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Cross-Linked DNA Generated by "Bis-click" Reactions with Bis-functional Azides: Site Independent Ligation of Oligonucleotides via Nucleobase Alkynyl Chains

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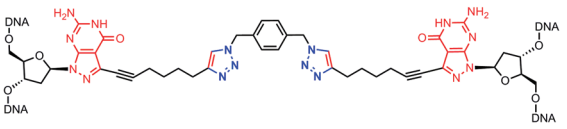

Template-free cross-linking of single-stranded DNA bearing octadiynyl side chains at the 7-position of 8-aza-7-deazapurine moieties with bisfunctional azides is reported employing a Cu(I)-catalyzed azide−alkyne "bis-click" reaction. Bis-adducts were formed when the bis-azide:oligonucleotide ratio was 1:1; monofunctionalization occurred when the ratio was 15:1. Four-stranded DNA consisting of two cross-linked duplexes was obtained after hydridization. Cross-linked duplexes are as stable as individual duplexes when ligation was introduced at terminal positions; ligation at a central position led to a slight duplex destabilization.

The copper-catalyzed azide−alkyne Huisgen−Meldal−Sharpless cycloaddition "click" reaction (CuAAC)[1−3] has been applied to cross-link DNA strands. Consequently, azido groups as well as terminal triple bonds were introduced at the termini of oligonucleotides and the "click" reaction was induced with copper(I) salts.[4] Even though the azides were introduced via phosphonate chemistry[5a] or by solid phase,[5b] the scope of azide introduction is very limited and postmodification of DNA is required, which restricts the click reaction mainly to the terminal positions of an oligonucleotide chain. This drawback ultimately led us to explore another possible methodology of cross-linking DNA strands.

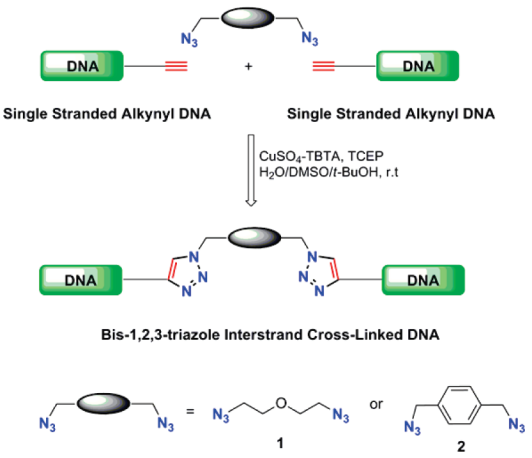

FIGURE 1. Schematic representation of Cu(I)-catalyzed interstrand cross-linking of DNA by the "bis-click" reaction.

As we wanted to be free in the selection of the cross-link positions−terminally or internally−we selected DNA strands which were modified by side chains with terminal triple bonds. These side chains can be adjusted in length and can be introduced at any position of DNA by using corresponding phosphoramidites.[6−9]

In this note, we report for the first time on the single-step, template-free internal interstrand cross-linking of DNA employing a "bis-click" protocol. Through this procedure, two identical strands can be linked together by using bis-functional azides **1** or **2** (Figure 1). The bis-azides were prepared as described in the literature.[10] A stepwise procedure was also developed, where in the first step only one azido group was reacted to give a triazole monofunctionalized oligonucleotide conjugate bearing still another reactive azido group. Consequently, this intermediate has the potential to be cross-linked in a second step with another strand of any type of DNA bearing an alkynyl group. Our protocol offers the freedom to synthesize both identical as well as nonidentical cross-linked oligonucleotides. The triazole monofunctionalization requires a large molar excess of the bis-azide (15:1), while

