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DNA-directed formation of peptide bond: a model study toward DNA-programmed peptide ligation

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ABSTRACT

A model study of DNA-directed peptide ligation has been developed by transferring fluorescent reporting group from small molecule thioester to a DNA strand (template DNA) in the presence of a thiol-functionalized DNA strand (auxiliary DNA), mimicking the Native Chemical Ligation (NCL) reaction. This DNA-directed transfer shows dependence on the sequence complementarity of the two DNA strands, with in situ generated 4-thiolphenylmethyl functionalized oligonucleotide as the auxiliary DNA strand, under mild basic condition (pH=8.5), and with tris(2-carboxyethyl) phosphine hydrochloride (TCEP) as a reducing agent. Reactions with different amino acid α-thioesters resulted in varied transfer efficiencies from glycine to α-substituted amino acids. This study has provided the basic foundation to use DNA-programmed chemistry toward the chemical synthesis or unnatural modification of protein molecules.

1. Introduction

Chemical synthesis and site-selective modification of large proteins call for versatile ligation strategies to assemble small peptide fragments generated from conventional solid-phase peptide synthesis (SPPS). Inspired by the thiol capture strategy developed by Kemp and co-workers,[1] several ligation methods have been introduced. Among them, Native Chemical Ligation (NCL)[2] is arguably the most powerful one and, due to its mild reaction condition, high efficiency, and excellent chemospecificity, has shown great utility and versatility in broad applications of protein chemical synthesis.[3] In NCL, the cysteine residue at the N-terminus of one peptide can capture the α-thioester of another peptide by thiol–thioester exchange, followed by the S to N shift, to afford the amide bond at the ligation site.[4] However, despite its wide applications, the requirement of an *N*-cysteine residue, a low abundance amino acid in natural proteins,[5] has limited NCL as a general method for protein chemical synthesis.

Therefore a variety of elegant strategies have been developed to either transform *N*-cysteine residue to other amino acid after ligation,[6] or to utilize template structures to fulfill the function of cysteine in order to overcome this limitation.[7–9] In NCL and other ligation methods, thiol–thioester exchange is usually considered as a fast step, and the kinetic advantage changes the slow intermolecular acyl transfer to a much faster intramolecular S to N shift (Scheme 1a), which is the origin of NCL's excellent chemospecificity for peptide chemical ligation where amino acid side chains remain unprotected.[4]

Among the numerous methods to control the reactivity in organic synthesis, DNA-templated synthesis (DTS)[10–18] has emerged as a novel approach to achieve such goal at very low reactant concentration while increasing the effective molarity of DNA-linked substrates through DNA hybridization. DTS has been systematically developed and applied in programmed synthesis of small molecule libraries, interrogation, and selection against biological targets, sequence specific modification of DNA/RNA, reaction discovery, bio-detection, as well as evolution and manipulation of DNA-conjugated molecules.[19–22] The high fidelity of Watson–Crick base pairing confers great selectivity and sequence specificity to DNA-templated chemical reactions.[14]

Previously, efforts have been focused on using DNA-templated acyl transfer reaction to generate peptide or peptidomimetic bond. Liu and co-workers have applied DNA-templated amide bond formation reactions in the synthesis of DNA-encoded libraries with peptidic structures.[22,23] Stulz group has systematically assessed the efficiencies of DNA-templated attack of thioesters by a variety of nucleophiles.[24] Using a DNA-conjugated peptide thioester, Joyce and co-workers have shown that the peptide component could be transferred to another DNA strand by DNA-templated acyl transfer reaction.[25] In addition, native chemical ligation has been used by Grossmann and Seitz group in the DNA-catalyzed transfer of

