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Nucleic Acids

A New Architecture for DNA-Templated Synthesis in Which Abasic Sites Protect Reactants from Degradation

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Abstract: The synthesis of artificial sequence-defined polymers that match and extend the functionality of proteins is an important goal in materials science. One way of achieving this is to program a sequence of chemical reactions between precursor building blocks by means of attached oligonucleotide adapters. However, hydrolysis of the reactive building blocks has so far limited the length and yield of product that can be obtained using DNA-templated reactions. Here, we report an architecture for DNA-templated synthesis in which reactants are tethered at internal abasic sites on opposite strands of a DNA duplex. We show that an abasic site within a DNA duplex can protect a nearby thioester from degradation, significantly increasing the yield of a DNA-templated reaction. This protective effect has the potential to overcome the challenges associated with programmable, sequence-controlled synthesis of long non-natural polymers by extending the lifetime of the reactive building blocks.

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*T*he ribosome mediates the concatenation of amino acids to form proteins following a genetic program (messenger RNA).[1] This translation process has been harnessed to enable the directed evolution of a range of useful products including, for example, antibodies with enhanced affinity and enzymes with enhanced activity, stability and selectivity.[2] However, the ribosome uses only a limited palette of molecular building blocks (the 20 canonical amino acids); although diversification is possible through ribosome engineering, it is highly challenging.[3] Thus, there is a need for alternative synthetic strategies applying chemistry similar to that of the ribosome implemented by programmable synthetic molecular machinery. The creation of such programmable molecular machinery capable of the autonomous synthesis of completely artificial, sequence-defined polymers —a synthetic ribosome—would enable the rapid exploration of vast new areas of chemical space.[4] A promising way to achieve this is to employ DNA as a supramolecular scaffold to promote chemical reactions by colocalizing reactants.[4b,5] In this way, information stored in the base sequences of DNA adapters, conjugated to a library of reactive moieties, can be used to determine which of the many possible chemical reactions will be selectively enhanced. However, for the multistep processes required to build protein-length products, this 'DNA-templated chemical synthesis' (DTS) has so far been limited by reactant degradation in water.[6] Reactions with water can be circumvented by transferring pre-hybridized DNA duplexes to organic solvents for the reaction step[7] but this is incompatible with autonomous, multistep processes which are programmed through dynamic control of DNA hybridization.[8] Unless the issue of reactant degradation is addressed, the synthesis of long, sequence-defined polymers by autonomous molecular machinery based on DTS will remain out of reach. Here, we report a strategy with the potential to address this problem. We show that an abasic site in a DNA duplex can protect a nearby thioester linkage from hydrolysis and that this protection can be switched on and off by DNA strand displacement. We use an acyl transfer reaction, such as might occur during polymer synthesis by a molecular machine, to confirm the potential of this approach to overcome the major limitation of autonomous DNA-templated synthesis.

Previous work has shown that DTS yield is sensitive to the linker between the reactive groups and their DNA adapters and to the positions at which they are conjugated

to the templating DNA duplex.[9] In most published implementations of DTS, reactants are tethered at the 3′ and 5′ termini of adapter oligonucleotides and the reaction takes place when reactants meet, either across the end of a DNA duplex formed by hybridization of the adapters or across the nick between adapters hybridized to a common template.[6b,c,9a,10] Here, we use a previously unreported architecture in which reactants are tethered at internal, abasic sites on opposite strands such that DTS takes place across one of the grooves of the DNA duplex (Figure 1a). This reaction geometry allows the effects of proximity on DTS yield to be explored simply by moving one of the reactive groups away from the other in single-nucleotide steps.

The DNA-templated reaction tested was an acyl transfer between a primary amine (the acceptor) and a thioester (the donor) (Figure 1b). Both the acceptor and donor oligonucleotides were purchased from Integrated DNA Technologies, Inc. with butylamine modifications at an internal C_3-spacer which acts as an abasic site (Uni-Link™ modifier Figure 1e, see Supporting Information for sequences). This amine modification was used as supplied as the acceptor. The complementary donor oligonucleotide was obtained by reacting the amine moiety with succinimidyl 3-(2-pyridyldithio)propionate (SPDP) to create a disulfide which, upon reduction and reaction with 6-carboxytetramethylrhodamine-succinimidyl ester (NHS-TAMRA), formed the thioester linkage between fluorophore TAMRA and the oligonucleotide (Figure 1b and d and Figure S1a). The product was purified by RP-HPLC and its identity verified by mass spectrometry (Figure S1b).

