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Synthesis and Polymerase Chain Reaction Amplification of DNA Strands Containing an Unnatural Triazole Linkage

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Abstract: DNA strands containing an unnatural T-triazole-T linkage have been synthesized by click DNA ligation between oligonucleotides with 3′-AZT and 5′-propargylamido dT and amplified efficiently by polymerase chain reaction (PCR) using several different polymerases. DNA sequencing of PCR amplicons and clones in two different sequence contexts revealed the presence of a single thymidine at the ligation site. The remarkable ability of thermostable polymerases to reproducibly copy DNA templates containing such an unnatural backbone opens up intriguing possibilities in gene synthesis, genetic analysis, biology, and nanotechnology.

Introduction

In the past 25 years the availability of synthetic oligonucleotides has led to major advances in molecular biology. In the field of genetic engineering the use of oligonucleotides, DNA polymerases, restriction enzymes, and ligases to produce synthetic genes is well established. However, there is an upper limit of around 150 bases on the length of oligonucleotides that can be used for this purpose. Beyond this point restrictions imposed by imperfect coupling efficiency and chemical modifications caused by side-reactions during solid-phase synthesis become prohibitive. Consequently oligonucleotides of this length cannot be used reliably and much shorter sequences are typically used, requiring polymerase chain reaction (PCR) with multiple primers and/or multiple enzymatic ligation reactions for gene assembly. Thus there is a need for inexpensive and simple methods to ligate shorter oligonucleotides in a controlled manner on a scale compatible with oligonucleotide synthesis ("large scale"). Libraries of synthetic oligonucleotides could then be synthesized and converted to longer fragments by chemical ligation. Purification to remove shorter fragments would yield large quantities of highly pure DNA of much greater length than those currently available. These DNA fragments could be stored for repeated use in the assembly of genes containing (for example) site-specific mutations. The two requirements for this are (i) a simple and efficient method to ligate oligonucleotide strands and (ii) a chemical linkage (modified DNA backbone) that is compatible with enzymatic processing, in particular PCR, the method of choice for sequence-specific DNA amplification.[1] We recently described a high-yielding DNA strand ligation method (click DNA ligation)[2−4] based on the CuAAC reaction[5,6] that fulfills the first criterion. This reaction is fast and efficient and proceeds smoothly in aqueous media.[7] Click DNA ligation can be performed in templated or nontemplated modes simply by changing the oligonucleotide concentration. However, use of this chemistry in the above applications is limited by the properties of the unnatural triazole linkage that is produced in the reaction, which unfortunately does not allow read-through by DNA polymerases. If a solution could be found to this problem such that DNA constructs containing triazole linkages could be copied during replication, one could contemplate the use of click chemistry to assemble functional synthetic genes or plasmids on large scale. In this context we have shown that circular single DNA strands and duplexes can be made in high yield by chemical methods.[2] Such constructs could also have uses in living cells; enzyme-free synthesis of genes would permit the incorporation of site-specific base analogues (e.g., for studying DNA replication/repair) or other chemical modifications (e.g., fluorescent dyes) for in vivo visualization. Reverse transcription of RNA analogues containing triazole linkages might also be envisaged. This methodology also has potential uses in genetic analysis, particularly when a combination of ligation and in vitro replication is necessary.[8−11]

Results and Discussion

In our search for a polymerase-compatible triazole linkage we were encouraged by the fact that normal or modified polymerases can amplify damaged DNA by reading through

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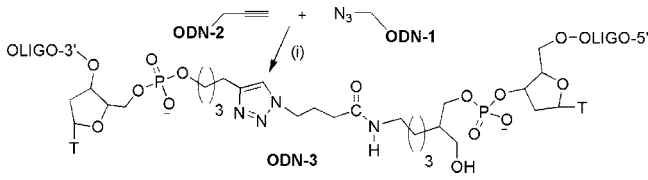
Scheme 1. Click DNA Ligation[a]



[a] (i) Cu(I), ligand.[14]

modified bases.[12,13] Our initial experiments focused on the triazole linkage (Scheme 1),[3] which was incorporated into ODN-3 (Table 1) by click ligation of azide ODN-1 and alkyne ODN-2 in the presence of a water-soluble Cu(I) binding ligand.[14] No discernible PCR amplification was observed from 5 ng of template ODN-3, not surprising as the triazole is flanked on both sides by alkyl chains and phosphodiester groups. Despite this a modest amount of amplicon was obtained when the PCR reaction was repeated with a large amount of this template (125 ng). We excluded linear copying of the 58 bases up to the triazole linkage as an origin of this product by comparing it with the product from a single primer complementary to alkyne-labeled ODN-2 (Supporting Information). Although this level of PCR amplification is of little practical value we reasoned that a shorter triazole linkage, similar to a normal phosphodiester, might allow more efficient read-through and yield an acceptable quantity of PCR product. Accordingly we designed a linkage based on 3′-azidothymidine (AZT) and the 4′-propargylamido-derivative of 5′-deoxymethylthymidine (Scheme 2). The similarity between the structure of this linkage and a normal phosphodiester is shown in Figure 1.

Synthesis of oligonucleotides with 3′-AZT required a non-standard approach because of the incompatibility of the azide group with P(III) chemistry (Scheme 2a). Previously an H-phosphonate approach has been used to synthesize trinucleotides with 3′-AZT,[15] but we needed a more efficient method of oligonucleotide assembly. Our strategy was to assemble the required sequence in the 5′- to-3′ direction using the 3′-DMT −5′-phosphoramidites of A, G, C, and T (reverse phosphoramidites) and then to add the 3′-AZT unit as a phosphotriester monomer **3** in the final step of oligonucleotide assembly. The partner alkyne oligonucleotides were made by coupling the propargylamido dT phosphoramidite **6**, synthesized from the carboxylic acid derivative of thymidine **4**[16,17] (Scheme 2b), to the 5′-end of the appropriate oligonucleotides by automated solid-phase synthesis. This methodology was used to prepare azide ODN-4 and alkyne ODN-5, template-mediated click DNA ligation of which proceeded smoothly to produce the 81-mer ODN-7 containing the AZT-based triazole linkage. Matrix-assisted laser desorption−ionization time-of-flight (MALDI-TOF) mass spectrometry was used to characterize the click product by synthesizing a shorter construct (ligation of ODN-4 to ODN-6 to give ODN-8; Found: 10513. Requires: 10515.).

