Supporting Information

Combined nucleobase and backbone modifications enhance DNA duplex stability and preserve biocompatibility

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Page

Oligonucleotide Synthesis and purification

General method for oligonucleotide synthesis

Standard DNA phosphoramidites, reverse phosphoramidites, solid supports, and additional reagents were purchased from Link Technologies and Applied Biosystems. 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 35 s, reverse A, G, C and T monomers were coupled for 180 s, and the coupling time for the G-clamp phosphoramidite monomer (Glen Research) was extended to 360 s. Unless it has been stated elsewhere, cleavage of 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. 3′-Alkyne dT oligonucleotides were synthesized using the 3′-propargylthymidine phosphoramidite monomer (figure S5) and assembling the required sequence in the 5′ to 3′-direction using the 3′-*O*-(4,4′-dimethoxytrityl)deoxyribonucleoside-5′-phosphoramidites of A, G, C and T (reverse phosphoramidites, Link Technologies). The oligonucleotides were then cleaved and deprotected by exposure to concentrated aqueous ammonia for 60 min at room temperature followed by heating in a sealed tube for 5 h at 55°C before HPLC purification as explained below.

The building blocks for the RNA analogues were prepared using 2'-TBS protected RNA phosphoramidite monomers with *t*-butylphenoxyacetyl protection of the A, G and C nucleobases and U unprotected on the base (Sigma-Aldrich). A solution of 0.3 M benzylthiotetrazole (BTT) in acetonitrile (Link Technologies) was used as the coupling agent, *t*-butylphenoxyacetic anhydride as the capping agent and 0.1 M iodine as the oxidizing agent (Sigma-Aldrich). All phosphoramidite monomers were dissolved in anhydrous acetonitrile to a concentration of 0.1 M immediately prior to use, and the coupling time for all monomers during RNA synthesis was 6 min. Stepwise coupling efficiencies were determined by automated trityl cation conductivity monitoring and in all cases were >97%. Cleavage of oligonucleotides from the solid support and deprotection were achieved by exposure to concentrated aqueous ammonia/ethanol (3/1 v/v) for 2 h at room temperature followed by heating in a sealed tube for 45 min at 55°C.

2'-TBS deprotection of oligoribonucleotides (RNA)

After cleavage from the solid support and deprotection of the nucleobases and phosphodiesters in ammonia/ethanol as described above, oligonucleotides were concentrated to a small volume in *vacuo*, transferred to 15 mL plastic tubes and freeze dried (lyophilised). The residue was dissolved in DMSO (300 μL) and triethylamine trihydrofluoride (300 μL) was added after which the reaction mixtures were kept at 65°C for 2.5 h. Sodium acetate (3 M, 50 μL) and butanol (3 mL) were added with vortexing and the samples were kept at -80°C for 30 min then centrifuged at 13,000 rpm at 4°C for 10 min. The supernatant was decanted and the precipitate was washed twice with ethanol (0.75 mL) then dried under vacuum.

Synthesis of the 3′-alkyne 5-methyl dC oligonucleotides

3′-Alkyne 5-methyl dC oligonucleotides were synthesized on the 1.0 μmole scale using 5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-propargyl-5-methyldeoxycytidine solid support (33 μmole/g loading on AM polystyrene, Applied Biosystems) which was prepared by the published method.[1] The resin was packed into a twist column (Glen Research) then used to assemble the required sequence in the 3′- to 5′-direction by standard phosphoramidite oligonucleotide synthesis. The oligonucleotides were then cleaved and deprotected by exposure to concentrated aqueous ammonia for 60 min at room temperature followed by heating in a sealed tube for 5 h at 55°C before purification by HPLC as explained below.

Synthesis of the 3′-alkyne dT oligonucleotides

3′-Alkyne dT oligonucleotides were synthesized using the 3′-propargylthymidine phosphoramidite monomer (figure S5) and assembling the required sequence in the 5′ to 3′-direction using the 3′-*O*-(4,4′-dimethoxytrityl)deoxyribonucleoside-5′-phosphoramidites of A, G, C and T (reverse phosphoramidites, Link Technologies). The oligonucleotides were then cleaved and deprotected by exposure to concentrated aqueous ammonia for 60 min at room temperature followed by heating in a sealed tube for 5 h at 55°C before HPLC purification as explained below.

