

## Construction and Assembly of Branched Y-Shaped DNA: “Click” Chemistry Performed on Dendronized 8-Aza-7-deazaguanine Oligonucleotides

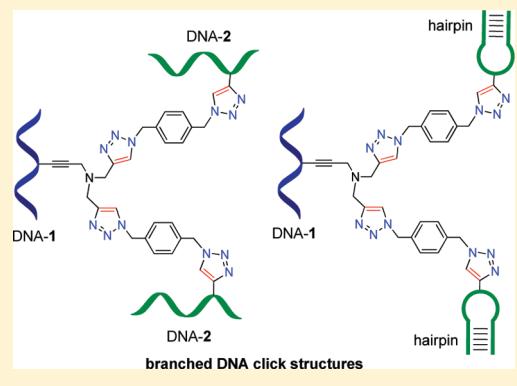
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### S Supporting Information

**ABSTRACT:** Branched DNA was synthesized from tripropargylated oligonucleotides by the Huisgen-Meldal-Sharpless cycloaddition using “stepwise and double click” chemistry. Dendronized oligonucleotides decorated with 7-tripropargylamine side chains carrying two terminal triple bonds were further functionalized with bis-azides to give derivatives with two terminal azido groups. Then, the branched side chains with two azido groups or two triple bonds were combined with DNA-fragments providing the corresponding clickable function. Both concepts afforded branched (Y-shaped) three-armed DNA. Annealing of branched DNA with complementary oligonucleotides yielded supramolecular assemblies. The concept of “stepwise and double click” chemistry combined with selective hybridization represents a flexible tool to generate DNA nanostructures useful for various purposes in DNA diagnostics, delivery, and material science applications.



### INTRODUCTION

Branched oligonucleotides have been found to represent an important class of molecules acting in nature as structural intermediates of RNA splicing.<sup>1–4</sup> They can form multistranded structures or hairpin motifs. Branched DNA was also prepared chemically following various concepts. One approach uses the self-assembly by hybridization of predesigned complementary oligonucleotides leading to DNA three- or four-way junctions, so-called X- or Y-shaped DNA.<sup>5–9</sup> In another more synthetic approach, branched DNA was synthesized with preformed branching elements including dendronized molecules. Nucleoside or non-nucleosidic building blocks were used for this purpose.<sup>10–22</sup> Dendronized materials find applications in catalysis, drug delivery, nanomedicine, and nanoscale electronics.<sup>23–30</sup> Branched or dendronized oligonucleotides were synthesized, modified, or further assembled to dendritic nucleic acid structures.<sup>31–33</sup> However, these approaches show drawbacks and are often laborious and low-yielding. The Huisgen-Meldal-Sharpless copper(I) cycloaddition<sup>34–36</sup>—“click” chemistry—was recently used to covalently connect DNA oligonucleotides.<sup>37–42</sup> Our laboratory introduced tripropargylamine side chains as branching units into the 5-position of pyrimidine nucleosides<sup>43</sup> as well as in the 7-position of 7-deazapurine and 7-deaza-8-azapurine 2'-deoxyribonucleosides<sup>44–46</sup> to generate dendronized DNA. Both terminal triple bonds were simultaneously “double clicked” to azido functions of azido reporter groups.<sup>43–46</sup> We also described DNA cross-

linking of dendronized DNA by a “bis-click” or “stepwise click” protocol.<sup>41,42</sup>

This manuscript reports on the “stepwise and double click” concept employing tripropargylated 3 as branching point for the defined construction of three-armed (Y-shaped) dendronized DNA. 8-Aza-7-deaza-2'-deoxyguanosine with short (1), long (2), and branched (3) linker arms together with *p*-azidoxylene 4<sup>47</sup> were used for this purpose. Azido-functionalized oligonucleotides incorporating 5, 6, or 7 were linked to alkynylated DNA-fragments affording branched oligonucleotides. Hybridization experiments of branched oligonucleotides and single-stranded chains were performed. The controlled synthesis via click chemistry and intra- or intermolecular base-pairing leads to self-assembly resulting in unique DNA nanoconstructs applicable to material science and nanomedicine.<sup>48,49</sup>

### EXPERIMENTAL PROCEDURES

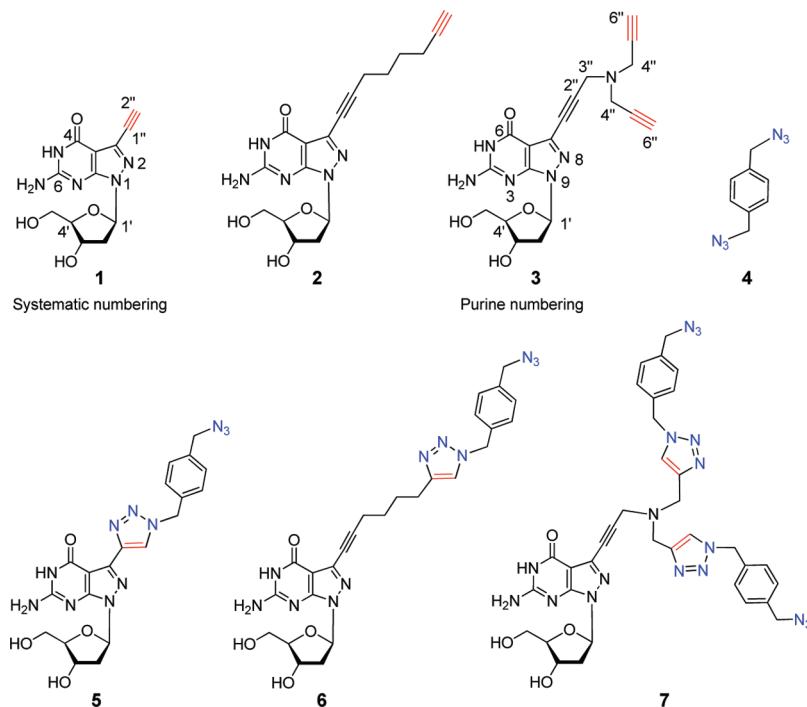
**General Methods and Materials.** All chemicals and solvents were of laboratory grade as obtained from Acros, Aldrich, Sigma, or Fluka (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) and were used without further purification. Standard phosphoramidites were purchased from

