

Cross-Linked DNA: Site-Selective “Click” Ligation in Duplexes with Bis-Azides and Stability Changes Caused by Internal Cross-Links

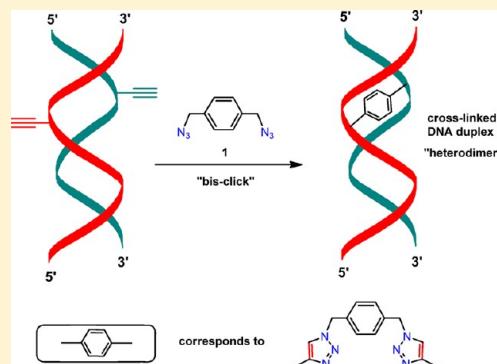
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S Supporting Information

ABSTRACT: Heterodimeric interstrand cross-linked DNA was constructed by the “bis-click” reaction carried out on preformed oligonucleotide duplexes with the bis-azide 1. For this, alkynylated 8-aza-7-deazapurine or corresponding 5-substituted pyrimidine nucleosides were synthesized. Cross-linking resulted in chemoselective formation of heterodimeric duplexes while homodimers were suppressed. For product identification, heterodimeric DNA was prepared by the “stepwise click” reaction, while noncomplementary homodimers were accessible by “bis-click” chemistry, unequivocally. Studies on duplex melting of complementary cross-linked duplexes (heterodimers) revealed significantly increased T_m values compared to the non-cross-linked congeners. The stability of this cross-linked DNA depends on the linker length and the site of modification. Cross-linked homodimers hybridized with single-stranded complementary oligonucleotides show much lower stability.



INTRODUCTION

Currently, our laboratory has reported on the cross-linking of oligonucleotide strands^{1,2} using the Huisgen-Meldal-Sharpless “click” chemistry.^{3–13} The reaction was performed on single-stranded oligonucleotides by two methods: (i) the “bis-click” reaction, which was limited to the cross-linking of identical oligonucleotide strands (homodimers) and (ii) the “stepwise click” protocol to cross-link nonidentical DNA strands (heterodimers) in a two-step method with a large excess of cross-linking agent.^{1,2} Both methods made use of DNA modified at the nucleobases with alkynyl side chains and bis-azides like 1, functioning as cross-linking reagents.^{14–16} As a result, internal cross-links were generated at various positions and with linkers of different lengths.^{17–35}

Now, the cross-linking of cDNA strands in duplex DNA will be investigated, thereby using duplex formation as a tool (“template”) for the selective generation of heterodimeric DNA duplexes by the “bis-click” reaction, while the “stepwise click” protocol will be used for heterodimer assignment. We show that a diversity of alkynylated nucleosides related to the four canonical DNA bases can provide terminal triple bonds to construct cross-linked DNA within a duplex.^{2,36–42}

In our study, cross-linking is performed by the “bis-click” protocol using a duplex with triple bonds in each of the complementary strands and the bis-azide 1.¹⁶ The “stepwise click” protocol was used for the unambiguous assignment of the heterodimers and was performed with one strand bearing the triple bond and the other one carrying the azido function.

The latter was previously used to cross-link nonidentical strands selectively. We show now that under optimal conditions side chain triple bonds can be connected in a chemoselective way by “bis-click” chemistry or for comparison by the “stepwise click” protocol (Figure 1). For this, it is a prerequisite that the size of the cross-linker and the distance between the triple bonds have to be of comparable length. Then, the linker is well-accommodated in the duplex structure and the formation of heterodimers is favored while homodimers are suppressed. It will be shown that DNA cross-links induce additional stability to duplex DNA as displayed by strongly increased T_m values of cross-linked species. In this context, the impact of a variety of modified alkynylated nucleobases and the influence of the side chain length and distance of triple bonds regarding duplex stability is evaluated. The cross-linking reaction using “bis-click” or “stepwise click” chemistry is applicable for duplex DNA and might be exerted to cross-link other DNA assemblies such as triplexes or quartets.

EXPERIMENTAL PROCEDURES

General Methods and Materials. All chemicals were purchased from Acros, Fluka, or Sigma-Aldrich (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany). Standard phosphoramidites were purchased from Sigma (Sigma-Aldrich Chemie

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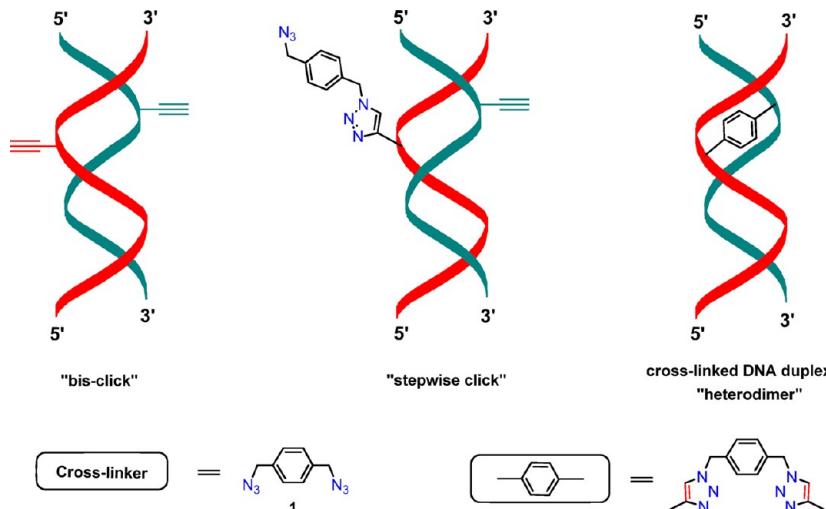
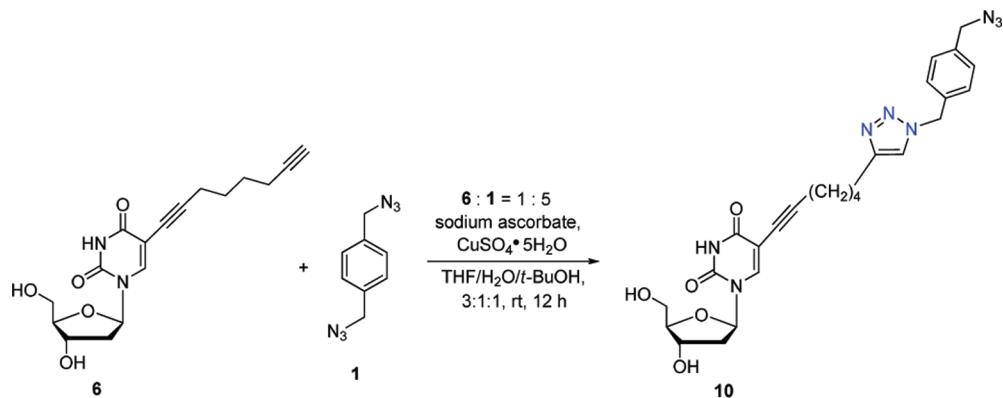


Figure 1. Cross-linked DNA formed by the “bis-click” or “stepwise click” reaction performed on duplex DNA. Red and green display complementary oligonucleotide strands.

Scheme 1. Synthesis of the Azido-Functionalized Conjugate 10



GmbH, Deisenhofen, Germany). Solvents were of laboratory grade. Thin layer chromatography (TLC) was performed on TLC aluminum sheets covered with silica gel 60 F254 (0.2 mm, VWR International, Germany). Flash column chromatography (FC): silica gel 60 (40–60 μm , VWR International, Germany) at 0.4 bar. UV spectra: U-3000 spectrophotometer (Hitachi, Japan); λ_{max} (ϵ) in nm, ϵ in $\text{dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$. NMR spectra: DPX 300 spectrometer (Bruker, Germany) at 300.15 MHz for ^1H and 75.48 MHz for ^{13}C . The J values are given in Hz; δ values in ppm relative to Me_4Si as internal standard. For NMR spectra recorded in DMSO, the chemical shift of the solvent peak was set to 2.50 ppm for ^1H NMR and 39.50 ppm for ^{13}C NMR. Elemental analysis was performed by the Mikroanalytisches Laboratorium Beller, Göttingen, Germany. Reversed-phase HPLC was carried out on a 250×4 mm RP-18 LiChrospher 100 column (Merck, Darmstadt, Germany) with a Merck-Hitachi HPLC pump connected with a variable wavelength monitor, a controller, and an integrator. Gradients used for HPLC chromatography (A = MeCN, B = 0.1 M (Et_3NH)OAc (pH 7.0)/MeCN, 95: 5): (I) 3 min 15% A in B, 12 min 15–50% A in B, and 5 min 50–10% A in B, flow rate 0.7 mL min^{-1} ; (II) 0–25 min 0–20% A in B, flow rate 0.7 mL min^{-1} ; (III) 0–15 min 0–20% A in B, 15–18 min 20–40% A in B, flow rate 0.7 mL min^{-1} ; (IV) 0–30 min 20–80% D in C with a flow rate of 0.75 mL min^{-1} . C = 25 mM Tris-HCl, 10% MeCN, pH 7.0; D = 25 mM Tris-HCl, 1.0 M NaCl, and 10%

MeCN, pH 7.0. The ion-exchange chromatography was performed on a 4×250 mm DNA PAC PA-100 column (P/N 043011; Dionex, USA) connected with a 50 mm NucleoPac PA-100 Guard (P/N 43018; Dionex, USA) precolumn using a Merck-Hitachi HPLC apparatus with a pump (model L-6200A) and a UV detector (model L-4000) connected with an integrator (model D-2000). Elution profiles were recorded at 260 nm. The molecular masses of oligonucleotides were determined by LC-ESI-TOF (Agilent 1200 Series, Bruker Micro-TOF Q2) or MALDI-TOF (MS Autoflex, Bruker and Voyager-DE PRO spectrometer, Applied Biosystems) in the linear negative or positive mode with 3-hydroxypicolinic acid (3-HPA) as a matrix. Melting curves were measured with a Cary-100 Bio UV-vis spectrophotometer (Varian, Australia) equipped with a Cary thermoelectrical controller with a heating rate of 1 $^\circ\text{C}/\text{min}$. Nanopure water (resistance <0.055 $\mu\text{S}/\text{cm}$) from MembranePure water system (Astacus) was used for all experiments.