DNA-templated acyl transfer was expected to result in the transfer of the TAMRA moiety from the donor to the acceptor adapter through aminolysis and formation of an amide bond (Figure 1b). Hydrolysis of the donor thioester to produce free TAMRA is a competing reaction. We designed the acceptor adapter oligonucleotide to be 10 nucleotides longer than the donor adapter, enabling transfer of the fluorescent TAMRA group between adapters to be monitored by denaturing polyacrylamide gel electrophoresis (PAGE). DTS reactions were performed at pH 11 for 15 hours at room temperature. The relative proportions of DTS reaction and thioester hydrolysis products were assessed by PAGE densitometry by comparing the intensities of TAMRA fluorescence from bands corresponding to the amide product, intact thioester and free TAMRA (Figure 1c and Supporting Information).

We expected the DTS yield to be highest when reactants are tethered as close to each other as possible. We describe the relative positions of acyl donor and acceptor by the offset n (measured in base pairs) between their attachment sites: for $n = 0$ the donor and acceptor are directly opposite each other in the duplex (i.e., replacing a single base pair); n increases as one of the reactants (here, the acceptor) moves towards the 5′ end of its DNA adapter and decreases as it moves towards the 3' end. On the basis of measurements
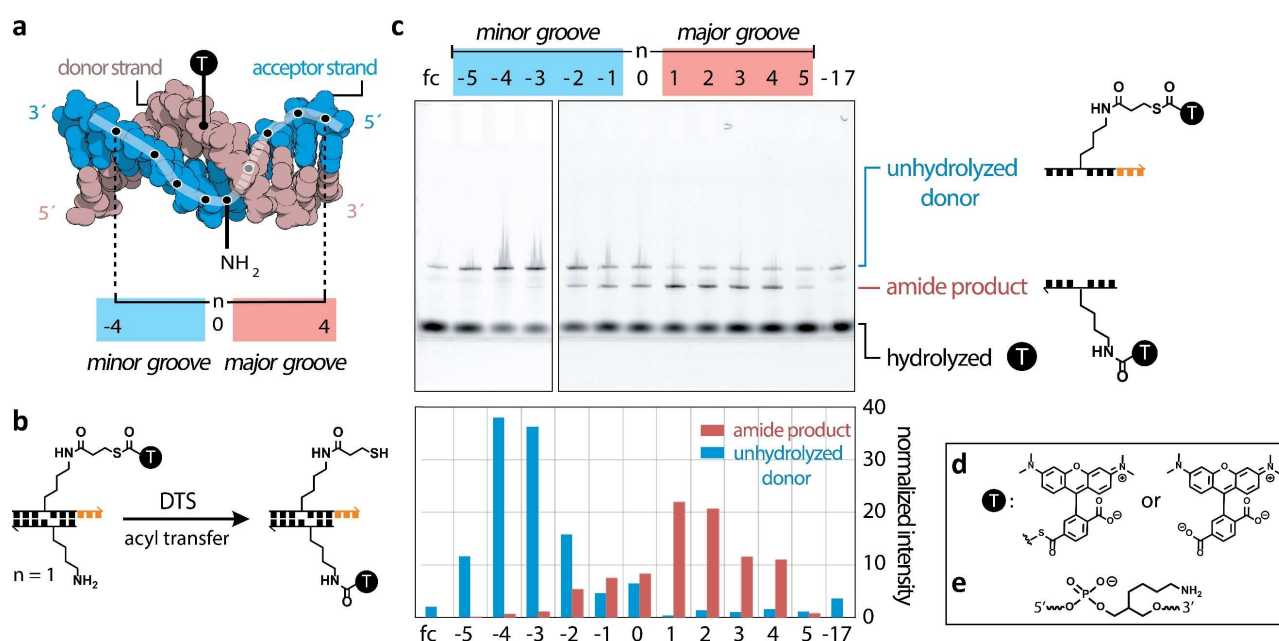