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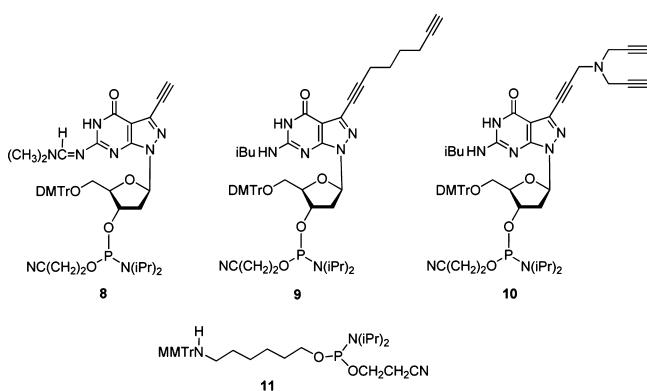
**Figure 1.** Structures of mono- and bisfunctionalized “clickable” nucleosides used in this study.

Sigma (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany). The 5'-MMT-amino-modifier C6-CE phosphoramidite **11** was purchased from Link Technologies (Bellshill, Lanarkshire, UK). 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  $\mu\text{M}$ , VWR International, Germany) at 0.4 bar. UV spectra were recorded on a U-3000 spectrophotometer (Hitachi, Japan);  $\lambda_{\text{max}}$  ( $\epsilon$ ) in nm,  $\epsilon$  in  $\text{dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$ . NMR spectra: DPX 300 spectrophotometer (Bruker, Germany) at 300.15 MHz for  $^1\text{H}$  and 75.48 MHz for  $^{13}\text{C}$ . The  $J$  values are given in Hz and  $\delta$  values in ppm relative to  $\text{Me}_3\text{Si}$  as internal standard. For NMR spectra recorded in DMSO, the chemical shift of the solvent peak was set to 2.50 ppm for  $^1\text{H}$  NMR and 39.50 ppm for  $^{13}\text{C}$  NMR. Elemental analysis was performed by the Mikroanalytisches Laboratorium Beller, Göttingen, Germany. Reversed-phase HPLC was carried out on a 250  $\times$  4 mm RP-18 LiChrospher 100 column with a HPLC pump connected with a variable wavelength monitor, a controller, and an integrator. Gradients used for HPLC chromatography are as follows: A = MeCN; B = 0.1 M ( $\text{Et}_3\text{NH}$ )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 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). 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 MembraPure water system (Astacus) was used for all experiments. ESI-TOF mass spectra of the nucleosides were measured with a Micro-TOF spectrometer. The molecular masses of oligonucleotides were determined by

LC-ESI-TOF (Agilent 1200 Series, Bruker Micro-TOF Q2) or MALDI-TOF (MS Autoflex, Bruker) mass spectrometry in the linear positive mode with 3-hydroxypicolinic acid (3-HPA) as a matrix.

**6-Amino-1-(2-deoxy- $\beta$ -D-erythro-pentofuranosyl)-1,5-dihydro-3-[di(1',2',3'-triazoyl-1-methyl-4-azidomethylbenzyl)propargylamino]-4H-pyrazolo[3,4-d]pyrimidin-4-one (7).** To a solution of compound **3**<sup>44</sup> (59.4 mg, 0.15 mmol) and **4**<sup>47</sup> (282 mg, 1.5 mmol) in THF/H<sub>2</sub>O/t-BuOH, **3**: 1: 1 (2 mL) was added a freshly prepared 1 M solution of sodium ascorbate (120  $\mu\text{L}$ , 0.12 mmol) in water, followed by the addition of copper(II) sulfate pentahydrate 7.5% in water (96  $\mu\text{L}$ , 0.0285 mmol). The reaction mixture was stirred in the dark at r.t. for 12 h. After completion of the reaction (monitored by TLC), the mixture was filtered. The solvent was evaporated, and the residue was applied to FC (silica gel, column 8  $\times$  3 cm, eluted with CH<sub>2</sub>Cl<sub>2</sub>/MeOH, 95:5  $\rightarrow$  90:10). Evaporation of the main zone gave **7** as a pale yellow solid (70 mg, 60%). TLC (CH<sub>2</sub>Cl<sub>2</sub>/MeOH, 90:10):  $R_f$  0.46; UV  $\lambda_{\text{max}}$  (MeOH)/nm 246 ( $\epsilon/\text{dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$  20 500), 260 (11 600), 280 nm (4800). Anal. (C<sub>35</sub>H<sub>36</sub>N<sub>18</sub>O<sub>4</sub>) C, H, N.  $^1\text{H}$  NMR (300 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  = 10.70 (s, 1H, NH), 8.21 (s, 2H, 2*X* H-triazole), 7.37–7.30 (m, 8H, 2*X* H-phenylene), 6.79 (br s, 2H, NH<sub>2</sub>), 6.30 (t,  $J$  = 6.3 Hz, 1H, 1'-H), 5.60 (s, 4H, 2*X* PhCH<sub>2</sub>), 5.24–5.23 (d,  $J$  = 4.5 Hz, 1H, 3'-OH), 4.73 (t,  $J$  = 5.7 Hz, 1H, 5'-OH), 4.43 (s, 4H, 2*X* PhCH<sub>2</sub>), 4.39–4.35 (m, 1H, 3'-H), 3.82–3.75 (m, 5H, 2*X* NCH<sub>2</sub>, 4'-H), 3.52–3.44 (m, 2H, NCH<sub>2</sub>), 3.40–3.36 (m, 2H, 5'-H), 2.63–2.72 (m, 1H, 2'-H<sub>β</sub>), 2.14–2.19 (m, 1H, 2'-H<sub>α</sub>). (ESI-TOF): *m/z* calcd for C<sub>35</sub>H<sub>36</sub>N<sub>18</sub>O<sub>4</sub> + Na<sup>+</sup>: 795.3067 [M + Na<sup>+</sup>]; found: 795.3059.

