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Stepwise Click Functionalization of DNA through a Bifunctional Azide with a Chelating and a Nonchelating Azido Group

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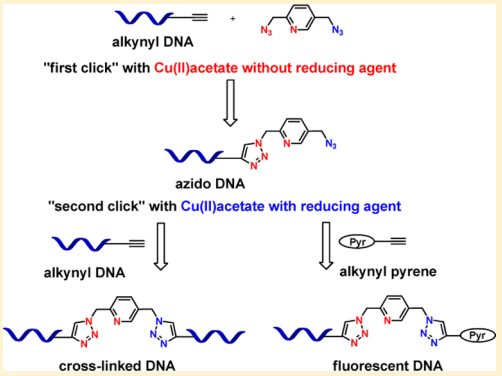
Supporting Information

ABSTRACT: A stepwise chemoselective click reaction was performed on nucleosides and oligonucleotides containing 7-octadiynyl-7-deaza-2′-deoxyguanosine and 5-octadiynyl-2′-deoxycytidine with unsymmetrical 2,5-bis(azidomethyl)pyridine using copper(II) acetate. The reaction is selective for the chelating azido group, thereby forming monofunctionalized adducts still carrying the nonchelating azido functionality. The azido-functionalized adduct was applied to a second click reaction, now performed in the presence of reducing agent, to generate cross-linked DNA or a pyrene click conjugate. The chelate-controlled stepwise click reaction is applicable to alkynylated nucleosides and oligonucleotides.



The copper-catalyzed azide−alkyne cycloaddition (CuAAC)[1] is one of the most prominent reactions currently available for the functionalization or cross-linking of oligonucleotides and other biomacromolecules.[2] Recently, several copper-free, strain-promoted azide−alkyne cycloaddition (SPAAC) protocols have been added that avoid the cytotoxicity caused by copper ions.[3]

Previously, our laboratory has introduced the so-called "bis-click" reaction for the functionalization and cross-linking of nucleosides and DNA/RNA chains.[4] Although the method is very efficient, it was limited to the cross-linking of identical strands to yield homodimers.[4a,b] Heterodimer construction was only possible in preformed duplexes with alkynyl and azido groups in sterically favored positions.[4c] Consequently, a stepwise click protocol was developed using a large excess of the symmetrical bis-azide over the alkynylated nucleoside or oligonucleotide to generate a monofunctionalized derivative, still carrying one of the two original azido groups.[5] By this means, the reaction becomes extremely versatile as the monofunctionalized derivative can be clicked to any other alkynylated molecule by a second click reaction.

Depending on the excess of the bifunctionalized azide and the reactivity of the reaction partner, small amounts of side products (homodimers) are formed occasionally. The removal of side products as well as the excess of bis-azide prompted us to undertake efforts to improve the stepwise click procedure by optimizing the selectivity of the bis-azide in the monofunctionalization step.

A recent advanced click protocol makes use of azides, which are capable to chelate copper ions.[6] The reaction proceeds in organic solvents and in aqueous solution in the presence of copper(II)acetate, but in the absence of an external reducing agent, e.g., ascorbic acid. This protocol has been described for monofunctional and bifunctional azides and was found to proceed chemoselectively, when unsymmetrical bis-azides, containing a chelating and a nonchelating azido group, were used.[6,7] The selectivity of the click reaction results from the increased reactivity of the chelating azido group in comparison to the less reactive, nonchelating azido functionality.[7]

Herein, we report on a novel and efficient stepwise click functionalization of nucleosides and oligonucleotides by using an unsymmetrical bis-azide with one chelating and one nonchelating azido group. More specifically, instead of the previously utilized 1,4-bis(azidomethyl)benzene,[4,5] we now employed the unsymmetrical 2,5-bis(azidomethyl)pyridine[7] in a stepwise click procedure (Scheme 1). For the chelate-assisted click functionalization of nucleosides, 7-octadiynyl-7-deaza-2′-deoxyguanosine (**1**)[2e,f] and 5-octadiynyl-dC (**2**),[2e] both constituents of a "dG-dC" base pair, were selected as alkynyl components. The unsymmetrical 2,5-bis(azidomethyl)pyridine **3** was synthesized according to a previously reported procedure[7] and was used in a 1:1 molar ratio with regard to the alkynylated nucleosides **1** or **2**. The click reaction with nucleosides was performed in a mixture of CH_2Cl_2/CH_3OH at room temperature using Cu(II)acetate as catalyst, and the azide **3** versus copper(II) ratio was 1:0.1. Addition of a reducing

