Chemical Biology

Nucleotide-Bearing Benzylidene-Tetrahydroxanthylium Near-IR Fluorophore for Sensing DNA Replication, Secondary Structures and Interactions

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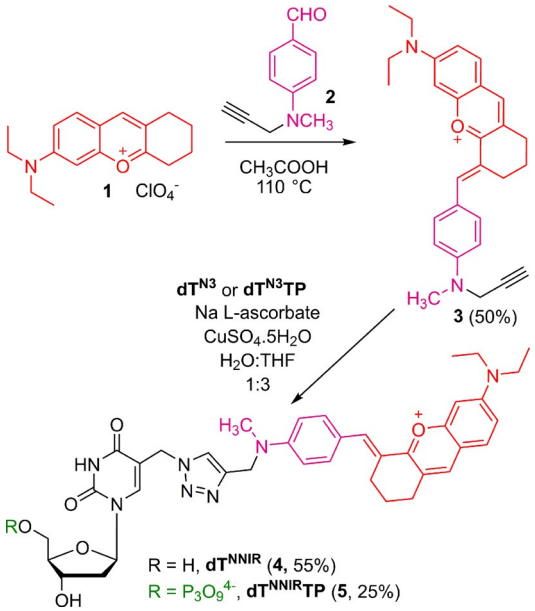
Abstract: Thymidine triphosphate bearing benzylidene-tetrahydroxanthylium near-IR fluorophore linked to the 5-methyl group via triazole was synthesized through the CuAAC reaction and was used for polymerase synthesis of labelled DNA probes. The fluorophore lights up upon incorporation to DNA (up to 348-times) presumably due to interactions in major groove and the fluorescence further increases in the single-stranded oligonucleotide. The labelled dsDNA senses binding of small molecules and proteins by a strong decrease of fluorescence. The nucleotide was used as a light-up building block in real-time PCR for detection of SARS-CoV-2 virus.

Fluorescent techniques using labelled nucleic acids are an important tool in many applications in chemical biology, diagnostics and imaging.[1] Environment-sensitive fluorophores are particularly useful because they can sense changes in the secondary structures of nucleic acids or some microenvironment changes when nucleic acids interact with other molecules.[2] The use of fluorescent nucleotide analogues or fluorophore-linked nucleotides were extensively used for detection of mismatches[3] or secondary structure changes.[4] Within our programme on detection of protein–DNA interactions,[5] we reported several nucleotides bearing environment-sensitive fluorophores based on substituted arylmethylidene-imidazolines[6] or -cyano-acetamides[7] or substituted bodipy[8] which either increased the fluorescence intensity or lifetime of fluorescence in more viscous environment upon binding of a protein. Also we reported nucleotides bearing solvatochromic push–pull fluorene, which changed colour in the less polar environment of a protein.[9] Most of these fluorophores gave blue or green emission and, in some cases, the changes were not strong enough for real applications.

Thiazole orange (TO) is a yellow emitting dye which strongly enhances fluorescence through intercalation into DNA duplex.[10] It has frequently been used as a nucleobase surrogate or label to construct oligonucleotide (ON) hybridization probes.[11,12] Related benzylidene-tetrahydroxanthyliums are red/nearIR fluorophores which were recently used for cell microscopy and sensing H_2S.[13,14] Herein, we report on a nucleotide labelled with this near-IR fluorophore and its use in sensing incorporation to DNA, secondary structure changes, and interactions with small molecules and proteins.

The synthesis of the modified nucleoside and nucleotide was achieved by copper-catalysed alkyne-azide 1,3-dipolar cycloaddition (CuAAC) reaction of 5-(azidomethyl)-2′-deoxyuridine (**dT^{N3}**) or the trisphosphate (**dT^{N3}TP**) with alkyne-linked fluorophore (**3**). The alkyne building block **3** was synthesized in 50% yield in analogy to the literature[13] through condensation of 6-(diethylamino)-1,2,3,4-tetrahydroxanthylium **1** with propargylamino-linked benzaldehyde **2** in acetic acid (Scheme 1). The labelled nucleoside **dT^{NNIR}** was obtained by CuAAC reaction of **dT^{N3}** with **3** in 55% yield, while the corresponding triphosphate **dT^{NNIR}TP** was obtained analogously



Scheme 1. Synthesis of labelled nucleoside and nucleotide.