**Synthesis, Purification, and Characterization of Oligonucleotides.** The syntheses of oligonucleotides were performed on a DNA synthesizer on a 1  $\mu\text{mol}$  scale in the trityl-on mode using the phosphoramidites **8–10**<sup>41,42,44</sup> (Figure 2) and the standard phosphoramidite building blocks following the synthesis protocol for 3'-O-(2-cyanoethyl)phosphoramidites.<sup>50</sup>



**Figure 2.** Structure of phosphoramidite building blocks **8–11** used in this study.

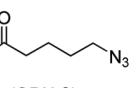
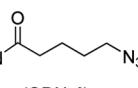
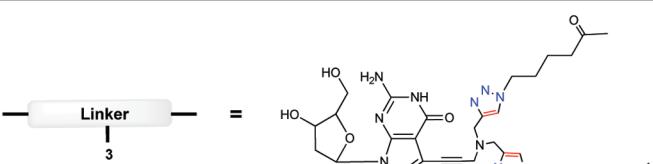
After cleavage from the solid support, the oligonucleotides were deprotected in 25% aq. ammonia solution in a sealed tube for 12–16 h at 60 °C. The 5'-aminoalkyl oligonucleotides were synthesized starting from 5'-MMT-amino-modifier C6-CE phosphoramidite **11** (Link Technologies Ltd., Bellshill, Lanarkshire, UK). The oligonucleotides were cleaved from the solid support and deprotected by heating the oligonucleotides in a 25% aq. ammonia solution in a sealed tube for 5 h at 55 °C. The purification of the “trityl-on”

oligonucleotides was carried out on reversed-phase HPLC (RP-18 column; gradient system I). The purified "trityl-on" oligonucleotides were treated with 2.5%  $\text{Cl}_2\text{CHCOOH}/\text{CH}_2\text{Cl}_2$  for 5 min at 0 °C to remove the mono- or dimethoxytrityl residues. The detritylated oligomers were purified by reversed-phase HPLC (gradient II). The oligomers were desalted on a short column using distilled water for elution of salt, while the oligonucleotides were eluted with  $\text{H}_2\text{O}/\text{MeOH}$  (2: 3). Then, the solvent was evaporated using a SpeedVac 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 (Table 1 and 2). Extinction coefficients  $\epsilon_{260}$  (MeOH) of nucleosides: dA, 15400; dG, 11700; dT, 8800; dC, 7300; 1, 10900;<sup>42</sup> 2, 11500;<sup>42</sup> 3, 14000;<sup>44</sup> 5, 26000;<sup>42</sup> 6, 23100;<sup>42</sup> 7, 11600.

**Ion-Exchange HPLC Analysis of Branched Oligonucleotides.** The ion-exchange chromatography was performed

**cleotides.** The ion-exchange chromatography was performed on a  $4 \times 250$  mm DNA PA-100 column with a precolumn using a HPLC apparatus. Elution profiles were recorded at 260 nm. The azidomethylbenzyl-labeled oligonucleotides as well as the nonbranched and branched oligonucleotides (0.2 A<sub>260</sub> units each) were dissolved in 100  $\mu\text{L}$  of water and were then directly injected into the HPLC apparatus. The compounds were eluted using gradient IV: 0–30 min

**Table 1.** Structures and Mass Spectrometric Data of Nonbranched Oligonucleotides ODN-1–ODN-5 and Branched Oligonucleotides BR-1 and BR-2

Oligonucleotides	MS [calc.] found	Oligonucleotides	MS [calc.] found
3'-d(TATATATATATA)-O-  -O(CH <sub>2</sub> ) <sub>6</sub> NH <sub>2</sub> (ODN-1)	[3820.6 <sup>a</sup> ] 3820.7 <sup>b</sup>	3'-d(TAGGTCAATACT)-O-  -O(CH <sub>2</sub> ) <sub>6</sub> NH <sub>2</sub> (ODN-2)	[3821.7 <sup>c</sup> ] 3821.7 <sup>d</sup>
3'-d(TATATATATATA)-O-  -O(CH <sub>2</sub> ) <sub>6</sub> NH  (ODN-3)	[3945.7 <sup>a</sup> ] 3944.8 <sup>b</sup>	3'-d(TAGGTCAATACT)-O-  -O(CH <sub>2</sub> ) <sub>6</sub> NH  (ODN-4)	[3946.8 <sup>c</sup> ] 3946.8 <sup>d</sup>
5'-d(A <sup>3T</sup> ATT GAC CTA) (ODN-5) <sup>44</sup>	[3772.6 <sup>a</sup> ] 3772.6 <sup>b</sup>	5'-d(AGTATTGACCTA) (ODN-6)	[3644.4 <sup>a</sup> ] 3640.3 <sup>b</sup>
5'-d(TAGGTCAATACT) (ODN-7)	[3644.4 <sup>a</sup> ] 3643.7 <sup>b</sup>		
 <p style="text-align: center;"><b>BR-1</b></p>			
 <p style="text-align: center;"><b>BR-2</b></p>			
			

