Bioorganic & Medicinal Chemistry Letters 23 (2013) 761–763

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Synthesis of DNA oligonucleotides containing C5-ethynylbenzenesulfonamide-modified nucleotides (EBNA) by polymerases towards the construction of base functionalized nucleic acids

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ARTICLE INFO

Article history:
Received 12 October 2012
Revised 15 November 2012
Accepted 20 November 2012
Available online 3 December 2012

Keywords:
Modified nucleotides
Enzymatic incorporation
Functionalized nucleic acids
Oligonucleotides

ABSTRACT

C5-Ethynylbenzenesulfonamide-modified nucleotide (EBNA) was investigated as substrate of various DNA polymerases. The experiments revealed that KOD, Phusion and Klenow DNA polymerases successfully accepted EBNA-T nucleotide as a substrate and yielded the fully extended DNA. KOD DNA polymerase was found to be the most efficient enzyme to furnish EBNA-T containing DNA in good yields. Phusion DNA polymerase efficiently amplified the template containing EBNA-T nucleotides by PCR.

Nucleic acid aptamers[1–5] are functional nucleic acids with significant potential in therapeutic development and bio-analysis. Introduction of various functional groups at the C5-position of the nucleobase is one approach to modify the DNA or RNA aptamers with additional functions (cross-coupling reactions to introduce fluorescent molecules, affinity capture tags etc.).[6] Aptamers containing modified nucleotides can be synthesized using a DNA synthesizer via standard phosphoramidite chemistry. However, there are some limitations to this approach such as incompatibility of chemical reagents, requirement of using additional protecting groups, reduced coupling yields etc. Enzymatic approach for synthesizing modified oligonucleotides can overcome these limitations, however, the recognition of modified nucleotide triphosphates by polymerases is often difficult due to their conformation and positioning in the enzyme active site. Aptamers are developed by a process called Systematic Evolution of Ligands by EXponential enrichment (SELEX).[7,8] In order to use modified nucleotides in SELEX processes, it is mandatory to investigate their enzymatic tolerance as it involves enzymatic steps.

We have recently reported the synthesis of C5-ethynylbenzenesulfonamide-modified (we use the abbreviation EBNA to address this modification in this report) nucleosides.[9] Four consecutive incorporations of EBNA into a DNA:RNA duplex led to the efficient stacking of sulfonamide substituted phenylalkynyl moiety in the major groove thereby, resulting in the very stable DNA:RNA duplexes.[9] Moreover, specificity of the recognition was also maintained as mismatch duplexes with single central mismatch against EBNA were found to be significantly less stable compared to the matched duplexes. [9] Herein, we report the enzymatic recognition capabilities of EBNA-T nucleotide (Fig. 1) and EBNA-T-modified templates for PCR amplification. First, we tested the incorporation

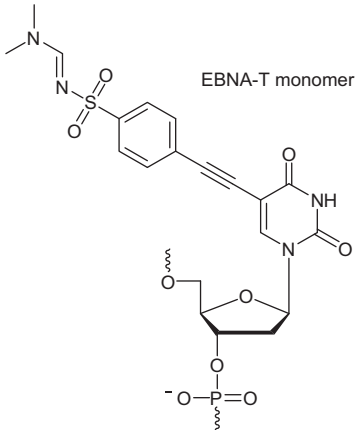

Figure 1. Structural representation of C5-ethynylbenzenesulfonamide-modified nucleotide (EBNA) monomer.

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http://dx.doi.org/10.1016/j.bmcl.2012.11.096

Table 1
Primer and template sequences. Incorporation sites are represented in bold and underlined

Name	Sequence	Length
T1	5′-**AAAAAAA**CCTATAGTGAGTCGTATTA-3′	26
T2	5′-CCGCTGGT**AAAAAAAA**CCCGGGGCCTATAGTGAGTCGTATTA-3′	42
T3	5′-GGTCTGGTCC**A**C**A**CCC**A**GCCGGGGCCTATAGTGAGTCGTATTA-3′	43
P1	5′-TAATACGACTCACTATAGG-3′	19
P2	5′-GGTCTGGTCCACACCCAGCC-3′	20