1-(2-Deoxy- β -D-erythro-pentofuranosyl)-5-{1-(4-azido-methylbenzyl)-1*H*-[1,2,3-triazol-4-yl]}hexylidene uracil (10) (Scheme 1). To a solution of compound 6^{38,39} (99.4 mg, 0.3 mmol) and 1¹⁶ (282 mg, 1.5 mmol) in THF/H₂O/*t*-BuOH, 3:1:1 (4.5 mL), was added a freshly prepared 1 M solution of sodium ascorbate (239 μL , 0.24 mmol) in water, followed by the addition of copper(II) sulfate pentahydrate 7.5% in water (192 μL , 0.057 mmol). The reaction mixture was stirred in the dark at room temperature for 12 h. After completion of the

reaction (monitored by TLC), the solvent was evaporated, and the residue was applied to FC (silica gel, column 8 × 3 cm, eluted with CH₂Cl₂/MeOH, 95:5 → 90:10). Evaporation of the main zone gave **10** as a pale yellow solid (80 mg, 51%). TLC (CH₂Cl₂/MeOH, 90:10): *R*_f 0.58; UV λ_{max} (MeOH)/nm 230 ($\varepsilon/\text{dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$ 17 800), 260 (3700), 292 (11 400). Anal. (C₂₆H₂₉N₁₁O₄) C, H, N. ¹H NMR (300 MHz, DMSO-*d*₆): δ = 1.47–1.56 (m, 2H, CH₂), 1.68–1.75 (m, 2H, CH₂), 2.09–2.12 (m, 2H, 2'-H_α + 2'-H_β), 2.38 (t, *J* = 7.2 Hz, 2H, CH₂), 2.64 (t, *J* = 7.2 Hz, 2H, CH₂), 3.50–3.63 (m, 2H, 5'-H), 3.77–3.79 (m, 1H, 4'-H), 4.22–4.23 (m, 1H, 3'-H), 4.43 (s, 2H, PhCH₂), 5.15 (br s, 1H, 5'-OH), 5.25 (br s, 1H, 3'-OH), 5.55 (s, 2H, PhCH₂), 6.11 (t, *J* = 6.6 Hz, 1H, 1'-H), 7.28–7.38 (m, 4H, H-phenylene), 7.92 (s, 1H, H-triazole), 8.13 (s, 1H, C6-H), 11.56 (s, 1H, HN).

Synthesis, Purification, and Characterization of Oligonucleotides. The syntheses of oligonucleotides was performed on a DNA synthesizer, model 392–08 (Applied Biosystems, Weiterstadt, Germany) on a 1 μmol scale using the phosphoramidites **67–72**^{2,37,39–43} (Figure S2, Supporting Information) and the standard phosphoramidite building blocks following the synthesis protocol for 3'-*O*-(2-cyanoethyl)-phosphoramidites.⁴⁴ After cleavage from the solid support, the oligonucleotides were deprotected in 25% aqueous ammonia solution for 12–16 h at 60 °C. The purification of the “trityl-on” oligonucleotides was carried out on reversed-phase HPLC (Merck-Hitachi-HPLC; RP-18 column; LiChrospher 250 × 5 mm, gradient system (*I*)). The purified “trityl-on” oligonucleotides were treated with 2.5% Cl₂CHCOOH/CH₂Cl₂ for 5 min at 0 °C to remove the 4,4'-dimethoxytrityl residues. The detritylated oligomers were purified by reversed-phase HPLC (gradient *II*). The oligomers were desalted by reversed-phase HPLC (RP-18, LiChrospher, 125 × 5 mm) using nanopure water for elution of salt, while the oligonucleotides were eluted with H₂O/MeOH (2:3). Then, they were lyophilized on a Speed Vac evaporator to yield colorless solids which were frozen at –24 °C. The molecular masses of the oligonucleotides were determined by LC-ESI-TOF or MALDI-TOF mass spectrometry in the linear negative or linear positive mode (see Tables 2, 3, and 5; and Tables S3 and S4, Supporting Information). Extinction coefficients ε_{260} (MeOH) of nucleosides: dA, 15400; dG, 11700; dT, 8800; dC, 7300; **2**, 10900;² **3**, 11500;⁴¹ **4**, 8500;⁴² **5**, 10500;⁴⁵ **6**, 3800,³⁹ **7**, 4900.⁴⁰

Ion-Exchange HPLC Analysis of Interstrand Cross-Linked Oligonucleotides. The ion-exchange chromatography was performed on a 4 × 250 mm DNA PA 100 column (P/N 043011; Dionex, USA) connected with a 50 mm NucleoPac PA-100 Guard (P/N 43018; Dionex, USA) precolumn using a Merck-Hitachi HPLC apparatus with a pump (model L-6200A), and a UV detector (model L-4000) connected with an integrator (model D-2000). Elution profiles were recorded at 260 nm. An artificial mixture of oligonucleotides was prepared as follows: 0.1–0.2 A₂₆₀ units of the oligonucleotides were dissolved in 20 μL of distilled water. Crude reaction mixtures were treated as follows before injection: the crude reaction mixture was concentrated in a Speed Vac, dissolved in 300 μL of bidistilled water, and centrifuged for 20 min at 12 000 rpm. Thirty microliters of the supernatant solution was then injected into the system, and gradient IV was used for elution: 0–30 min with 20–80% D in C with a flow rate of 0.75 mL min⁻¹ (C: 25 mM Tris-HCl, 10% MeCN, pH 7.0; D: 25 mM Tris-HCl, 1.0 M NaCl, 10% MeCN, pH 7.0).

T_m Measurements. For the T_m measurements, the oligonucleotides were dissolved in 1 M NaCl, 100 mM MgCl₂, and 60 mM Na cacodylate buffer, pH 7.0. For non-cross-linked oligonucleotide duplexes 5 μM + 5 μM single-strand concentration was used. Hybridization experiments on cross-linked duplexes were carried out with 5 μM of the cross-linked oligonucleotide. The melting curves were measured with a UV-vis spectrophotometer equipped with a thermoelectrical controller. The temperature was measured continuously in the reference cell with a Pt-100 resistor with a heating rate of 1 °C min⁻¹, and the absorbance at 260 nm was recorded as a function of the temperature. The thermodynamic data of duplex formation were calculated by the *MeltWin* (v 3.0) program using the curve fitting of the melting profiles according to a two-state model.⁴⁶

The thermal hypochromicity (*h*% = [($\varepsilon_{\text{monomer}} - \varepsilon_{\text{polymer}}$) · ($\varepsilon_{\text{monomer}}^{-1}$)] × 100%) of the oligonucleotides was determined from the melting curves. The extinction coefficients of the oligonucleotides were calculated from the sum of the extinction coefficients of the monomeric 2'-deoxyribonucleosides divided by the hyperchromicity.⁴⁷

T_m Measurements under the Reaction Conditions used for the “Bis-click” and the “Stepwise Click” reaction: For the T_m measurements under “click” reaction conditions, the oligonucleotides were dissolved in 0.4 M NaCl, 40 mM NaHCO₃ containing 6 vol % *t*-BuOH (“bis-click”). In the case of the “stepwise click” reaction conditions, the measurements were performed without adding NaCl (40 mM NaHCO₃ containing 6 vol% *t*-BuOH). These measurements were performed with 5 μM + 5 μM oligonucleotide single-strand concentration.

Denaturing Polyacrylamide Gel Electrophoresis (PAGE) of Oligonucleotides. Analysis of oligonucleotides was carried out by polyacrylamide gel electrophoresis (17% polyacrylamide gel, 19:1, acrylamide: bisacrylamide, containing 7 M urea (8.4 g in 8.5 mL bisacrylamide/acrylamide (1:19) solution). In order to catalyze polymerization, 20 μL of TEMEDA (*N,N,N',N'*-tetramethylethylenediamine) and 60 μL of 10% ammonium persulfate (APS) were added. After complete polymerization (1 h), the gel (10 × 10 cm) was prerun for 30 min at r.t using 1 × tris-borate-EDTA (TBE) buffer containing 20 mM MgCl₂ (pH 8.4). The individual oligonucleotides (0.15–0.30 A₂₆₀ units) were dissolved in 2–5 μL of distilled water at r.t. Ten microliters of gel loading buffer containing formamide was added, and the oligonucleotide solution was loaded onto the gel. Electrophoresis was run at r.t. for 6 h using 1 × TBE buffer containing 20 mM MgCl₂ (pH 8.4) at a constant field strength of 12 V/cm. The gel was stained with 0.02% methylene blue for 20 min and was then incubated in water for 1 h to remove excess dye from the background.

General Procedure for the Cross-Linking of Complementary Oligonucleotides (Heterodimers) using the “Bis-Click” Cycloaddition. A solution of the alkynylated complementary oligonucleotide strands (2.0 A₂₆₀ units each) in aq. NaCl (100 μL of a 1 M stock solution) was heated at 80 °C for 5 min, then slowly cooled down to 20 °C using a water-bath. Then, CuSO₄-TBTA (tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine) ligand complex (25 μL of a 20 mM stock solution in H₂O/DMSO/*t*-BuOH 4:3:1 for TBTA; 25 μL of a 20 mM stock solution in H₂O/DMSO/*t*-BuOH 4:3:1 for CuSO₄) was added to the solution. Next, TCEP (tris(2-carboxyethyl)phosphine, 25 μL of a 20 mM stock solution in water), sodium bicarbonate (20 μL of a 200 mM aq. solution), bis-azide **1**¹⁶ (1.5 μL of a 20 mM stock solution in dioxane/H₂O 1:1),

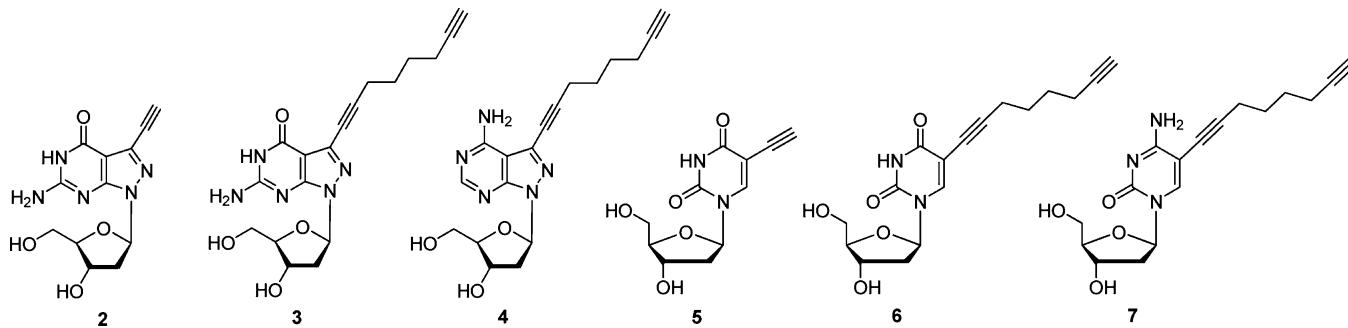


Figure 2. Alkynylated nucleosides 2–7 incorporated into oligonucleotides.