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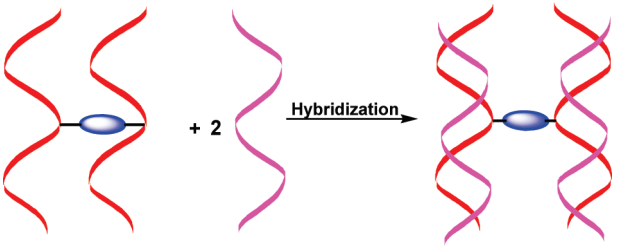
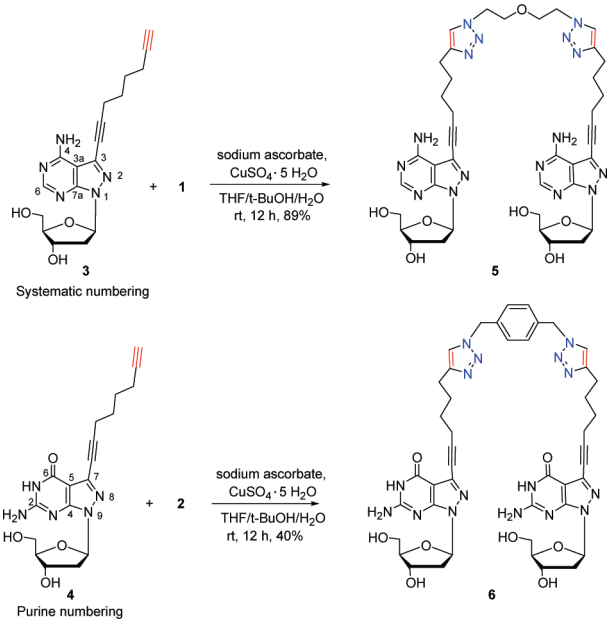


FIGURE 2. Schematic representation to generate four-stranded DNA consisting of two cross-linked double helices by hybridization with two complementary chains. Red and pink chains are complementary.

SCHEME 1. Cu(I)-Catalyzed "Bis-click" Reaction Performed with Nucleosides 3 or 4

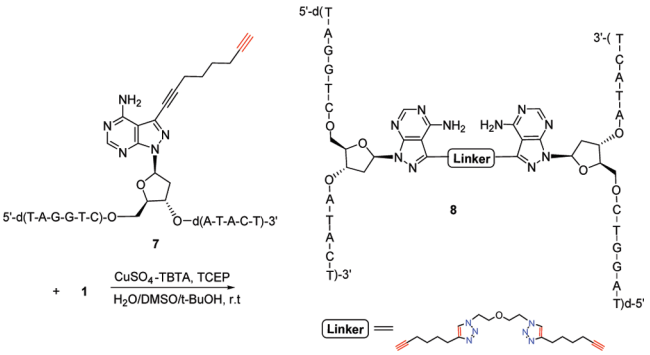


cross-linked oligonucleotides are formed almost exclusively in one step, when the molar excess of azide is small (1:1). Through this "bis-click" methodology, we cross-linked DNA at a central as well as at a terminal position with high efficacy (Figure 1). Cross-linked oligonucleotides with identical chains can be hybridized with complementary strands to form four-stranded DNA consisting of two duplexes ligated by a linker unit (Figure 2).

This cross-linking protocol works well on both the nucleoside and the oligonucleotide level. For this purpose, 8-aza-7-deazapurine 2′-deoxyribonucleosides related to dA and dG (**3** and **4**) were chosen. These alkynyl nucleosides were synthesized by *Sonogashira* cross-coupling according to the literature.[6,7] Then the CuAAC click reaction was performed on both nucleosides **3** and **4** with respective azides[10] to give the adducts **5** and **6**, respectively (Scheme 1). The reaction of **3** and **4** was performed with the respective azides in THF/*t*-BuOH/H_2O, which led to the expected products. Nucleosides were characterized by ^{1}H and ^{13}C NMR spectra. ^{13}C NMR chemical shift assignments were performed on the basis of ^{1}H−^{13}C gated decoupled and DEPT-135 spectra.