Purification of oligonucleotides (DNA or RNA)

The fully deprotected oligonucleotides were purified by reversed-phase HPLC on a Gilson system using a Luna 10μ C8 100Å pore Phenomenex 10x250 mm column with a gradient of acetonitrile in triethylammonium acetate or ammonium acetate (0% to 50% buffer B over 20 min, flow rate 4 mL/min), (buffer A: 0.1 M triethylammonium acetate, pH 7.0, buffer B: 0.1 M triethylammonium

acetate, pH 7.0, with 50% acetonitrile). Elution was monitored by UV absorption at 295 nm. After HPLC purification, oligonucleotides were desalted using NAP-25 then NAP-10 columns (GE Healthcare). For short oligonucleotides (less than 10 bases in length), HPLC using the volatile buffer triethylammonium bicarbonate buffer (buffer A: 0.1 M triethylammonium bicarbonate, pH 7.5, buffer B: 0.1 M triethylammonium bicarbonate, pH 7.5, with 50% acetonitrile) was used. The fractions from HPLC were evaporated without need for additional desalting.

DNA sequence analysis of amplified G-clamp triazole DNA

The PCR amplicon obtained from ODN-20 was purified by 2% agarose gel electrophoresis and extracted from the gel using a QIAquick Gel Extraction kit. It was then sent to Eurofins Medigenomix GmbH (Eurofins MWG Operon) for TOPO-TA cloning and subjected to automated Sanger DNA sequencing. No mutations were found around the triazole C_tC linkage. Only two mutations were identified in total (cytosine deletions, indicated below in blue) and these were 27 bases remote from the original site of the triazole linkage.

DNA sequencing of 23 clones

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Ref             GCATTCGAGCAACGTAAGATCGXXAGCACACAATCTCACACTCTGGAATTCACACTGACAATACTGCCGACACACATAACC
21_A-100109_A10 GCATTCGAGCAACGTAAGATCGCCAGCACACAATCTCACACTCTGGAATTCACACTGACAATACTGCCGACACACATAACC
23_A-100109_C10 GCATTCGAGCAACGTAAGATCGCCAGCACACAATCTCACACTCTGGAATTCACACTGACAATACTGCCGACACACATAACC
24_A-100109_D10 GCATTCGAGCAACGTAAGATCGCCAGCACACAATCTCACACTCTGGAATTCACACTGACAATACTGCCGACACACATAACC
25_A-100109_E10 GCATTCGAGCAACGTAAGATCGCCAGCACACAATCTCACACTCTGGAATTCACACTGACAATACTGCCGACACACATAACC
27_A-100109_G10 GCATTCGAGCAACGTAAGATCGCCAGCACACAATCTCACACTCTGGAATTCACACTGACAATACTGCCGACACACATAACC
28_A-100109_H10 GCATTCGAGCAACGTAAGATCGCCAGCACACAATCTCACACTCTGGAATTCACACTGACAATACTGCCGACACACATAACC
31_A-100109_C11 GCATTCGAGCAACGTAAGATCGCCAGCACACAATCTCACACTCTGGAATTCACACTGACAATACTGCCGACACACATAACC
33_A-100109_E11 GCATTCGAGCAACGTAAGATCGCCAGCACACAATCTCACACTCTGGAATTCACACTGACAATACTGCCGACACACATAACC
34_A-100109_F11 GCATTCGAGCAACGTAAGATCGCCAGCACACAATCTCACACTCTGGAATTCACACTGACAATACTGCCGACACACATAACC
35_A-100109_G11 GCATTCGAGCAACGTAAGATCGCCAGCACACAATCTCACACTCTGGAATTCACACTGACAATACTGCCGACACACATAACC
38_A-100109_B12 GCATTCGAGCAACGTAAGATCGCCAGCACACAATCTCACACTCTGGAATTCACACTGACAATACTGCCGACACACATAACC
39_A-100109_C12 GCATTCGAGCAACGTAAGATCGCCAGCACACAATCTCACACTCTGGAATTCACACTGACAATACTGCCGACACACATAACC
40_A-100109_D12 GCATTCGAGCAACGTAAGATCGCCAGCACACAATCTCACACTCTGGAATTCACACTGACAATACTGCCGACACACATAACC
41_A-100254_A6  GCATTCGAGCAACGTAAGATCGCCAGCACACAATCTCACACTCTGGAATTCACACTGACAATACTGCCGACACACATAACC
42_A-100254_B6  GCATTCGAGCAACGTAAGATCGCCAGCACACAATCTCACACTCTGGAATTCACACTGACAATACTGCCGACACACATAACC
43_A-100254_C6  GCATTCGAGCAACGTAAGATCGCCAGCACACAATCTCACACTCTGGAATTCACACTGACAATACTGCCGACACACATAACC
44_A-100254_D6  GCATTCGAGCAACGTAAGATCGCCAGCACACAATCTCACACTCTGGAATTCACACTGACAATACTGCCGACACACATAACC
45_A-100254_E6  GCATTCGAGCAACGTAAGATCGCCAGCACACAATCTCACACTCTGGAATTCACACTGACAATACTGCCGACACACATAACC
46_A-100254_F6  GCATTCGAGCAACGTAAGATCGCCAGCACACAATCTCACACTCTGGAATTCACACTGACAATACTGCCGACACACATAACC
47_A-100254_G6  GCATTCGAGCAACGTAAGATCGCCAGCACACAATCTCACACTCTGGAATTCACACTGACAATACTGCCGACACACATAACC
48_A-100254_H6  GCATTCGAGCAACGTAAGATCGCCAGCACACAATCTCACACTCTGGAATTCACACTGACAATACTGCCGACACACATAACC
49_A-100254_A7  GCATTCGAGCAACGTAAGATCGCCAGCACACAATCTCACACTCTGGAAT-ACACTGACAATACTGCCGACACACATAACC
50_A-100254_B7  GCATTCGAGCAACGTAAGATCGCCAGCACACAATCTCACACTCTGGAAT-ACACTGACAATACTGCCGACACACATAACC
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Ultraviolet Melting Studies