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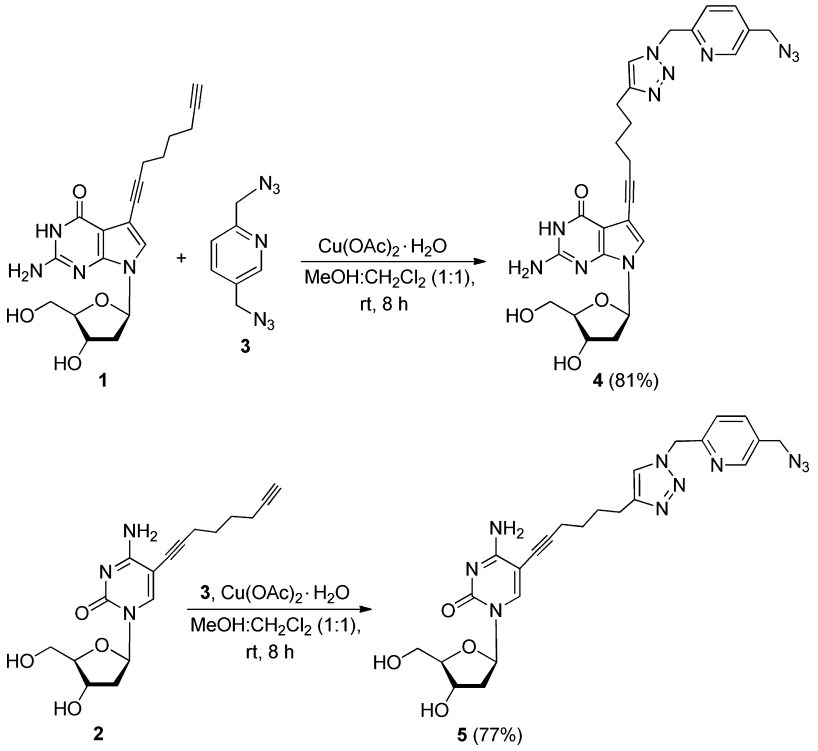
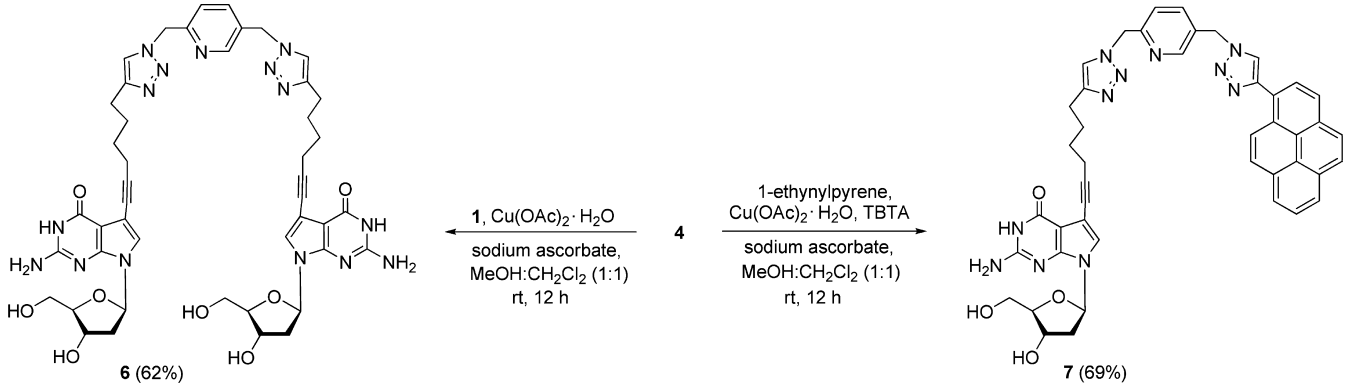
Scheme 1. Chelate-Assisted Click Reactions (First Step) Performed in the Presence of Cu(II) Acetate without Sodium Ascorbate



Scheme 2. Nonchelating Click Reactions (Second Step) Performed in the Presence of Cu(II) Acetate and Sodium Ascorbate



agent (ascorbic acid), as used in the classical protocol, was not necessary (Scheme 1). The alkynylated nucleoside **1** was consumed in less than 8 h to give exclusively the monofunctionalized click adduct **4** (81% yield), still carrying the nonchelating azido functionality. Functionalization of 5-octadiynyl-dC (**2**)[2e] with bis-azide **3** was performed as described for **1**, affording the monofunctionalized azido-nucleoside **5** in 77% yield. In both cases, bifunctionalized click products were not observed.

The reactions were monitored by TLC. Repeated TLC development (CH_2Cl_2: MeOH, 9:1) was necessary as the mobilities of nucleoside **1** (faster) and the monofunctionalized product **4** (slower) are nearly the same. The bifunctionalized compound **6** (Scheme 2), which migrates significantly slower, was not detected.

The exclusive formation of monofunctionalized click adduct **4** is attributed to the formation of a chelate complex between copper(II) ions and 2,5-bis(azidomethyl)pyridine (**3**). Due to a chelating effect, the reactivity of the chelated azido group is strongly increased over the nonchelating one. Apparently, the catalytic active Cu(I) species, which is needed to perform the cycloaddition, emerges from alcohol oxidation or oxidative homocoupling of terminal alkyne residues by $Cu(OAc)_2$.[6a,8] As the copper concentration is low (0.1 equiv) with respect to the azide (**3**; 1 equiv), all metal ions probably exist in the chelated state. Thus, a second click reaction of the less reactive, nonchelateing azido group is prevented.

Next, the second step of the stepwise click reaction was performed. For this, the monofunctionalized 7-deaza-2′-deoxyguanosine derivative **4** and octadiynyl nucleoside **1** were selected. Now, sodium ascorbate (0.2 equiv) and $Cu(OAc)_2$ (0.1 equiv) were added to the reaction mixture affording the bifunctionalized adduct **6** in 62% yield (Scheme 2). Contrary to the chelate-assisted click reaction (first step), the reaction time was now extended to 12 h compared to less than 8 h for the first step. In order to show the scope of the stepwise click