Table 1. T_m Values of Duplexes Containing the Alkynylated Nucleosides 2–7 as well as Azido-Functionalized Nucleoside Derivatives 8–12

Duplexes	T_m^a [°C]	ΔT_m^b [°C]	% h ^c	$\Delta G^{\circ}_{310}d$ [kcal/mol]	Duplexes	T_m^a [°C]	ΔT_m^b [°C]	% h ^c	$\Delta G^{\circ}_{310}d$ [kcal/mol]
5'-d(TAG GTC AAT ACT) (13) 3'-d(ATC CAG TTA TGA) (14)	51.0 (49.0)	---	18 15	-11.4 -10.8	5'-d(TAG 2TC AAT ACT) (16) 3'-d(ATC CA2 TTA TGA) (24)	57.0	+6	18	-12.8
5'-d(TAG 2TC AAT ACT) (16) 3'-d(ATC CA3 TTA TGA) (25)	54.5	+3.5	18	-12.2	5'-d(TAG 6AG GTC AAT ACT) (21) 3'-d(ATC 7AG TTA TGA) (27)	53.0	+2	17	-12.2
5'-d(TAG 3TC AAT ACT) (18) 3'-d(ATC CA3 TTA TGA) (25)	55.5	+4.5	19	-13.0	5'-d(TAG GT7 AAT ACT) (23) 3'-d(ATC 7AG TTA TGA) (28)	52.5	+1.5	18	-11.8
5'-d(TA2 GTC AAT ACT) (15) 3'-d(ATC CA2 TTA TGA) (24)	55.0	+4	19	-12.8	5'-d(TAG GT7 AAT ACT) (23) 3'-d(ATC 7AG TTA TGA) (27)	54.0	+3	17	-12.2
5'-d(TAG GT7 AAT ACT) (23) 3'-d(ATC CA2 TTA TGA) (24)	55.5	+4.5	18	-13.0	5'-d(TAG 2TC AAT ACT) (16) 3'-d(ATC CA8 TTA TGA) (35)	54.5	+3.5	19	-12.3
5'-d(TA2 GTC AAT ACT) (15) 3'-d(AT7 CAG TTA TGA) (27)	54.5	+3.5	18	-12.4	5'-d(TAG 2TC AAT ACT) (16) 3'-d(ATC CA9 TTA TGA) (36)	52.0	+1.0	17	-11.8
5'-d(TAG 2TC AAT ACT) (16) 3'-d(AT7 CAG TTA TGA) (27)	55.0	+4	19	-12.7	5'-d(TAG 9TC AAT ACT) (31) 3'-d(ATC CA3 TTA TGA) (25)	53.5 (52.0)	+2.5 +3.0	18 17	-11.8 -11.5
5'-d(TAG 3TC AAT ACT) (18) 3'-d(AT7 CAG TTA TGA) (27)	53.5	+2.5	19	-12.3	5'-d(10AG GTC AAT ACT) (37) 3'-d(ATC CA2 TTA TGA) (24)	55.5	+4.5	19	-12.8
5'-d(TAG G6C AAT ACT) (22) 3'-d(ATC CA2 TTA TGA) (24)	54.5	+3.5	18	-12.8	5'-d(TAG G10C AAT ACT) (32) 3'-d(ATC CA3 TTA TGA) (25)	50.0 (48.5)	-1.0 -0.5	15 14	-11.2 -10.9
5'-d(TAG G6C AAT ACT) (22) 3'-d(ATC CA3 TTA TGA) (25)	52.0	+1	18	-11.8	5'-d(5AG GTC AAT ACT) (20) 3'-d(A10C CAG TTA TGA) (38)	49.5	-1.5	18	-11.2
5'-d(5AG GTC AAT ACT) (20) 3'-d(A6C CAG TTA TGA) (26)	50.0 (49.0)	-1.0 0	18	-11.0 -10.9	5'-d(TAG GT11 AAT ACT) (33) 3'-d(ATC CA3 TTA TGA) (25)	50.0 (48.0)	-1.0 -1.0	17 17	-11.1 -10.5
5'-d(TAG GTC 2AT ACT) (17) 3'-d(ATC CA2 TTA TGA) (24)	47.0	-4	17	-10.4	5'-d(TAG GTC 12AT ACT) (34) 3'-d(ATC CA3 TTA TGA) (25)	49.5 (47.5)	-1.5 -1.5	15 14	-11.0 -10.5
5'-d(TAG2TCAACT) (16) 3'-d(A ₆ ATCCA3TTATGAT ₆) (30)	55.5	+4.5	14	-12.4	5'-d(TAG2TCAACT) (16) 3'-d(A ₆ ATCCA3TTATGAT ₆) (29)	55.0	+4	15	-12.6

^aMeasured at 260 nm in a 1 M NaCl solution containing 100 mM MgCl₂ and 60 mM Na cacodylate (pH 7.0) with 5 μ M + 5 μ M single-strand concentration. Data in parentheses were measured at a 2 μ M + 2 μ M single-strand concentration. ^bRefers to the contribution of the modified residues. ^c%h refers to the percentage of hypochromicity. ^d ΔG°_{310} were determined from the melting curves by using the software MELTWIN v 3.0 (J. A. Mc Dowell, 1996). The ΔG°_{310} values are given within an error of 15%.

and 30 μ L of DMSO were introduced, and the reaction mixture was kept at room temperature for 12 h. The reaction mixture was concentrated in a Speed Vac, and dissolved in 300 μ L bidistilled water and centrifuged for 20 min at 12 000 rpm. The supernatant was collected and further purified by reversed-phase HPLC using gradient III. Using this procedure, the cross-linked oligonucleotides ICL-39, ICL-40, ICL-43, ICL-44, ICL-46, and ICL-47 to ICL-59 were obtained in 40–50% yield. The reversed-phase HPLC profiles of the crude and purified reaction mixtures for selected cross-linked oligonucleotides obtained after the “bis-click” reaction are shown in Figure S7–S9 (Supporting Information). The molecular masses of the cross-linked oligonucleotides were determined by MALDI-TOF or LC-ESI-TOF mass spectrometry (Tables 2 and 3; and Table S4 and Figures S21–S43, Supporting Information).

General Procedure for the Cross-Linking of Identical Oligonucleotides (Homodimers ICL-60 to ICL-65) Using the “Bis-Click” Huisgen-Sharpless-Meldal [3 + 2] Cyclo-addition. To a solution of the alkynylated oligonucleotide (5.0 A_{260} units) in 20 μ L of water were added CuSO₄-TBTA ligand complex (50 μ L of a 20 mM stock solution in H₂O/DMSO/t-BuOH 4:3:1 for TBTA; 50 μ L of a 20 mM stock solution in H₂O/DMSO/t-BuOH 4:3:1 for CuSO₄). Next, TCEP (50 μ L of a 20 mM stock solution in water), sodium bicarbonate (20 μ L of a 200 mM aq. solution), bis-azide 1 (1.5 μ L of a 20 mM stock solution in dioxane/H₂O 1:1), and 30 μ L of DMSO were introduced, and the reaction mixture was kept at room temperature for 12 h. The reaction mixture was concentrated in a Speed Vac, and dissolved in 300 μ L bidistilled water and centrifuged for 20 min at 12 000 rpm. The supernatant was collected and further purified by reversed-phase HPLC using

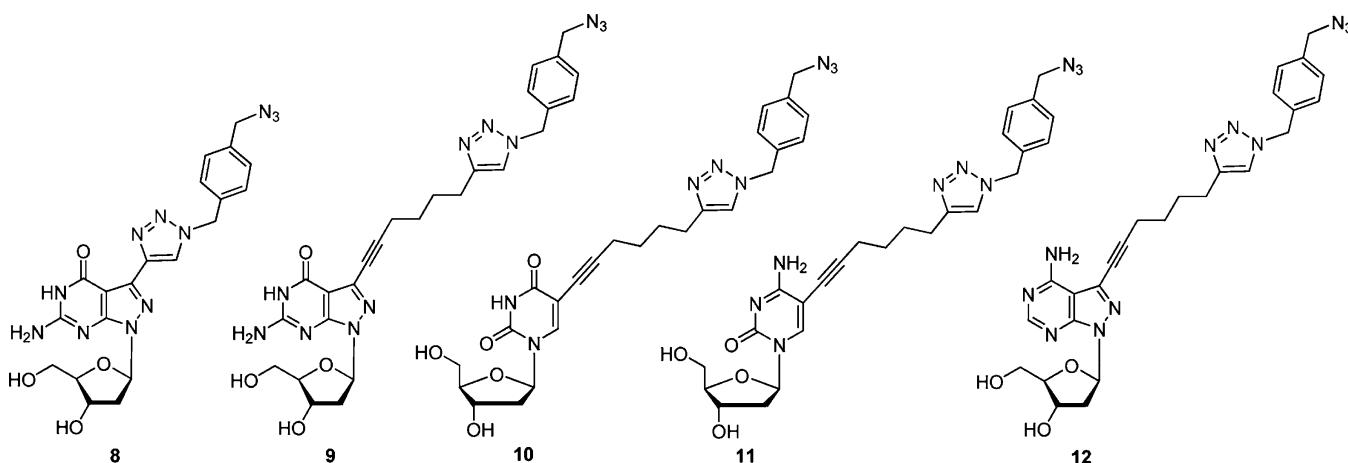
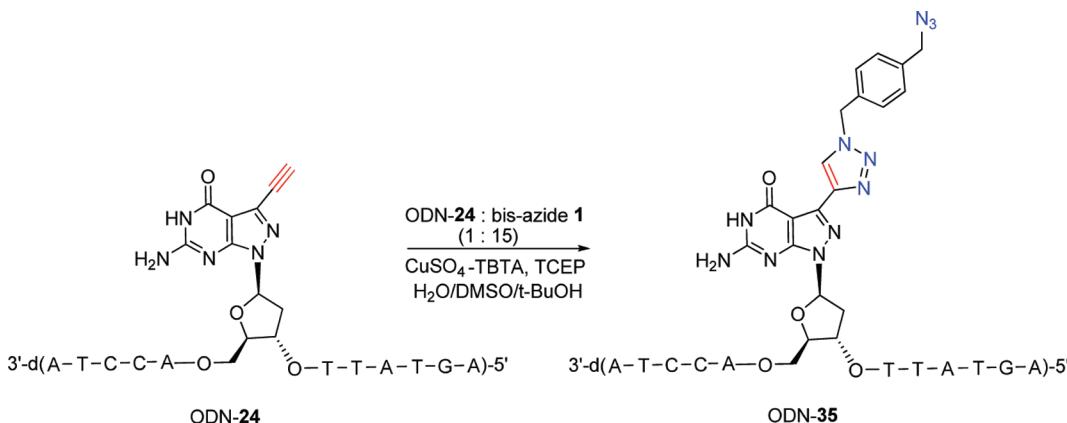