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from **dT^{N3}TP** in a lower 25% yield, which was presumably caused by partial hydrolysis and difficult separation of the modified dNTP.

The photophysical properties of nucleoside **dT^{NNIR}** and triphosphate **dT^{NNIR}TP** are summarized in Tables S1 and S2 in the Supporting Information. The absorption (622–694 nm) and emission maxima (749–760 nm) of the nucleoside depended on the polarity, and the quantum yields increased (by 2.5–11%) with viscosity of the solvent. The fluorescence in water or aqueous buffers was very low.

Triphosphate **dT^{NNIR}TP** was then tested as a substrate for KOD XL DNA polymerase in primer extension (PEX) (Figure 1A, B).[5,15] This polymerase was able to incorporate the labelled nucleotide into a 19-mer in the PEX reaction (Figure 1B and Figure S3A), but PEX using a 31-mer template encoding for 4 modifications failed (Figure S3A). However, a longer PEX product could be prepared by single-nucleotide incorporation[16] followed by PEX with natural dNTPs (Figure S4). The PEX product **DNA19_1T^{NNIR}** was characterized by determining the UV melting temperature ($T_m = 79.5\,^{\circ}C$) and compared with natural dsDNA ($T_m = 79.6\,^{\circ}C$), showing only a negligible influence of modification on stability of DNA duplex (Figure S5). PCR amplification using **dT^{NNIR}TP** instead of dTTP did not proceed (Figure S7) but we obtained a PCR amplicon when a max. 10% of **dT^{NNIR}TP** was used in the mixture with natural dTTP. Modified DNA containing **dT^{NNIR}** can be also prepared through the CuAAC reaction of DNA containing 5-azidomethyluracil (**dT^{N3}**), which can be easily prepared by PEX or PCR using **dT^{N3}TP**,[17] with alkyne **3** (see Scheme S2 and Figure S9). Cell-based experiments using the **dT^{NNIR}TP** with cyclodextrine-based synthetic nucleotide triphosphate transporter (SNTT)[18] did not show incorporation of the labelled nucleotide into genomic DNA, presumably because of the combination of low substrate activity and high cytotoxicity of the nucleotide (Figure S11).

The modified dsDNA **DNA19_1T^{NNIR}** synthesized by PEX exerted a shift of the absorption maximum from 623 nm (for **dT^{NNIR}TP**) to 690 nm (Figure 1C and Figure S6). The fluorescence of **DNA19_1T^{NNIR}** was strongly enhanced ($\Phi = 6.8\%$). When excited at 690 nm, the enhancement of fluorescence intensity in dsDNA (compared to triphosphate **dT^{NNIR}TP**) was 59-fold (Figure 1D), but when excited at 720 nm, the enhancement was 348-fold (Figure S13). Then we prepared the modified single-stranded ON through PEX with biotinylated template followed by magnetoseparation on streptavidine magnetic beads[19] and denaturation or through the PEX with 5′-phosphorylated template followed by λ exonuclease digestion of the phosphorylated template.[20] The resulting **ON19_1T^{NNIR}** exerted ca. 2-fold stronger fluorescence compared to dsDNA. Then we re-hybridized **ON19_1T^{NNIR}** with a 5-phosphorylated complementary ssON again and the fluorescence dropped to ca. 50%. After addition of λ exonuclease, the fluorescence was enhanced again to the original level of **ON19_1T^{NNIR}** (Figure 1E, F).

Apparently, the NIR-fluorophore lights-up when the nucleotide is incorporated to DNA. We assumed that the enhancement can be due to interaction of the fluorophore with DNA in major- or minor groove or through intercalation. Therefore, we treated **DNA19_1T^{NNIR}** with TO (intercalator),[21] 4′,6-diamidino-2-phenylindole (DAPI, minor-groove binder),[22] or methyl green (MG, major-groove binder)[23] and measured the emission spectra. Figure 1G shows that TO and DAPI had only minor influence, whereas addition of MG strongly decreased the fluorescence suggesting that the major-groove interaction of the fluorophore with DNA is the main reason for the significant enhancement of fluorescence.