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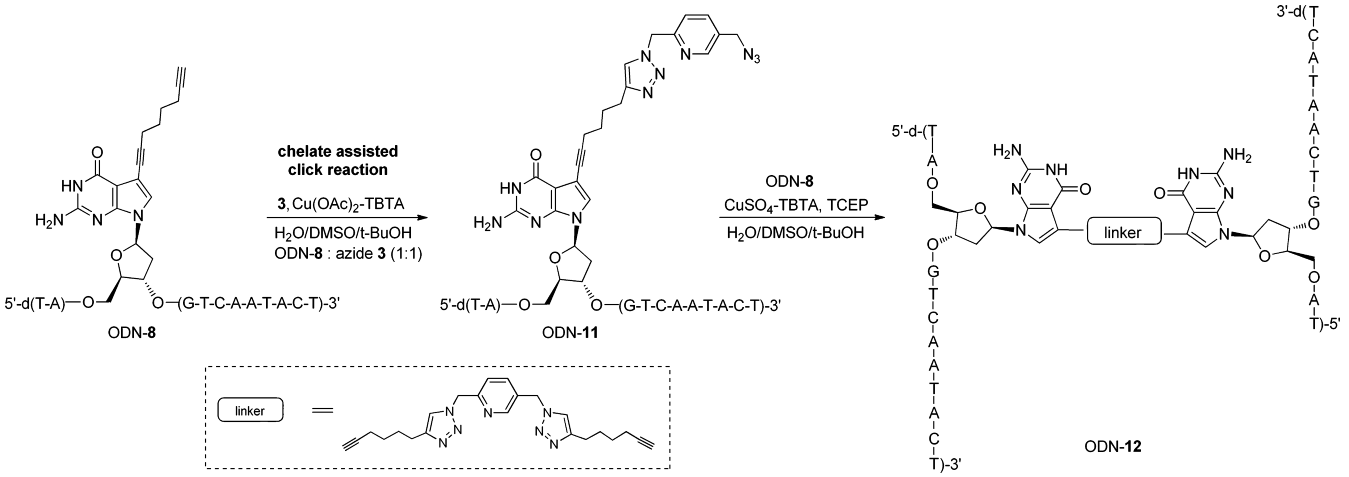
Scheme 3. Synthesis of Monofunctionalized and Cross-Linked Oligonucleotide Click Conjugates



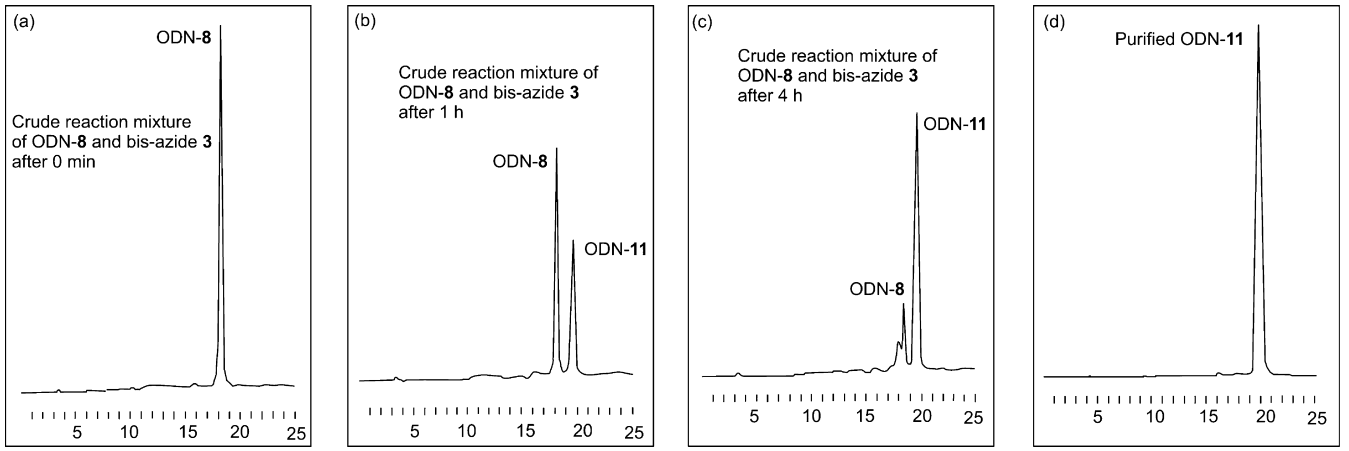


Figure 1. Reversed-phase HPLC elution profiles of (a) the crude reaction mixture of ODN-**8** and bis-azide **3** after 0 h, (b) 1 h, and (c) 4 h and (d) of the purified monofunctionalized ODN-**11** monitored at 260 nm using gradient system II.

procedure using the bis-azide **3**, the second click reaction was also performed with 1-ethynylpyrene as fluorescent dye reporter to give the conjugate **7** in 69% yield (Scheme 2).

All compounds were characterized by mass spectroscopy as well as by ^{1}H and ^{13}C NMR spectra. NMR chemical shifts were assigned by ^{1}H-^{13}C gated-decoupled spectra as well as by DEPT-135 NMR spectra. The monofunctionalized adducts, e.g., **4** can be easily distinguished from the bis-click product **6** as the ratio of the ^{1}H NMR aromatic signals (7.24, 7.81, 8.54 ppm) vs the triazole signal (7.98 ppm) is 3:1 for the monofunctionalized click adduct **4** and 3:2 (3 × pyridinyl-H and 2 × triazole-H) for the bifunctionalized derivative **6** (Figure S4 and S12, Supporting Information).

In a subsequent study, the chemoselective and chelate assisted click reaction was performed on oligonucleotides carrying alkynyl side chains. For this, oligonucleotides ODN-**8** to ODN-**10** with terminal triple bonds were prepared from phosphoramidites of **1** and **2** by solid-phase synthesis.[2e] The synthesized oligonucleotides were characterized by MALDI-TOF mass spectra (Table S1, Supporting Information).

The first click reaction of the stepwise procedure was carried out with alkynylated ODN-**8** and bis-azide **3** in aqueous solution containing *t*-BuOH, DMSO with $Cu(OAc)_2$−TBTA, and $NaHCO_3$ at room temperature. The ratio of bis-azide **3** and ODN-**8** was 1:1. As described for the nucleosides, this click reaction was performed in the absence of an external reducing agent (Scheme 3).