Figure 1. Position-dependent DTS with reactants tethered at internal DNA modifications. a) Space-filling 3D model of the central region of the DNA duplex employed in this study indicating possible positions of the acceptor amine relative to the donor TAMRA thioester (T). b) Schematic representation of the DNA-templated acyl transfer reaction. Squares represent nucleotides and missing squares represent abasic sites. The single-stranded overhang on the donor strand (orange) allows the two strands to be distinguished using PAGE. c) Denaturing PAGE gels imaged using TAMRA fluorescence (top) and reaction progression quantified by densitometry (bottom) for DTS reactions between the thioester and the amine at relative positions $-5 \leq n \leq 5$ and $n = -17$. The fully complementary control (fc) has no acceptor modification. d) Structures of the TAMRA fluorophore before and after hydrolysis of the thioester linkage. e) Structure of the abasic site with butylamine modification.

from crystal and NMR structures (Figure S2), we predicted that DTS yield would be highest at positions $n = -3, -4$ at which the sites where reactants are tethered are closest, directly across the minor groove. To test our hypothesis, we explored all positions of the acceptor relative to the donor between $n = -5$ and $n = 5$ (Figure 1c) alongside negative control reactions with a fully complementary (fc) strand with no acceptor and a strand in which the donor and acceptor are too far apart ($n = -17$) for the reaction to occur by intramolecular transfer.

As expected, DTS was observed to be strongly position-dependent (Figure 1c, samples $n = -5$ to 5) with maximum transfer observed when the acceptor was displaced by 1–2 nucleotides in the 5′ direction (positions $n = 1, 2$) from the donor. No transfer was observed with $n = -17$, indicating that there was no significant intermolecular DTS between reactants tethered to different duplexes (Figure 1c, $n = -17$). The results confirmed that DTS is possible in this previously unreported architecture in which reactants are tethered to opposite strands of a duplex at internal, abasic sites. However, our prediction that maximal yield would be observed when the separation between reactants is minimal proved to be incorrect: almost no DTS was observed for $n < -2$. Instead, this configuration was associated with effective protection of the donor thioester from hydrolysis (Figure 1c, samples $n = -3, -4$). This protective effect was also found to be strongly position-dependent, with almost no protection for offsets $n > 0$ at which DTS occurred most readily. These first results suggest that the distance between reactive groups is not the only factor that is critical for a successful acyl transfer reaction: a protective interaction between the TAMRA thioester and the DNA duplex with its abasic sites is also important.

To explore whether the amine group (the reactive acceptor) was involved in the protective effect, we compared the rates of thioester hydrolysis in duplexes with, at offset $n = -3$, either the amine-functionalized abasic site used in the experiments described above, an abasic site incorporating 1′,2′-dideoxyribose (with no acceptor modification), or an unmodified deoxyriboadenosine forming a fully complementary duplex (Figure 2a). The rate of thioester hydrolysis was reduced by over 100-fold in the presence of either colocalized abasic site, with the thioester being stable for over 48 hours at pH 11. Approximately equal protection was afforded by both abasic modifications, indicating that the internal abasic site, rather than the amine, is the necessary feature that confers protection. When TAMRA was replaced by a biotin group, DTS was observed but protection from hydrolysis was not (Figure S3): protection depends on the nature of the thioester-linked moiety and its interactions with the environment created by the surrounding DNA duplex.

The position-dependence of the protection effect was confirmed by using RP-HPLC to quantify degradation of the TAMRA thioester in the presence of an abasic site (C_3-spacer) in the opposite strand (Figure 2b, 2c and Figure S4). Results are broadly consistent with data presented in Figure 1c, for which reaction with an amine tethered at the abasic site competed with hydrolysis. Significant protection

of the TAMRA thioester was observed for $n = -1$ to -3, i.e. positions for which the abasic site was located across the minor groove from the thioester modification. Increased thioester stability for $n = -3$ was also observed when the thiol linker was extended (Figure S5). However, protection at $n = -4$ was weaker than at $n = -3$. As inferred from Figure 1c, TAMRA-thioester protection was generally weak (comparable to the fully complementary duplex control) when the abasic site was located in the range $n = 0$ to 4, corresponding to the donor positions at which maximal acyl transfer was observed. Protection at $n = 2$ is anomalously effective: this could have been masked by competing aminolysis in the experiment presented in Figure 1c.

The RP-HPLC retention time is significantly reduced when the abasic site is located at $n = -3$, where protection is maximal, compared to $n = 1$, where thioester hydrolysis is most severe (Figure 2b). No change in retention time was observed in controls utilizing the reduced thiol linker lacking the TAMRA moiety (Figure S6). The fluorescence signal strength of the tethered TAMRA moiety is significantly enhanced when the abasic site is located at $n = -3$.