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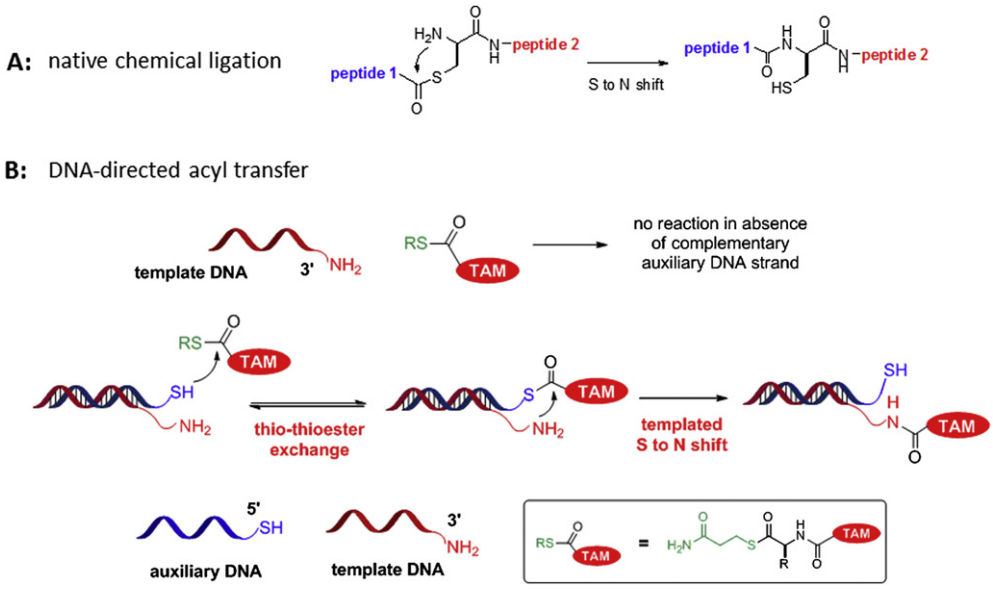

Scheme 1. A: Native chemical ligation (NCL); B: DNA-directed formation of peptide bond by acyl transfer. After the thiol–thioester exchange between the thiol-functionalized auxiliary DNA with the small molecule thioester labeled with fluorescent dye (TAM: TAMRA (tetramethyl-6-carboxyrhodamine)), DNA-templated S to N shift transfers TAM to the template DNA.

peptide from PNA-linked peptide thioester to another PNA-linked peptide.[26] These previous DNA-templated acyl transfer reactions highlight their potential application in the controlled synthesis of polypeptides.

However, previously demonstrated DNA-templated acyl transfer reactions are limited by the requirement of an existing DNA-conjugated thioester and the fact that such reactions only proceeded in a DNA-templated coupling format. Enabling the acyl transfer from free, non-DNA-conjugated thioesters to peptides in a DNA-directed manner will significantly expand the scope of DNA-directed synthesis of peptide bond and hold further potential for DNA-programmed peptide ligation and protein chemical synthesis, especially modifications by unnatural amino acid thioesters. NCL relies on the irreversible intramolecular S to N shift to capture the transferred acyl group and a series of studies have shown auxiliary groups can be used as surrogates in the absence of a terminal cysteine.[9] Here in this report, we propose to use the hybridization of two complementary oligonucleotides to enable the intramolecular S to N shift to form the peptide bond. As shown in Scheme 1b, a thiol-modified oligonucleotide (auxiliary DNA) and a complementary amine-bearing oligonucleotide (template DNA) form a DNA duplex. The thiol–thioester exchange brings the thioester in close proximity to the amine group on the template DNA, eventually leading to intramolecular S to N shift and formation of the peptide bond, analogous to NCL. Different from other DNA-based approaches, this design does not require a DNA-conjugated thioester and the ligation specificity depends on the sequence complementarity of the two participating oligonucleotides.

Reaction yield of NCL is mainly influenced by amino acid residues at the ligation site;[2] therefore, in our study, the amine-modified DNA and the TAMRA (TAM)-linked amino acid thioester could mimic the two amino acid residues at the ligation site. And the conjugated TAMRA group permits easy monitoring and quantification of reaction yield by polyacrylamide gel electrophoresis and fluorescent densitometry.

2. Results and discussion

Mechanistic studies show that aryl-thioesters are more reactive than alkyl-thioesters in native chemical ligation reactions, and (4-carboxymethyl)thiophenol (MPAA) was usually added as catalyst to generate the reactive thioester species in situ.[4] Therefore, we synthesized the 2-(4-(pyridin-2-yldisulfanyl)phenyl)acetic acid modified oligonucleotides as the auxiliary DNA. Reduction of the disulfide bond will generate the reactive thiol-modified DNA in situ. We envisioned that three major factors will affect the reaction yield. First, the intermolecular nature of thiol–thioester exchange requires higher concentration of the small molecule thioester and the template DNA for greater reaction yield; second, the nucleophilicity of the amino and thiol group on the DNA strands are heavily influenced by the pH of the reaction solution: protonation of the nitrogen at low pH will substantially slow down the acyl transfer reaction; while high pH should also be avoided to prevent the reaction from competing hydrolysis of the thioester; third, reducing agent should be added to reduce the disulfide bond and protect the in situ generated thiol group against oxidation.