Next, the "bis-click" reaction was performed on the oligonucleotide level (Scheme 2). The reaction route is outlined in

SCHEME 2. Interstrand Cross-Linking of Oligonucleotides by Cu(I)-Catalyzed "Bis-click" Reaction



Scheme 2. For this, oligonucleotides with terminal triple bonds were prepared with regular and modified phosphoramidites.[6−9] The "bis-click" reaction was carried out at room temperature in an aqueous solution containing *t*-BuOH and DMSO to yield the cross-linked oligonucleotide **8**, which was isolated by reversed phase HPLC. Although the reaction is template-free, it proceeds smoothly and almost quantitatively to yield **8** when the bis-azide:oligonucleotide ratio was 1:1 (corresponding to 2 equiv of the respective azido groups of the bis-azide to 1 equiv of oligonucleotide). Along with the expected cross-linked oligonucleotide, a small amount (9:1) of the monofunctionalized oligonucleotide still carrying one azido group was formed. In contrast, this monofunctionalized oligonucleotide becomes the major product when the azide ratio was 15:1. On the other hand, Scheme 3 outlines the stepwise "bis-click" reaction performed with oligonucleotide **9**. At first, the triazole monofunctionalized conjugate **10** was isolated. In a second step, the intermediate **10** was further converted to the cross-linked oligonucleotide **11**. However, as found for conversion of **7** → **8**, the same "bis-click" adduct **11** was isolated when the azide ratio was 1:1. The molecular masses obtained by ESI-TOF mass spectrometery were used to identify the adducts and conjugates. For further characterization data, see the Supporting Information.

Analytical ion-exchange chromatography (4 × 250 mm DNA PAC PA-100 column) outlines the particular properties of the cross-linked adducts **8** and **17** compared to the corresponding non-cross-linked oligonucleotides **7** and **15**, respectively. Ion-exchange chromatography separates molecules according to their negative phosphodiester charges. The dimeric character of the cross-linked oligonucleotides shown in the mobility shift analysis relates to 6−7 min longer retention times of the cross-linked oligonucleotides **8** and **17** compared to the non-cross-linked single strands **7** and **15**, respectively (Figure 3). Furthermore, the retention times of cross-linked oligonucleotide **8** and **17** are very similar to that of the duplex **12**·**13** illustrating that the number of charges of the cross-linked oligonucleotides is twice as high as that of the non-cross-linked oligonucleotides (data not shown).[11]

Generally, interstrand cross-links have both positive and negative effects on the stability of a cross-linked duplex.[12−17] Thus, to assess the influence of interstrand cross-links on

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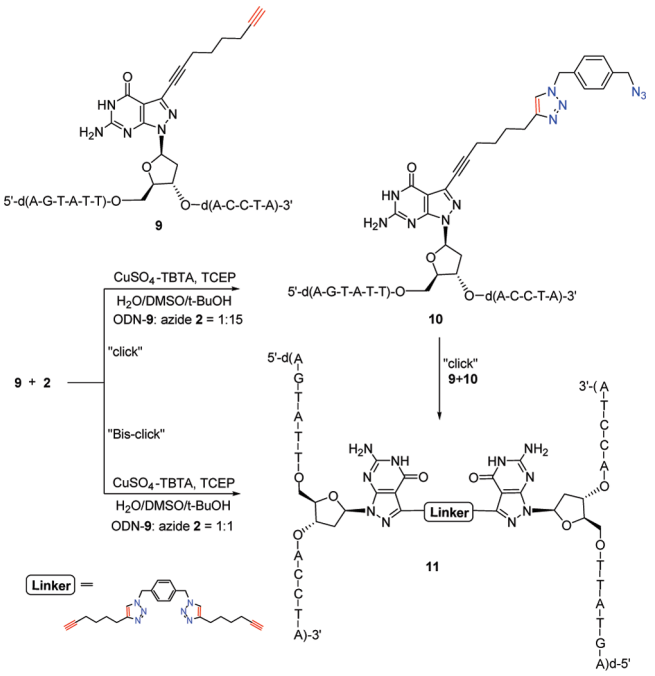
SCHEME 3. Interstrand Cross-Linking of Oligonucleotide 9 by Bis-click and Stepwise Click Reactions



duplex stability, cross-linked oligonucleotides of two identical strands were hybridized with complementary chains and T_m measurements were performed. According to Table 1, the two cross-linked duplexes **12·16·12** and **12·17·12** showed a very similar thermal stability compared to individual, non-cross-linked duplexes (**12·14** and **12·15**). When the cross-linking position is situated at the center of the duplex (**13·8·13** and **12·11·12**) it results in a 4 to 6 °C decrease of T_m values most likely induced by steric crowding. This affects base pair stability at central positions more than that at terminal ones.