The reaction was monitored at different time intervals by injecting authentical samples of the crude reaction mixture of ODN-**8** and bis-azide **3** onto a reversed-phase HPLC (RP-18) column (Figure 1). Already after 1 h formation of the monofunctionalized product ODN-**11** was clearly visible from the HPLC elution profile of the crude reaction mixture. The reaction was completed within 8 h. The monofunctionalized oligonucleotide ODN-**11** still carrying the nonchelated azido group was obtained in 75% yield (Scheme 3).

Next, the purified, monofunctionalized ODN-**11** (Figure 1d) was subjected to the second click reaction. For this, a mixture of $CuSO_4$−TBTA, TCEP as well as benzoic acid was added to an aqueous solution of *t*-BuOH and DMSO containing ODN-**8** and ODN-**11**, resulting in the formation of the cross-linked oligonucleotide ODN-**12** (Scheme 3).

Using this protocol, functionalized oligonucleotides containing alkynylated derivatives of dG (**1**) or dC (**2**) at a terminal or central position (ODN-**8**, ODN-**9**, ODN-**10**) were obtained with high efficacy (→ ODN-**11**, ODN-**13**, ODN-**14**) (Scheme 4). For the second click reaction, either alkynylated oligonucleotides (ODN-**8**, ODN-**9**) or 1-ethynylpyrene was used, affording cross-linked oligonucleotides (ODN-**12**, ODN-**15**) (Figure S1 and Table S2, Supporting Information) or

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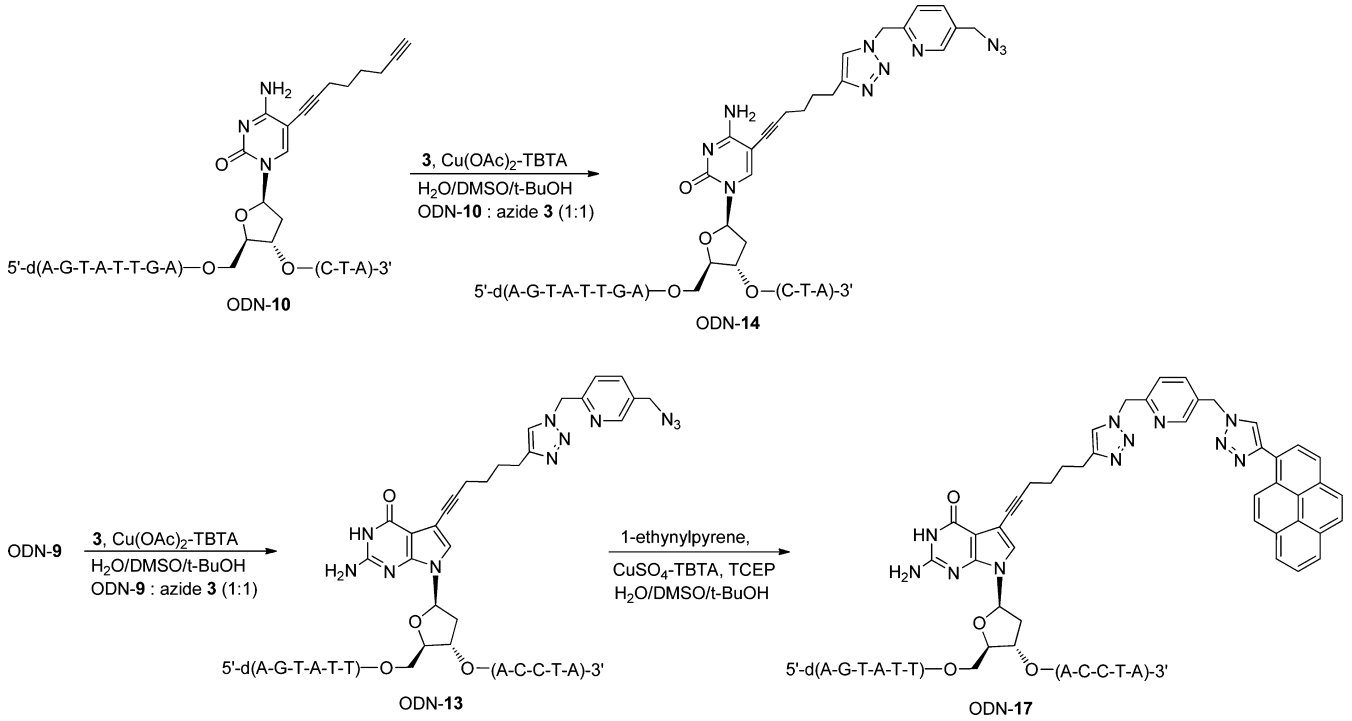
Scheme 4. Synthesis of Mono- and Bifunctionalized Oligonucleotide Click Conjugates



pyrene-labeled oligonucleotides (ODN-**16**, ODN-**17**). Formation of the functionalized and cross-linked oligonucleotides was confirmed by ion-exchange chromatography and MALDI-TOF mass spectrometry (Figure S2 and Table S1, Supporting Information). Pyrene-functionalized nucleoside **7** and oligonucleotides ODN-**16** and ODN-**17** showed monomer fluorescence emission (Figure S3, Supporting Information).[9] Duplexes containing one pyrene residue in each strand show excimer emission, resulting from the intermolecular interaction of proximal pyrene residues.[10]

To study the influence of mono- and bifunctionalization on duplex stability of oligonucleotides, mono- and bifunctionalized oligonucleotides were hybridized with complementary strands (ODN-**18** and ODN-**19**) and T_m measurements were performed. Monofunctionalized (still containing one azido group) and cross-linked duplexes show a slight destabilizing influence on the T_m value compared to the unmodified oligonucleotide duplexes (Table S2, Supporting Information).[5,11] More stable duplexes were formed by the pyrene modified oligonucleotides compared to the parent alkynylated oligonucleotide duplexes.