Next we examined the influence of binding of some other small molecules or proteins to DNA. When the **DNA19_1T^{NNIR}** was incubated with bovine serum albumin (BSA) as an example of non-DNA binding protein, the effect on fluorescence was negligible. Also the influence of spermine (minor-groove binder)[24] exerted only a minor decrease of fluorescence. On the other hand, addition of H2A histone (2 equiv) resulted in a significant (5-fold) decrease of fluorescence. Addition of protamine (mixture of strongly DNA-binding proteins)[25] resulted in almost complete quenching of fluorescence (Figure 1H). When the complex of **DNA19_1T^{NNIR}** with histone (2 equiv) was treated with proteinase K (which digested the histone) the fluorescence was enhanced back to the level of dsDNA. The same effect was observed when we added 1 equiv of 98-mer dsDNA, which displaced the **DNA19_1T^{NNIR}** from the complex with histone. Similarly, the non-emitting complex of **DNA19_1T^{NNIR}** with protamine was treated with heparin (which strongly binds protamine)[26] and the high emission of **DNA19_1T^{NNIR}** was restored.

Finally, we wanted to apply turn-on fluorescence properties of **dT^{NNIR}TP** for monitoring enzymatic incorporation to DNA and for application in real-time PCR (rtPCR),[27] which is one of the most frequently used methods for detection of small quantities of DNA in diagnostic or forensic applications. Classical rtPCR typically uses an external intercalating dye[28] or labelled primers/hybridization probes.[29] To the best of our knowledge, no light-up nucleotide has been reported in this technique. First we tested whether we can monitor single nucleotide incorporation (SNI) of **dT^{NNIR}TP** in real time. Figure S22 shows that fluorescence intensity increased with time as the reaction proceeds and after 2 minutes the intensity was constant, indicating that the reaction was finished. Control experiments without enzyme or without template showed no change in fluorescence with time. This result clearly indicated that observed increase of fluorescence was due to the successful incorporation of modified nucleotide. Encouraged by these results we moved further to investigate utility of **dT^{NNIR}TP** in real-time PCR (rtPCR) for detection of SARS-CoV-2 virus,[30] the cause of COVID-19 pandemic. In this study, the RdRP gene was chosen as the target. DNA standard (Temp2^{PCR}) was prepared by amplification of the target region of cDNA with specific primers followed by purification with nucleospin columns. Aliquots of DNA (Temp2^{PCR}) were prepared in 10-fold serial dilutions (5.71×10^{7} to 5.71×10 DNA copies) and were used for generation of a standard curve (Figure S25A). We used 5% of **dT^{NNIR}TP** in the mixture with dTTP and other natural dNTPs. Figure 2A shows the amplification curve of real-time PCR. Efficiency of the PCR was calculated to be 60.5% and was able to detect a minimum of 5.71×10^{3} DNA copies. The performance