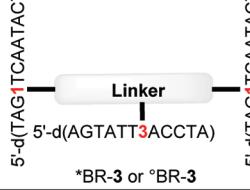
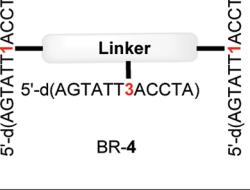
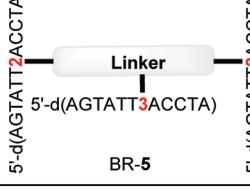
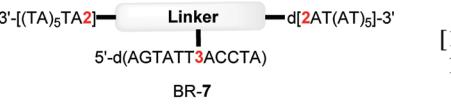
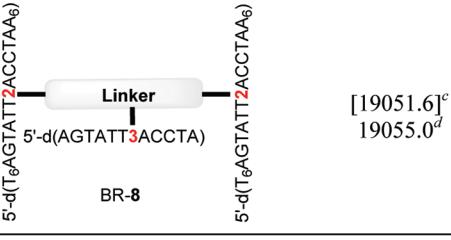
<sup>a</sup>Calculated on the basis of molecular weight as [M+1]<sup>+</sup>. <sup>b</sup>Determined by Maldi-TOF mass spectrometry as [M+1]<sup>+</sup> in the linear positive mode.  
<sup>c</sup>Calculated on the basis of exact mass. <sup>d</sup>Determined by LC-ESI-TOF mass spectrometry.

<sup>c</sup>Calculated on the basis of exact mass. <sup>d</sup>Determined by LC-ESI-TOF mass spectrometry.

Table 2. Structure and Mass Spectrometric Data of Nonbranched and Branched Oligonucleotides<sup>e</sup>

Oligonucleotides	MS [calc.] found	ODN Intermediates with Azido Groups	MS [calc.] found
5'-d(TAG <sup>1</sup> TCAATACT) (ODN-8) <sup>42</sup>	[3666.7] <sup>c</sup> 3666.6 <sup>d</sup>	5'-d(TAG <sup>5</sup> TCAAT ACT) (ODN-14) <sup>42</sup>	[3855.6] <sup>a</sup> 3854.5 <sup>b</sup>
5'-d( <sup>1</sup> ATATATATATATAT) (ODN-9)	[3994.7] <sup>a</sup> 3995.1 <sup>b</sup>	5'-d( <sup>5</sup> ATATATATATAT) (ODN-15)	[4181.8] <sup>c</sup> 4181.8 <sup>d</sup>
5'-d( <sup>2</sup> ATATATATATAT) (ODN-12)	[4074.8] <sup>a</sup> 4075.0 <sup>b</sup>	5'-d( <sup>6</sup> ATATATATATAT) (ODN-16)	[4261.9] <sup>c</sup> 4261.9 <sup>d</sup>
5'-d(AGTATT <sup>3</sup> ACCTA) (ODN-13) <sup>44</sup>	[3772.6] <sup>a</sup> 3772.0 <sup>b</sup>	5'-d(AGT ATT <sup>7</sup> AC CTA) (ODN-17)	[4147.9] <sup>c</sup> 4147.9 <sup>d</sup>
5'-d(T <sub>6</sub> AGTATT <sup>2</sup> ACCTAA <sub>6</sub> ) (ODN-18) <sup>53</sup>	[7452.0] <sup>a</sup> 7452.4 <sup>b</sup>	5'-d(T <sub>6</sub> AGTATT <sup>6</sup> ACCTA A <sub>6</sub> ) (ODN-19)	[7641.2] <sup>a</sup> 7642.4 <sup>b</sup>
5'-d(T <sub>6</sub> AGTATTGACCTAA <sub>6</sub> ) (ODN-20)	[7398.9] <sup>a</sup> 7398.9 <sup>b</sup>		
5'-d(AGTATT <sup>1</sup> ACCTA) (ODN-10) <sup>42</sup>	[3667.4] <sup>a</sup> 3667.2 <sup>b</sup>		
5'-d(AGTATT <sup>2</sup> ACCTA) (ODN-11) <sup>41</sup>	[3747.6] <sup>a</sup> 3746.9 <sup>b</sup>		

Branched oligonucleotides	Branched oligonucleotides	
 <p>*BR-3 or °BR-3</p>	 <p>BR-4</p>	
	[11481.5] <sup>c</sup> 11483.2 <sup>d,*</sup> 11483.2 <sup>d,°</sup>	
 <p>BR-5</p>	 <p>BR-6</p>	[11481.5] <sup>c</sup> 11482.0 <sup>d</sup>
	[11641.3] <sup>c</sup> 11643.4 <sup>d</sup>	[12135.3] <sup>c</sup> 12137.3 <sup>d</sup>
 <p>BR-7</p>	 <p>BR-8</p>	[12295.4] <sup>c</sup> 12297.4 <sup>d</sup>
		[19051.6] <sup>c</sup> 19055.0 <sup>d</sup>

<sup>a</sup>Calculated on the basis of molecular weight as [M+1]<sup>+</sup>. <sup>b</sup>Determined by MalDI-TOF mass spectrometry as [M+1]<sup>+</sup> in the linear positive mode.<sup>c</sup>Calculated on the basis of exact mass. <sup>d</sup>Determined by LC-ESI-TOF mass spectrometry.<sup>\*</sup>BR-3 from azido-ODN-14 and <sup>°</sup>BR-3 from azido-ODN-17. <sup>e</sup>1-3-1 (branched-short) and 2-3-2 (branched-long) represent the cross-linked nucleosides formed by the cycloaddition of the azido group within one side chain and the terminal triple bond of the other side chain.

with 20–80% D in C with a flow rate of 0.75 mL min<sup>-1</sup> (C: 25 mM Tris-HCl, 10% MeCN, pH 7.0; D: 25 mM Tris-HCl, 1.0 M NaCl, 10% MeCN, pH 7.0).