The 81-mer ODN-7 was evaluated as a template in PCR using GoTaq DNA polymerase and primers ODN-10 and ODN-11, where it produced an amplicon of the expected 81 bp length (lane 3 of Figure 2). Linear copying of the 58 bases up to the triazole linkage was excluded as the origin of this product. We demonstrated that this gave a faster migrating band than the real PCR amplicon and required a much greater quantity of template (lane 5 of Figure 2). The amplicon gave a band of similar intensity to that of an unrelated PCR product from an unmodified template (ODN-14) under the same conditions (25 cycles of PCR using primers ODN-15 and ODN-16). For ODN-7 the product was visible after 15 cycles and strong after 20 cycles (Supporting Information), whereas for the unmodified template a strong band was obtained after 15 cycles. In another PCR reaction on template ODN-7, a second pair of primers (ODN-12 and ODN-13) with 17-mer tails at the 5′-end (M13 forward and reverse sequence primer sites) was used to produce a longer 115 bp amplicon (lane 2 of Figure 2). Primitive polymerases of family A (Taq) and B (Pfu) also produced clean amplicons from ODN-7 (Supporting Information). In each PCR reaction 5 ng of template was used, but subsequent experiments showed that 5 pg is adequate. The stark difference in amplification efficiency of ODN-7 and ODN-3 confirms that polymerase copying through the T-triazole-T linkage is more favorable if the modified backbone is comparable in length to a normal phosphodiester.

The sequence-dependence of amplification was examined by coupling azide ODN-4 to a different alkyne oligonucleotide (ODN-17) to produce ODN-18. This template was used successfully in PCR reactions with GoTaq (lacking 5′-exonuclease activity) and Pfu (with proofreading activity) using primers ODN-12 and ODN-19. To gain insight into the precise nature of the copying process, sequencing was carried out on the PCR products from ODN-7 with short and long primers using GoTaq polymerase and on clones containing amplicons produced from ODN-7 with two different polymerases, GoTaq (57 clones) and DyNAzyme EXT (62 clones). Remarkably, in every single case T-triazole-T in the template behaved as a single thymidine (Figures 3 and 4). To investigate the effect of the sequence around the triazole linkage on copying, template ODN-18 was amplified with GoTaq, and the sequences of 8 clones were determined. The results showed that in every case CT-triazole-TTC at the click linkage was converted to CTTC during the PCR (Figure 4). In summary, a total of 127 clones and 2 PCR products were sequenced using two different templates and in every case T-triazole-T was read as T. Our current explanation of this phenomenon is that the primer-polymerase complex inserts an adenine opposite to the 3′-thymidine (i.e., T1) of T1-triazole-T2 (Scheme 2c), ignores T2, and then progresses to make a complementary copy of the rest of the template. An alternative mechanism would produce the same amplicon by ignoring 3′-T1 and using T2 as a template base. In order to confirm which of the two adjacent thymidines is copied it will be necessary to carry out experiments on DNA templates with triazole backbones assembled from ODNs containing propar-

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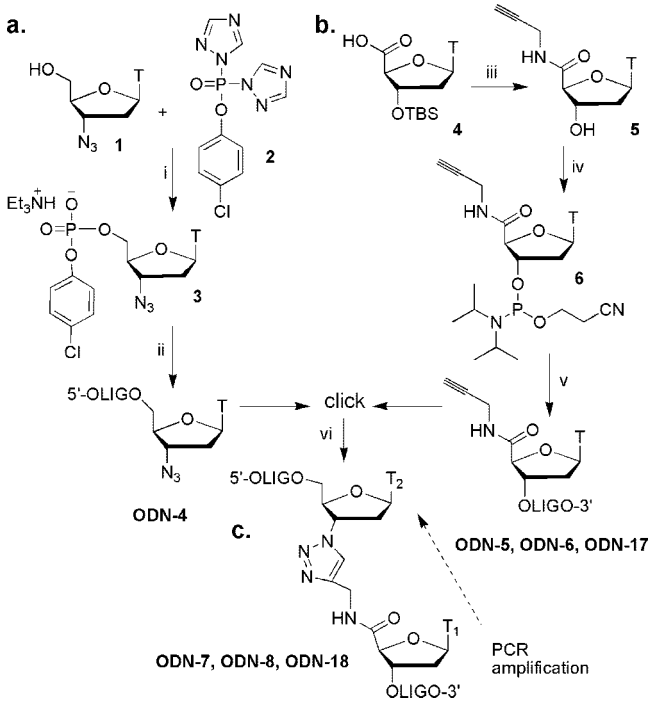
Table 1. Oligonucleotide Sequences[a]