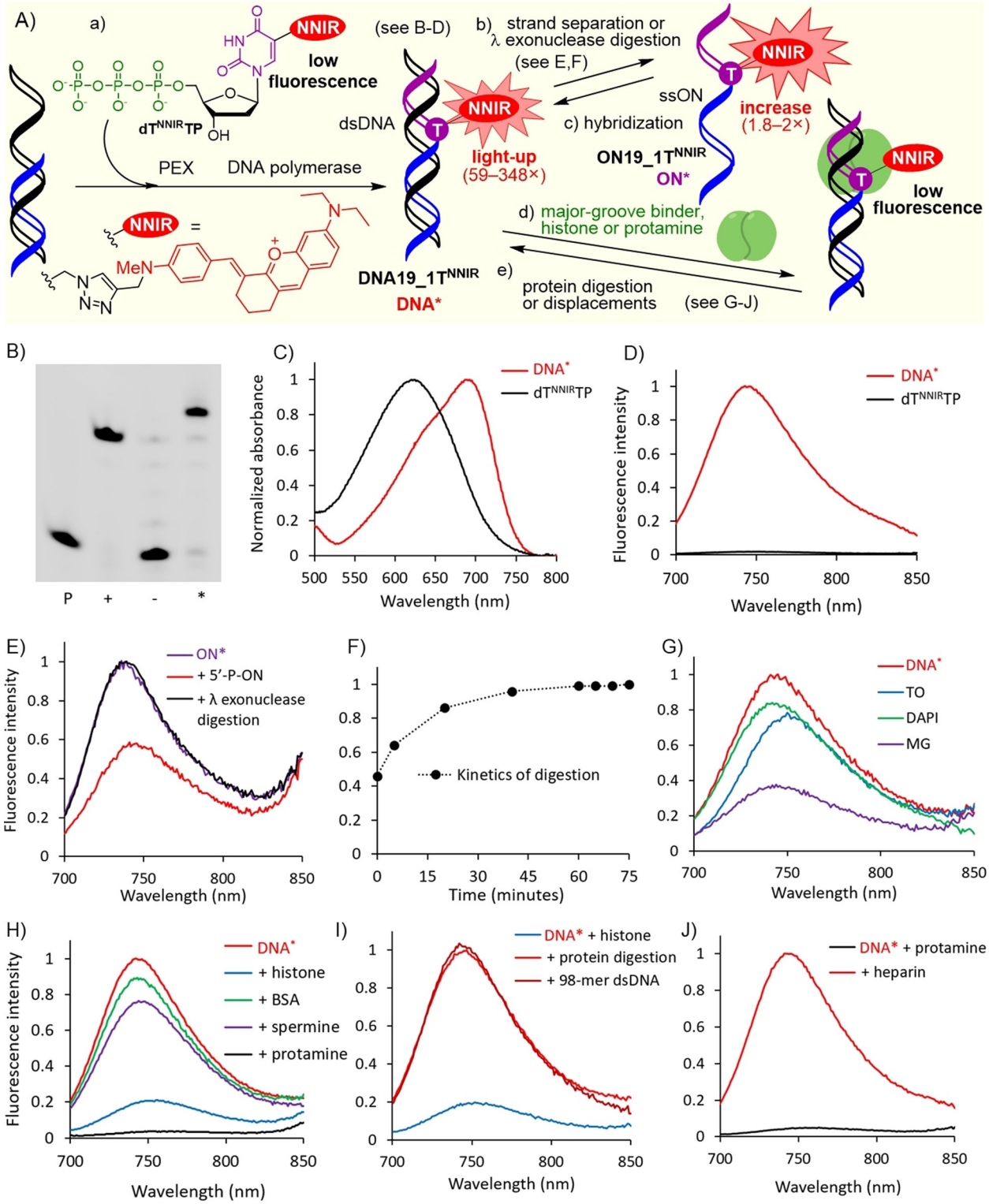

Figure 1. A) Scheme representing: a) light-up response after incorporation of **dT^{NNIR}TP** into DNA, fluorescence response of **DNA19_1T^{NNIR}** after b) strand separation/digestion, and c) subsequent hybridization with the complementary strand, d) fluorescence decrease of **DNA19_1T^{NNIR}** after interaction with major-groove binder, histone or protamine and e) subsequent recovery of fluorescence after protein digestion or displacements. B) PAGE analysis of PEX using **dT^{NNIR}TP**, KOD XL DNA polymerase, 15-mer Prim1^{PEX}-FAM and 19-mer Temp2^{PEX} template. P: primer, (+): positive control (PEX with all natural dNTPs), (−): negative control (PEX in absence of dTTP), (*): PEX with **dT^{NNIR}TP**. C) UV/Vis absorption spectra of **DNA19_1T^{NNIR}** (DNA*; λ_{abs} = 690 nm) compared to **dT^{NNIR}TP** (λ_{abs} = 623 nm). D) Fluorescence spectra of **DNA19_1T^{NNIR}** compared to **dT^{NNIR}TP** (λ_{ex} = 690 nm). E) Hybridization and λ exonuclease digestion. The purple line represents **ON19_1T^{NNIR}** (ON*), the red line shows fluorescence upon annealing with complementary strand Temp2^{PEX}-P-5′, and the black line shows the response after subsequent digestion of the 5′-P strand. F) Kinetics of digestion in real time. G) Fluorescence spectra of **DNA19_1T^{NNIR}** after addition of 20 equiv of TO, DAPI, MG or H) histone (2 equiv), BSA (4 equiv), spermine (4 equiv) or protamine (1.5 equiv). I) Fluorescence spectra of complex **DNA19_1T^{NNIR}** with histone (blue line) and after digestion of protein with proteinase K (red line) or after addition of 98-mer dsDNA (1 equiv, dark red line). J) Fluorescence spectra of complex of **DNA19_1T^{NNIR}** with protamine (black line) and after addition of heparin (0.7 equiv, red line).