UV DNA melting was monitored on Cary 4000 Scan UV-Visible Spectrophotometer (Varian) at 3 μM concentration of each oligonucleotide (unless otherwise stated) in 10 mM phosphate buffer, 200 mM NaCl at pH 7.0. Spectra were recorded at 260 nm. The samples were initially denatured by heating to 85°C (or 90°C) at 10°C min^{-1} then cooled to 20°C at 1°C min^{-1} and heated to 85°C (or 90°C) at 1°C min^{-1}. Eight successive melting curves were measured and T_m values were calculated from their derivatives using Cary Win UV Thermal application Software.

Representative UV melting curves and derivatives of oligonucleotides containing C_xC step

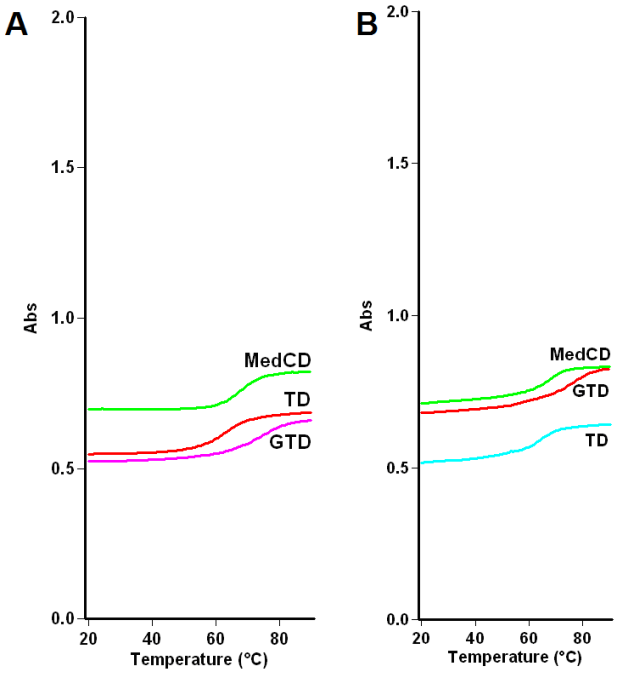

Figure S1. UV melting curves of DNA:DNA duplexes and DNA:RNA hybrid duplexes containing a C_xC step: UV melting curves were recorded at 260 nm. A); DNA:DNA type duplexes, TD = triazole duplex (ODN-6 + ODN-7, T_m = 63.0°C), GTD = G-clamp triazole duplex (ODN-3 + ODN-7, T_m = 75.0°C), MedCD = unmodified control duplex (ODN-4 + ODN-7, T_m = 68.5°C). B); DNA:RNA hybrid type duplexes, TD = triazole duplex (ODN-6 + ODN-14, T_m = 64.8°C), GTD = G-clamp triazole duplex (ODN-3 + ODN-14, T_m = 77.5°C), MedCD = unmodified control duplex (ODN-4 + ODN-14, T_m = 68.1°C)