On the basis of these observations, we propose a mechanism for protection based on insertion of TAMRA within the DNA duplex, mediated by hydrophobic interaction, at the nearby abasic site. We hypothesize that the positioning of the thioester bond close to or within the hydrophobic core of the DNA duplex hinders the approach of water molecules and thereby limits hydrolysis. This hypothesis is consistent with previous work showing that rhodamine dyes interact with guanine and cytosine residues (which flank the abasic site in most of our sequences),[11–12] and that insertion can be promoted by the presence of an abasic site.[13] Insertion of the hydrophobic TAMRA moiety in the DNA duplex is consistent with the decrease in RP-HPLC retention and enhancement of TAMRA fluorescence (associated with a reduction in the polarity of the environment of the dye[11]) that are observed to correlate with thioester protection. The lack of protection of a thioester linked to a hydrophilic biotin also points to the significance of hydrophobic interaction between TAMRA and the hydrophobic core of the duplex. A molecular model of the DNA duplex (Figure S7) confirms that the linker employed to connect the thioester is sufficiently long to allow the TAMRA moiety to insert into an abasic site in the -3 position.

As proof of principle that a strategically positioned abasic site can protect reactants and enhance the yield of a multi-step DTS reaction, we simulated an acyl transfer reaction occurring at a late stage in sequence-defined polymer synthesis by a molecular machine (Figure 3).