**Denaturing Polyacrylamide Gel Electrophoresis (PAGE) of Oligonucleotides.** Analysis of nonbranched and branched oligonucleotides as well as cross-linked oligonucleotide ODN-22<sup>42</sup> was carried out by polyacrylamide gel electrophoresis (17% polyacrylamide gel, 19:1, acryl:bisacrylamide, containing 7 M urea). The gel was prerun for 30 min at rt using 1× tris-borate-EDTA (TBE) buffer containing 20 mM MgCl<sub>2</sub> (pH 8.4). The individual oligonucleotides (1–2 μM) were dissolved in 2–5 μL of distilled water at rt. Then, 10 μL of gel loading buffer containing formamide was added, and the oligonucleotide solution was loaded onto the gel. Electrophoresis was run at rt for 6 h using 1× TBE buffer containing 20 mM MgCl<sub>2</sub> (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.

**Native Polyacrylamide Gel Electrophoresis (PAGE).** Analysis of hybridization of nonbranched and branched oligonucleotides was carried out by native polyacrylamide gel electrophoresis (15% polyacrylamide gel, 19:1 acryl:bisacrylamide). The gel was prerun for 30 min at 0 °C using 1× tris-borate-EDTA (TBE) buffer containing 20 mM MgCl<sub>2</sub> (pH 8.4). The individual oligonucleotides (2 μM) were dissolved in 2–5 μL of 10× tris-borate-EDTA buffer containing 20 mM MgCl<sub>2</sub> (pH 8.4), and the solutions were heated to 85 °C for 10 min. The oligonucleotides were annealed by cooling the solution to room temperature followed by incubation at rt for 1 h. Then, 10 μL of gel loading buffer containing 30% glycerol was added, and the oligonucleotide solution was loaded onto the gel. Electrophoresis was run at 0 °C for 6 h using 1× TBE buffer containing 20 mM MgCl<sub>2</sub> (pH 8.4) at a constant field strength of 10 V/cm. The gel was stained with 0.02% methylene blue for 20 min and then incubated in water for 1 h to remove excess dye from the background.

**T<sub>m</sub> Measurements.** For the T<sub>m</sub> measurements, the oligonucleotides were dissolved in 1 M NaCl, 100 mM MgCl<sub>2</sub>, and 60 mM Na-cacodylate buffer, pH 7.0. For the self-complementary oligonucleotides ODN-3, ODN-15, and ODN-16, 4 μM of single-strand concentration was used. Hairpin-forming oligonucleotides were measured with 2.0 μM and 5.0 μM single-strand concentration (Table S3, Supporting Information). Hybridization experiments with branched oligonucleotides containing self-complementary oligonucleotide strands (BR-1, BR-6, or BR-7) were performed with 2 μM single-strand concentration. Hybridization experiments with complementary oligonucleotide strands were carried out with 2 μM of the branched oligonucleotide (BR-1, BR-3–BR-7) and 2 μM, 4 μM, or 6 μM of the complementary single-stranded oligonucleotide. The melting temperature 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<sup>-1</sup>, and the absorbance at 260 nm was recorded as a function of the temperature. For melting profiles, see Figures S9–13 (Supporting Information).

**Synthesis of 5'-Azidovaleate-Labeled Oligonucleotides ODN-3 and ODN-4.** The C6-aminoalkyl oligonucleotide ODN-1 or ODN-2 (10 A<sub>260</sub> units) in 160 μL of 0.5 M Na<sub>2</sub>CO<sub>3</sub>/NaHCO<sub>3</sub> buffer (pH 8.7) was incubated for 5 h at room temperature with 34 μmol of succinimidyl-5'-azidovaleate

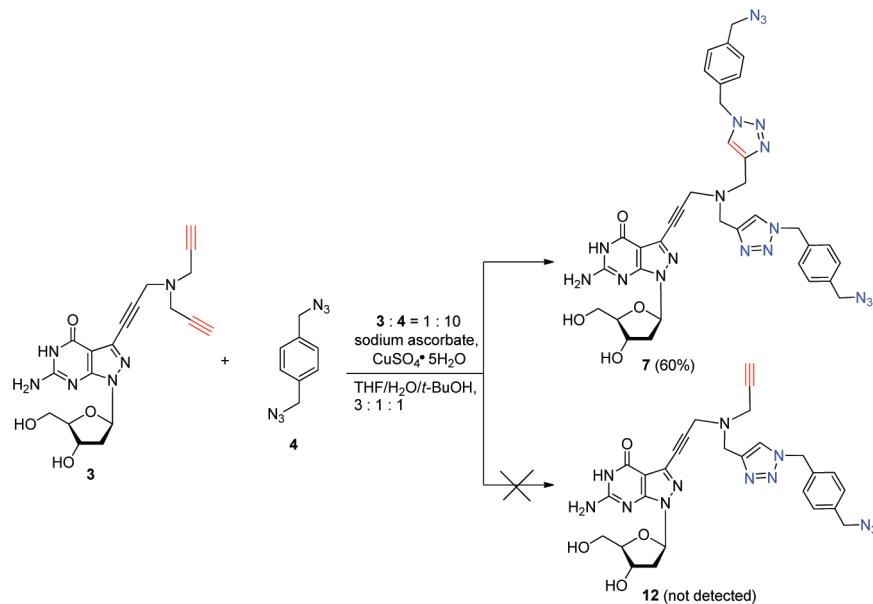
13 in 40 μL of DMSO. The reaction mixture was concentrated in a SpeedVac, dissolved in 1 mL bidistilled water, and centrifuged for 20 min at 12 000 rpm. The supernatant was collected and further purified by reversed-phase HPLC (gradient III) to give about 90% isolated yield of the 5'-azidovaleate-labeled oligonucleotide ODN-3 or ODN-4 (Supporting Information, Figure S3a,b). The molecular masses of ODN-3 and ODN-4 were determined by LC-ESI-TOF or MALDI-TOF mass spectrometry (Table 1).