code	sequences (5′−3′)
ODN-1	GCATTCGAGCAACGTAAGATCCT-azide (Scheme 1)
ODN-2	alkyne-TACCACACAATCTCACACTCTGGAATTCACACTGACAATACTGCCGACACACATAACC (Scheme 1)
ODN-3	ODN-1-triazole-ODN-2 (Scheme 1)
ODN-4	C3-GCATTCGAGCAACGTAAGATCC-t-azide (Scheme 2)
ODN-5	alkyne-t-ACCACACAATCTCACACTCTGGAATTCACACTGACAATACTGCCGACACACATAACC (Scheme 2)
ODN-6	alkyne-t-ACCACACAAT (Scheme 2)
ODN-7	ODN-4-triazole-ODN-5
ODN-8	ODN-4-triazole-ODN-6
ODN-9	TGTGTGGTAAGGATCTTA template for click reactions ODN-4 + ODN-5 and ODN-1 + ODN-2
ODN-10	GCATTCGAGCAACGTAAG primer
ODN-11	GGTTATGTGTGTCGGCAG primer
ODN-12	CAGGAAACAGCTATGACGCATTCGAGCAACGTAAG primer
ODN-13	GTAAAACGACGGCCAGTGGTTATGTGTGTCGGCAG primer
ODN-14	GCATTCGAGCATCCTACCACACAAAATCTCACAGTCAGTGTCTGACACACACAAAATGCACGACACACATAACC
ODN-15	GCATTCGAGCATCCTACC primer
ODN-16	GGTTATGTGTGTCGTGCA primer
ODN-17	alkyne-t-TCACAGTCAGTGTCTGACACACACAAAACAAGCGACACACATAACC
ODN-18	ODN-4-triazole-ODN-17
ODN-19	GTAAAACGACGGCCAGTGGTTATGTGTGTCGCTTG primer
ODN-20	TGACTGTGAAAGGATCTTAC template for click reaction ODN-4 + ODN-17
ODN-21	CGCGCCATGGGCATTCGAGCAACGTAAG primer
ODN-22	CGCGCTCGAGGGTTATGTGTGTCGGCAG primer

[a] Structure of alkyne-t and azide-t shown in Scheme 2. C3 = 5′-hydroxypropylphosphate (C3 SynBase, Link Technologies).

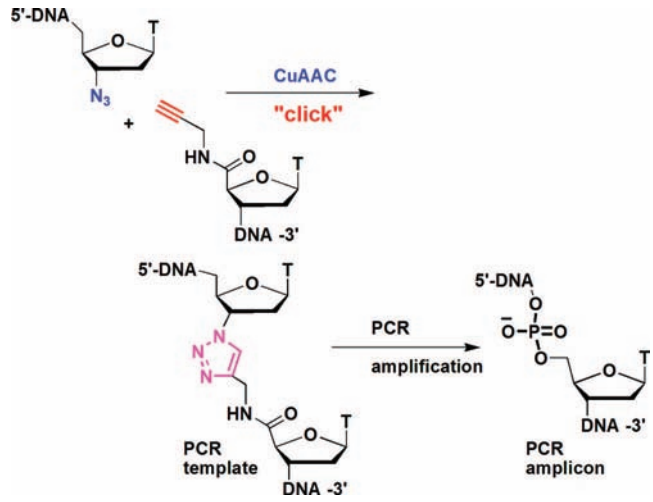
Scheme 2. Synthesis of Monomers and ODNs[a]



[a] (a) (i) THF, RT, 4 h, 1 M triethylammonium bicarbonate buffer, 71%; (ii) ODN on column, MSNT, 1-methylimidazole, pyridine. (b) (iii) Propargylamine, DIPEA, HOBt, EDC, DMF, RT, overnight, then Dowex 50W (H+), 67%; (iv) 2-O-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite, DIPEA, THF, RT, 2 h, 84%; (v) phosphoramidite ODN synthesis; (vi) Cu(I), ligand.[14] (c) PCR amplification of template ODNs.

gylamide or azide analogues of A, G, or C. Regardless of the mechanism we have shown that T-triazole-T is a template mimic of thymidine, suggesting that PCR templates can be synthesized by ligation of an oligonucleotide with 3′-azide **3** to a partner with 5′-alkyne **6** at a suitable thymidine site in the desired sequence. This is fortunate, because although a modified thymidine nucleoside is required in both oligonucleotides to promote the click ligation reaction, it is not necessary to locate a TpT step at a suitable ligation point in the desired template; only a single T is required (Scheme 3). In the future it should be possible to ligate oligonucleotides containing other alkynes and azides to produce a variety of triazole linkages carefully designed to mimic of phosphodiester groups.[18] It would be interesting to study a range of these and compare their properties as PCR templates.

Scheme 3. Click DNA Ligation of Alkyne and Azide Oligonucleotides Produces a PCR Template in which T-triazole-T is Copied as T



Conclusions

Oligonucleotides containing an AZT-based T-triazole-T linkage have been synthesized and used as PCR templates. Amplification proceeded efficiently with a variety of polymerases and DNA sequencing revealed the presence of a single thymidine at the ligation site in two different sequence contexts. This is the first example of highly efficient nonenzymatic DNA strand ligation combined with reproducible amplification. The ability of thermostable polymerases to read through such an

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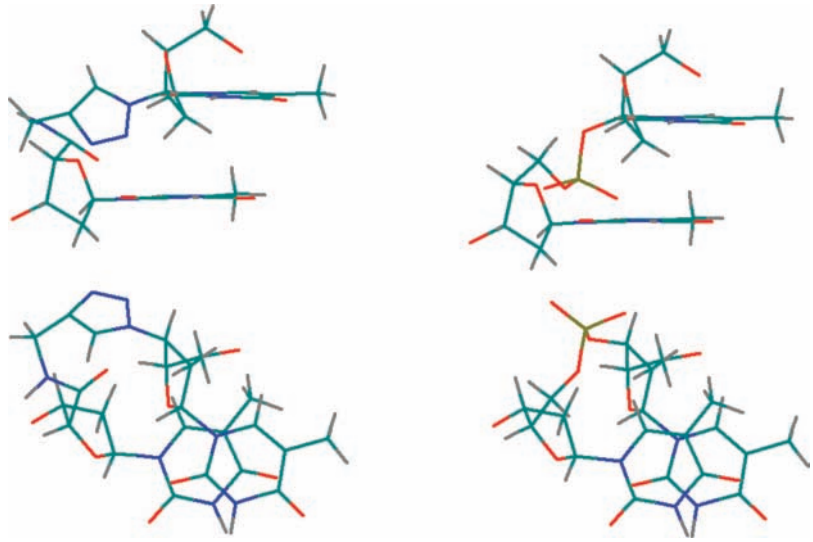

Figure 1. AZT-based triazole linkage (left) and normal phosphodiester linkage (right). Top view in plane of bases and bottom view perpendicular. Modeled in HyperChem 7 with geometry optimization.