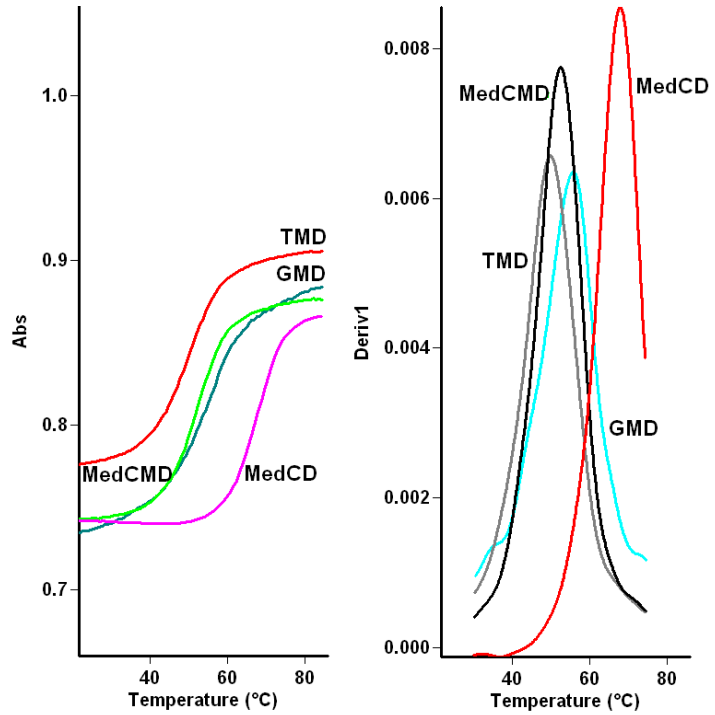

Figure S2. UV melting curves (A) and derivatives (B) of DNA:DNA mismatched duplexes containing a C_xC step: C.T mismatch in which C is MedC, triazole C or G-clamp triazole C

UV melting curves were recorded at 260 nm. TMD = triazole mutant duplex (ODN-6 + ODN-13, T_m = 50.2°C), GMD = G-clamp triazole mutant duplex (ODN-3 + ODN-13, T_m = 55.8°C), MedCMD = mutant duplex (ODN-4 + ODN-13, T_m = 52.2°C), MedCD = control duplex (ODN-4 + ODN-7, T_m = 68.5°C)

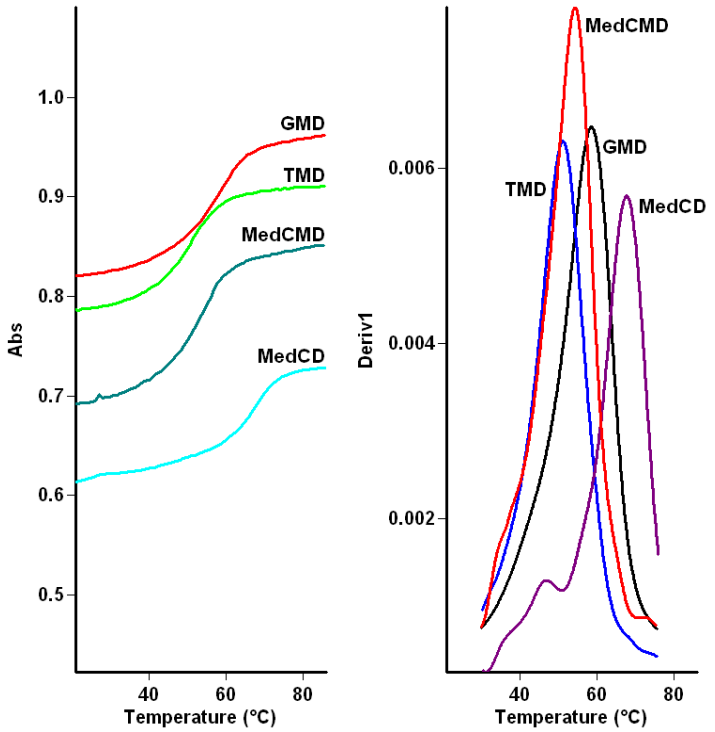

Figure S3. UV melting curves (A) and derivatives (B) of DNA:RNA mismatched duplexes containing a C_xC step: All mismatched duplexes contain a single C.A mismatch