**Synthesis of Branched BR-1.** To ODN-5<sup>44</sup> (1.2 A<sub>260</sub> units) and 5'-azidovaleate-labeled ODN-3 (2.8 A<sub>260</sub> units), CuSO<sub>4</sub>-TBTA ligand complex (25 μL of a 20 mM stock solution in H<sub>2</sub>O/DMSO/t-BuOH, 4:3:1 for TBTA; 25 μL of a 20 mM stock solution in H<sub>2</sub>O/DMSO/t-BuOH 4:3:1 for CuSO<sub>4</sub>), tris(carboxyethyl)phosphine (TCEP; 25 μL of a 20 mM stock solution in water), sodium bicarbonate (10 μL of a 200 mM aq. solution), and 30 μL of DMSO were added, and the reaction proceeded at room temperature for 12 h. The reaction mixture was concentrated in a SpeedVac, dissolved in 0.3 mL bidistilled water and centrifuged for 20 min at 12 000 rpm. The supernatant was collected and further purified by reversed-phase HPLC (gradient III). The peak containing the “click” product BR-1 was collected (Figure S3c, Supporting Information) and concentrated to dryness in a SpeedVac. The integrity of BR-1 was confirmed by MALDI-TOF mass spectrometry (Table 1).

**Synthesis of Branched BR-2.** To ODN-5 (1.2 A<sub>260</sub> units) and 5'-azidovaleate-labeled ODN-4 (2.8 A<sub>260</sub> units), CuSO<sub>4</sub>-TBTA ligand complex (25 μL of a 20 mM stock solution in H<sub>2</sub>O/DMSO/t-BuOH, 4:3:1 for TBTA; 25 μL of a 20 mM stock solution in H<sub>2</sub>O/DMSO/t-BuOH, 4:3:1 for CuSO<sub>4</sub>), tris(carboxyethyl)phosphine (TCEP; 25 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 were added, 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 SpeedVac, dissolved in 0.3 mL bidistilled water and centrifuged for 20 min at 12 000 rpm. The supernatant was collected and further purified by reversed-phase HPLC (gradient III) to give the “click” product BR-2 (Figure S3d, Supporting Information). The integrity of BR-2 was confirmed by LC-ESI-TOF mass spectrometry (Table 1).

**General Protocol for the Combined “Stepwise Click” and “Double Click” Procedure: “First Click”: Synthesis of Azidomethylbenzyl-Labeled Oligonucleotides ODN-14–ODN-17 and ODN-19 from Alkynylated Oligonucleotides ODN-8, ODN-9, ODN-12, ODN-13, and ODN-18 with Bis-Azide 4.** To the single-stranded oligonucleotide (5 A<sub>260</sub> units), a mixture of CuSO<sub>4</sub>-TBTA ligand complex (50 μL of a 20 mM stock solution in H<sub>2</sub>O/DMSO/t-BuOH, 4:3:1 for TBTA; 50 μL of a 20 mM stock solution in H<sub>2</sub>O/DMSO/t-BuOH, 4:3:1 for CuSO<sub>4</sub>), tris(carboxyethyl)phosphine (TCEP; 50 μL of a 20 mM stock solution in water), 1,4-bis-azidomethylbenzene 4<sup>47</sup> (37.5 μL of a 20 mM stock solution in dioxane/H<sub>2</sub>O, 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 12 h. The reaction mixture was concentrated in a SpeedVac, dissolved in 1 mL bidistilled water, and centrifuged for 20 min at 12 000 rpm. The supernatant was collected and further purified by reversed-phase HPLC (gradient III) to give about 80% isolated yield of the azidomethylbenzyl oligonucleotides ODN-14–ODN-17

Scheme 1. Synthesis of the Double-Functionalized “Click” Conjugate 7



and ODN-19. For the preparation of ODN-17, 70  $\mu$ L of 1,4-bis-azidomethylbenzene 4 was used. The molecular masses of the azidomethylbenzyl oligonucleotides ODN-14–ODN-17 and ODN-19 were determined by MALDI-TOF or LC-ESI-TOF mass spectrometry (Table 2).

**“Second Click”: Synthesis of Branched Y-shaped Oligonucleotides BR-3–BR-8 from Azidomethylbenzyl Oligonucleotides ODN-14–ODN-17 and ODN-19 with Alkynylated Oligonucleotides ODN-8–ODN-13 and ODN-18.** To the alkynylated oligonucleotide (1.2  $A_{260}$  units) and azidomethylbenzyl oligonucleotide (2.8  $A_{260}$  units), a mixture of the CuSO<sub>4</sub>–TBTA ligand complex (25  $\mu$ L of a 20 mM stock solution in H<sub>2</sub>O/DMSO/t-BuOH, 4:3:1 for TBTA; 25  $\mu$ L of a 20 mM stock solution in H<sub>2</sub>O/DMSO/t-BuOH, 4:3:1 for CuSO<sub>4</sub>), tris(carboxyethyl)phosphine (TCEP; 25  $\mu$ L of a 20 mM stock solution in water), benzoic acid (10  $\mu$ L of a 100 mM stock solution in DMSO), and 30  $\mu$ L of DMSO were added, and the reaction proceeded at room temperature for 20 min. Then, sodium bicarbonate (100  $\mu$ L of a 200 mM aq. solution) was used to neutralize the excess of benzoic acid. The reaction mixture was concentrated in a SpeedVac, dissolved in 1 mL bidistilled water, and centrifuged for 20 min at 12 000 rpm. The supernatant was collected and further purified by reversed-phase HPLC (gradient III) to give the branched oligonucleotide. After purification by reversed-phase HPLC, the branched oligonucleotides BR-3–BR-8 were isolated in 50–60% yield. The molecular masses of branched BR-3–BR-8 were determined by MALDI-TOF or ESI-TOF mass spectrometry (Table 2).