Figure 3. Azidomethylbenzyl-labeled nucleosides 8–12 obtained after the “first click” of the “stepwise click” reaction performed on oligonucleotides

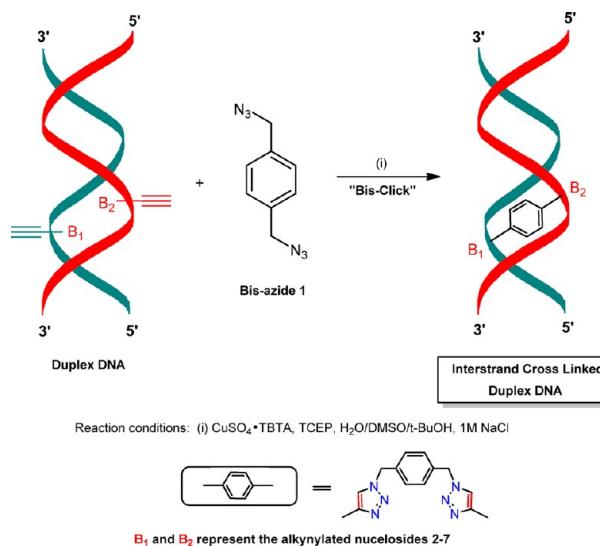
Scheme 2. Typical Example for the Synthesis of Mono-Functionalized Azido-Oligonucleotides Synthesized by the “Stepwise Click” Chemistry



gradient III. Using this procedure, the cross-linked oligonucleotides ICL-60 to ICL-65 were obtained. Their molecular masses were determined by MALDI-TOF or LC-ESI-TOF mass spectrometry (Tables 3 and 5).

General Procedure for the “Stepwise Click” Cycloaddition. Synthesis of the Azidomethylbenzyl-Labeled Oligonucleotides ODN-31 to ODN-38 Using Oligonucleotides ODN-18, ODN-19, and ODN-21 to ODN-26 and Bis-Azide 1 (“First Click”). To the single-stranded oligonucleotide (5 A₂₆₀ units), a mixture of the CuSO₄-TBTA ligand complex (50 μL of a 20 mM stock solution in H₂O/DMSO/t-BuOH, 4:3:1 for TBTA; 50 μL of a 20 mM stock solution in H₂O/DMSO/t-BuOH, 4:3:1 for CuSO₄), tris(carboxyethyl)-phosphine (TCEP; 50 μL of a 20 mM stock solution in water), 1,4-bis-azidomethylbenzene **1**¹⁶ (37.5 μL of a 20 mM stock solution in dioxane/H₂O, 1:1), sodium bicarbonate (50 μL of a 20 mM aq. solution) and 30 μL of DMSO were added, and the reaction proceeded at room temperature for 2 h. The reaction mixture was concentrated in a Speed Vac, and dissolved in 300 μL bidistilled water and centrifuged for 20 min at 12 000 rpm. The supernatant solution was collected and further purified by reversed-phase HPLC (gradient *III*) to give about 80% isolated yield of the azidomethylbenzyl oligonucleotide. The reversed-phase HPLC profiles of the crude and purified reaction mixtures for monofunctionalized intermediates ODN-31 to ODN-38 obtained after the “first click” reaction are shown in Figure S5–S6 (Supporting Information). Their molecular

Scheme 3. Principle of Duplex DNA Assisted Cross-Linking Using the “Bis-Click” Reaction^a



^aRed and green display complementary oligonucleotide strands both bearing terminal triple bonds.

masses were determined by MALDI-TOF or LC-ESI-TOF mass spectrometry (Table S3, Supporting Information).

Scheme 4. “Bis-Click” Cross-Linking on Duplex DNA Using the Complementary Ethynylated Oligonucleotides ODN-16 and ODN-24 as Starting Materials

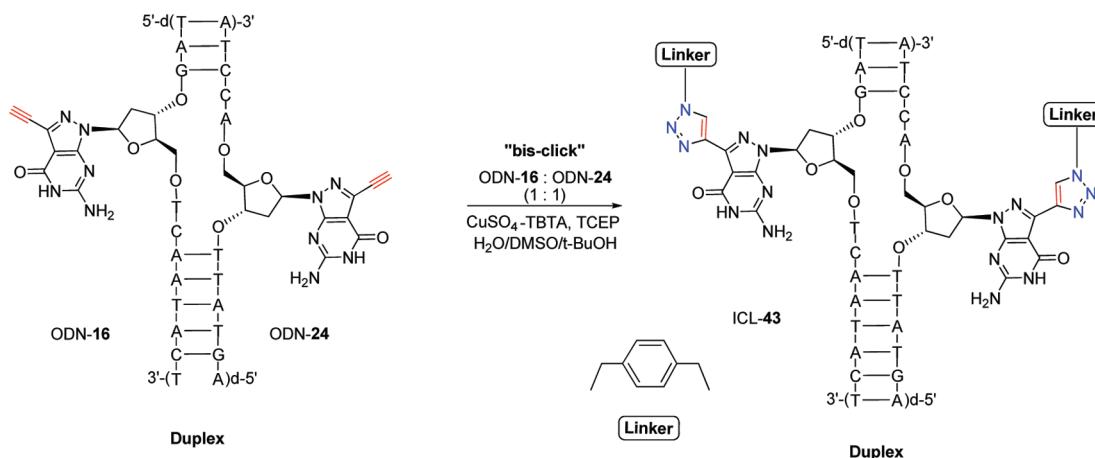


Table 2. Structure and Mass Spectrometric Data of Interstrand Cross-Linked Oligonucleotides (ICL 39 to 59) Prepared by “Bis-Click” or “Stepwise Click” Chemistry on Duplexes

Duplex DNA (ICL)	MS (calc.) found	Duplex DNA (ICL)	MS (calc.) found
5'-d(TAG 3TC AAT ACT) 3'-d(ATC CA3 TTA TGA) (39)*	[7681.5] ^c 7685.5 ^d	5'-d(TAG 2TC AAT ACT) 3'-d(A ₆ ATCCA3TTATGA T ₆) (50)*	[11308.6] ^c 11309.0 ^d
5'-d(TAG G6C AAT ACT) 3'-d(ATC CA3 TTA TGA) (40)*	[7667.5] ^c 7669.4 ^d	5'-d(TAG GT7 AAT ACT) 3'-d(ATC CA2 TTA TGA) (51)*	[7604.2] ^e 7604.2 ^f
5'-d(TAG GT7 AAT ACT) 3'-d(ATC CA3 TTA TGA) (41) [#]	[7685.3] ^a 7688.3 ^b	5'-d(TA2 GTC AAT ACT) 3'-d(AT7 CAG TTA TGA) (52)*	[7601.5] ^c 7605.4 ^d
5'-d(TAG GTC 4AT ACT) 3'-d(ATC CA3 TTA TGA) (42) [#]	[7686.3] ^a 7689.2 ^b	5'-d(TAG 2TC AAT ACT) 3'-d(AT7 CAG TTA TGA) (53)*	[7601.5] ^c 7603.5 ^d
5'-d(TAG 2TC AAT ACT) 3'-d(ATC CA2 TTA TGA) (43)*	[7521.4] ^c 7524.5 ^d	5'-d(TAG 3TC AAT ACT) 3'-d(AT7 CAG TTA TGA) (54)*	[7680.5] ^c 7685.5 ^d
5'-d(TAG 2TC AAT ACT) 3'-d(ATC CA3 TTA TGA) (44)*	[7601.5] ^c 7604.5 ^d	5'-d(TAG G6C AAT ACT) 3'-d(AT7 CAG TTA TGA) (55)*	[7587.5] ^c 7589.5 ^d
5'-d(6AG GTC AAT ACT) 3'-d(ATC CA2 TTA TGA) (45) [#]	[7594.2] ^a 7599.7 ^b	5'-d(TAG G6C AAT ACT) 3'-d(AT7 CAG TTA TGA) (56)*	[7667.5] ^c 7673.5 ^d
5'-d(SAG GTC AAT ACT) 3'-d(A6C CAG TTA TGA) (46)*	[7578.1] ^a 7575.0 ^b	5'-d(6AG GTC AAT ACT) 3'-d(AT7 CAG TTA TGA) (57)*	[7667.5] ^c 7670.3 ^d
5'-d(TA2G TC AAT ACT) 3'-d(ATC CA2 TTA TGA) (47)*	[7521.4] ^c 7523.3 ^d	5'-d(TAG G T7 AAT ACT) 3'-d(AT7 CAG TTA TGA) (58)*	[7681.5] ^c 7682.3 ^d
5'-d(TAG GTC 2AT ACT) 3'-d(ATC CA2 TTA TGA) (48)*	[7537.4] ^c 7539.3 ^d	5'-d(TAG GT7 AAT ACT) 3'-d(AT7 CAG TTA TGA) (59)*	[7681.5] ^c 7682.5 ^d
5'-d(TAG 2TC AAT ACT) 3'-d(T ₆ ATCCA3TTATGA T ₆) (49)*	[11250.0] ^c 11250.9 ^d		

^aCalculated on the basis of molecular weight as [M+1]⁺. ^bDetermined by MALDI-TOF mass spectrometry as [M+1]⁺ in the linear positive mode.

^cCalculated on the basis of exact mass. ^dDetermined by LC-ESI-TOF mass spectrometry. ^eCalculated on the basis of molecular weight as [M-1]⁻.

^fDetermined by MALDI-TOF mass spectrometry as [M-1]⁻ in the linear negative mode. *Refers to interstrand cross-linked oligonucleotides prepared by the “bis-click” reaction. #Refers to interstrand cross-linked oligonucleotides prepared by the “stepwise click” reaction. The particular cross-linked nucleobases, e.g., 2-2, are shown in the Supporting Information (Figure S3 and Figure S4).