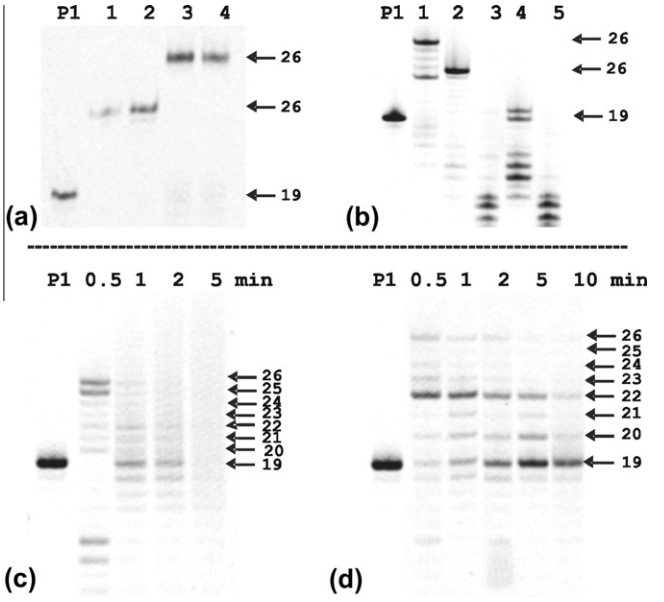

Figure 2. Enzymatic incorporation of modified EBNA-T using KOD DNA polymerase and template T1. (a) Successive incorporation of EBNA-T; lane P1: Primer, lane 1 and 2: Incorporation using dT at 30 s and 1 min respectively, lane 3 and 4: Incorporation of EBNA-T at 1 and 2 min, respectively. (b) Fidelity test; lane P1: primer, lane 1: extension with EBNA-T, lane 2: extension with dT, lane 3: extension with dA, lane 4: extension with dG, lane 4: extension with dC. (c and d) Stability of extension products with dT and EBNA-T nucleotides respectively at different incubation time.

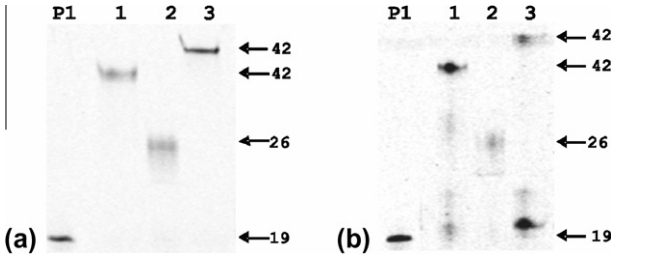

Figure 3. Multiple successive incorporation of EBNA-T nucleotide using T2. (a) Incorporation using KOD DNA polymerase; lane P1: primer, lane 1: extension with natural dNTPs, lane 2: negative control (dATP, dGTP, dCTP), lane 3: EBNA-T incorporation (dATP, dGTP, dCTP, EBNA-TTP). (b) Incorporation using KOD DNA polymerase; lane P1: primer, lane 1: extension with natural dNTPs, lane 2: negative control (dATP, dGTP, dCTP), lane 3: EBNA-T incorporation (dATP, dGTP, dCTP, EBNA-TTP).

of EBNA-T nucleotides by primer extension experiments for which the triphosphate derivative of EBNA was synthesized as previously reported.[10] Four different enzymes, KOD, Phusion, Klenow and *Taq* DNA polymerases were screened for their ability to accept EBNA-T nucleotide as a substrate. Template T1 (Table 1) was designed to