In conclusion, we developed a simple and efficient, template-free ligation method for interstrand cross-linking of DNA, through CuAAC "bis-click" reaction, independent of base composition and sequence. It is applicable to all DNAs or RNAs bearing alkynyl chains. This includes purines, pyrimidines, or other nucleoside residues and all building blocks of DNA and RNA.[6−9] DNA duplex structure was disturbed significantly neither by alkynyl modification nor by cross-linking, as the side chains were introduced at the 7-position of 8-aza-7-deazapurines, which gave the residues steric freedom similar to those of 5-modified pyrimidines or 7-substituted 7-deazapurines.[6−9] The evolved methodology is applicable to any single stranded DNA as well to duplexes and multi-stranded structures.

Cross-linked single strands or cross-linked duplexes of this type may find application in nanobiotechnology and material science. The method can be extended for creating novel self-assembled DNA nanostructures in solution or on surfaces; new connectivities can be introduced in scaffolded-DNA-origamis.[18] As we were also able to generate monofunctionalized conjugates (**10**) from bis-functional azides, interstrand cross-links can be generated between nonidentical chains including those having complementary sequences. This stepwise route which is under current investigation will widen the application of bifunctional reagents in chemical biology of DNA as it was reported for other bifunctional reagents such as psoralen,[19] mitomycin,[20] nitrogen mustards, nitrosoureas, or platinum

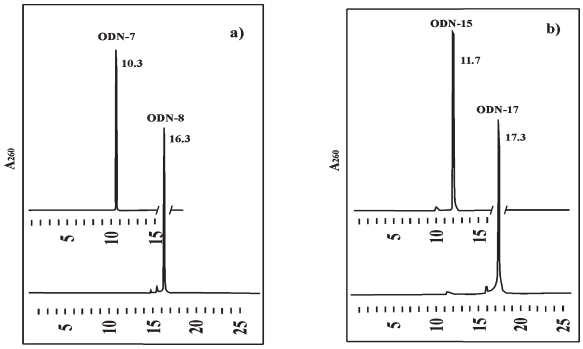


FIGURE 3. Ion-exchange HPLC elution profiles of (a) oligonucleotide **7** and interstrand cross-linked **8** and (b) oligonucleotide **15** and interstrand cross-linked **17** on a 4 × 250 mm DNA Pac PA-100 column, using the following buffer system: (A) 25 mM Tris-HCl, 10% MeCN, pH 7.0; (B) 25 mM Tris-HCl, 1.0 M NaCl, and 10% MeCN, pH 7.0. Elution gradient: 0−30 min 20−80% B in A with a flow rate of 0.75 mL min^{-1}.

TABLE 1. T_m Values of Duplexes and Cross-Linked Duplexes[a]