In conclusion, the chelate-assisted stepwise click procedure is chemoselective for one of the azido groups. The first click was performed with Cu(II)acetate in the absence of a reducing agent, while the addition of sodium ascorbate or TCEP was required for the second click reaction. The advantage of this protocol results from the reactivity differences of the azido groups. As the reaction of the chelated azido group proceeds much faster, an excess of the bis-azide can be avoided during the first step of functionalization. Consequently, this method represents a significant improvement to our earlier protocol for the stepwise functionalization of nucleosides and oligonucleotides using azido groups with identical reactivity.[5] The stepwise click reaction with a chelating and a nonchelating azido group is applicable to any alkynylated nucleoside or oligonucleotide for functionalization or cross-linking. This method reduces the load of copper ions which can cleave DNA phosphodiester bonds or can be otherwise harmful to the cellular machinery. The functionalization can be performed at any position of a DNA strand bearing an alkynyl side chain.

EXPERIMENTAL SECTION

General Methods and Materials. All chemicals and solvents were of laboratory grade as obtained from commercial suppliers and were used without further purification. Thin-layer chromatography (TLC) was performed on TLC aluminum sheets covered with silica gel 60 F254 (0.2 mm). Flash column chromatography (FC): silica gel 60 (40−60 μM) at 0.4 bar. UV spectra were recorded on a U-3000 spectrophotometer; λ_{max} (ε) in nm, ε in dm^3 mol^{-1} cm^{-1}. NMR spectra: measured at 300.15 MHz for ^{1}H 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 ^{1}H NMR and 39.50 ppm for ^{13}C NMR. The ^{13}C NMR signals were assigned on the basis of DEPT-135 and ^{1}H-^{13}C gated-decoupled NMR spectra. Reversed-phase HPLC was carried out on a 250 × 4 mm RP-18 LiChrospher 100 column with a HPLC pump connected with a variable wavelength monitor, a controller and an integrator. ESI-TOF mass spectra of the nucleosides were measured. The molecular masses of the oligonucleotides were determined by MALDI-TOF mass spectrometry in the linear negative mode with 3-hydroxypicolinic acid (3-HPA) as a matrix. Melting curves were measured with a UV−vis spectrophotometer equipped with a thermoelectrical controller with a heating rate of 1 °C/min. Gradients used for HPLC chromatography: A = MeCN; B = 0.1 M (Et_3NH)OAc (pH 7.0)/MeCN, 95:5. (I): 0−3 min 10−15% A in B, 3−15 min 15−50% A in B, flow rate 0.8 mL min^{-1}; (II): 0−20 min 0−20% A in B, 20−25 min 20% A in B, flow rate 0.8 mL min^{-1}.

Ion-exchange chromatography was performed on a 4 × 250 mm DNA PA-100 column with a precolumn using a HPLC apparatus. Elution profiles were recorded at 260 nm. The alkynyl modified ss-oligonucleotide ODN-**9**, azidomethylpyridine-labeled ss-oligonucleotide ODN-**13**, and cross-linked oligonucleotide ODN-**15** (0.1 A_{260} units each) were dissolved in 100 μL of water and then directly injected into the apparatus. The compounds were eluted using the following gradient: 0−30 min with 20−80% B in A with a flow rate of

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0.75 mL min^{-1} (A: 25 mM Tris-HCl, 10% MeCN, pH 7.0; B: 25 mM Tris-HCl, 1.0 M NaCl, 10% MeCN, pH 7.0).

2-Amino-7-(2-deoxy-β-D-*erythro*-pentofuranosyl)-3,7-dihydro-5-[6-[1-[[5-(azidomethyl)pyridin-2-yl]methyl]-1*H*-1,2,3-triazol-4-yl]hex-1-ynyl]-4*H*-pyrrolo[2,3-*d*]pyrimidin-4-one (4). Nucleoside **1**[2e] (0.076 g, 0.21 mmol) and 2,5-bis(azidomethyl)-pyridine[7] (0.039 g, 0.21 mmol) were dissolved in the mixed solvents CH_3OH/CH_2Cl_2 (1:1, 1 mL), followed by addition of an aqueous solution of $Cu(OAc)_2·H_2O$ (50 μL, 0.4 M in H_2O, 0.02 mmol). The reaction mixture was stirred in the dark at room temperature for 8 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, CH_2Cl_2/MeOH, 90:10) to give **4** (0.093 g, 81%) as a yellow solid: TLC (CH_2Cl_2/MeOH, 90:10) R_f 0.21; UV λ_{max} (MeOH)/nm 260 (ε/dm^3 mol^{-1} cm^{-1} 11800), 271 (13600), 290 (9800); ^{1}H NMR (DMSO-d_6, 300 MHz) (δ, ppm) 1.52−1.57 (m, 2H, CH_2), 1.73−1.77 (m, 2H, CH_2), 2.05−2.06 (m, 1H, $H_α$-2′), 2.30−2.41 (m, 3H, $H_ß$-2′, CH_2), 2.67 (t, J = 7.2 Hz, 2H, CH_2), 3.46−3.50 (m, 2H, 2 × H-5′), 3.73−3.75 (m, 1H, H-4′), 4.26−4.27 (m, 1H, H-3′), 4.51 (s, 2H, CH_2), 4.91 (t, J = 5.4 Hz, 1H, 5′-OH), 5.20 (d, J = 3.6 Hz, 1H, 3′-OH), 5.67 (s, 2H, CH_2), 6.24−6.30 (m, 3H, H-1′, NH_2), 7.14 (s, 1H, H-8), 7.24 (d, J = 8.1 Hz, 1H, Ar-H), 7.80, 7.83 (dd, J = 2.4 Hz, 1H, Ar-H), 7.98 (s, 1H, triazole-H), 8.54 (d, J = 1.8 Hz, 1H, Ar-H), 10.40 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75.4 MHz) (δ, ppm): 158.0 (C6), 155.2 (Ar−C), 153.1 (C2), 150.2 (C4), 149.2 (Ar-CH), 147.1 (triazole-C), 137.4, (Ar-CH), 130.9 (Ar-C), 122.9 (triazole-CH), 122.0 (Ar-CH), 121.1 (C8), 99.5 (C5), 99.5 (C7), 89.9 (≡C), 87.1 (C4′), 82.3 (C1′), 74.7 (≡C), 71.0 (C3′), 62.0 (C5′), 54.1 (CH_2), 50.7 (CH_2), 28.2 (CH_2), 28.0 (CH_2), 24.6 (CH_2), 18.8 (CH_2); ^{1}H-^{13}C coupling constants in Hz (DMSO-d_6, 75.4 MHz) 5.2 [^3J (C4, H-C8)], 194.5 [^1J (triazole-C, H-triazole)], 190.1 [^1J (C8, H-C8)], 165.2 [^1J (C1′, H-C1′)], 149.3 [^1J (C3′, H-C3′)], 149.6 [^1J (C4′, H-C4′)], 140.2 [^1J (C5′, H-C5′)]; ESI-TOF m/z calcd for $C_{26}H_{29}N_{11}O_4$ [M + Na^+] 582.2301, found 582.2296.