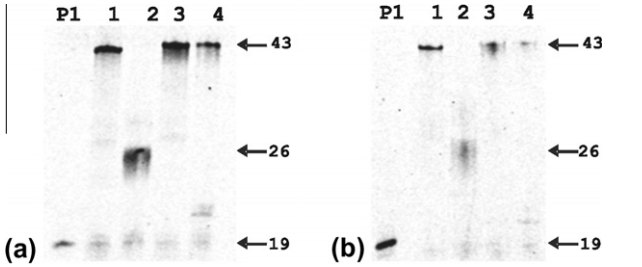

Figure 4. Incorporation of EBNA-T nucleotide using T3. (a) Incorporation using KOD DNA polymerase; lane P1: Primer, lane 1: extension with natural dNTPs, lane 2: negative control (dATP, dGTP, dCTP), lane 3 and 4: EBNA-T incorporation (dATP, dGTP, dCTP, EBNA-TTP) at 5 and 10 min, respectively. (b) Incorporation using Phusion DNA polymerase; lane P1: primer, lane 1: extension with natural dNTPs, lane 2: negative control (dATP, dGTP, dCTP), lane 3 and 4: EBNA-T incorporation (dATP, dGTP, dCTP, EBNA-TTP) at 5 and 10 min, respectively.

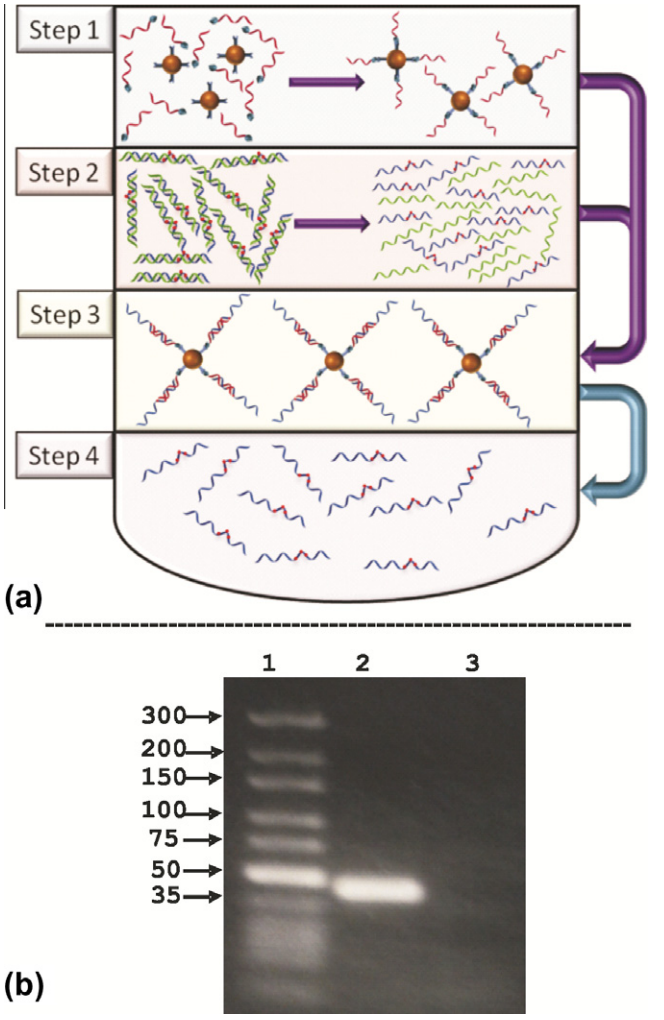

Figure 5. Purification of the primer extended DNA and subsequent PCR amplification. (a) Purification and isolation of the primer extended ssDNA with EBNA-T nucleotide incorporations; Step 1: Immobilization of a capture probe DNA onto a streptavidin coated magnetic bead, step 2: phenol–chloroform extraction of the primer extension product followed by heat denaturation, step 3: Primer extended ssDNA with EBNA-T incorporations of EBNA-T nucleotides are captured by the magnetic bead immobilized probe DNA, step 4: Isolation of the extension product by gentle heating. (b) PCR amplification using the purified primer extension product with EBNA-T nucleotide by Phusion DNA polymerase; lane 1: Marker DNA, lane 2: amplification using the ssDNA containing EBNA-T nucleotides, lane 3: amplification without using a template (negative control).

incorporate seven consecutive EBNA-T nucleotides and annealed to a 5′-FAM-labeled 19 nucleotide (nt) primer P1 (Table 1).