We first examined the effects of reactant concentration. As expected, reaction yield increases with higher concentrations of TAM-Gly-thioester (Fig. 1a, lane 1, 4, 5). At 50 μM (lane 1), the reaction yield reaches the plateau and further increasing thioester concentration did not improve the yield, suggesting saturation and the S to N shift is now the limiting step. In contrast, very little product was observed in the absence of tris(2-carboxyethyl) phosphine hydrochloride (TCEP) (Fig. 1a, lane 3), indicating the necessity of activation of the auxiliary DNA by disulfide cleavage to generate the free thiol group. Importantly, as a unique feature for DNA-directed reactions, with a mismatched auxiliary DNA (labeled MR in Fig. 1a, lane 2), also very little product was formed, proving the intramolecular nature of the acyl transfer between the two DNA strands. The faint band observed with mismatched DNA is possibly due to the background direct aminolysis between the template DNA and the high concentration thioester.[27] Moreover, consistent with reported NCL, reaction yield was achieved at mildly basic pH=8.5 (Fig. 1a, lane 1 and lane 6–9), which seems to be the balanced condition conferring enough nucleophilicity to the amino group while keeping the hydrolysis of thioester sufficiently slow.

The overall reaction yield is still lower than other reported auxiliary-mediated ligation methods.[7–9] To understand the underlying reasons, we first performed the reaction with organic

