Eco Chemie, The Netherlands) connected to a VA-Stand 663 (Metrohm, Switzerland). Electrochemical responses of TTF, modified nucleosides and dNTPs were measured in a conventional in situ mode (with the analyte dissolved in background electrolyte) on a glassy carbon electrode (GCE). The PEX products were analyzed by ex situ (adsorptive transfer stripping) SWV with a basal-plane pyrolytic graphite electrode as described in ref.[9–11]. All measurements were performed in 0.2 M sodium acetate, pH 5.0, with an initial potential of –1.0 V, a final potential +1.5 V, a pulse amplitude of 50 mV, a frequency of 200 Hz and a potential step of 5 mV at ambient temperature in a three-electrode set-up (using Ag/AgCl/3 M KCl as reference and a platinum wire as a counter-electrode). The voltammograms were baseline-corrected by means of a moving average algorithm (GPES 4 software, EcoChemie).

Primer Extension Incorporation of Tetrathiafulvalene-Modified Nucleoside Triphosphates into Oligonucleotides

Materials: Synthetic oligonucleotides were purchased from VBC Genomics (Austria). Dynabeads® M-270 Streptavidin (DBStv) were obtained from Dynal A.S. (Norway), Vent (*exo*-), Pwo, Dynazyme and Klenow DNA polymerases and T4 polynucleotide kinase were from New England Biolabs (Great Britain), unmodified nucleoside triphosphates (dATP, dTTP, dCTP and dGTP) from Sigma and γ-32P-ATP from MP Empowered Discovery (USA). Other chemicals were of analytical grade.

Primer Extension: The reaction mixture contained primer (0.5 μM), template (0.5 μM), dNTPs (200 μM or 2 mM in experiments with a 10-fold higher concentration), buffer and DNA polymerase (0.2 U). The reaction mixtures were incubated for 30 min at 60 °C. For polyacrylamide gel electrophoresis, primers were ^{32}P-prelabelled at the 5′-end to allow radiographic detection. For electrochemical detection, unlabelled primers and biotinylated templates were used to allow magnetoseparation.

Polyacrylamide Gel Electrophoresis: The products of the primer extension reactions were mixed with loading buffer (80% formamide, 10 mM EDTA, 1 mg mL^{-1} xylene cyanol, 1 mg mL^{-1} Bromphenol Blue) and subjected to electrophoresis in 15% denaturing polyacrylamide gel containing 1xTBE buffer (pH = 8) and 7 M urea at 25 W for 50 min. The gels were dried, autoradiographed and visualized by using a phosphorimager.

Isolation of Single-Strand Oligonucleotides by the DBStv Magnetoseparation Procedure: Reaction mixture (50 μL) containing 0.3 M NaCl was added to DBS_{tv} [25 μL of the stock solution washed three times by 150 μL of buffer (0.3 M NaCl, 10 mM TRIS, pH = 7.4)]. The suspension was shaken at room temperature to allow the oligonucleotides to bind to the DBStv beads. The DBStv beads were washed three times with 200 μL of PBS solution (0.14 M NaCl, 3 mM KCl, 4 mM sodium phosphate, pH = 7.4) with 0.01% Tween 20 and then three times by 200 μL of buffer (0.3 M NaCl, 10 mM TRIS, pH = 7.4) and finally by ddH_2O (200 μL). Single-strand oligonucleotides were released by shaking and heating the sample at 75 °C for 2 min. Each medium exchange was performed by using a magnetoseparator (Dynal, Norway).

Supporting Information (see also the footnote on the first page of this article): Selected NMR spectra.

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