## RESULTS AND DISCUSSION

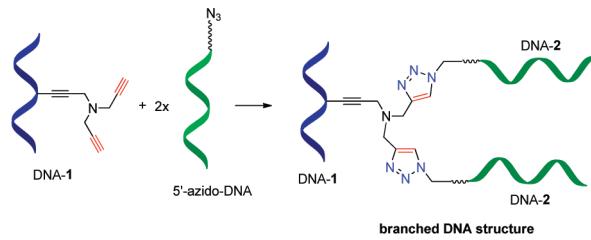
**Synthesis and Characterization of Monomers.** Recently, we have reported on the “stepwise click” reaction of modified oligonucleotides containing one terminal triple bond with the bis-azido compound 4.<sup>42</sup> Now, this protocol is applied to the construction of branched oligonucleotides. To evaluate the efficiency of the “double click” reaction as the crucial step (“first click”) in the “stepwise click” protocol, this reaction was first performed on monomeric nucleosides. For that, tripropargylated 8-aza-7-deaza-2'-deoxyguanosine (3)<sup>44</sup> and

bis-azido 4<sup>47</sup> were employed as starting materials. The “double click” reaction was performed on nucleoside 3 with bis-azido 4 in a mixture of THF/H<sub>2</sub>O/t-BuOH at r.t. using CuSO<sub>4</sub>·5H<sub>2</sub>O as catalyst and sodium ascorbate as reducing agent. The starting material 3 was consumed within 12 h. Under these conditions, double-functionalization occurred when the molar excess of the bis-azido 4 over nucleoside 3 was 10:1 (Scheme 1). We found that both terminal triple bonds were functionalized simultaneously by two bis-azido residues 4, even though they are space demanding. Formation of the monofunctionalized derivative 12 was not observed. “Double click” functionalization might result from the enhanced catalytic action of copper(I) being bound to the tripropargylamine side chain or to a monofunctionalized intermediate, thereby increasing the reaction rate for the “second click” reaction. The double-functionalized compound 7 was characterized by elemental analyses, as well as by <sup>1</sup>H and <sup>13</sup>C NMR spectra. The <sup>13</sup>C NMR chemical shifts are listed in the Supporting Information (Table S1). <sup>13</sup>C NMR chemical shift signals were assigned by <sup>1</sup>H–<sup>13</sup>C gated-decoupled spectra (Table S2, Supporting Information) as well as by DEPT-135 NMR spectra (Figure S16, Supporting Information).

**Construction and Base Pair Stability of Branched Oligonucleotides.** *Principles of Construction.* For the construction of branched oligonucleotides by the “click” reaction, triple bonds as well as azido groups have to be introduced into individual oligonucleotides. While triple bonds are compatible to solid-phase phosphoramidite chemistry, azido groups are prone to react with phosphites used in phosphoramidite chemistry (Staudinger reaction). Consequently, azido groups were introduced into oligonucleotides by postmodification. Our first concept for the construction of branched DNA comprises the preparation of monoazido functionalized oligonucleotides (azido-DNA) following a reported protocol.<sup>37,42</sup> The azido-DNA was employed in a subsequent “double click” reaction to yield branched DNA structures as illustrated in Scheme 2.

For our second concept, a combination of the “stepwise click” and “double click” reaction was used to generate azido-modified oligonucleotides. Two routes were evaluated: (i) the “stepwise click” reaction was performed on dendronized

**Scheme 2. Illustration of the First Concept for the Construction of Branched DNA Structures: DNA Conjugation of Dendronized DNA-1 with 5'-Azido-DNA**



tripropargylamine oligonucleotides (“double click”) with bis-azido-*p*-xylene (**4**) resulting in the formation of bis-functionalized azido-DNA carrying two azido groups at the branching position (Scheme 3, route 1). (ii) The triple bond of monoalkynylated oligonucleotides was functionalized with the bis-azido linker **4** to yield monoazido DNA (Scheme 3, route 2).<sup>37,42</sup> In both cases, “second click” reactions were performed with alkynylated oligonucleotides yielding branched DNA oligonucleotides.

**Construction of Branched Oligonucleotides from 5'-Azido-oligonucleotides and Oligonucleotides with Tripropargylamine Side Chains by the “Double Click” Reaction.** Following our first concept, 5'-alkylamino-modified oligonucleotides (ODN-1 and ODN-2) were synthesized from MMT (monomethoxytrityl) protected 5'-aminolink oligonucleotides by removal of the MMT protecting group (2.5% DCA/CH<sub>2</sub>Cl<sub>2</sub>). Following a literature protocol, the oligomers were treated with succinimidyl-5-azidovaleate **13**<sup>51</sup> in a mixture of DMSO/bicarbonate buffer (pH 8.7) to give the 5'-azidovaleate-functionalized oligonucleotides ODN-3 and ODN-4 (Scheme 4 and Table 1).<sup>37</sup> The starting oligonucleotides and the 5'-azido oligonucleotides were purified by reversed-phase HPLC and characterized by LC-ESI-TOF or MALDI-TOF mass spectrometry (Table 1). For additional details, see the Experimental section and the Supporting Information.