UV melting curves were recorded at 260 nm. TMD = triazole mutant duplex (ODN-6 + ODN-15, T_m = 51.1°C), GMD = G-clamp triazole mutant duplex (ODN-3 + ODN-15, T_m = 58.2°C), MedCMD = mutant duplex (ODN-4 + ODN-15, T_m = 53.3°C), MedCD = control duplex (ODN-4 + ODN-14, T_m = 68.1°C)

Studies on oligonucleotides containing TC step at the triazole linkage

To determine whether duplex stabilisation by triazole G-clamp is a general effect, or a special case applying only to the C_tC^C step, oligonucleotide ODN-25 with a T_tC^C step at the site of ligation was prepared (Table S1) from 3′-alkyne dT oligonucleotide ODN-24 and 5′-azide G-clamp ODN-23. The synthesis of ODN-24 was carried out using the 3′-O-(4,4′-dimethoxytrityl) deoxyribonucleoside-5′-phosphoramidites of A, G, C and T (reverse phosphoramidites), assembling the required sequence in the 5′- to 3′-direction and finally adding the 3′-propargylthymidine-5′-phosphoramidite monomer (Figure S5).[1] The resultant alkyne oligonucleotide (ODN-24) was reacted with ODN-23 to form the ligated product ODN-25. Control triazole oligonucleotide (ODN-27) without the G-clamp modification was prepared from ODN-24 and ODN-26 by the same method.