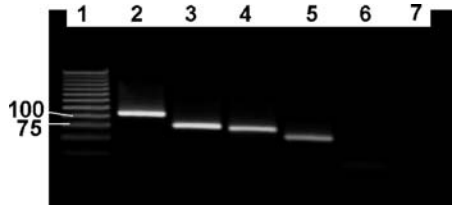

Figure 2. PCR from template ODN-7 (5 ng). Lane 1, 25 bp DNA ladder; lane 2, PCR using long primers ODN-12 and ODN-13; lane 3, PCR using short primers ODN-10 and ODN-11; lane 4, linear copying of the alkyne moiety ODN-5 (125 ng) using long primer ODN-13; lane 5, linear copying of the alkyne moiety ODN-5 (125 ng) using short primer ODN-11; lane 6, control without the template ODN-7 using long primers ODN-12 and ODN-13; lane 7, control without the template ODN-7 using short primers ODN-10 and ODN-11. 25 cycles of PCR were used throughout. 2% agarose gel (w/v) in 1 X TBE buffer, ethidium bromide (50 μL, 1 mg/mL), was added after heating and before pouring the gel.

unnatural backbone opens up intriguing possibilities in biology and nanotechnology. In order to fully exploit this discovery it will be necessary to develop methods to synthesize oligonucleotides bearing a suitable alkyne at one terminus and an azide at the other. If this can be achieved the size range of synthetic oligonucleotide analogues available for gene synthesis and related applications could be greatly extended.

Experimental Section

All reagents were purchased from Aldrich, Avocado, Fluka, or Link Technologies and used without purification with the exception of THF (distilled over sodium wire and benzophenone), DCM, DIPEA, and pyridine (distilled over calcium hydride). All reactions were carried out under an atmosphere of argon using oven-dried glassware. NAP columns were purchased from GE Healthcare and used according to the manufacturer's instructions. Column chromatography was carried out under argon pressure using Fisher scientific DAVISIL 60A (35−70 μm) silica. Thin-layer chromatography was performed using Merck Kieselgel 60 F24 plates (0.22 mm thickness, aluminum backed). ^{1}H NMR spectra were measured at 300 MHz on a Bruker AV300 spectrometer, and ^{13}C NMR spectra were measured at 75 MHz. Chemical shifts are given in ppm relative to tetramethylsilane, and J values are quoted in Hz, correct to within 0.5 Hz. All spectra were internally referenced to the appropriate residual undeuterated solvent signal. Multiplicities of ^{13}C signals were determined using DEPT spectral editing technique. ^{31}P NMR spectra were recorded on a Bruker AV300 spectrometer at 121 MHz and were externally referenced to 85% phosphoric acid in deuterated water. Low-resolution mass spectra were recorded using the electrospray technique on a Fisons VG platform instrument or a Waters ZMD quadrupole mass spectrometer in acetonitrile (high-performance liquid chromatography (HPLC) grade). High-resolution mass spectra were recorded in acetonitrile, methanol, or water (HPLC grade) using the electrospray technique on a Bruker APEX III FT-ICR mass spectrometer. MALDI-TOF MS were recorded using a ThermoBio- Analysis Dynamo MALDI-TOF mass spectrometer in positive ion mode using oligonucleotide (oligo-dT) standards.[19]

Synthesis of the AZT Phosphotriester Monomer 3. Triethylammonium[3′-azido-3′-deoxythymidine-5′-O-(4-chlorophenylphosphate)] (3). A stoppered sintered glass filtration unit (50 mL) with a side neck was attached to the vapor duct of a rotary evaporator and slowly rotated under an atmosphere of argon. 1,2,4-Triazole (dried in a desiccator over P_2O_5) (0.28 g, 4.00 mmol), dry THF (10 mL), and dry Et_3N (0.49 mL, 3.50 mmol) were added to the filtration unit and when they had dissolved 4-chlorophenyl phosphorodichloridate (0.24 mL, 1.50 mmol) was added. A white precipitate of triethylammonium chloride was formed immediately and the reaction mixture was rotated for 30 min under argon. The filtrate was collected in a flask under argon, and the white precipitate was washed with dry THF and collected in the same flask. AZT **1** (0.27 g, 1.00 mmol) was coevaporated with dry pyridine three times before being dried under vacuum for 1 h and then dissolved in dry THF (2 mL). The above prepared solution of 4-chlorophenyl phosphorodi(triazolide) **2** (1.50 mmol) in dry THF (10 mL) was added to the flask containing the dry AZT solution under argon. The reaction was left to stir for 4 h before it was terminated by the addition of 1 M triethylammonium bicarbonate buffer (20 mL). After 10 min the reaction mixture was extracted with DCM and the volume of the aqueous layer was reduced and re-extracted with DCM. The organic layers were collected, washed with 0.5 M triethylammonium bicarbonate buffer, dried over sodium sulfate, and filtered, and the solvent was removed in vacuo. The crude compound was purified by reversed-phase HPLC using a C8 (octyl) HPLC column and a gradient of 0−100% acetonitrile in water to give the title compound **3** as a white foam (0.4 g, 71%).

δ_H (300 MHz, DMSO-d_6) 11.3 (1H, s, thymidine-NH), 9.5 (1H, bs, CH_3CH_2-NH), 7.8 (1H, d, J = 1.1, H-6), 7.3 (2H, d, J = 8.8

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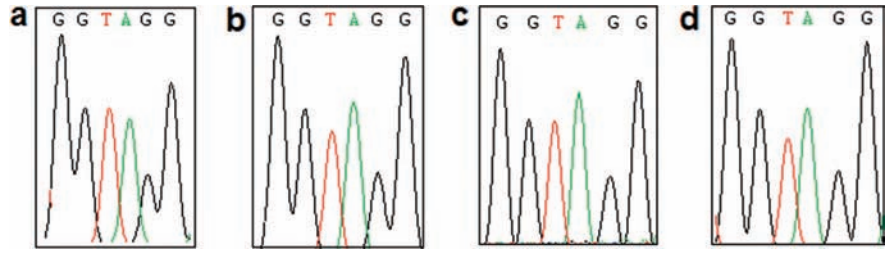

Figure 3. Sequencing of (a) amplicon from ODN-7 and short primers ODN-10 and ODN-11 using GoTaq. (b) amplicon from ODN-7 and long primers ODN-12 and ODN-13 using GoTaq. (c) clone from GoTaq amplicon. (d) clone from DyNAzymeTM EXT amplicon. In all cases only one of the two thymidines at the triazole linkage is copied during PCR. An ABI 3730 XL sequencer was used with Big Dye Terminator chemistry.