[6a,14] We incubated the thioester donor adapter, hybridized to a complementary 'keeper' strand with or without an abasic protective site, at pH 11 for up to 48 hours to mimic the time required for previous reaction steps in the programmed multi-step DTS (Figure 3a). The complementary strand was then displaced by an acceptor adapter by toehold-mediated strand-displacement[15] to trigger DTS during a subsequent reaction period of up to 24 hours (Figure S8). In the resulting donor-acceptor duplex the abasic site carrying the
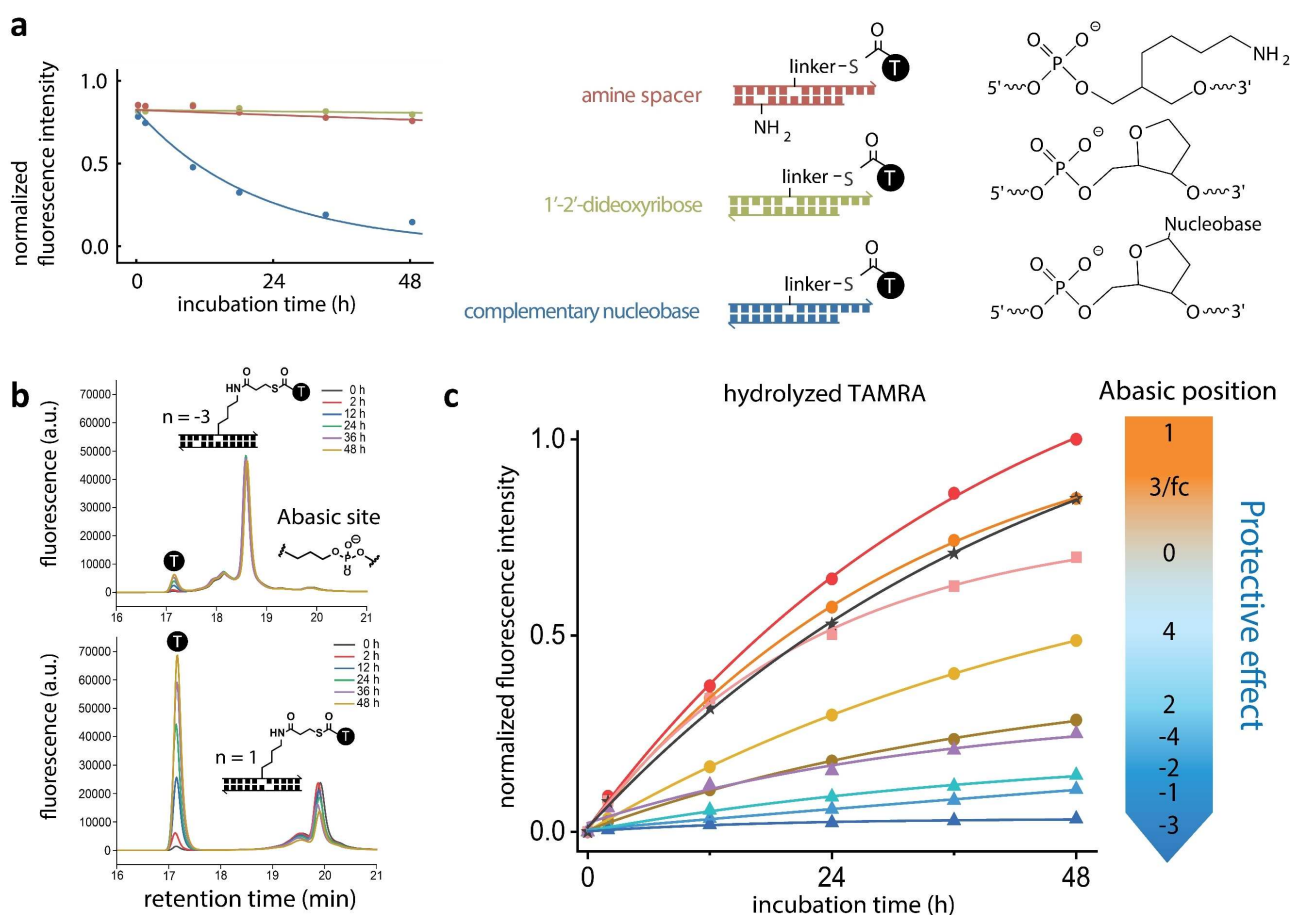
Figure 2. Protection of a TAMRA thioester by an abasic site in the complementary strand. Hydrolysis of the thioester was measured by RP-HPLC after incubation at 5 °C, pH 11 for 0–48 h. TAMRA fluorescence was measured with λ_{ex}:550 nm and λ_{em}:580 nm. a) Protective effects of each of the three modifications shown at the $n = -3$ position in the complementary strand. The proportion of unhydrolyzed thioester was calculated by comparing the areas under the RP-HPLC peaks corresponding to the DNA-thioester and free TAMRA. b) Comparison of protection afforded by complementary strands with the abasic site (C_3-spacer) located in the −3 position (maximum protection, top) and in the 1 position (minimum protection, bottom). c) Position-dependence ($n = 4$ to −4) of the protective effect of an abasic site (C_3-spacer) monitored by RP-HPLC time course using the integrated fluorescence intensity of hydrolyzed TAMRA as an indicator. A fully complementary opposite strand (fc) was used as a control.