1-(2-Deoxy-β-D-*erythro*-pentofuranosyl)-5-[6-[1-[[5-(azidomethyl)pyridin-2-yl]methyl]-1*H*-1,2,3-triazol-4-yl]hex-1-ynyl]-cytosine (5). As described for **4**, nucleoside **2**[2e] (0.066 g, 0.20 mmol), 2,5-bis(azidomethyl)pyridine[7] (0.038 g, 0.20 mmol), and $Cu(OAc)_2·H_2O$ (50 μL, 0.4 M in H_2O, 0.02 mmol) were used. Purification by FC (silica gel, column 8 × 3 cm, CH_2Cl_2/MeOH, 90:10) gave **5** (0.080 g, 77%) as a yellow solid: TLC (CH_2Cl_2/MeOH, 90:10) R_f 0.33; UV λ_{max} (MeOH)/nm 260 (ε/dm^3 mol^{-1} cm^{-1} 8000), 299 (7700); ^{1}H NMR (DMSO-d_6, 300 MHz) (δ, ppm): 1.53−1.62 (m, 2H, CH_2), 1.66−1.73 (m, 2H, CH_2), 1.95−2.02 (m, 1H, $H_α$-2′), 2.08−2.14 (m, 1H, $H_β$-2′), 2.43 (t, J = 7.2 Hz, 2H, CH_2), 2.66 (t, J = 7.2 Hz, 2H, CH_2), 3.55−3.60 (m, 2H, 2 × H-5′), 3.77−3.78 (m, 1H, H-4′), 4.18−4.20 (m, 1H, H-3′), 4.52 (s, 2H, CH_2), 5.08 (t, J = 4.8 Hz, 1H, 5′-OH), 5.20 (d, J = 4.2 Hz, 1H, 3′-OH), 5.67 (s, 2H, CH_2), 6.11 (t, J = 6.6 Hz, 1H, H-1′), 6.73 (br s, 1H, NH), 7.26 (d, J = 7.8 Hz, 1H, Ar-H), 7.67 (br s, 1H, NH), 7.81, 7.84 (dd, J = 2.1 Hz, 1H, Ar-H), 7.94 (s, 1H, triazole-H), 8.08 (s, 1H, H-6), 8.54 (d, J = 1.5 Hz, 1H, Ar-H); ^{13}C NMR (DMSO-d_6, 75.4 MHz) (δ, ppm): 164.8 (C4), 155.6 (Ar-C), 154.0 (C2), 149.7 (Ar-CH), 147.3 (triazole-C), 144.0 (C6), 137.8 (Ar-CH), 131.3 (Ar-C), 123.2 (triazole-CH), 122.4 (Ar-CH), 95.9 (C5), 90.8 (≡C), 87.8 (C4′), 85.7 (C1′), 72.6 (≡C), 70.6 (C3′), 61.5 (C5′), 54.5 (CH_2), 51.0 (CH_2), 28.7 (CH_2), 28.0 (CH_2), 24.9 (CH_2), 19.3 (CH_2); ^{1}H-^{13}C coupling constants in Hz (DMSO-d_6, 75.4 MHz): 5.7 [^3J (C2, H−C6)], 7.9 [^3J (C4, H-C6)], 182.9 [^1J (C6, H-C6)], 194.7 [^1J (triazole-C, H-triazole)], 172.1 [^1J (C1′, H-C1′)], 147.3 [^1J (C3′, H-C3′)], 147.3 [^1J (C4′, H-C4′)], 140.5 [^1J (C5′, H-C5′)]; ESI-TOF m/z calcd for $C_{24}H_{28}N_{10}O_4$ [M + Na^+] 543.2192, found 543.2189.