Duplexes	T_m [°C]	Duplexes		T_m [°C]
5'-d(TAG GTC AAT ACT) **12** 3'-d(ATC CAG TTA TGA) **13**	51			
5'-d(TAG GTC **3**AT ACT) **7** 3'-d(ATC CAG TTA TGA) **13**	51	3'-d(ATC CAG TTA TGA) **13** 5'-d(TAG GTC AAT ACT) | 5'-d(TAG GTC AAT ACT) 3'-d(ATC CAG TTA TGA) **13**	**8**	47
5'-d(TAG GTC AAT ACT) **12** 3'-d(ATC CAG TTA TG**3**) **14**	52	5'-d(TAG GTC AAT ACT) **12** 3'-d(ATC CAG TTA TGA) | 3'-d(ATC CAG TTA TGA) 5'-d(TAG GTC AAT ACT) **12**	**16**	52
5'-d(TAG GTC AAT ACT) **12** 3'-d(ATC CA**4** TTA TGA) **9**	51	5'-d(TAG GTC AAT ACT) **12** 3'-d(ATC CAG TTA TGA) | 3'-d(ATC CAG TTA TGA) 5'-d(TAG GTC AAT ACT) **12**	**11**	45
5'-d(TAG GTC AAT ACT) **12** 3'-d(ATC CAG TTA T**4**A) **15**	53	5'-d(TAG GTC AAT ACT) **12** 3'-d(ATC CAG TTA TGA) | 3'-d(ATC CAG TTA TGA) 5'-d(TAG GTC AAT ACT) **12**	**17**	52

[a]Measured at 260 nm in 1 M NaCl, 100 mM $MgCl_2$, and 60 mM Na-cacodylate (pH 7.0). **A**−**A** corresponds to **5** and **G**−**G** to **6**.

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compounds which are used to covalently connect nucleobases of DNA.[21]

Experimental Section

Synthesis of Bis-adduct 5 by Cross-Linking of Nucleoside 3 with 1-Azido-2-(2-azidoethoxy)ethane (5). To a solution of compound **3**[6] (213 mg, 0.6 mmol) and **1**[10] (47 mg, 0.3 mmol) in THF−H_2O−*t*-BuOH (3:1:1, 5 mL) was added a freshly prepared 1 M solution of sodium ascorbate (498 μL, 0.5 mmol) in water, followed by the addition of copper(II) sulfate pentahydrate 7.5% in water (401 μL, 0.12 mmol), and the reaction mixture was stirred at room temperature for 12 h. After completion of the reaction (monitored by TLC), the reaction mixture was evaporated, and flash chromatography (FC) (silica gel, column 10 × 3 cm, CH_2Cl_2−MeOH, 85:15) was applied. From the main zone compound **5** (231 mg, 89%) was isolated as a colorless solid. TLC (silica gel, CH_2Cl_2−MeOH, 90:10): R_f 0.3. λ_{max} (MeOH)/nm 248 (ε/dm^3 mol^{-1} cm^{-1} 27 200), 287 (25 300). ^{1}H NMR (DMSO-d_6, 300 MHz): δ 1.63−1.75 (m, 6H, 3 × CH_2), 2.18−2.26 (m, 2H, 2 × C2′−H_α), 2.56−2.66 (m, 6H, 3 × CH_2), 2.72−2.80 (m, 2H, 2 × C2′-H_β), 3.47−3.53 (m, 2H, 2 × C5′-H), 3.74−3.80 (m, 4H, 2 × C5′-H, 2 × C4′-H), 4.38−4.45 (m, 4H, 2 × CH_2), 4.78 (br s, 1H, 2 × C5′−OH), 5.27 (br s, 1H, 2 × C3′−OH), 6.49−6.54 (t, J = 6.3 Hz, 2H, 2 × C1′−H), 7.68 (s, 2H, 2 × triazole-H), 8.00 (br s, 1H, NH_2), 8.21 (s, 2H, 2 × C2−H). Anal. Calcd for $C_{40}H_{50}N_{16}O_7$ (866.9): C, 55.42; H, 5.81; N, 25.85. Found: C, 55.38; H, 5.79; N, 25.71. ESI-TOF calcd for $C_{40}H_{50}N_{16}O_7Na$ (M+Na$^+$) 889.3946; *m*/*z* found 889.3935.