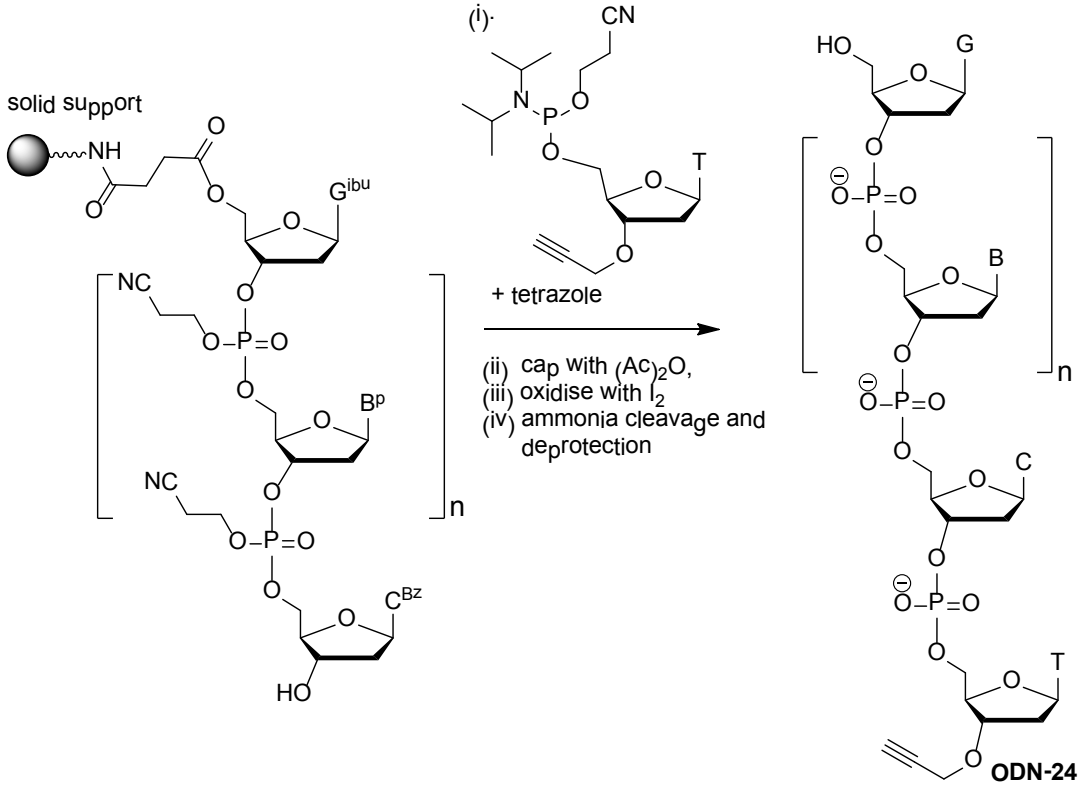

Figure S5. Synthesis of 3′-propargyl oligonucleotide ODN-24

After purification of the click products by HPLC, ultraviolet melting studies were performed with complementary DNA strand ODN-29 (Table S2). The stability of the duplex formed by ODN-25 and its unmodified DNA complement (ODN-29) was compared to the equivalent unmodified canonical duplex (ODN-28/ODN-29) and to the duplex containing a triazole linkage and a cytosine base in place of G-clamp (ODN-27/ODN-29). Incorporation of the G-clamp increased the UV melting temperature (T_m) of the triazole DNA duplex by 3.5°C compared to the unmodified duplex, and by 7.8° C compared to the triazole duplex with cytosine in place of the G-clamp.

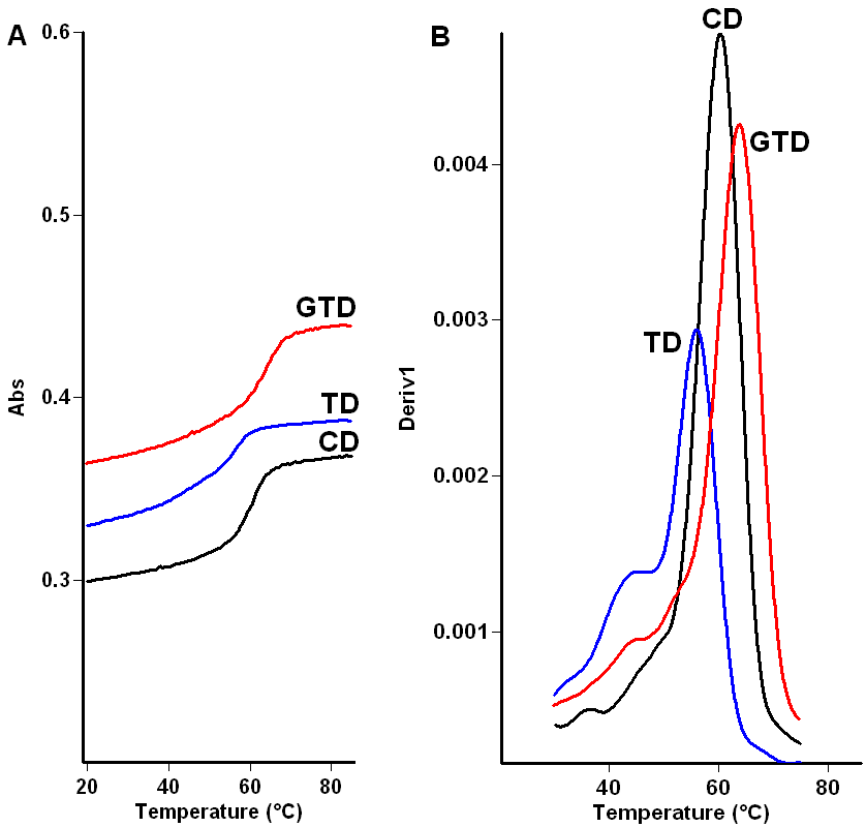

Figure S6. UV melting curves (A) and derivatives (B) of DNA duplexes containing a C_xT step:
UV melting curves were recorded at 260 nm (at 0.7 μM concentration of each oligonucleotide). TD = triazole duplex (ODN-27 + ODN-29, T_m = 55.8°C), GTD = G-clamp triazole duplex (ODN-25 + ODN-29, T_m = 63.6°C). CD = control duplex (ODN-28 + ODN-29, T_m = 60.1°C)

Table S1. Oligonucleotides used in this study containing a T_xC step
T^Y = 3'-propargyl-T, C^Z = 5'-azido dC, C^{CZ} = 5'-azido G-clamp, $_t$ = triazole linkage. F = amidohexylfluorescein

Oligo code	Sequence 5′ to 3′
ODN-23	$\underline{C}^{CZ}$ACCACACAAT
ODN-24	GCATTCGAGCAACGTAAGATCCT^Y
ODN-25	GCATTCGAGCAACGTAAGATCCT_tC^CACCACACAAT
ODN-26	C^ZACCACACAAT
ODN-27	GCATTCGAGCAACGTAAGATCCT_tCACCACACAAT
ODN-28	GCATTCGAGCAACGTAAGATCCTCACCACACAAT
ODN-29	FTGTGTGGTGAGGATCTTA

Table S2. Ultraviolet melting studies on oligonucleotides containing T_xC step
ΔT_m = (T_m duplex – T_m control duplex) , $_t$ = triazole linkage, C^C = G-clamp, F = amidohexylfluorescein
ΔT_m = T_m relative to unmodified control duplex.