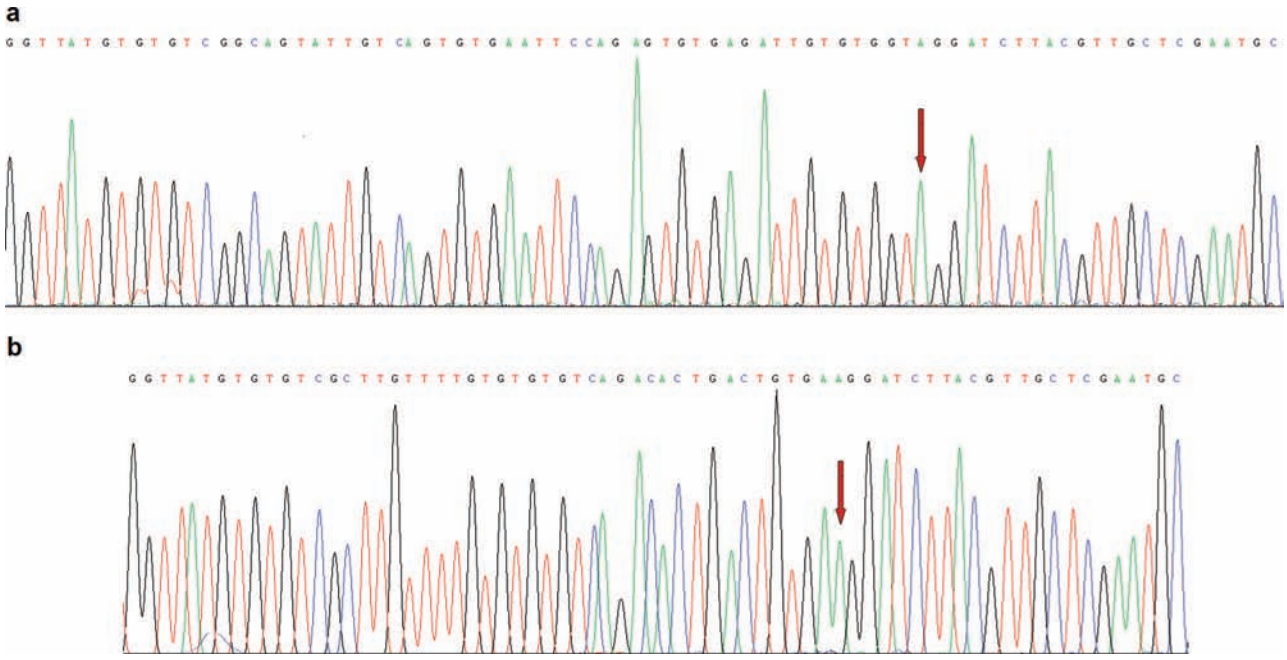

Figure 4. DNA sequences of cloned PCR products from (a) template ODN-7 and (b) template ODN-18. In both cases the strand that is complementary to the original template is shown. The arrow indicates the adenine base that has been copied from T-triazole-T. In both cases the sequence of the PCR product that appears in the clones is otherwise identical to that of the original template. PCR amplification was carried out using GoTaq DNA polymerase and an ABI 3730 XL sequencer was used with Big Dye Terminator chemistry.

Hz, Ar−H), 7.2 (2H, d, J = 8.8 Hz, Ar−H), 6.1 (1H, t, J = 6.8, H′-1), 4.4 (1H, m, H′−3), 3.9 (3H, m, H′−4, H′−5), 3.1 (6H, m, CH_3CH_2-NH), 2.3 (2H, m, H′−2), 1.8 (3H, d, J = 1.1, thymidine-CH_3), 1.2 (9H, t, J = 7.3, CH_3CH_2−NH); δ_C (75 MHz, DMSO-d_6) 164.2 (C-4), 153.4 and 150.9 (C-2 and C−Ar), 136.5 (C-6), 129.1 and 122.0 (*C*H−Ar), 126.1 (C−Ar), 110.3 (C-5), 83.9 (C-1′), 83.1 (C-4′), 65.1 (C-5′), 61.1 (C-3′), 46.1 (CH_3CH_2NH), 36.6 (C-2′), 12.5 (thymidine CH_3), 9.0 (CH_3CH_2NH); δ_P (300 MHz, DMSO-d_6) −4.5. *m/z* LRMS [ES$^-$, MeCN] 456 (M - Et_3NH, 100%). HRMS (M - Et_3NH) ($C_{16}H_{16}ClN_5O_7P$) calcd. 456.0481, found 456.0476.