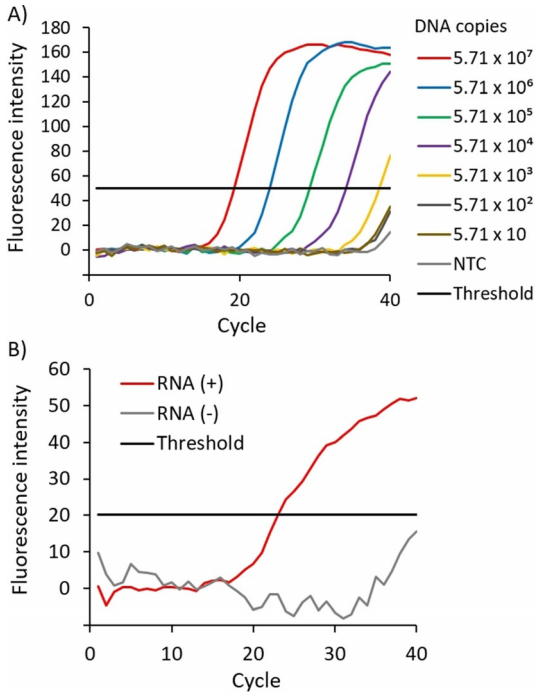

Figure 2. A) Amplification curve of rtPCR with **dT^{NNIR}TP** using 10-fold serial dilutions (5.71×10^7 to 5.71×10 DNA copies) of Temp2^{PCR} template. B) One-step RT-rtPCR; the red line represents the experiment in the presence and grey line in absence of the viral RNA. NTC = no template control.

of our unoptimized rtPCR assay was somewhat less efficient compared to the standard rtPCR assay (using excess of SYBR Green intercalating dye),[28b] which showed the PCR efficiency of 77% (Figure S27). One-step reverse transcription (RT) rtPCR using **dT^{NNIR}TP** and isolated RNA from a real sample also showed amplification and a detectable signal with Ct (threshold cycle) value of 22.7 (Figure 2B).

In conclusion, we designed and synthesized a new nucleoside triphosphate **dT^{NNIR}TP** bearing a near-IR fluorophore. The **dT^{NNIR}TP** is rather a poor substrate for DNA polymerases but can be used for PEX incorporation to DNA where it lights-up (up to 348-times depending of the excitation wavelength) due to interactions in the major groove. We assume that the light-up is due to a combination of the restricted rotation of the fluorophore and less polar environment in the major groove of DNA. Unfortunately, **dT^{NNIR}TP** is not suitable for in cellulo imaging due to its cytotoxicity. However, the labelled DNA probes respond to secondary structure changes, for example, they further enhance fluorescence after separation or digestion of the complementary strand. They can also be used for detection of binding of small molecules (MG or spermine) or proteins (histone or protamine) to DNA. The light-up after incorporation to dsDNA was used for application of **dT^{NNIR}TP** in rtPCR as a new approach to directly visualize the DNA synthesis. Further modification of the linker tethering the fluorophore to the nucleotide will be pursued in order to decrease the toxicity and improve the substrate activity of the nucleotide building block for practical applications.

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Conflict of interest

The authors declare no conflict of interest.