Oligo code	**Sequence 5′ to 3′**	**Oligo code**	**Sequence 5′ to 3′**	**Central base pairs**	**T_m**	**ΔT_m**
ODN-28	GCATTCGAGCAACGTAA GATCCTCACCACACAAT	ODN-29	dFTGTGTGGT GAGGATCTTA	TA-CG **unmodified**	60.1	
ODN-27	GCATTCGAGCAACGTAA GATCCT$_t$CACCACACAAT	ODN-29	dFTGTGTGGT GAGGATCTTA	TA-CG **triazole**	55.8	-4.3
ODN-25	GCATTCGAGCAACGTAA GATCCT$_t$C^{C}ACCACACAAT	ODN-29	dFTGTGTGGT GAGGATCTTA	TA-C^{C}G **G-clamp triazole**	63.6	+3.5

Templated click ligation to synthesise double triazole G-clamp ODN-37 and double triazole ODN-38

The three short oligonucleotides (ODN-32 + ODN-33 + ODN-34) or (ODN-32 + ODN-35 + ODN-36) and the template (ODN-39) [50.0 nmol of each in 0.2 M NaCl (7.45 mL)] (sequences in Table S3) were annealed by heating at 90 °C for 5 min then cooled slowly to room temperature. A solution of Cu^I click catalyst was prepared from *tris*-hydroxypropyltriazole ligand[2] (8.75 µmol in 0.2 M NaCl, 12.5 µL), sodium ascorbate (12.5 µmol in 0.2 M NaCl, 25.0 µL) and $CuSO_4.5H_2O$ (1.25 µmol in 0.2 M NaCl, 12.5 µL). This solution was added to the annealed oligonucleotides and the reaction mixture was kept at room temperature for 2 h. Reagents were removed using 3 x NAP-25 gel-filtration columns and the ligated DNA was analysed and purified by denaturing 20% polyacrylamide gel electrophoresis.

Ultraviolet melting studies on double triazole-containing DNA duplexes

The duplex formed by 13-mer oligonucleotide ODN-37 (containing two units of triazole G-clamp) and its complementary strand ODN-41 was subjected to analysis by UV melting under the conditions described above. This was repeated for the control MedC duplex ODN-40/ODN-41 and the double triazole control duplex ODN-38/ODN-41. The stability of the G-clamp triazole DNA duplex (T_m = 73.0 °C) was found to be significantly higher than the unmodified and the triazole modified duplexes (T_m = 60.8 °C and 50.3 °C respectively). These very large increases in T_m (12.2 °C and 22.7 °C respectively) confirm the potential of triazole G-clamp as a modification in antisense oligonucleotides.

Linear copying of G-clamp triazole modified template (ODN-20) and normal template (ODN-30) using DNA Polymerase I, Large Klenow fragment

A reaction mixture was prepared by mixing 4 µL of 10 X *NEB buffer2** in a total reaction volume of 40 µL with template ODN-20 + primer ODN-22F or template ODN-30 + primer ODN-31F (44 pmol of each), 0.2 mM dNTP and 0.4 µL of DNA Polymerase I, Large Klenow fragment (5u/µL). Each reaction mixture was divided into two fractions and left at 37 ºC for 2 min or 5 min. Samples were mixed with 2 µL EDTA (100 µM) and 20 µL of formamide then loaded onto a 20% polyacrylamide gel.

**(10 X NEB buffer2 was supplied with the enzyme).1 X NEB buffer 2 = 50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT (pH 7.9 at 25 °C).*

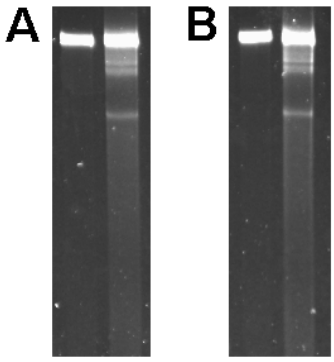

Figure S7. Linear copying of G-clamp triazole and normal DNA templates at 37 °C as described above
Lane 1 extension of unmodified template, lane 2 extension of triazole G-clamp modified template.
A: 2 min. B: 5 min. Minor band in lane 2 suggests some pausing at the triazole linkage.