reactive amine acceptor was at position $n = 1$, favouring DNA-templated acyl transfer. Results of experiments with fixed 48-hour incubation time and variable reaction time are shown in Figure 3b, and of experiments with variable incubation time and fixed 24 hour reaction time in Figure 3c. Without protection by an abasic site, hydrolysis of the starting material during the initial incubation period decreases the final DTS yield; with protection, yield is approximately independent of incubation time. Protection of the thioester during the incubation period increases synthesis yield significantly. These results demonstrate that this new architecture can be used to enable protection of a reactant from degradation until selectively displaced from its protective site by a hybridization reaction that is part of the sequence of reactions controlling synthesis. This general mechanism could be incorporated in a wide range of DNA-controlled mechanisms for sequence-controlled synthesis, including mechanisms based on chain-insertion reactions.[10a]

In conclusion, we report a new architecture for DTS in which reactants are tethered to complementary oligonucleotide adapters through internal DNA modifications. Proof-of-concept experiments using a TAMRA fluorophore linked to a DNA adaptor by a thioester led to the unexpected discovery that an abasic site in a DNA duplex can protect a thioester from hydrolysis. Control experiments suggest that protection is associated with insertion of the planar, hydrophobic fluorophore into the abasic site, positioning the thioester within or close to the hydrophobic core of the DNA duplex. This result expands the potential roles of the DNA adapters used in DTS from reactant carriers and reaction templates to protectors of reactive building blocks, analogous to the elongation factor EF–Tu that protects aminoacyl-tRNA during ribosomal peptide synthesis.[16] The utility of the approach will depend on how well the protective effect can be extended to building blocks that do not insert stably in the DNA duplex. We note that it is specifically the labile linker between monomer and DNA
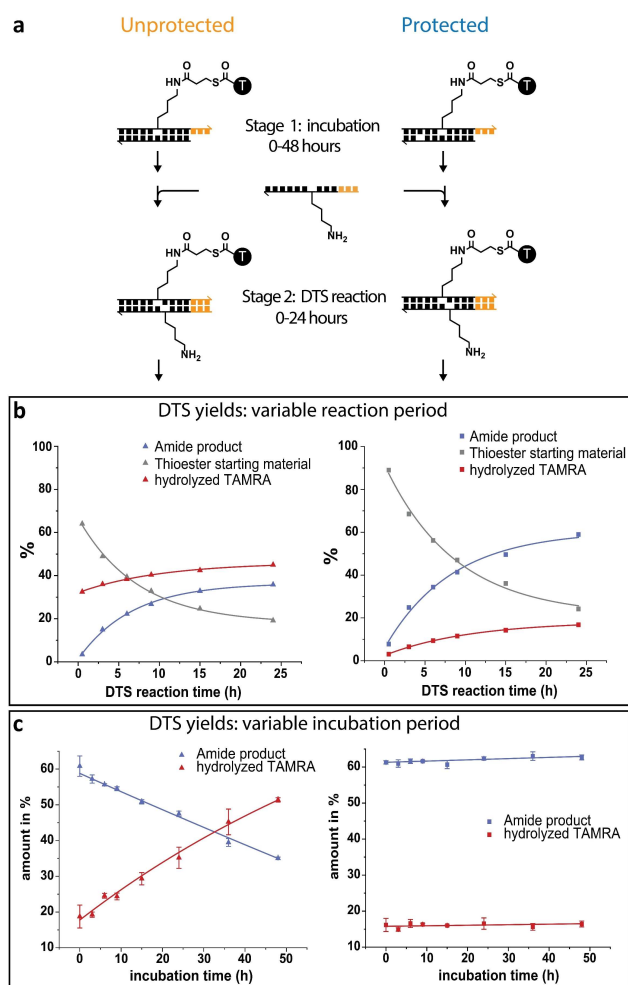
Figure 3. Exploiting the protection strategy. a) Stage 1 (incubation). Donor adapters, hybridized to a complementary keeper strand with or without an abasic protective site at position $n=-3$, were incubated at pH 11 at 5°C for between 0 and 48 h. Stage 2 (reaction). Reactions were initiated using toehold-mediated strand exchange to replace the keeper strand with an acceptor strand forming a duplex with an amine acceptor at the $n=1$ position. DTS was performed at 15°C for 24 hours. b) DTS reaction progress for the unprotected and protected systems as functions of reaction time after 48 h incubation at stage 1. DTS reaction progression was assessed by RP-HPLC using the TAMRA fluorescence channel (λ_{ex}:550 nm and λ_{em}:580 nm) (Figure S9). c) As b) but with fixed 24 h reaction time and with variable incubation time. DTS reactions were performed in triplicate: error bars represent the standard deviation of the yield.

tag that is in need of protection: it may be possible to incorporate hydrophobic elements in a universal linker that drive insertion and consequent protection at an abasic site, even for hydrophilic monomers. Protection of reactive building blocks until they are required for reaction is an important attribute of molecular machinery capable of sequence-defined polymer synthesis and a significant step towards new technologies that will enable the power of directed evolution to be brought to bear on artificial materials.[4b]

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: DNA-templated synthesis · colocalization · hydrolysis · abasic · DNA

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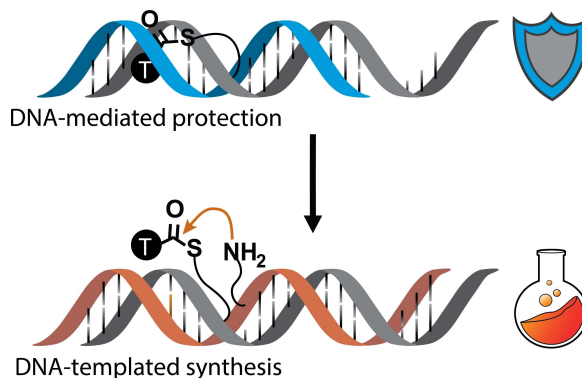## *Zuschriften*

Nucleic Acids

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A New Architecture for DNA-Templated
Synthesis in Which Abasic Sites Protect
Reactants from Degradation

DNA-mediated protection

DNA-templated synthesis

An abasic site within a DNA duplex reduces hydrolysis of a colocalised DNA-thioester. This forms the basis for an architecture for DNA-templated synthe-sis which protects DNA-tagged building blocks until a DNA strand exchange reaction removes the protective site and colocalizes a second reactive group.