Synthesis of Bis-adduct 6 by Cross-Linking of Nucleoside 4 with 1,4-Bisazidomethylbenzene (6). To a solution of **4**[7] (74.2 mg, 0.2 mmol) and **2**[10] (18.8 mg, 0.1 mmol) in THF−H_2O−*t*-BuOH, 3:1:1, (3 mL) were added a freshly prepared 1 M solution of sodium ascorbate (159 μL, 0.16 mmol) in water and copper(II) sulfate pentahydrate 7.5% in water (128 μL, 0.038 mmol), then the reaction mixture was stirred at room temperature for 12 h. After completion of the reaction (monitored by TLC) the solvent was evaporated, and the remaining residue was loaded on a silica gel column FC (silica gel, column 10 × 3 cm, eluted with CH_2Cl_2/MeOH 95:5 → 85:15). From the main zone compound **6** was obtained as a colorless solid (37 mg, 40%). TLC (CH_2Cl_2/MeOH, 85:15): R_f 0.25. λ_{max} (MeOH)/nm 243 (ε/dm^3 mol^{-1} cm^{-1} 51 200), 280 (sh) (13 100). ^{1}H NMR (DMSO-d_6, 300 MHz): δ 1.54−1.61 (m, 4H, 2 × CH_2), 1.68−1.76 (m, 4H, 2 × CH_2), 2.09−2.17 (m, 2H, 2 × C2′−H_α), 2.44−2.46 (m, 4H, 2 × CH_2), 2.61−2.66 (m, 6H, 2 × CH_2, 2 × C2′−H_β), 3.45−3.49 (m, 2H, 4 × C5′−H), 3.73−3.78 (m, 2H, 2 × C4′−H), 4.34 (s, 2H, 2 × C3′−H), 4.74 (s, 2H, 2 × C5′−OH), 5.23 (s, 2H, 2 × C3′−OH), 5.51 (s, 4H, 2 × NCH_2), 6.24−6.29 (t, J = 6.3 Hz, 2H, 2 × H−C1′), 6.74 (br s, 4H, 2 × H_2N), 7.26 (s, 4H, arom. H), 7.92 (s, 2H, 2 × triazole−H), 10.69 (s, 2H, 2 × HN). ESI-TOF calcd for $C_{44}H_{50}N_{16}O_8Na$ (M + Na$^+$) 953.3895; *m*/*z* found 953.3908.

General Procedure for Cross-Linking Oligonucleotides. To the solution of oligonucleotide **9** (5.0 A_{260} units, 50 μmol) in 20 μL of water were added a mixture of a $CuSO_4$−TBTA ligand complex (50 μL, 1 mmol; from stock solution I; 50 μL; from stock solution II), tris(carboxyethyl)phosphine (TCEP; 50 μL, 1 mmol; from stock solution III), sodium bicarbonate (50 μL, 1 mmol; from stock solution IV), the bis-azide **2** (2.5 μL, 50 μmol; from stock solution V), and 30 μL of DMSO, then the reaction mixture was stirred at room temperature for 12 h. The reaction mixture was concentrated in a SpeedVac and dissolved in 1 mL of bidistilled water and centrifuged for 20 min at 12 000 rpm. The supernatant was collected and further purified by reversed-phase HPLC, in gradient: 0−15 min 0−20% B in A, 15−18 min 20−40% B in A, 18−25 min 40−0% B in A, flow rate 0.7 mL min^{-1}; A: 0.1 M (Et_3NH)OAc (pH 7.0)/MeCN 95:5; B: MeCN) to give the cross-linked oligonucleotide **11** (3.0 A_{260} units). The other cross-linked oligonucleotides were prepared in a similar way. The monofunctionalized oligonucleotide conjugate was prepared in the same way but by using the ratio of azide to oligonucleotide as 15:1. A 37.5 μL (0.75 mmol) sample of stock solution V instead of 2.5 μL was used to yield the monofunctionalized oligonucleotide conjugate **10**. For stock solutions see the Supporting Information.

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Supporting Information Available: General descriptions, oligonucleotide synthesis and purification, mass spectra, HPLC profiles, ESI-TOF masses and purification profiles of the oligonucleotides, ^{1}H, ^{13}C, DEPT-135 and ^{1}H−^{13}C NMR gated decoupled spectra of the nucleoside derivatives and click conjugates, and ^{1}H−^{13}C coupling constants for peak assignment. This material is available free of charge via the Internet at http://pubs.acs.org.

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