Cross-Linked Complementary Oligonucleotides Using Azidomethylbenzyl Oligonucleotides ODN-31 to ODN-38 and Alkynylated Oligonucleotides (“Second Click”). To the azidomethylbenzyl oligonucleotides ODN-31 to ODN-38 (2.0 A_{260} units) and complementary alkynylated oligonucleotides (1.5 A_{260} units), a mixture of the $\text{CuSO}_4\text{-TBTA}$ ligand complex (50 μL of a 20 mM stock solution in $\text{H}_2\text{O}/\text{DMSO}/t\text{-BuOH}$, 4:3:1 for TBTA; 25 μL of a 20 mM stock solution in

$\text{H}_2\text{O}/\text{DMSO}/t\text{-BuOH}$, 4:3:1 for CuSO_4), TCEP (30 μL of a 20 mM stock solution in water), sodium bicarbonate (10 μL of a 200 mM aq. solution), and 20 μL of DMSO were added, and the reaction proceeded at room temperature for 12 h. The reaction mixture was concentrated in a Speed Vac, dissolved in 300 μL bidistilled water, and centrifuged for 20 min at 12 000 rpm. The supernatant solution was collected and further purified by reversed-phase HPLC (gradient III) to give

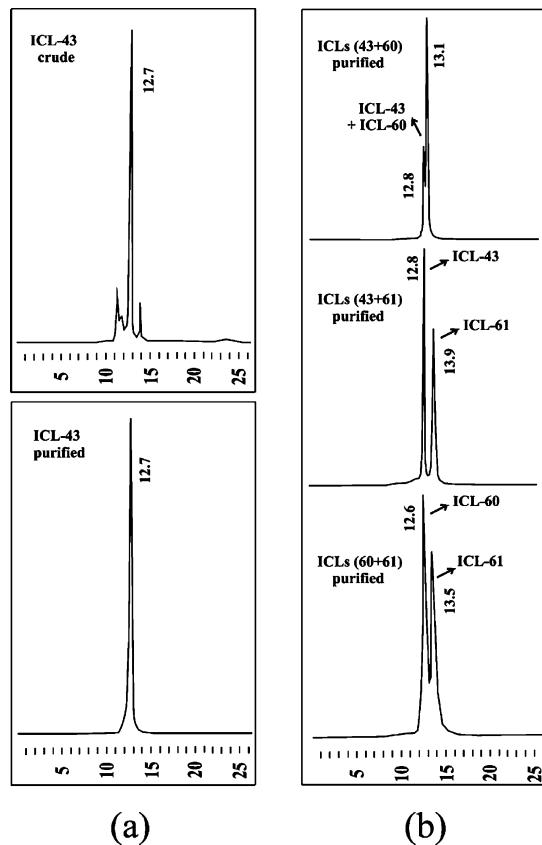


Figure 4. Reversed-phase HPLC profiles of (a) crude ICL-43 obtained after the “bis-click” reaction; (b) artificial mixtures of purified ICL-43 (heterodimer), ICL-60, and ICL-61 (homodimers) measured at 260 nm. The following gradient system was used: MeCN (A) and 0.1 M (Et_3NH)OAc (pH 7.0) (B). Gradient III: 0–15 min 0–20% A, 15–18 min 20–40% A in B, 20–25 min 40–0% A in B with a flow rate of 0.7 mL min^{-1} .

about 40–50% isolated yield of the cross-linked oligonucleotide. ICL-43 and ICL-45 were prepared according to a procedure published earlier.² SI Figures S5 and S6 depict the reversed-phase HPLC profiles of the crude and purified reaction mixtures of cross-linked oligonucleotides ICL-39 to ICL-46 obtained after the “second click” reaction. Their molecular masses were determined by MALDI-TOF or ESI-TOF mass spectrometry (Table 2).

RESULTS AND DISCUSSION

Synthesis of Oligonucleotide Cross-Link Precursors with Modified Nucleobases and Side Chains of Different Length and Their Duplex Stability. The alkynylated nucleosides 2–7 (Figure 2) are representing analogues of the canonical nucleosides 2'-deoxyguanosine, 2'-deoxyadenosine, 2'-de oxythymidine, and 2'-deoxycytidine and are bearing ethynyl or octadiynyl side chains at the 7-position of 8-aza-7-deazapurines or the 5-position of pyrimidine nucleosides (purine numbering is used throughout the discussion part).^{2,36–42} They have been incorporated into the 12-mer oligonucleotides 5'-d(TAG GTC AAT ACT) (13) and 3'-d(ATC CAG TTA TGA) (14) which served as reference duplex. For this, phosphoramidites of the monomeric building blocks were synthesized by existing procedures^{2,37,39–43} and oligonucleotides have been prepared using modified as well as standard phosphoramidites (Table 1 and Table S3, Supporting Information).

Additionally, monofunctionalized oligonucleotides (ODN-31 to ODN-38) incorporating nucleosides 8–12 (Figure 3) were prepared. They were obtained by the “stepwise click” reaction from the alkynylated oligonucleotides ODN-18, ODN-19, and ODN-21 to ODN-26 by using an excess of the bis-azide 1¹⁶ (15:1) (Scheme 2, Table 1, and Table S3, Supporting Information). The reaction was performed at room temperature in aqueous solution ($\text{H}_2\text{O}/\text{DMSO}/t\text{-BuOH}$) employing a premixed 1:1 complex of $\text{CuSO}_4\text{--TBTA}$ (tris(benzyltriazoylethyl)amine), TCEP (tris(carboxyethyl)phosphine), and NaHCO_3 leading to the azido oligonucleotides ODN-31 to ODN-38 (Table 1). The use of excess of bis-azide 1 guarantees that only one azido group is employed in the cycloaddition, thereby yielding almost exclusively the monofunctionalized “click” intermediates ODN-31 to ODN-38 with a terminal azido group in the side chain as shown in Figure 3. Compound 10 was newly synthesized (experimental part) while 8² and 9² were reported earlier. Compounds 11 and 12 are solely components of oligonucleotides (Table 1, SI Table S3, and Figure 3).

All oligonucleotides have been characterized by LC-ESI-TOF or MALDI-TOF mass spectrometry (for details, see Supporting Information). Hybridization experiments were performed on the non-cross-linked functionalized oligonucleotides ODN-15 to ODN-18 and ODN-20 to ODN-38, which served as starting materials for cross-linking using “stepwise” or “bis-click” chemistry (Table 1). It will be shown later that particular duplexes can be cross-linked in a chemoselective way. The T_m values, hypochromicities, as well as thermodynamic data of duplexes used for cross-linking experiments are presented in Table 1 (for additional T_m values see Table S3, Supporting Information).

From the T_m values displayed in Table 1 (see also SI Table S3), it was concluded that duplexes incorporating the alkynylated nucleosides 2–7 in one strand at central positions show similar T_m values as the reference duplex, while azido-functionalized nucleosides 8–12 (Figure 3) at the same positions slightly decreased the duplex stability. The destabilizing effect is less pronounced when the monofunctionalized nucleosides are located at a terminal position. Duplexes modified in both strands with alkynylated nucleosides showed increased T_m values. These findings coincide with earlier observations made on side chain modifications in DNA duplexes.^{2,38–41,44}

Cross-Linking Performed on Oligonucleotide Duplexes with a Diversity of Nucleobases Bearing Short and Long Linkers at Proximal and Distant Positions. DNA cross-linking with bis-azides was already reported and was performed on oligonucleotides with identical strands by the “bis-click” reaction and on nonidentical strands by the “stepwise click” protocol. The work was carried out exclusively on DNA single strands.^{1,2} Now, cross-linking was performed on duplex DNA by the “bis-click” reaction and the identity of the compounds was unambiguously confirmed by the “stepwise click” reaction. We assumed that the cross-linking reaction can be controlled by a possible template effect of a preformed duplex and thus resulting in the chemoselective formation of heterodimers, while homodimers are suppressed. Until now, the efficiency of internal cross-linking in duplex DNA employing bis-azides is unexplored. However, it has been shown that template-assisted “click” reactions at the terminus of an oligonucleotide duplex are accelerated compared to single strand reactions.^{25,26} In this case, the “clickable” residues are flexible, and by duplex formation, their local concentration is

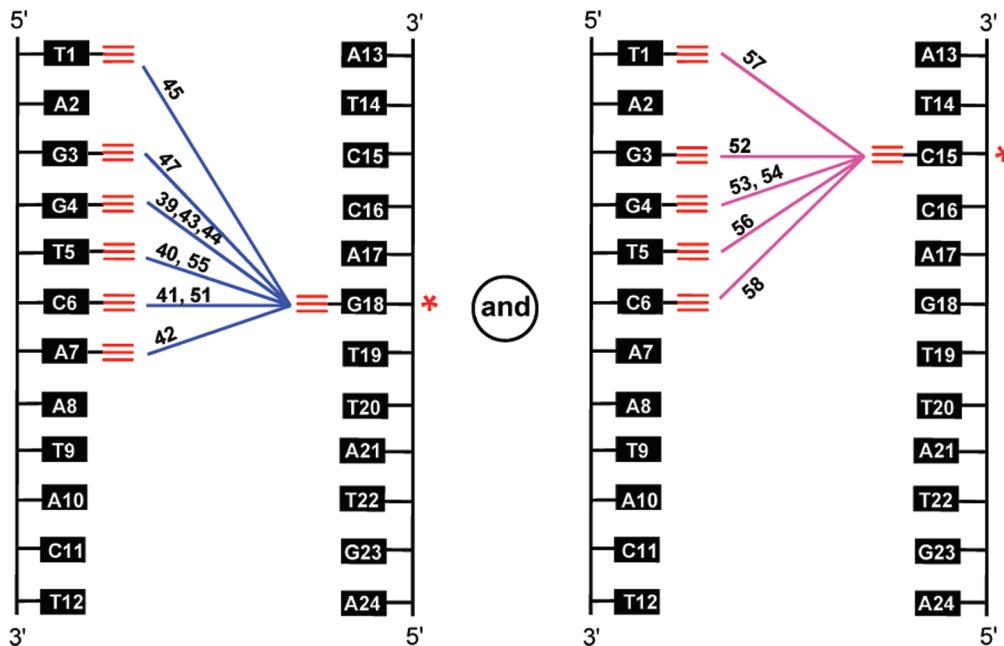


Figure 5. Linking positions of the cross-linked duplexes ICL 39 to 45 (left side) and ICL 52 to 58 (right side) prepared by “bis-click” or “stepwise click” chemistry. The cross-link is located between a central 7-(octa-1,7-diynylated) or 7-ethynylated 8-aza-7-deaza-2'-deoxyguanosine (G18)* or an octa-(1,7-diynylated) dC (C15*) residue in one oligonucleotide chain and a dG, dU, dC, or dA derivative in the complementary strand. The numbers on the arrows represent the individual ICLs.