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[1] a) W. Xu, K. M. Chan, E. T. Kool, *Nat. Chem.* **2017**, *9*, 1043–1055; b) R. W. Sinkeldam, N. J. Greco, Y. Tor, *Chem. Rev.* **2010**, *110*, 2579–2619; c) A. A. Tanpure, M. G. Pawar, S. G. Srivatsan, *Isr. J. Chem.* **2013**, *53*, 366–378.
[2] B. Y. Michel, D. Dziuba, R. Benhida, A. P. Demchenko, A. Burger, *Front. Chem.* **2020**, *8*, 112.
[3] Examples: a) D. D. Burns, K. L. Teppang, R. W. Lee, M. E. Lokensgard, B. W. Purse, *J. Am. Chem. Soc.* **2017**, *139*, 1372–1375; b) Y. Saito, A. Suzuki, Y. Okada, Y. Yamasaka, N. Nemoto, I. Saito, *Chem. Commun.* **2013**, *49*, 5684–5686; c) M. Yanagi, A. Suzuki, R. H. E. Hudson, Y. Saito, *Org. Biomol. Chem.* **2018**, *16*, 1496–1507; d) A. Okamoto, K. Kanatani, I. Saito, *J. Am. Chem. Soc.* **2004**, *126*, 4820–4827; e) H. Gardarsson, A. S. Kale, S. T. Sigurdsson, *ChemBioChem* **2011**, *12*, 567–575.
[4] a) T. Kanamori, A. Takamura, N. Tago, Y. Masaki, A. Ohkubo, M. Sekine, K. Seio, *Org. Biomol. Chem.* **2017**, *15*, 1190–1197; b) T. Kanamori, Y. Masaki, Y. Oda, H. Ohzeki, A. Ohkubo, M. Sekine, K. Seio, *Org. Biomol. Chem.* **2019**, *17*, 2077–2080.
[5] M. Hocek, *Acc. Chem. Res.* **2019**, *52*, 1730–1737.
[6] a) J. Riedl, P. Ménová, R. Pohl, P. Orság, M. Fojta, M. Hocek, *J. Org. Chem.* **2012**, *77*, 8287–8293; b) M. Kuba, R. Pohl, M. Hocek, *Tetrahedron* **2018**, *74*, 6621–6629.
[7] D. Dziuba, R. Pohl, M. Hocek, *Chem. Commun.* **2015**, *51*, 4880–4882.
[8] a) D. Dziuba, P. Jurkiewicz, M. Cebecauer, M. Hof, M. Hocek, *Angew. Chem. Int. Ed.* **2016**, *55*, 174–178; *Angew. Chem.* **2016**, *128*, 182–186; b) P. Güixens-Gallardo, J. Humpolickova, S. P. Miclea, R. Pohl, T. Kraus, P. Jurkiewicz, M. Hof, M. Hocek, *Org. Biomol. Chem.* **2020**, *18*, 912–919.
[9] D. Dziuba, P. Pospíšil, J. Matyašovský, J. Brynda, D. Nachtigallová, L. Rulíšek, R. Pohl, M. Hof, M. Hocek, *Chem. Sci.* **2016**, *7*, 5775–5785.
[10] A. N. Glazer, H. S. Rye, *Nature* **1992**, *359*, 859–861.
[11] a) F. Hövelmann, O. Seitz, *Acc. Chem. Res.* **2016**, *49*, 714–723; b) F. Hövelmann, I. Gaspar, A. Ephrussi, O. Seitz, *J. Am. Chem. Soc.* **2013**, *135*, 19025–19032; c) A. Knoll, S. Kankowski, S. Schöllkopf, J. C. Meier, O. Seitz, *Chem. Commun.* **2019**, *55*, 14817–14820; d) S. Berndl, S. D. Dimitrov, F. Menacher, T. Fiebig, H.-A. Wagenknecht, *Chem. Eur. J.* **2016**, *22*, 2386–2395; e) C. Holzhauser, R. Liebl, A. Goepferich, H.-A. Wagenknecht, M. Breunig, *ACS Chem. Biol.* **2013**, *8*, 890–894; f) S. Ikeda, A. Okamoto, *Chem. Asian J.* **2008**, *3*, 958–968; g) S. Ikeda, T. Kubota, M. Yuki, A. Okamoto, *Angew. Chem. Int. Ed.* **2009**, *48*, 6480–6484; *Angew. Chem.* **2009**, *121*, 6602–6606; h) M. Kovaliov, M. Segal, P. Kafri, E. Yavin, Y. Shav-Tal, B. Fischer, *Bioorg. Med. Chem.* **2014**, *22*, 2613–2621.
[12] P. Klimkowski, S. De Ornellas, D. Singleton, A. H. El-Sagheer, T. Brown, *Org. Biomol. Chem.* **2019**, *17*, 5943–5950.
[13] L. Zhou, Q. Wang, Y. Tan, M. J. Lang, H. Sun, X. Liu, *Chem. Eur. J.* **2017**, *23*, 8736–8740.
[14] L. Zhou, D. Lu, Q. Wang, S. Liu, Q. Lin, H. Sun, *Biosens. Bioelectron.* **2017**, *91*, 699–705.
[15] a) A. Hottin, A. Marx, *Acc. Chem. Res.* **2016**, *49*, 418–427; b) M. Hollenstein, *Molecules* **2012**, *17*, 13569–13591.
[16] P. Ménová, H. Cahová, M. Plucnara, L. Havran, M. Fojta, M. Hocek, *Chem. Commun.* **2013**, *49*, 4652–4654.