The experiment revealed that KOD (Fig. 2a), and Klenow (Fig. S1a), DNA polymerases successfully extended the primer to full-length whereas Phusion and *Taq* DNA polymerase could only incorporate up to three and one EBNA-T nucleotide respectively (Fig. S1b and c). KOD DNA polymerase was found to be the most efficient one as it yielded the full-length product in good yields. Interestingly, we observed a shift in the migration of products between the positive control reaction using all natural nucleotides and EBNA-T containing reaction product. This might be due to the successive incorporation of the bulky EBNA moiety affecting the mobility of the modified DNA. An extension experiment was also performed to check the fidelity of EBNA-T incorporation using other four individual natural nucleotides by KOD DNA polymerase. The results showed that the polymerase followed the necessary fidelity in recognizing EBNA-T nucleotides (Fig. 2b).

The stability of EBNA-T containing extension product was compared with the product containing natural nucleotides by performing an extension experiment using template T1. KOD DNA polymerase was used for this experiment as it has a very high degree of 3′→5′ exonuclease activity. The reactions mixtures were incubated at 72 °C and monitored at 50 s, 1, 2, 5 and 10 min. Extension product with natural nucleotide was degraded in 2 min whereas the product containing EBNA-T was still present even at 10 min of incubation (Fig. 2c and d). Later, we investigated the successive incorporation of EBNA-T nucleotides using a long DNA template, T2 in presence of other three natural nucleotides. In parallel to the positive control and the EBNA-T reactions, a negative control reaction that lacks 'T' nucleotide in the dNTP mixture was also performed. The designed template T2 (Table 1) directs eight consecutive sites of incorporations. KOD and Phusion DNA polymerase incorporated eight EBNA-T nucleotides successively and extended the primer onwards to full-length (Fig. 3a and b). Notably, both Klenow and *Taq* DNA polymerase failed to extend the primer after the first EBNA-T incorporations (data not shown). As shift in the migration of EBNA-T containing product was again observed in line to the reaction using template T1.

Another extension experiment was conducted with the aim of generating EBNA-T modified DNA as a template for polymerase chain reaction amplification (PCR) as it is the first enzymatic step involved in the aptamer selection process. For this experiment, the designed template T3 (Table 1) directs three EBNA-T nucleotide incorporations. KOD, Phusion (Fig. 4a and b) and Klenow (Fig. S2) DNA polymerases afforded the full-length extension product with EBNA-T nucleotides. Using the conditions applied for KOD, the reactions were scaled up for purifying the extended single-stranded DNA as KOD DNA polymerase was found to be the efficient enzyme.

To isolate the primer extended ssDNA containing EBNA-T nucleotides, we adopted a magnetic bead-based purification approach (Fig. 5a). First, a 21 nt probe DNA complementary to the 3′-end of the primer extended top strand of the extension product was immobilized to a streptavidin containing magnetic bead. Next, the crude primer extension reaction mixture was subjected to phenol–chloroform extraction and the aqueous phase containing DNA was heat dissociated and immediately mixed with the magnetic bead immobilized capture probe DNA. The resulting mixture was gently heated at 55–60 °C and collected the clean primer extended EBNA-T containing ssDNA while capturing the bead immobilized capture probe on the magnet. PCR amplification was performed using the purified primer extension product with three EBNA-T nucleotides furnished by KOD DNA polymerase from template T2. Primers P1 and P2 (Table 1) were used as forward and reverse primers respectively. Phusion DNA polymerase yielded the PCR product in good yields (Fig. 5b).

In summary, we have demonstrated that KOD DNA polymerase is an efficient enzyme to accept EBNA-T nucleotide as a substrate. Klenow and Phusion DNA polymerases can also incorporate EBNA-T nucleotides. Phusion DNA polymerase efficiently amplified an EBNA-T modified DNA template by PCR. The results reported here clearly suggest that EBNA-T nucleotide can now be used in SELEX processes in deriving functional nucleic acids such as aptamers with additional functionality.

Acknowledgment

This work was supported by the UQ Fellowship scheme awarded to RNV.

Supplementary data

Supplementary data (supporting information with additional gel images and experimental protocols) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.11.096.

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