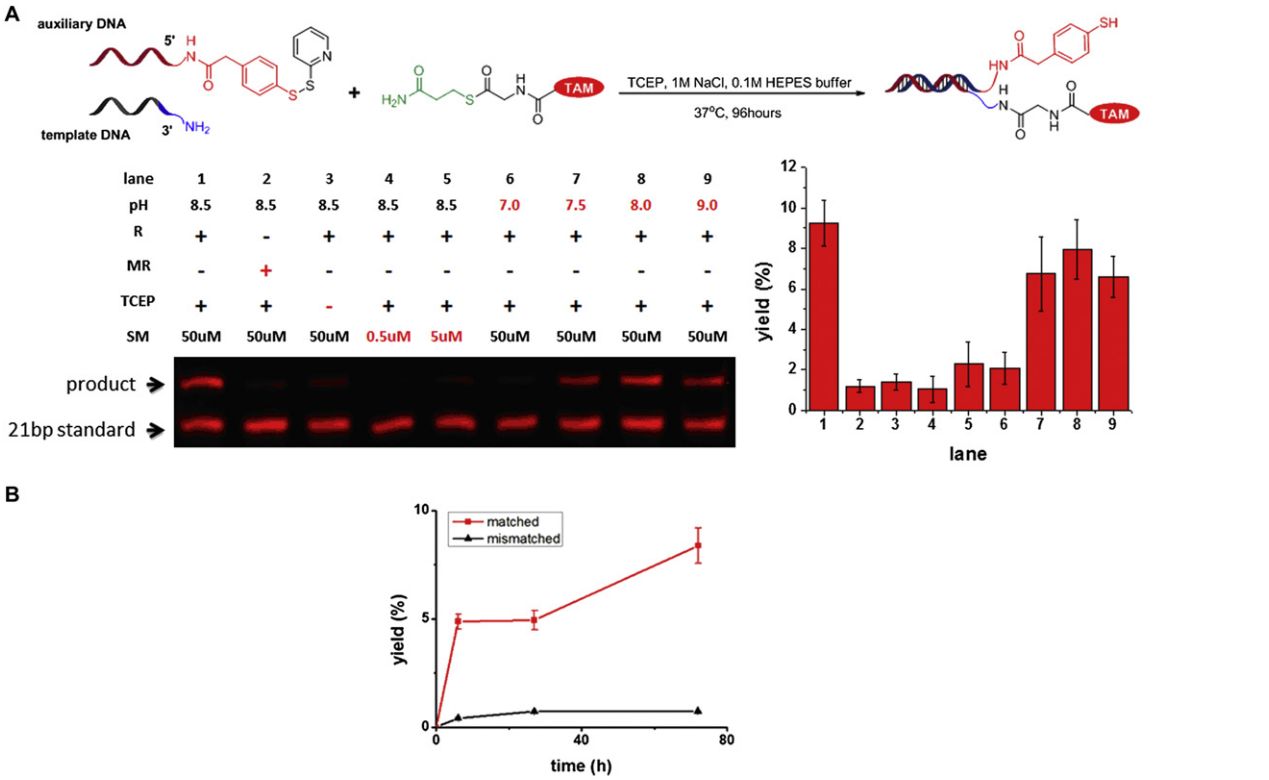

Fig. 1. Investigation of reaction conditions. R: matched auxiliary DNA; MR: mismatched auxiliary DNA; SM: TAM-Gly-thioester. Reactions were carried out in 1 M NaCl solution, 0.1 M HEPES buffer, 5 mM TCEP, with DNA concentration of 0.5 μM for each strand at 37 °C after 96 h. 0.05 μM 21 bp TAMRA-linked oligonucleotide was added to each reaction mixture as internal standard for reaction yield quantification. Yield was calculated from the ratio of fluorescent intensity of product bands to internal 21 bp standard bands. A: denaturing PAGE analysis of reactions performed in different conditions. B: reactions with matched auxiliary DNA and mismatched auxiliary DNA were analyzed at different time points.

co-solvent, previously used to reduce competing thioester hydrolysis.[9g] However, addition of several organic solvents did not lead to improved yield (supplementary data, Fig. S1), suggesting thioester availability is not the limiting factor in our reaction. Second, we performed direct NCL between thioester and cysteine-labeled template DNA strand as a comparison (supplementary data, Fig. S2). Indeed, the reaction yield increased significantly to 45–50%, comparable to regular NCL.[3] In addition, a DNA-directed acyl transfer similar to Fig. 1 but with a cysteine-labeled template DNA instead of a simple amine also gives improved yield (38.4%, 47.7%, supplementary data, Fig. S2). Collectively, the data strongly suggest that the S to N shift is rate-limiting in our system, in consistency with other NCL-based ligation methods.[2,7–9] We reason that possibly the formation of a large 44-memebered macrocyclic transition state is entropically much less favored than the five-membered ring in NCL (see supplementary data, Fig. S3), therefore leading to slower reaction kinetics.[9g,h] We also prepared an auxiliary DNA with a shortened linker (supplementary data, Fig. S2, auxiliary DNA A2); the reaction also gave even lower yield, suggesting steric or conformational factors may also be implicated. Wong and co-workers have reported ~20% to ~80% yields with either a 15- or 18-membered transition states in Sugar-Assisted Ligation (SAL),[9f–h] providing the possibility of yield improvement via further optimization of the DNA and linker structures in our system.

In agreement with previous studies on DNA-templated acyl transfer reaction between DNA-linked amine and thioester,[12] the reaction efficiency is lower than that of DNA-templated amide bond formation with *N*-hydroxylsuccinimidyl (NHS) activated ester as the electrophile,[23] presumably due to the lower electrophilicity of thioester compared with NHS activated ester. Indeed, previous reports show that direct intermolecular aminolysis of the thioester with amine has very slow kinetics, while reactions with NHS activated ester proceed to near completion rapidly.[23,27]

Typically, DNA-templated reactions have high sequence specificity.[14] We further compared the kinetics of the reactions with matched and mismatched auxiliary DNAs under conditions developed above. The initial rate of reaction with matched auxiliary DNA is 15-fold faster than that of auxiliary DNA bearing mismatches. (k_{app}=7.5×10^{-5} μM^{-1} s^{-1} vs k_{app}=7.5×10^{-5} μM^{-1} s^{-1} Fig. 1b). These results clearly showed the necessity of DNA strand sequence complementarity and the template effect in our system.

To evaluate the substrate scope, we prepared other two TAM-linked amino acid α-thioesters bearing the residues of Leu and Pro (see supplementary data for details). Together with TAM-Gly-thioester, these three thioesters represent three sterically different types of amino acids. We also conjugated different types of amino acids to the amine-modified template DNA strand. Similar to NCL and other ligation methods, reaction yield decreases with amino acids that have bulkier side chains (Fig. 2). Higher reaction yields were observed with TAM-Gly-thioester, while almost no product was formed when TAM-Pro-thioester was used. Reactions with oligonucleotides modified with sterically hindered amino acids (Val, Pro, Phe) showed lower yields. Polar or charged residues (Gln, Glu) did not show significant effects on the ligation yields. Interestingly, Ala-Gly pair gives higher yield than Gly-Gly, indicating effecting parameters other than simple steric hindrance. It should be noted that the desired product was observed even with phenylglycine-linked oligonucleotides, which suggested the potential for incorporating unnatural functionality into the peptide linkage using our DNA-directed ligation method.