Table S3. Additional oligonucleotide sequences used in this study

Description	Code No	Oligonucleotide sequence (5′ to 3′)
Templates and primers for linear copying		
Triazole G-clamp template for linear copying experiment	ODN-20	GCATTCGAGCAACGTAAGATCG^{Me}C$_t$C^{C}AGCACA CAATCTCACACTCTGGAATTCACACTGACAATA CTGCCGACACACATAACC
Primer for triazole G-clamp template for linear copying experiment	ODN-22F	F-GGTTATGTGTGTCGGCAG
Normal template for linear copying experiment	ODN-30	GCATTCGAGCAACGTAAGATCGCCAGCACACA ATCTCACACTCTGGAATTCACACTGACAATACC AATACACACAGCCGTC
Primer for normal template for linear copying experiment	ODN-31F	F-GACGGCTGTGTGTATTGG
Oligonucleotides for click ligation to make/study double triazole oligonucleotides		
Alkyne ODN for double G clamp triazole and double triazole ODNs	ODN-32	CGA^{Me}C^Y
Azide ODN for double G clamp triazole ODN	ODN-33	C^{CZ}AGC
Middle ODN for double G clamp triazole ODN	ODN-34	C^{CZ}TTT^{Me}C^Y
Azide ODN for double triazole ODN	ODN-35	C^{Z}AGC
Middle ODN for double triazole ODN	ODN-36	C^{Z}TTT^{Me}C^Y
Double G-clamp triazole ODN	ODN-37	CGA^{Me}C$_t$C^{C}TTT^{Me}C$_t$C^{C}AGC
Double triazole ODN	ODN-38	CGA^{Me}C$_t$CTTT^{Me}C$_t$CAGC
Template for click ligation of double G clamp triazole and double triazole ODNs	ODN-39	F-TTTTTTTTTTTGCTGGAAAGGTCG
MedC control for double G-clamp triazole and double triazole ODNs	ODN-40	CGA^{Me}CCTTT^{Me}CCAGC
Complement for double G-clamp triazole and double triazole ODNs	ODN-41	GCTGGAAAGGTCG

^{Me}C^Y = 3′-propargyl-5-methyl dC, C^Z = 5′-azido dC, C^{CZ} = 5′-azido G-clamp, $_t$ = triazole linkage. F = amidohexylfluorescein

Table S4. Mass spec analysis of oligonucleotides used in this study

Mass spectra were recorded on a Bruker micrOTOF^{TM} II focus ESI^{-}TOF MS instrument in ES^{-} mode

Oligo code	Calc. Mass	Found. Mass
ODN-1	2255	2255
ODN-2	1830	1828
ODN-3	4085	4083
ODN-4	3921	3919
ODN-5	2106	2105
ODN-6	3936	3935
ODN-7	3992	3990
ODN-8	3976	3974
ODN-9	3952	3950
ODN-10	3967	3965
ODN-11	3976	3974
ODN-12	3952	3950
ODN-13	3967	3965
ODN-14	4171	4170
ODN-15	4155	4154
ODN-16	4155	4154
ODN-18	7110	7109
ODN-19	17779	17778
ODN-20	24889	24888
ODN-23	3428	3427
ODN-24	7071	7070
ODN-25	10499	10499
ODN-26	3279	3278
ODN-27	10350	10349
ODN-28	10349	10348
ODN-29	6137	6137
ODN-32	1212	1210
ODN-33	1333	1333
ODN-34	1655	1654
ODN-35	1184	1183
ODN-36	1507	1505
ODN-37	4200	4198
ODN-38	3903	3900
ODN-39	7922	7922
ODN-40	3898	3897
ODN-41	4040	4038

References

[1] A. H. El-Sagheer, A. P. Sanzone, R. Gao, A. Tavassoli and T. Brown, *Proc. Natl. Acad. Sci. U. S. A.* **2011**, *108*, 11338-11343.
[2] T. R. Chan, R. Hilgraf, K. B. Sharpless and V. V. Fokin, *Org. Lett.* **2004**, *6*, 2853-2855.