Synthesis of the Alkyne Monomer 6. 4′-N-(Prop-2-ynyl)formamido-5′-deoxymethylthymidine, 5. The 4′-carboxylic acid derivative of thymidine[16,17] **4** (0.57 g, 1.55 mmol) was coevaporated twice with anhydrous DMF before being dissolved in DMF (20 mL). DIPEA (1.35 mL, 7.75 mmol), HOBt (0.31 g, 2.30 mmol), propargylamine (0.32 mL, 4.65 mmol), and EDC (0.59 g, 3.10 mmol) were added, and the reaction was left stirring overnight. Methanol (40 mL) was then added, and the mixture was passed through Dowex 50W (H^+) to remove amines and TBS cations. The resin was washed with 6 volumes of MeOH. Methanol was combined and evaporated in vacuo, and the residue was purified by silica-gel column chromatography (93:7, DCM:MeOH). The title compound was isolated as a white foam (0.3 g, 67%), δ_H (300 MHz, DMSO-d_6) 11.3 (1H, s, thymidine-NH), 8.7 (1H, t, J = 5.5 Hz, amide-NH), 8.0 (1H, d, J = 1.1 Hz, H-6), 6.3 (1H, dd, J = 8.4, 5.8 Hz, H-1′), 5.6 (1H, d, J = 3.8 Hz, OH-3′), 4.3 (1H, m, H-3′), 4.2 (1H, d, J = 1.5 Hz, H-4′), 3.9 (2H, dd, J = 5.5, 2.6 Hz, CH_2NH), 3.2 (1H, t, J = 2.6 Hz, alkyne-H), 2.1 (2H, m, H-2′), 1.8 (3H, d, J = 1.1 Hz, CH_3); δ_C (75 MHz, DMSO-d_6) 170.1 (amid-CO), 163.7 (C-4), 150.6 (C-2), 136.7 (C-6), 109.5 (C-5), 85.2 and 85.1 (C-1′ and C-4′), 80.6 (alkyne-C), 73.4 and 73.2 (C-3′ and alkyne-CH), 38.4 (C-2′), 27.9 (CH_2NH), 12.4 (CH_3); *m/z* LRMS [ES$^+$, MeCN] 316 (M + Na$^+$, 100%). HRMS (M + Na$^+$) ($C_{13}H_{15}N_3NaO_5$) calcd. 316.0904, found 316.0906.

4′-N-(Prop-2-ynyl)formamido-5′-deoxymethylthymidine-3′-O-(2-cyanoethyl-N,N-diisopropyl)phosphoramidite, 6. 4′-*N*-(Prop-2-ynyl)formamido-5′-deoxymethylthymidine, **5** (0.15 g, 0.51 mmol), was dissolved in THF (10 mL) under an atmosphere of argon. DIPEA (0.22 mL, 1.28 mmol) was added followed by 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite (0.14 mL, 0.61 mmol) dropwise. The reaction mixture was left to stir at room temperature for 2 h, and the volume of the solvent was then reduced to 3 mL by flushing argon through the reaction mixture. Ethyl acetate (50 mL) was added, and the mixture was transferred under argon into a separating funnel and washed with degassed saturated aqueous potassium chloride (20 mL). The organic layer was separated, dried over sodium sulfate, and filtered, and the solvent was removed in vacuo. Upon purification by silica gel column chromatography under argon pressure (80:20 ethyl acetate:hexane, 0.5% pyridine), the product was isolated as a white foam (0.21 g, 84%); δ_P (300 MHz, DMSO-d_6) 150.2, 149.4; *m/z* LRMS [ES$^+$,

MeCN] 494 (M + H^+, 20%), 516 (M + Na^+, 100%). HRMS (M + Na^+) ($C_{22}H_{32}N_5NaO_6P$) calcd. 516.1982, found 516.1981.

Oligonucleotide Synthesis. Standard DNA phosphoramidites, solid supports, and additional reagents were purchased from Link Technologies and Applied Biosystems Ltd. All oligonucleotides were synthesized on an Applied Biosystems 394 automated DNA/RNA synthesizer using a standard 0.2 or 1.0 μmol phosphoramidite cycle of acid-catalyzed detritylation, coupling, capping, and iodine oxidation. Stepwise coupling efficiencies and overall yields were determined by the automated trityl cation conductivity monitoring facility and in all cases were >98.0%. All β-cyanoethyl phosphoramidite monomers were dissolved in anhydrous acetonitrile to a concentration of 0.1 M immediately prior to use. The coupling time for normal A, G, C, and T monomers was 25 s, and the coupling time for the alkyne phosphoramidite monomer **6** was extended to 360 s. Cleavage of the oligonucleotides from the solid support and deprotection was achieved by exposure to concentrated aqueous ammonia solution for 60 min at room temperature followed by heating in a sealed tube for 5 h at 55 °C. The oligonucleotides were purified by reversed-phase HPLC[2] and analyzed by gel electrophoresis.

Synthesis of the Azide Oligonucleotide Using AZT-Phosphotriester Monomer 3. ODN-4 was synthesized by assembling the required sequence in the 5′- to-3′ direction on a C3 spacer SynBase column (Link Technologies) using the 3′-DMT-5′-phosphoramidites of A, G, C, and T (reverse phosphoramidites). Incorporation of AZT at the 3′ end was achieved by manual addition of AZT phosphotriester monomer **3** to the solid support on the oligonucleotide column. To accomplish this, 1-(2-mesitylenesulphonyl)-3-nitro-1,2,4-triazole (MSNT) (60.0 mg, 202.4 μmol) was dissolved in dry pyridine (300.0 μL) and then transferred to a flask containing the AZT-phosphotriester monomer **3** (30.0 mg, 53.8 μmol). 1-Methylimidazole (30.0 μL, 378.1 μmol) was added, and the mixture was transferred via a 1-mL syringe, which was then connected to the oligonucleotide column. A second 1-mL syringe was connected to the other end of the column, and the solution was passed through the column periodically by back and forward movement of the two syringes. After 30 min the column was washed three times with dry pyridine followed by DNA grade acetonitrile. The oligonucleotide was then cleaved from the solid support and deprotected by heating the resin in concentrated aqueous ammonia solution in a sealed tube for 5 h at 55 °C. Purification of the oligonucleotide was carried out by reversed-phase HPLC.[2]