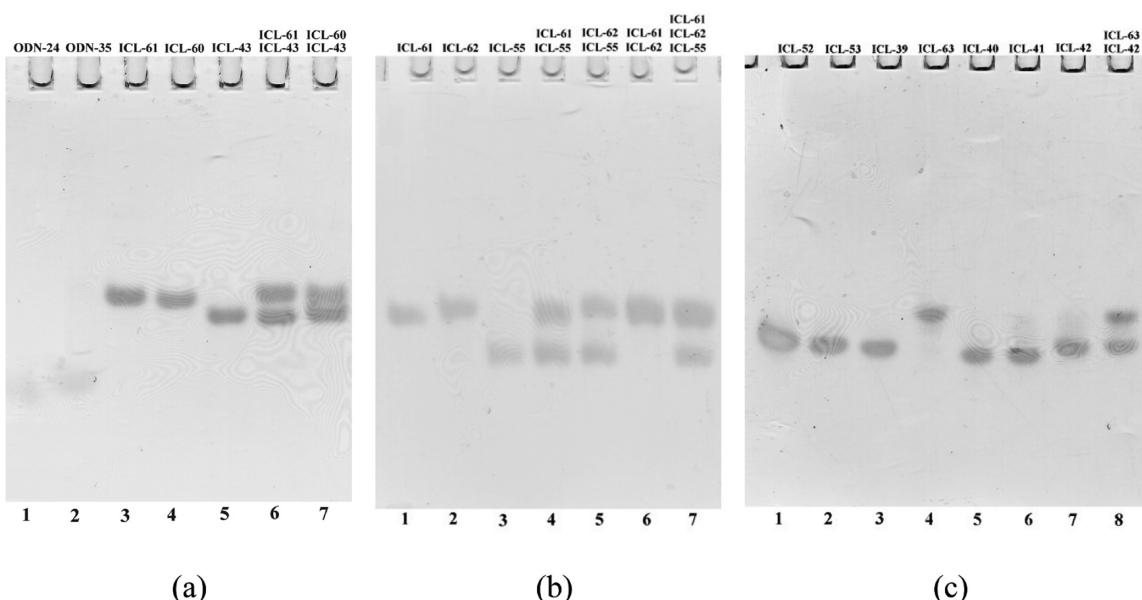


Figure 6. Denaturing PAGE analysis of oligonucleotides on a 17% polyacrylamide/7 M urea gel. (a) Lane 1: 12-mer ODN-24; lane 2: 12-mer ODN-35; lane 3: 24-mer homodimer ICL-61;² lane 4: 24-mer homodimer ICL-60;² lane 5: 24-mer heterodimer ICL-43; lane 6: artificial mixture of ICL-61 + ICL-43; lane 7: artificial mixture of ICL-60 + ICL-43. (b) Lane 1: 24-mer homodimer ICL-61; lane 2: 24-mer homodimer ICL-62; lane 3: 24-mer heterodimer ICL-55; lane 4: artificial mixture of ICL-61 + ICL-55; lane 5: artificial mixture of ICL-62 + ICL-55; lane 6: artificial mixture of ICL-61 + ICL-62; lane 7: artificial mixture of ICL-61 + ICL-62 + ICL-55. (c) Lane 1 → lane 3: 24-mer heterodimers (ICL-52, ICL-53, ICL-39); lane 4: 24-mer homodimer ICL-63;^{1,2} lane 5 → lane 7: 24-mer heterodimers (ICL-40, ICL-41, ICL-42); lane 8: artificial mixture of ICL-63 + ICL-42.

increased. On the contrary, when “click” cross-linking is performed on an internal side chain of the duplex the terminal triple bonds are located in the major groove of DNA duplexes. Consequently, their accessibility and reactivity might be different. A positive template effect can be expected, when the triple bonds are freely accessible by the bis-azide **1**, and the final linker fits perfectly into the gap of the two alkyne functionalities. In order to study the outcome of cross-linking

reactions performed on duplex DNA using “click” chemistry, the nucleobases, the linker length, and the linking positions were altered, and the stability of the various cross-linked duplexes was compared.

Oligonucleotide Cross-Linking Performed on Duplex DNA Using “Bis-Click” Chemistry. Cross-linking of complementary strands by the “bis-click” reaction was performed using DNA duplexes as a tool (“template”). Standard reaction conditions

Scheme 5. Cross-Linking on DNA Duplexes by the “Stepwise Click” Reaction Using the Complementary Oligonucleotides ODN-16 (ethynylated) and azido-ODN-35 as Starting Materials

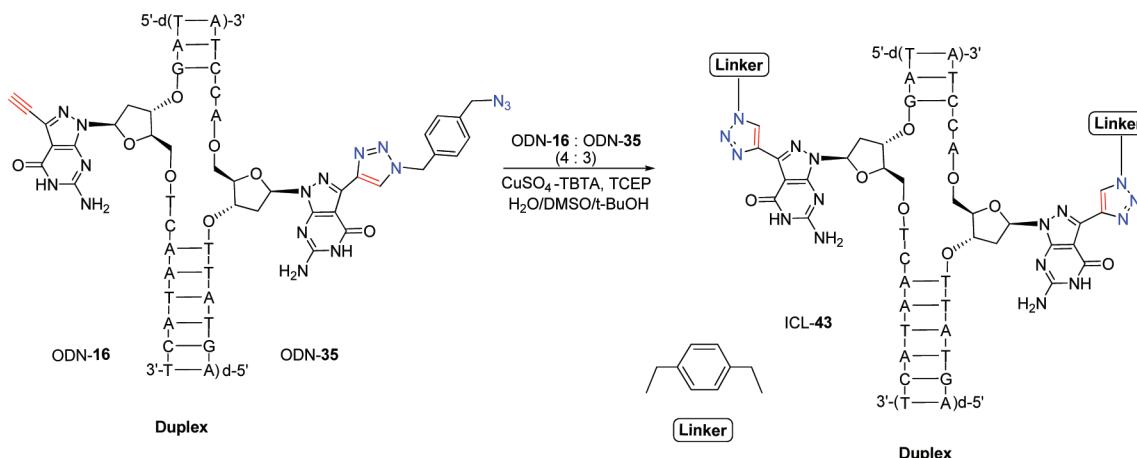


Table 3. Structure of Interstrand Cross-Linked Complementary Oligonucleotides (ICL) Synthesized by “Bis-Click” or “Stepwise Click” Reaction

Duplexes	MS [calc.] found	ICL Duplex	MS [calc.] found
5'-d(TAG G ⁶ C AAT ACT) (22) 3'-d(ATC CA ³ TTA TGA) (25)	[3735.5] ^a 3733.0 ^b (ODN-22)	5'-d(TAG G ⁶ C AAT ACT) (40)* 3'-d(ATC CA ³ TTA TGA) (heterodimer)	[7670.3] ^e 7669.4 ^d
	[3747.6] ^e 3746.9 ^f (ODN-25)	5'-d(TAG G ⁶ C AAT ACT) (62)* 5'-d(TAG G ⁶ C AAT ACT) (homodimer)	[7658.3] ^a 7658.0 ^b
		3'-d(ATC CA ³ TTA TGA) (63) ^{*1,2} 3'-d(ATC CA ³ TTA TGA) (homodimer)	[7681.5] ^c 7685.3 ^d
		5'-d(TAG G ⁶ C AAT ACT) (40) [#] 3'-d(ATC CA ³ TTA TGA) (control heterodimer)	[7670.3] ^e 7670.2 ^f
5'-d(TAG G ¹⁰ C AAT ACT) (32) 3'-d(ATC CA ³ TTA TGA) (25)	[3923.7] ^a 3922.2 ^b (ODN-32)	5'-d(TAG G ⁶ C AAT ACT) (40) [#] 3'-d(ATC CA ³ TTA TGA) (control heterodimer)	[7670.3] ^e 7670.2 ^f
5'-d(TAG 2TC AAT ACT) (16) 3'-d(ATC CA ² TTA TGA) (24)	[3666.7] ^c 3668.6 ^d (ODN-16)	5'-d(TAG 2TC AAT ACT) (43)* 3'-d(ATC CA ² TTA TGA) (heterodimer)	[7521.4] ^c 7524.5 ^d
	[3667.4] ^e 3667.2 ^f (ODN-24)	5'-d(TAG 2TC AAT ACT) (60) ^{*2} 5'-d(TAG 2TC AAT ACT) (homodimer)	[7521.4] ^c 7521.3 ^d
		3'-d(ATC CA ² TTA TGA) (61) ^{*2} 3'-d(ATC CA ² TTA TGA) (homodimer)	[7521.4] ^c 7524.3 ^d
		5'-d(TAG 2TC AAT ACT) (43) [#] 3'-d(ATC CA ² TTA TGA) (control heterodimer)	[7521.4] ^c 7525.5 ^d
5'-d(TAG 2TC AAT ACT) (16) 3'-d(ATC CA ⁸ TTA TGA) (35)	[3855.6] ^e 3855.1 ^f (ODN-35)		

^aCalculated on the basis of molecular weight as [M+1]⁺. ^bDetermined by MALDI-TOF mass spectrometry as [M+1]⁺ in the linear positive mode.

^cCalculated on the basis of exact mass. ^dDetermined by LC-ESI-TOF mass spectrometry. ^eCalculated on the basis of molecular weight as [M-1]⁻.

^fDetermined by MALDI-TOF mass spectrometry as [M-1]⁻ in the linear negative mode. *Refers to interstrand cross-linked oligonucleotides prepared by the “bis-click” reaction. #Refers to interstrand cross-linked oligonucleotides prepared by the “stepwise click” reaction. The particular cross-linked nucleobases, e.g., 2-2, are shown in the Supporting Information Figure S3 and Figure S4.

were used for the “bis-click” reaction except that the solution contains 1 M NaCl (see Experimental Procedures). Under these conditions, stable duplexes were formed in all cases with T_m values 10–15 °C lower than those reported in Table 1, but all above 40 °C (Supporting Information, Figure S19) which guarantees that the “bis-click” reaction occurs on duplex DNA. A schematic illustration of the reaction is outlined in Scheme 3.