5,5′-[Pyridine-2,5-diylbis(methylene-1*H*-1,2,3-triazole-1,4-diyl-hexyne-6,1-diyl)]bis[2-amino-7-(2-deoxy-β-D-*erythro*-pentofuranosyl)-3,7-dihydro-4*H*-pyrrolo[2,3-*d*]pyrimidin-4-one] (6). Nucleoside **1**[2e] (0.015 g, 0.04 mmol) and nucleoside **4** (0.022 mg, 0.04 mmol) were dissolved in CH_3OH/CH_2Cl_2 (1:1, 2 mL), followed by the addition of a freshly prepared 1 M solution of sodium ascorbate in water (8 μL, 8 μmol) in water and $Cu(OAc)_2·H_2O$ (21 μL, 0.4 M in H_2O, 4 μmol). 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, CH_2Cl_2/MeOH, 75:25) to give **6** (0.023 g, 62%) as a colorless solid: TLC (CH_2Cl_2/MeOH, 75:25) R_f 0.39; UV λ_{max} (MeOH)/nm 260 (ε/dm^3 mol^{-1} cm^{-1} 18900), 272 (22700), 289 (17800). ^{1}H NMR (DMSO-d_6, 300 MHz) (δ, ppm): 1.48−1.54 (m, 4H, 2 × CH_2), 1.66−1.72 (m, 4H, 2 × CH_2), 2.01−2.07 (m, 2H, 2 × $H_α$-2′), 2.24−2.38 (m, 6H, 2 × $H_ß$-2′, 2 × CH_2), 2.59−2.63 (m, 4H, 2 × CH_2), 3.42−3.51 (m, 4H, 4 × H-5′), 3.72−3.73 (m, 2H, 2 × H-4′), 4.24−4.25 (m, 2H, 2 × H-3′), 4.90 (t, J = 4.8 Hz, 2H, 2 × 5′-OH), 5.19 (d, J = 3.3 Hz, 2H, 2 × 3′-OH), 5.56 (s, 2H, CH_2), 5.61 (s, 2H, CH_2), 6.22−6.29 (m, 6H, 2 × H-1′, 2 × NH_2), 7.12 (s, 2H, 2 × H-8), 7.19 (d, J = 8.1 Hz, 1H, Ar-H), 7.67, 7.70 (dd, J = 2.1 Hz, 1H, Ar-H), 7.93, 7.96 (2s, 2H, 2 × triazole-H), 8.51 (d, J = 1.8 Hz, 1H, Ar-H), 10.39 (s, 2H, 2 × NH). ^{13}C NMR (DMSO-d_6, 75.4 MHz) (δ, ppm) 157.9 (C6), 155.1 (Ar-C), 153.0 (C2), 150.1 (C4), 148.9 (Ar-CH), 147.2 (triazole-C), 146.9 (triazole-C), 136.9 (Ar-CH), 131.3 (Ar-C), 122.7 (triazole-CH), 122.1 (C8), 122.0 (Ar-CH), 121.0 (triazole-CH), 99.4 (C5), 99.4 (C7), 89.7 (≡C), 87.1 (C4′), 82.2 (C1′), 74.6 (≡C), 70.9 (C3′), 61.9 (C5′), 54.0 (CH_2), 49.8 (CH_2), 28.1 (CH_2), 28.0 (CH_2), 24.5 (CH_2), 18.7 (CH_2); ^{1}H−^{13}C coupling constants in Hz (DMSO-d_6, 75.4 MHz) 5.9 [^3J (C4, H-C8)], 188.4 [^1J (C8, H-C8)], 194.4, 193.0 [^1J (triazole-C, H-triazole)], 166.7 [^1J (C1′, H-C1′)], 149.4 [^1J (C3′, H-C3′)], 148.6 [^1J (C4′, H-C4′)], 139.8 [^1J (C5′, H-C5′)]; ESI-TOF m/z calcd for $C_{45}H_{51}N_{15}O_8$ [M + Na^+] 952.3943, found 952.3937.