Click Ligation Reactions. Nontemplated Click Ligation of Alkyne and Azide Oligonucleotides. To a solution of tris-hydroxypropyl triazole ligand[14] (0.7 μmol in 40 μL of 200 mM NaCl) under argon was added sodium ascorbate (1.0 μmol in 2.0 μL 200 mM NaCl) followed by $CuSO_4 \cdot 5H_2O$ (0.1 μmol in 1 μL 200 mM NaCl). The alkyne oligonucleotide ODN-5 or ODN-6 (2.2 nmol in 5 μL 200 mM NaCl) was added to the above solution followed by the azide oligonucleotide ODN-4 (2.0 nmol in 5 μL of 200 mM NaCl). The reaction mixture was kept under argon at room temperature for 2 h and was then made up to 1 mL with water, and a disposable NAP-10 gel-filtration column was used to remove reagents (GE Healthcare). The long ligated oligonucleotide ODN-7 was purified by gel electrophoresis to give 30% isolated yield.[2] Short ligated ODN-8 was purified by reversed-phase HPLC using a C8 (octyl) column and a gradient of acetonitrile in 0.1 M ammonium acetate followed by NAP-10 gel filtration to give 40% isolated yield. Successful click ligation of azide ODN-4 to alkyne ODN-6 to yield ODN-8 was confirmed by MALDI-TOF mass spectrometry. Azide oligonucleotide ODN-4: calcd. 7195 found 7199. Alkyne oligonucleotide ODN-6: calcd. 3320, found 3324. Ligated ODN-8: calcd. 10515, found 10513.

Templated Click Ligation of Alkyne and Azide Oligonucleotides. Sodium ascorbate solution (2.0 μmol in 4.0 μL of 200 mM NaCl) was added to a solution of the tris-triazole ligand[14] (1.4 μmol in 80 μL 200 mM NaCl) under argon followed by $CuSO_4 \cdot 5H_2O$ (0.2 μmol in 2 μL of 200 mM NaCl). A mixture of the click template ODN-9 (4.0 nmol), alkyne oligonucleotide (4.5 nmol), and azide oligonucleotide (4.0 nmol) in 100 μL of 200 mM NaCl was annealed by heating at 80 °C for 5 min followed by slow cooling then added to the above solution. The reaction mixture was left under argon at room temperature for 2 h and then made up to 1 mL with water, and a disposable NAP-10 gel-filtration column was used to remove reagents (GE Healthcare). The ligated oligonucleotides were analyzed and purified by denaturing 20% polyacrylamide gel electrophoresis. The templated click reaction proceeded to completion and gave, after purification by gel electrophoresis, ~35% isolated yield of ODN-3 (from ODN-1 + ODN-2) and ODN-7 (from ODN-4 + ODN-5). PCR template ODN-18 was synthesized from azide ODN-4 + alkyne ODN-17 using click template ODN-20 under the same conditions, except that the reaction was carried out at 4 °C for 1 h followed by 1 h at room temperature.

PCR Reactions. PCR products of ~81 and ~115 bp were generated in this study. Because of the tendency of polymerases to add 1 or 2 extra bases to the 3′ end of the template the length of these amplicons is not precise. PCR reactions were analyzed by 2% agarose gel electrophoresis (Supporting Information).

GoTaq DNA Polymerase. Four μL of 5 X buffer (green buffer) was used in a total reaction volume of 20 μL with 5 ng of the DNA template, 0.5 μM of each primer, 0.2 mM dNTP, and 0.5 unit of GoTaq. Fifteen μL of this reaction mixture was loaded onto a 2% agarose gel in 1 X TBE buffer. PCR cycling conditions for ~70, ~81 and ~115 base PCR products: 95 °C (initial denaturation) for 2 min then 25 cycles of 95 °C (denaturation) for 15 s, 54 °C (annealing) for 20 s and 72 °C (extension) for 30 s. Five X Promega green PCR buffer was provided with the enzyme (Promega GoTaq DNA polymerase) at pH 8.5 containing 7.5 mM $MgCl_2$ for a final concentration of 1.5 mM. The buffer contains tris-HCl, KCl, and two dyes (blue and yellow) that separate during electrophoresis to monitor the migration process. These conditions were used for template ODN-7 and primers ODN-10 and ODN-11.

Taq DNA Polymerase. Two μL of 10 X buffer was used in a total reaction volume of 20 μL with 5 ng of the DNA template, 0.5 μM of each primer, 0.2 mM dNTP, and 0.5 unit of Taq DNA polymerase. (10 X standard Taq reaction buffer = 100 mM tris-HCl, 500 mM KCl, 15 mM $MgCl_2$ at pH 8.3). PCR cycling conditions: 95 °C (initial denaturation) for 2 min and then 25 cycles of 95 °C (denaturation) for 15 s, 54 °C (annealing) for 20 s, and 72 °C (extension) for 30 s. This was followed by one cycle at 72 °C for 2 min according to the manufacturer's instructions. These conditions were used for template ODN-7 and primers ODN-10 and ODN-11.

Pfu DNA Polymerase. Two μL of 10 X buffer was used in a total reaction volume of 20 μL with 5 ng of the DNA template, 0.5 μM of each primer, 0.2 mM dNTP, and 1.0 unit of Pfu DNA polymerase. (Pfu DNA polymerase 10 X reaction buffer = 200 mM tris-HCl at pH 8.8, 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 20 mM $MgSO_4$, 1.0% Triton X-100, and 1 mg/mL nuclease-free BSA). PCR cycling conditions: 95 °C (initial denaturation) for 2 min and then 25 cycles of 95 °C (denaturation) for 15 s, 54 °C (annealing) for 20 s, and 72 °C (extension) for 30 s. This was followed by one cycle at 72 °C for 2 min according to the manufacturer's instructions. These conditions were used for template ODN-7 and primers ODN-10 and ODN-11.

A PCR reaction was also carried out as above on ODN-18 using the two long primers ODN-12 and ODN-19 and 1.5 units of Pfu DNA polymerase. A two-step program was used, 95 °C (initial denaturation) for 2 min, then 8 cycles of 95 °C (denaturation) for 15 s, 54 °C (annealing) for 20 s, and 72 °C (extension) for 30 s, and then 17 cycles of 95 °C (denaturation) for 15 s, 65 °C (annealing) for 20 s, and 72 °C (extension) for 30 s.