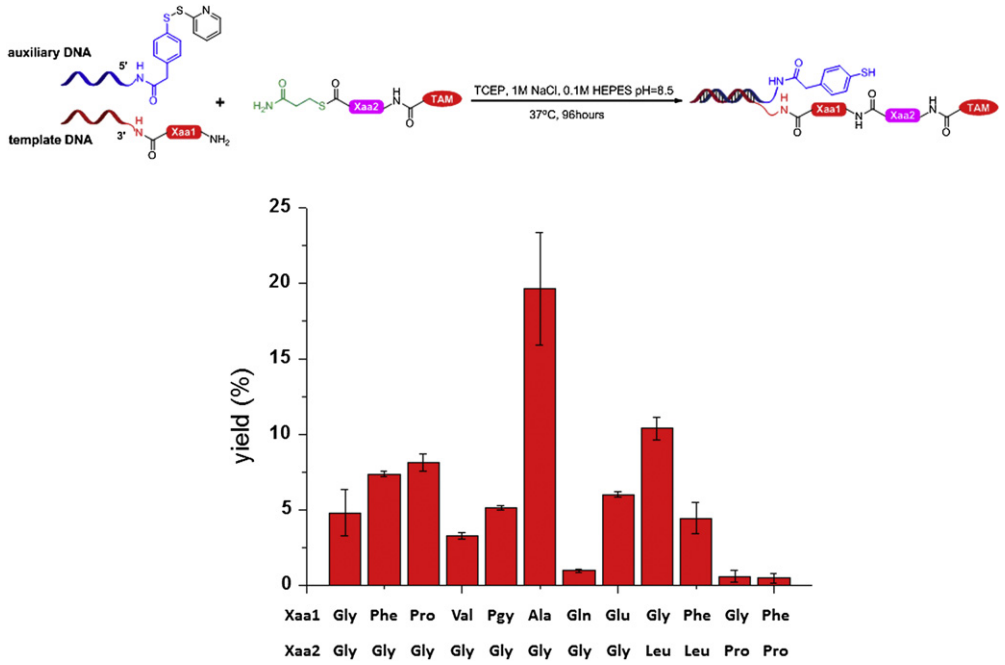

Fig. 2. DNA-directed formation of peptide bond with various terminal amino acid residues on the template DNA (Xaa1) and small molecule thioester (Xaa2). Reaction conditions are the same as those developed in Fig. 1a.

3. Conclusion

In summary, we have demonstrated the feasibility of using DNA to direct the ligation of small molecule thioester with complementary amine-modified oligonucleotides. Phenylthiol-linked oligonucleotide was generated in situ and used for thiol–thioester exchange with small molecule thioesters, following S to N shift transferred the fluorescent reporting group to the complementary DNA strand. The DNA-directed acyl transfer and peptide bond formation shows excellent specificity on the DNA sequence complementarity, providing the potential of performing multiplexed peptide ligation in a single solution directed by different DNA strands. The DNA-directed reaction's substrate scope was investigated and, as expected, ligation yield decreases with amino acids that have bulky side chains. Collectively, this study provides the potential applicability of this method in cysteinyl-free ligation of peptides directed by DNA. Data show that the rate-limiting step in our system is the intramolecular S to N shift, possibly due to the formation of an entropically disfavored macrocyclic transition state between the two DNA strands, instead of the five-membered ring in NCL. Further studies will focus on improving the reaction kinetics, including: (1) chemically modifying the DNA and linker structure to reduce the entropic loss in the S to N shift; and (2) shifting the hybridization position of the auxiliary DNA on the template to optimize the conformation and steric effects in the S to N shift.

4. Experimental section

4.1. General methods

Unless otherwise noted, all reagents and solvents were purchased from commercial sources and used as received. DNA oligonucleotides were synthesized on an Applied Biosystem 3940 DNA synthesizer using standard phosphoramidite protocols and purified by reverse-phase HPLC with triethylammonium acetate (TEAA)/CH_3CN gradient. Oligonucleotides bearing 3′-amino group were synthesized using 3′-amino-CPG. 5′-amino was incorporated to DNA oligonucleotides using 5′-amino-modifier. Oligonucleotides were quantitated by UV using BioTek Epoch UV–vis spectrometer. Reaction yields were quantitated by denaturing polyacrylamide gel electrophoresis (PAGE) followed by measuring fluorescent intensity using BioRad Gel-Doc system. Relative yields were calculated as the ratio of fluorescent volume between the product bands and the internal standard bands.