2-Amino-7-(2-deoxy-β-D-*erythro*-pentofuranosyl)-3,7-dihydro-5-[6-[1-[[5-[[4-(pyren-1-yl)-1*H*-1,2,3-triazol-1-yl]methyl]-pyridin-2-yl]methyl]-1*H*-1,2,3-triazol-4-yl]hex-1-ynyl]-4*H*-pyrrolo[2,3-*d*]pyrimidin-4-one (7). Nucleoside **4** (0.030 g, 0.05 mmol), 1-ethynylpyrene (0.018 g, 0.08 mmol), and TBTA (0.0032 g, 6 μmol) were dissolved in CH_3OH/CH_2Cl_2 (1:1, 2 mL), followed by the addition of a freshly prepared 1 M solution of sodium ascorbate (10 μL, 0.01 mmol) in water and $Cu(OAc)_2·H_2O$ solution (50 μL, 0.4 M in H_2O, 5 μmol). 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, CH_2Cl_2/MeOH, 90:10) to give **7** (0.029 g, 69%) as a yellow solid: TLC (CH_2Cl_2/MeOH, 90:10) R_f 0.21; UV λ_{max} (MeOH)/nm 260 (ε/dm^3 mol^{-1} cm^{-1} 20800), 268 (28500), 277 (35900), 343 (22500); ^{1}H NMR (DMSO-d_6, 300 MHz) (δ, ppm) 1.51−1.59 (m, 2H, CH_2), 1.69−1.77 (m, 2H, CH_2), 2.03−2.08 (m, 1H, $H_α$-2′), 2.26−2.40 (m, 3H, $H_ß$-2′, CH_2), 2.66 (t, J = 7.2 Hz, 2H, CH_2), 3.46−3.50 (m, 2H, 2 × H-5′), 3.72−3.75 (m, 1H, H-4′), 4.26−4.27 (m, 1H, H-3′), 4.91 (t, J = 5.7 Hz, 1H, 5′-OH), 5.21 (d, J = 3.9 Hz, 1H, 3′-OH), 5.68 (s, 2H, CH_2), 5.83 (s, 2H, CH_2), 6.24−6.31 (m, 3H, H-1′, NH_2), 7.14 (s, 1H, H-8), 7.30 (d, J = 8.1 Hz, 1H, Ar-H), 7.89, 8.93 (dd, J = 2.4 Hz, 1H, Ar-H), 7.98 (s, 1H, triazole-H), 8.23−8.34 (m, 8H, 8 × Ar-H), 8.72 (d, J = 1.8 Hz, 1H, Ar-H), 8.84 (d, 1H, J = 9.3 Hz, Ar-H), 8.91 (s, 1H, triazole-H), 10.41 (br s, 1H, NH); ^{13}C NMR (DMSO-d_6, 75.4 MHz) (δ, ppm): 157.9 (C6), 155.4 (Ar-C), 153.1 (C2), 150.2 (C4), 149.2 (Ar-CH), 147.0 (triazole-C), 146.5 (triazole-C), 137.3 (Ar-CH), 131.1 (Ar-C), 130.9 (Ar-C), 130.6 (Ar-C), 130.4 (Ar-C), 128.1 (Ar-CH), 127.8 (Ar-CH), 127.5 (Ar-C), 127.4 (Ar-CH), 127.1 (Ar-CH), 126.5 (Ar-CH), 125.6 (Ar-CH), 125.3 (Ar-C), 125.2 (Ar-CH), 125.1 (Ar-CH), 124.8 (Ar-CH), 124.3 (Ar-C), 123.9 (Ar-C), 122.8 (triazole-CH), 122.2 (triazole-CH), 121.1 (C8), 99.4 (C5), 99.4 (C7), 89.8 (≡C), 87.1 (C4′), 82.2 (C1′), 74.7 (≡C), 71.0 (C3′), 61.9 (C5′), 54.1 (CH_2), 50.3 (CH_2), 28.1 (CH_2), 28.0 (CH_2), 24.6 (CH_2), 18.8 (CH_2); ^{1}H−^{13}C coupling constants in Hz (DMSO-d_6, 75.4 MHz) 163.9 [^1J (C1′, H-C1′)], 148.8 [^1J (C3′, H-C3′)], 145.6 [^1J (C4′, H-C4′)], 139.3 [^1J (C5′, H-C5′)]; ESI-TOF m/z calcd for $C_{44}H_{39}N_{11}O_4$ [M + Na^+] 808.3083, found 808.3079.

General Procedure for the "Stepwise Click" Huisgen−Meldal−Sharpless [3 + 2] Cycloaddition. *Synthesis of the Azidomethylpyridine-Labeled Oligonucleotides **11**, **13**, and **14** ("First Click").* To the single-stranded oligonucleotide **8**, **9**, or **10** (3 A_{260} units) were added a mixture of the TBTA−$Cu(OAc)_2·H_2O$ ligand complex (30 μL of a 20 mM stock solution in H_2O/DMSO/*t*-BuOH,

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4:3:1 for TBTA; 20 μL of a 20 mM stock solution in H_2O/DMSO/*t*-BuOH, 4:3:1 for $Cu(OAc)_2 \cdot H_2O$), 2,5-bis(azidomethyl)pyridine (3 μL of a 20 mM stock solution in dioxane/H_2O, 1:1), sodium bicarbonate (20 μL of a 200 mM aq solution), and 30 μL of DMSO, and the reaction proceeded at room temperature for 8 h. The reaction mixture was concentrated in a Speed-Vac and dissolved in 200 μL of bidistilled water and centrifuged for 20 min at 14000 rpm. The supernatant solution was collected and further purified by reversed-phase HPLC (gradient *II*) to give about 70−75% isolated yield of the azidomethylpyridine oligonucleotides **11**, **13**, or **14**. The molecular masses of the azidomethylpyridine oligonucleotides were determined by MALDI-TOF mass spectrometry (Table S1, Supporting Information).

*Synthesis of the Cross-Linked or Bifunctionalized Oligonucleotides **12**, **15**, **16**, and **17** ("Second Click").* To the azidomethylpyridine-labeled oligonucleotide **11** or **13** (2 A_{260} units) and alkynylated oligonucleotide **8** or **9** (1.5 A_{260} units) or 1-ethynylpyrene [3 μL from 20 mM stock solution in dioxane:water (1:1)] were added a mixture of the TBTA-$CuSO_4$ ligand complex (30 μL of a 20 mM stock solution in H_2O/DMSO/*t*-BuOH, 4:3:1 for TBTA; 20 μL of a 20 mM stock solution in H_2O/DMSO/*t*-BuOH, 4:3:1 for $CuSO_4$), tris-(carboxyethyl)phosphine (TCEP; 20 μL of a 20 mM stock solution in water), benzoic acid (10 μL of a 100 mM stock solution in DMSO), and 30 μL of DMSO, and the reaction proceeded at room temperature for 20 min. Then, sodium bicarbonate (100 μL of a 200 mM aq solution) was used to neutralize the excess of benzoic acid. The reaction mixture was concentrated in a Speed-Vac, dissolved in 200 μL bidistilled water, and centrifuged for 20 min at 14000 rpm. The supernatant was collected and further purified by reversed-phase HPLC (gradient II for ODNs **12** and **15** and gradient I for ODNs **16** and **17**) to give about 50−55% isolated yield of the bis-click oligonucleotides. The molecular masses of the bifunctionalized oligonucleotides were determined by MALDI-TOF mass spectrometry (Table S1, Supporting Information).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Molecular masses of oligonucleotides, T_m values of duplexes, fluorescence spectra of pyrene-modified oligonucleotides, and copies of ^1H and ^{13}C NMR, DEPT-135, and ^1H-^{13}C gated-decoupled spectra of the click conjugates. 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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