Preparation of PCR Products for DNA Sequencing Analysis. For the 81 bp PCR product, template ODN-7, the short primers ODN-10, ODN-11, and GoTaq DNA polymerase were used under the conditions described above for GoTaq. For the 115 bp

PCR product, template ODN-7, the long primers ODN-12, ODN-13, and GoTaq DNA polymerase were used under the following conditions: A two-step program was used: 95 °C (initial denaturation) for 2 min, then 8 cycles of 95 °C (denaturation) for 15 s, 54 °C (annealing) for 20 s, and 72 °C (extension) for 30 s, and then 17 cycles of 95 °C (denaturation) for 15 s, 65 °C (annealing) for 20 s, and 72 °C (extension) for 30 s. After running the gel, the bands were cut under a UV lamp and extracted using a QIAquick, gel extraction kit (50), Cat.No.28704 (QIAGEN) following the manufacturer's instructions.

Cloning and Sequencing of the PCR Product Derived from Template ODN-7 Using GoTaq DNA Polymerase. A PCR reaction (50 μL) was carried out under the conditions described above under "PCR reactions -GoTaq DNA polymerase" using the two primers ODN-21 and ODN-22 which encode XhoI and NcoI restriction sites. The PCR product was then purified using a QIAquick PCR purification kit (50), (Qiagen, catalog no. 28104) according to the manufacturer's instructions. Purified PCR product (42 μL) was then double digested using XhoI and NcoI restriction enzymes (Promega) according to the manufacturer's instructions. The digested product was purified by extraction from 2% agarose gel using a QIAquick gel extraction kit (50) (Qiagen, catalog no. 28704) according to the manufacturer's instructions.

An overnight ligation reaction containing XhoI and NcoI digested pBAD vector (Invitrogen) and XhoI and NcoI digested PCR product was set up using T4 DNA ligase following the manufacturer's instructions. Ligation reaction mixture (10 μL) was added to an aliquot of TOP10 competent *E. coli* cells (100 μL) (Invitrogen) previously thawed on ice for 15 min. Cells were kept on ice for a further 30 min and heat shocked in a water bath at 42 °C for 45 s. They were then returned to the ice for 2 min, and SOC media (250 μL) was added. After incubation with orbital shaking (37 °C, 180 rpm) for one hour, the cells (100 μL) were plated out onto 2YT agar supplemented with 100 μg/mL of ampicillin. Plates were incubated at 37 °C overnight allowing transformants to develop into single colonies, which were used to inoculate overnight cultures of 2YT media (5 mL) supplemented with 100 μg/mL ampicillin. Plasmid was harvested from the cultures, using the Promega Wizard plus SV mini prep DNA purification system according to the manufacturer's instructions. Sequencing was performed by Cogenics using Big Dye Terminator chemistry (Applied Biosystems) and analyzed on an Applied Biosystems 3730 XL DNA sequencer.

Cloning and Sequencing of the PCR Product Derived from Template ODN-7 Using DyNAzyme EXT DNA Polymerase. A PCR reaction (50 μL) was performed using DyNAzyme EXT (Finnzymes cat No. F-505s) (35 cycles) according to the manufacturer's instructions using Primers ODN-10 and ODN-11. A 30-min extension time at 72 °C was inserted at the end of the cycling program to allow the addition of deoxyadenosine (dA) to the ends of the PCR products. The PCR product (2 μL) was then directly ligated to pCR4-TOPO vector from the TOPO TA Cloning Kit for Sequencing (Invitrogen cat No. K4575-J10) following the manufacturer's instructions. Ligation reaction mixture (2 μL) was added to an aliquot of TOP10 competent *E. coli* cells provided with the cloning kit, according to the manufacturer's instructions. Cells were thawed;, the ligation mixture was added, and the cells were kept on ice for 30 min. They were then heat shocked in a water bath at 42 °C for 30 s and returned to the ice for 2 min. SOC media (250 μL) was then added. Following incubation with orbital shaking (37 °C, 180 rpm) for one hour, the cells (50 μL) were plated out onto 2YT agar supplemented with 50 μg/mL of ampicillin. Plates were incubated at 37 °C overnight allowing transformants to develop into single colonies. These single colonies were used to inoculate overnight cultures of 2YT media (5 mL) supplemented with 50 μg/mL ampicillin. Plasmid was harvested from the cultures using the Qiagen Plasmid Mini Kit according to the manufacturer's instructions. The M13 Forward Vector primer was used to sequence the plasmids. Sequencing was performed using Big Dye Terminator chemistry (Applied Biosystems) and analyzed on an Applied Biosystems 3730 XL DNA sequencer.

Cloning and Sequencing of the PCR Product Derived from Template ODN-18. A PCR reaction was carried out on ODN-18 using the two primers ODN-12 and ODN-19 with GoTaq DNA polymerase. A two-step program was used: 95 °C (initial denaturation) for 2 min, then 8 cycles of 95 °C (denaturation) for 15 s, 54 °C (annealing) for 20 s, and 72 °C (extension) for 30 s, and then 17 cycles of 95 °C (denaturation) for 15 s, 65 °C (annealing) for 20 s, and 72 °C (extension) for 30 s. After running the gel, the bands were cut under a UV lamp and extracted using a QIAquick, gel extraction kit (50), catalog no. 28704 (QIAGEN) following the manufacturer's instructions. Cloning was carried out using a TOPO TA Cloning Kit (Invitrogen) as above. Sequencing was performed using Big Dye Terminator chemistry (Applied Biosystems) and analyzed on an Applied Biosystems 3730 XL DNA sequencer.

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Supporting Information Available: HPLC purification of AZT triester monomer and ODNs, capillary electrophoresis analysis of oligonucleotides, polyacrylamide gel electrophoresis analysis of click ligation reaction, agarose gel-electrophoresis of PCR reactions, enzyme digestion of PCR amplicons, DNA sequencing, and NMR spectra of AZT phosphotriester and alkyne monomers. This material is available free of charge via the Internet at http://pubs.acs.org.

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