4.2. Oligonucleotide functionalization

4.2.1. 2-(4-(Pyridin-2-yldisulfanyl)phenyl)acetic acid modified oligonucleotides. After automated DNA synthesis, the CPG was suspended in 3% TCA in DCM for 10 min to remove the 5′-MMTr protecting group, the resin was washed thoroughly with DCM and dried under vacuum. For 35 mg CPG resin (up to 1 μmol DNA loaded), 2-(4-(pyridin-2-yldisulfanyl)phenyl)acetic acid (11 mg, 50 μmol), HOBt (7 mg, 50 μmol), HBTU (17 mg, 45 μmol), DIPEA (20 μL, 115 μmol) and anhydrous DMF (1.2 mL) were added, and the reaction mixture was agitated at 25 °C for 12 h. The resin was washed with DMF and then thoroughly with DCM and dried under vacuum. Treatment for 50 min with AMA (NH_4OH/CH_3NH_2, v/v=1/1.4) at 55 °C fully deprotected the oligonucleotide and liberated the disulfide protected auxiliary DNA. The functionalized oligonucleotide was purified by reverse-phase HPLC and characterized by MALDI-TOF mass spectrometry. Matched auxiliary DNA in Figs. 1 and 2: observed as reduced thiol-modified auxiliary DNA, observed mass=4839±5 (expected: 4836). Mismatched auxiliary DNA in Figs. 1 and 2: observed as reduced thiol-modified auxiliary DNA, observed mass=4813±5 (expected: 4809).

4.2.2. Amino acid-conjugated oligonucleotides. FmocNH-Xaa-COOH (90 μmol) (For Glu-conjugated DNA, FmocNH-Xaa(OFm)-COOH was used), Sulfo-NHS (20 mg, 90 μmol), EDCI (18 mg, 90 μmol) in anhydrous DMF (100 μmol) were agitated for 2 h to generate the Sulfo-NHS esters in situ. The DMF solution (50 μL) was added to 3′-amino template DNA (up to 10 nmol) in 200 mM phosphate

(pH=7.0) at room temperature under sonication for 2 h. The Fmoc-Xaa-OH functionalized oligonucleotides were purified by gel filtration and reverse-phase HPLC and lyophilized. The Fmoc protecting group was removed by suspending the purified oligonucleotides in 0.1 M NaOH for 1 h, the amino acids conjugated oligonucleotides were purified by gel filtration and reverse-phase HPLC and characterized by MALDI-TOF mass spectrometry (using the mixture of 3-hyroxylpyridine-2-carboxylic acid (3-HPA) and ammonium citrate as matrix). Glycine conjugated oligonucleotide: observed mass 10,806±11 (expected: 10,801 $[M+citrate+H]^+$); valine conjugated oligonucleotide: observed mass 10,809±11 (expected: 10,811 $[M+matrix+Na]^+$); proline conjugated oligonucleotide: observed mass 10,765±11 (expected: 10,788 $[M+matrix+H]^+$); alanine conjugated oligonucleotide: observed mass 10,653±11 (expected: 10,645 $[M+Na]^+$).

4.2.3. TAM-linked internal standard oligonucleotide. 21 bp 3′-Amino DNA oligonucleotide (up to 20 nmol) was combined with TAM NHS ester (1 mg) in 200 mM sodium phosphate (pH=7.0) at room temperature for 2 h. The functionalized oligonucleotide was purified by gel filtration and reverse-phase HPLC.

4.3. DNA-templated reactions

Reactions were performed as described in Figs. 1 and 2. Starting materials and products were ethanol-precipitated from the reaction mixture, analyzed by denaturing PAGE, quantified as described above.

4.4. Oligonucleotide sequences

Sequences are as follows:

Template DNA in Fig. 1: 5′AGTGGGGATTGTGAGGGCTTGGGAATT CAGCTC-NH_2;

Template DNA in Fig. 2: 5′AGTGGGGATTGTGAGGGCTTGGGAATT CAGCTC-NHCO-Xaa1-NH_2; Matched auxiliary DNA in Figs. 1 and 2: 5′ protected thiol-CONH-GACCTGAATTCCCAA; Mismatched auxiliary DNA in Fig. 1: 5′ protected thiol-CONH-GTCCTCTATTGCCTA;

TAM-linked internal standard DNA in Figs. 1 and 2: 5′ GACGGCTTGGGAATTCAGGTC-NH-TAM.

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Supplementary data

Supplementary data related to this article can be found online at doi:10.1016/j.tet.2012.04.032.

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