For the synthesis of the cross-linked oligonucleotides, equimolar amounts of two complementary oligonucleotide strands were dissolved in 1 M NaCl solution. Then, CuSO₄-TBTA complex in H₂O/DMSO/t-BuOH was added, followed by TCEP, aq. NaHCO₃, the bis-azide 1, as well as DMSO (Scheme 4). As a typical example, the cross-linking reaction of two complementary oligonucleotide strands (ODN-16 and

Table 4. T_m Values of Interstrand Cross-Linked Oligonucleotide Duplexes Prepared by the “Bis-Click” or “Stepwise Click” Reaction

Cross-linked Duplexes	T_m^a [°C]	ΔT_m^b [°C]	% h ^c	Cross-linked Duplexes	T_m^a [°C]	ΔT_m^b [°C]	% h ^c	Cross-linked Duplexes	T_m^a [°C]	ΔT_m^b [°C]	% h ^c
5'-d(TAG ² TC AAT ACT) (43)*#	> 80.0 (>80.0)	> 29 > 29	- -	5'-d(TA ² G TC AAT ACT) (47)*	> 80.0	> 29	-	5'-d(TAG GT ⁷ AAT ACT) (51)*	> 80.0	> 29	-
3'-d(ATC CA ² TTA TGA)	56.0*	+5	11	3'-d(ATC CA ² TTA TGA)				3'-d(ATC CA ² TTA TGA)			
5'-d(TAG ² TC AAT ACT) (44)*#	75.5 (75.5)	+24.5 +24.5	19 18	5'-d(TAG ³ TC AAT ACT) (39)*#	75.5 (75.0)	+24.5 +26	18 19	5'-d(TAG G ⁶ C AAT ACT) (40)*#	75.5 (75.0)	+24.5 +26	17 17
3'-d(ATC CA ³ TTA TGA)	50.0*	-1.0	19	3'-d(ATC CA ³ TTA TGA)				3'-d(ATC CA ³ TTA TGA)			
5'-d(TAG ² TC AAT ACT) (53)*	74.0	+23	17	5'-d(TA ² GTC AAT ACT) (52)*	73.0	+22	16	5'-d(TAG GT ⁷ AAT ACT) (41)*#	73.5 (73.5)	+22.5 +24.5	17 17
3'-d(AT ⁷ CAG TTA TGA)				3'-d(AT ⁷ CAG TTA TGA)				3'-d(ATC CA ³ TTA TGA)			
5'-d(TAG G ⁶ C AAT ACT) (55)*	73.0	+22	16	5'-d(6AG GTC AAT ACT) (45)*#	72.5	+21.5	16	5'-d(TAG ³ TC AAT ACT) (54)*	72.0	+21	16
3'-d(ATC CA ² TTA TGA)				3'-d(ATC CA ² TTA TGA)				3'-d(AT ⁷ CAG TTA TGA)			
5'-d(6AG GTC AAT ACT) (57)*	75.5	+24.5	15	5'-d(5AG GTC AAT ACT) (46)*#	76.5 (76.5)	+25.5 +27.5	19 19				
3'-d(AT ⁷ CAG TTA TGA)				3'-d(A ⁶ C CAG TTA TGA)							
5'-d(TAG ² TC AAT ACT) (49)*	76.5	+25.5	14	5'-d(TAG ² TC AAT ACT) (50)*	75.0	+24	17				
3'-d(T ₆ ATCCA ³ TTATGA T ₆)				3'-d(A ₆ ATCCA ³ TTATGAT ₆)							
5'-d(TAG GT ⁷ AAT ACT) (59)*	67.0	+16	16	5'-d(TAG G ⁶ C AAT ACT) (56)*	68.0	+17	16	5'-d(TAG G T ⁷ AAT ACT) (58)*	62.5	+11.5	15
3'-d(ATC CA ² TTA TGA)				3'-d(AT ⁷ CAG TTA TGA)				3'-d(AT ⁷ CAG TTA TGA)			
5'-d(TAG GTC ⁴ AT ACT) (42)*#	68.5 (68.5)	+17.5 +19.5	16 15					5'-d(TAG GTC ² AT ACT) (48)*	61.0	+10	16
3'-d(ATC CA ³ TTA TGA)								3'-d(ATC CA ² TTA TGA)			

^aMeasured at 260 nm in a 1 M NaCl solution containing 100 mM MgCl₂ and 60 mM Na cacodylate (pH 7.0) with 5 μ M cross-linked DNA concentration. Data in parentheses were measured with 2 μ M cross-linked DNA concentration. T_m values marked with an asterisk were measured in 1:1 (v/v) mixture of the above salt buffer and formamide with the identical ICL duplex concentration (5 μ M). ^b ΔT_m refers to the increase/decrease of the T_m value compared to the reference duplex 13•14 (see Table 1). ^c% h refers to percentage hypochromicity. *Refers to interstrand cross-linked oligonucleotides prepared by the “bis-click” reaction. #Refers to interstrand cross-linked oligonucleotides prepared by the “stepwise click” reaction. The particular cross-linked nucleobases, e.g., 2-2, are shown in the Supporting Information Figure S3 and Figure S4.

ODN-24) both bearing a 7-ethynyl-7-deaza-8-azapurine residue is depicted in Scheme 4.

The reaction proceeded to completion in 12 h, and the cross-linked oligonucleotides were purified by reversed-phase HPLC. This protocol was applied to cross-link a whole series of oligonucleotides. The interstrand cross-linked duplexes obtained by this method are summarized in Table 2. The reaction yield was in the range 40–50% yield; in a typical example, 4 OD of starting material gave 2 OD of cross-linked duplex. The products were characterized by LC-ESI-TOF or MALDI-TOF mass spectrometry, ion-exchange HPLC, as well as gel-electrophoresis (Figures 4 and 6 and Supporting Information).

Comparing the “bis-click” reaction performed earlier on identical single strands and the cross-linking of complementary strands in duplex DNA described now, we found that the reaction is significantly faster for single-stranded DNA than for duplex DNA. Steric reasons might account for these differences. Compared to DNA single strands, duplex DNA is compact and inflexible, so that the cross-linker has to overcome these limitations, when the cross-link is introduced in an internal stable region of the duplex (central position).

Analytical Characterization of Heterodimers and Homodimers. Theoretically, cross-linking of complementary oligonucleotide strands by the “bis-click” reaction can lead to a mixture of three different compounds. One species is the desired heterodimer connecting two complementary strands (duplex); the others are homodimers composed from identical single strands. The statistic ratio of heterodimer to homodimer formation in the crude reaction mixture would be 50% heterodimer to 25% of each homodimer. However, by comparing the reversed-phase HPLC profiles (Figure 4a,b) of the crude reaction

mixtures it is obvious that the dominating product is the heterodimer and the corresponding homodimers are suppressed. The formation of homodimers might occur with remaining free single strands in solution or by reactions among two duplexes in close proximity. For example, almost exclusive formation of cross-linked heterodimers was observed for the cross-linked ICL-43 as well as for the duplexes ICL-44 and ICL-49 (Figure 4a and Figures S7b and S8a, Supporting Information).

In other cases, small amounts of side products were observed in the reaction mixture, which were identified as homodimers. These observations were exemplarily confirmed by ion-exchange HPLC and gel electrophoresis (Figures 4 and 6). Following the approach of “bis-click” cross-linking on duplexes, we were able to cross-link duplexes using different connectivities which varied in linker length, position in the duplex, and type of nucleobase. Figure 5 displays the various connectivities formed by the “click” cross-linking with G18* (8-aza-7-deazapurine analogue) or C15* (cytosine analogue) as central anchoring sites. The cross-link is formed between an 7-(octa-1,7-diynylated) or 7-ethynylated 8-aza-7-deaza-2'-deoxyguanosine (G18)*, as well as an octa-(1,7-diynylated) dC (C15*) residue in one oligonucleotide chain and a dG, dU, dC, or dA derivative in the complementary strand.

In order to confirm the structure of heterodimers prepared by the “bis-click” protocol on duplexes, they were also synthesized by the “stepwise click” reaction. For this, corresponding oligonucleotides shown in Table 1 were used. The “stepwise click” reaction carried out to form heterodimers (complementary sequences) differs from that performed to generate homodimers. As oligonucleotides are now complementary, they form duplexes in the reaction medium which was

Table 5. T_m Values of Interstrand Cross-Linked Oligonucleotides ICL-60 to ICL-65 (Homodimers)

Duplexes	T_m^a [°C]	ΔT_m^b [°C]	MS [calc.] found	Duplexes	T_m^a [°C]	ΔT_m^b [°C]	MS [calc.] found
5'-d(TAG GTC AAT ACT) (13)	51.0	-		3'-d(ATC CAG TTA TGA) (14)	(49.0)	-	
3'-d(ATC CAG TTA TGA) (14)				3'-d(ATC CAG TTA TGA) (14)			
5'-d(TAG 2 TC AAT ACT) 5'-d(TAG 2 TC AAT ACT)	41.0 (39.0)	-10 -10	[7521.4] ^e 7521.3 ^f	5'-d(ATC GT 7 AAT ACT) 5'-d(ATC GT 7 AAT ACT)	46.0 (43.5)	-5.0 -5.5	[7683.3] ^c 7688.0 ^d
3'-d(ATC CAG TTA TGA) (14)				3'-d(ATC CAG TTA TGA) (14)			
5'-d(TAG GTC AAT ACT) (13)				5'-d(TAG GTC AAT ACT) (13)			
3'-d(ATC CA 2 TTA TGA) 3'-d(ATC CA 2 TTA TGA)	34.0 (31.0)	-17 -18	[7521.4] ^e 7524.3 ^f	3'-d(ATC 7 AG TTA TGA) 3'-d(ATC 7 AG TTA TGA)	48.5 (45.5)	-2.5 -3.5	[7683.3] ^c 7688.0 ^d
5'-d(ATC CA 2 TTA TGA)				5'-d(TAG GTC AAT ACT) (13)			
5'-d(TAG GTC AAT ACT) (13)							
3'-d(ATC CA 3 TTA TGA) 3'-d(ATC CA 3 TTA TGA)	45.0 (43.5)	-6.0 -5.5	[7681.5] ^e 7685.3 ^f	3'-d(ATC CAG TTA TGA) (14) 5'-d(TAG G 6 C AAT ACT)	46.5 (45.0)	-4.5 -4.0	[7658.3] ^c 7658.0 ^d
5'-d(ATC CA 3 TTA TGA)				5'-d(TAG G 6 C AAT ACT)			
5'-d(TAG GTC AAT ACT) (13)				3'-d(ATC CAG TTA TGA) (14)			
5'-d(TAG 2 TC AAT ACT) 3'-d(ATC CA 2 TTA TGA) 3'-d(ATC CA 2 TTA TGA)				5'-d(TAGG 6 C AAT ACT) 3'-d(ATC C 1 A 2 TTA TGA) 3'-d(ATC C 1 A 2 TTA TGA)	49.0	-2.0	
5'-d(TAG 2 TC AAT ACT)				5'-d(TAGG 6 C AAT ACT)			
3'-d(ATC CA 3 TTA TGA) 3'-d(ATC CA 3 TTA TGA)	56.0	+5.0		3'-d(ATC C 1 A 3 TTA TGA) 3'-d(ATC C 1 A 3 TTA TGA)	62.0	+11.0	
5'-d(TAG 2 TC AAT ACT)				5'-d(TAGG 6 C AAT ACT)			
3'-d(ATC CA 3 TTA TGA) 3'-d(ATC CA 3 TTA TGA)	57.0 (56.0)	+6.0 +7.0					
5'-d(TAG 2 TC AAT ACT)							
5'-d(TAG GT 7 AAT ACT) 3'-d(ATC 7 A TTA TGA) 3'-d(ATC 7 A TTA TGA)	47.0 (45.5)	-4.0 -3.5					
5'-d(TAG GT 7 AAT ACT)							