[17] a) X. Ren, A. H. El-Sagheer, T. Brown, *Analyst* **2015**, *140*, 2671 – 2678; b) X. Ren, A. H. El-Sagheer, T. Brown, *Nucleic Acids Res.* **2016**, *44*, e79; c) A. B. Neef, N. W. Luedtke, *ChemBioChem* **2014**, *15*, 789 – 793; d) M. Tera, S. M. K. Glasauer, N. W. Luedtke, *ChemBioChem* **2018**, *19*, 1939 – 1943; e) M. Tera, Z. Harati Taji, N. W. Luedtke, *Angew. Chem. Int. Ed.* **2018**, *57*, 15405 – 15409; *Angew. Chem.* **2018**, *130*, 15631 – 15635.
[18] a) Z. Zawada, A. Tatar, P. Mocilac, M. Buděšínský, T. Kraus, *Angew. Chem. Int. Ed.* **2018**, *57*, 9891 – 9895; *Angew. Chem.* **2018**, *130*, 10039 – 10043; b) P. Güixens-Gallardo, Z. Zawada, J. Matyašovský, D. Dziuba, R. Pohl, T. Kraus, M. Hocek, *Bioconjugate Chem.* **2018**, *29*, 3906 – 3912.
[19] M. Fojta, P. Kostecka, M. Trefulka, L. Havran, E. Palecek, *Anal. Chem.* **2007**, *79*, 1022 – 1029.
[20] J. W. Litte, *J. Biol. Chem.* **1967**, *242*, 679 – 686.
[21] D. L. Boger, W. C. Tse, *Bioorg. Med. Chem.* **2001**, *9*, 2511 – 2518.
[22] J. Kapuscinski, *Biotechnic Histochem.* **1995**, *70*, 220 – 233.
[23] S. K. Kim, B. Nordén, *FEBS Lett.* **1993**, *315*, 61 – 64.
[24] A. Kabir, G. Suresh Kumar, *PLoS One* **2013**, *8*, e70510.
[25] S. Gupta, N. Tiwari, M. Munde, *Sci. Rep.* **2019**, *9*, 5891.
[26] S. Pang, S. Liu, X. Su, *RSC Adv.* **2014**, *4*, 25857.
[27] M. L. Wong, J. F. Medrano, *BioTechniques* **2005**, *39*, 75 – 85.
[28] a) H. J. Karlsson, M. Eriksson, E. Perzon, B. Akerman, P. Lincoln, G. Westman, *Nucleic Acids Res.* **2003**, *31*, 6227 – 6234; b) H.-M. Nam, V. Srinivasan, B. E. Gillespie, S. E. Murinda, S. P. Oliver, *Int. J. Food Microbiol.* **2005**, *102*, 161 – 171.
[29] a) N. Thelwell, S. Millington, A. Solinas, J. Booth, T. Brown, *Nucleic Acids Res.* **2000**, *28*, 3752 – 3761; b) D. Whitcombe, J. Theaker, S. P. Guy, T. Brown, S. Little, *Nat. Biotechnol.* **1999**, *17*, 804 – 807.
[30] V. M. Corman, O. Landt, M. Kaiser, R. Molenkamp, A. Meijer, D. K. Chu, T. Bleicker, S. Brünink, J. Schneider, M. L. Schmidt, D. G. Mulders, B. L. Haagmans, B. van der Veer, S. van den Brink, L. Wijsman, G. Goderski, J.-L. Romette, J. Ellis, M. Zambon, M. Peiris, H. Goossens, C. Reusken, M. P. Koopmans, C. Drosten, *Eurosurveillance* **2020**, *25*, 23 – 30.

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