^aMeasured at 260 nm in a 1 M NaCl solution containing 100 mM MgCl₂ and 60 mM Na cacodylate (pH 7.0) with 2.5 μ M of cross-linked DNA and 5 μ M of the single-stranded complementary oligonucleotides. Data in parentheses were measured at a 1 μ M of cross-linked DNA and 2 μ M of the single-stranded complementary oligonucleotides. ^b ΔT_m refers to the increase/decrease of the T_m value compared to the reference duplex **13**•**14** (see Table 1). ^cCalculated on the basis of molecular weight as [M+1]⁺. ^dDetermined by MALDI-TOF mass spectrometry as [M+1]⁺ in the linear positive mode. ^eCalculated on the basis of exact mass. ^fDetermined by LC-ESI-TOF mass spectrometry. The particular cross-linked nucleobases, e.g., **2-2**, are shown in the Supporting Information Figure S3 and Figure S4.

proven by T_m measurements. For example, for ODN-16 and ODN-35 duplex melting was observed under the conditions of the “click” reaction. This does not interfere with the exclusive formation of heterodimers but increases the reaction time significantly. A typical example for the “stepwise click” reaction is shown in Scheme 5. Similar to the “bis-click” reaction the cross-link is formed internally within a DNA duplex (\rightarrow ICL-43, Scheme 5). The cross-linked duplexes ICL-39 to ICL-46 were synthesized as proofs according to the “stepwise click” protocol (Table 2).²

A central feature for the identification of heterodimers and homodimers results from their unambiguous characterization. In principle, two methods were used to identify the various cross-linked oligonucleotides, mass spectrometry and gel-electrophoresis. Mass spectroscopy was used in cases when the masses of homodimers and heterodimers are different. This is exemplified for selected compounds shown in Table 3. A complementary cross-linked duplex (ICL-40*, heterodimer) is formed from ODN-22 and ODN-25 as well as the cross-linked homodimers ICL-62 and ICL-63. The heterodimer ICL-40* and the two homodimers have different molecular masses. Thus, they can be distinguished. As a control, the heterodimer was also synthesized by the “stepwise click” reaction. On the contrary, in the second example homodimers ICL-60 and

ICL-61 and heterodimer ICL-43* cannot be distinguished due to identical masses.

Unexpectedly, we found that cross-linked homodimers and heterodimers of identical molecular weight can be easily distinguished by denaturing polyacrylamide gel electrophoresis (PAGE) as depicted in Figure 6a–c (for further examples and experimental details, see Supporting Information as well as Experimental Procedures). Cross-linked heterodimers migrate significantly more rapidly than cross-linked homodimers. Although it is not possible to distinguish between two different homodimers, the desired heterodimers can be nicely characterized and/or separated by this method. A final proof results from the comparison of T_m values of cross-linked homomeric and heteromeric duplexes, which will be discussed in the next section.

Stability of Cross-Linked Duplexes. Next, the influence of cross-links on the duplex stability was investigated. For this, T_m values of cross-linked duplexes were measured and were compared to those of nonligated duplexes. T_m values marked with an asterisk were measured in a 1:1 (v/v) mixture of 1 M NaCl buffer (pH 7.0)/formamide mixture, when the T_m value was too high. When canonical nucleosides were replaced by those providing the linker, the Watson–Crick base pairing was always kept intact, indicated by the T_m values of Table 1

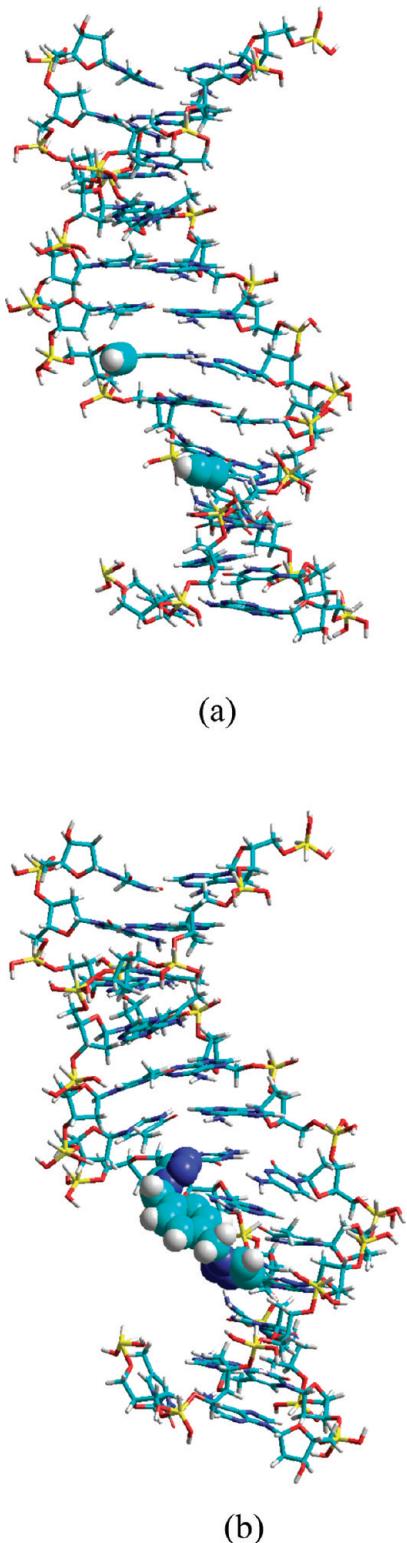


Figure 7. Comparison of cross-linked and non-cross-linked duplexes. Molecular models of (a) duplex 16•24 and (b) ICL-43. The modification sites are presented as colored space-filling overlapping spheres. The models were constructed using *Hyperchem* 7.0/8.0 and energy-minimized by using *AMBER* calculations.

(Table S3, Supporting Information). Hypochromicities (% h) are similar for cross-linked and non-cross-linked duplexes.

It can be seen that the T_m values of the cross-linked duplexes are significantly (20–29 °C) increased compared to those of the

non-cross-linked ones. This results from the different stoichiometries of duplex melting. From the identical number of base pairs, one can expect that all cross-linked duplexes should show similar T_m values. However, the cross-link generates steric stress on the duplex, which depends on the linker length and the linking position. These phenomena are reflected by the T_m changes shown in Table 4. Regarding duplex stability, the cross-linked oligonucleotides can be divided into three groups displaying the following various influences by the cross-links.

(1) The highest duplex stabilization ($T_m > 80$ °C) is obtained when the ethynylated compound 2 was cross-linked with another compound 2 residue (ICL-43 and ICL-47) or with the 5-octa-(1,7-diynylated)-dC (7) (ICL-51). The latter consists of a modified dG-dC base pair which is cross-linked and stabilized. In these cases, the duplex structure of the cross-linked species does not seem to be significantly perturbed and the cross-link is nicely integrated in the duplex structure. For these duplexes, the cross-linked nucleobases are in close proximity separated by at least one base pair.

(2) Most of the duplexes are represented by the second group. They are covered by cross-links which are different in linker length, type of alkyne, nucleobase, and position in the duplex. The T_m values for these duplexes are all in the range 72–76 °C. A partial disruption of base pairs is most likely induced by a cross-link not having the ideal spatial demands to be well accommodated in the DNA duplex.

(3) The duplexes with the lowest T_m values (62–68 °C) are represented by the third group. Here, a certain disorder of the duplex is present, in which at least one or more base pairs are disturbed. This is proved by comparison of ICL-58 ($T_m = 61$ °C) with ICL-48 ($T_m = 61$ °C) bearing a 2-dT mismatch, both showing similar duplex stability.

Contrary to the heterodimers, homodimers with identical strands do not form duplexes. Nevertheless, duplexes can be generated when they are hybridized with individual single strands complementary to the homodimers. Examples for cross-linked homodimers with two complementary strands are shown in Table 5. The duplex stabilization of heterodimeric cross-linked duplexes was significantly higher as observed for cross-linked heterodimeric duplexes when paired with complementary strands. This is also true when double duplexes of homodimers were generated by introducing two cross-links between two helices (Table 5).

CONCLUSION AND OUTLOOK

A diversity of complementary oligonucleotides incorporating alkynylated 8-aza-7-deazapurine or corresponding 5-alkynylated pyrimidine nucleosides (2–7) which are related to the four canonical DNA bases were synthesized. The “bis-click” reaction was performed on oligonucleotide duplexes with the bis-azide 1 which resulted in the chemoselective formation of interstrand cross-linked DNA. Preformed DNA duplexes served as a tool (“template”) for the selective generation of heterodimers, while homodimer formation was suppressed. The chemoselective formation of heterodimers is likely when the linker length and the linking positions are optimal as shown during the formation of cross-linked DNA ICL-43. The formation of homodimers increases under nonoptimal conditions. Internal “bis-click” cross-linking was always slower on duplex DNA compared to reactions performed on noncomplementary strands due to the rigid duplex structure. This is different from cross-linking reactions performed at the terminus of a duplex.^{25–27} Due to

the higher flexibility of the strands, these ligation reactions are accelerated.

Studies on interstrand cross-linked heterodimeric duplexes revealed significantly increased T_m values compared to non-cross-linked congeners. It was shown that the increase of duplex stability depends on the nucleobase, the linker length, and the linking positions. As an example, molecular dynamic simulations using Amber MM+ force field (*Hyperchem* 7.0/8.0; Hypercube Inc., Gainesville, FL, USA, 2001) was performed for the duplex **16•24** and the resulting interstrand cross-linked heterodimer ICL-43 (Figure 7). Obviously, the DNA duplex structure is not perturbed and the cross-link seems to be well accommodated in the major groove of the DNA duplex. This corresponds to the high T_m value of the duplex, while others are more obstructed resulting in lower T_m values. Consequently, the interstrand cross-linking reaction using “bis-click” chemistry is applicable for duplex DNA and can be exerted to the generation of very stable cross-linked DNA. We anticipate that this internal cross-linking protocol will find application in chemical biology and the construction of nanoassemblies.

■ ASSOCIATED CONTENT

S Supporting Information

Additional T_m values of alkynylated and azido-functionalized DNA duplexes, structures, and analytical data of cross-linked duplexes, reversed-phase HPLC profiles of cross-linked and non-cross-linked oligonucleotides, ion-exchange HPLC profiles of cross-linked and non-cross-linked oligonucleotides, denaturing PAGE analysis of oligonucleotides, melting profiles, MALDI-TOF and LC-ESI-TOF data of oligonucleotides. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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