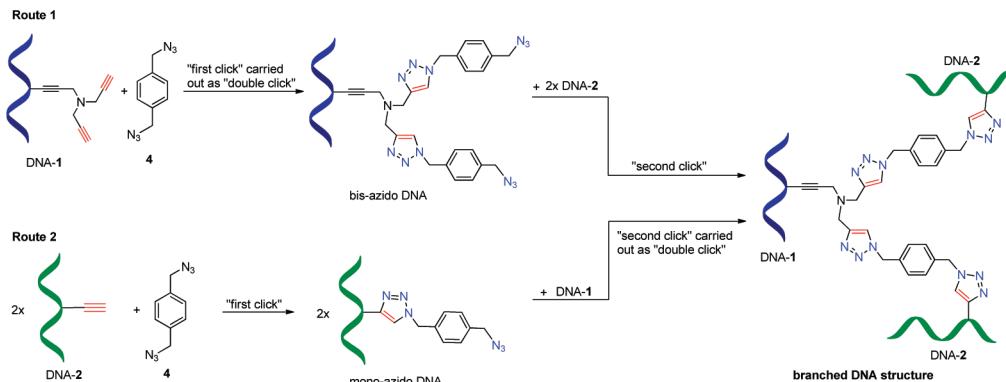
Next, the “double click” reaction was performed with 5'-azido-labeled oligonucleotide ODN-3 and dendronized ODN-5 bearing a tripropargylamine side chain which resulted in the selective formation of the three-armed (Y-shaped) oligonucleotide BR-1. The reaction was carried out at r.t. in a mixture of H<sub>2</sub>O/DMSO/*t*-BuOH employing a premixed 1:1 complex of CuSO<sub>4</sub>-TBTA (tris(benzyltriazoylethyl)amine), TCEP (tris(carboxyethyl)phosphine), and NaHCO<sub>3</sub>. The 5'-azido oligonucleotide ODN-3 was used in a slight excess (7:3) compared

to the tripropargylated ODN-5. By this, chromatographic purification was simplified as ODN-5 was completely consumed. The reaction proceeded smoothly, and a reaction time of 12 h was necessary to drive the reaction to completeness (Scheme 4). The branched BR-1 was obtained as the sole product confirmed by HPLC (Supporting Information, Figure S3c) and MALDI-TOF mass spectrometry (Table 1).

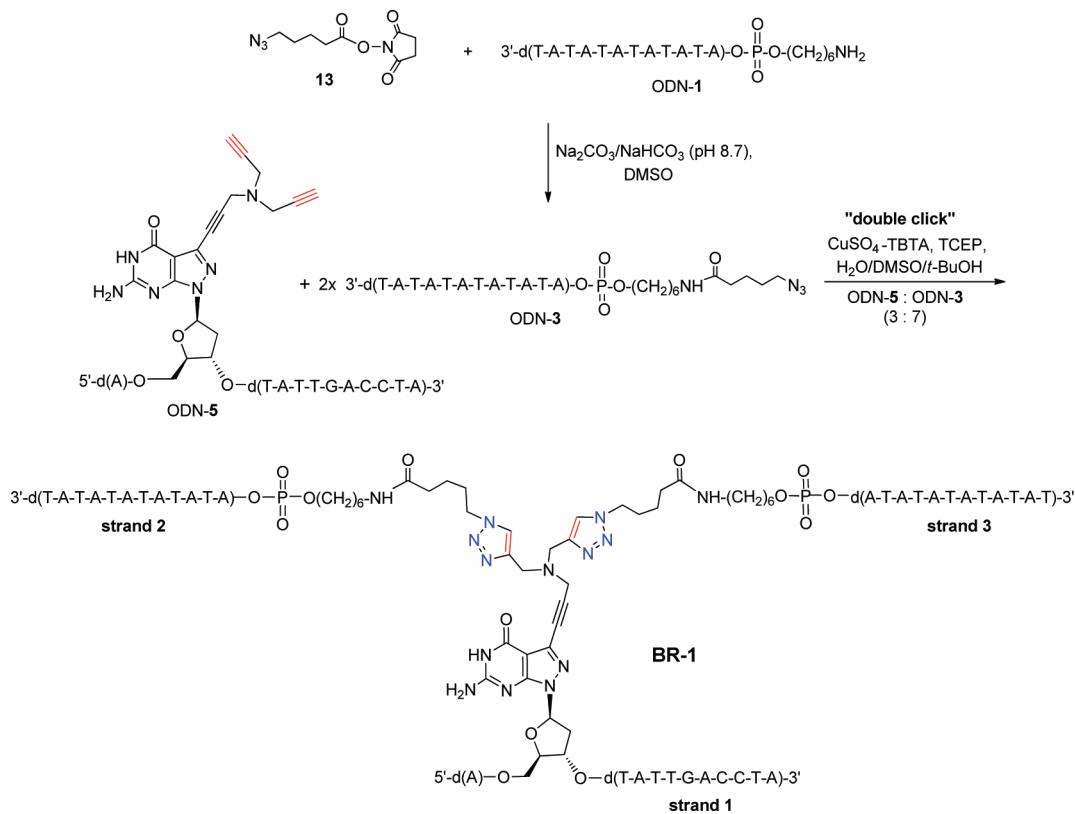
Recently, it was shown that the “click” reaction can be performed with high efficiency by the catalysis of carboxylic acids as demonstrated on small molecules.<sup>52</sup> Now, this method was applied for the synthesis of branched oligonucleotide BR-2. Benzoic acid was used as catalyst instead of an aq. NaHCO<sub>3</sub> solution for the “double click” reaction of ODN-5 containing a tripropargylamine side chain and two entities of 5'-azido-labeled oligonucleotide ODN-4. Compared to the synthesis of the Y-shaped branched BR-1 (NaHCO<sub>3</sub>), the reaction time for the synthesis of the Y-shaped BR-2 (benzoic acid) was now significantly shortened. The branched oligonucleotide BR-2 was characterized by RP-18 HPLC (Figure S3d, Supporting Information), and its integrity was confirmed by LC-ESI-TOF mass spectrometry (Table 1). Table 1 displays the structures of the synthesized oligonucleotides following the first concept for the construction of branched DNA and compiles the molecular masses obtained from mass spectrometry.

**Construction of Branched Oligonucleotides BR-3–BR-8 by a Combined “Stepwise Click” and “Double Click” Approach.** Next, the synthesis of a variety of three-armed branched DNA structures employing branching points at different positions and linkers of different length was performed following the protocol of a combined “stepwise click” and “double click” reaction according to our second concept. For that, oligonucleotides with short or long linkers and various linking positions were functionalized to monoazido intermediates. Two different routes were employed. Route 1 utilized ODN-8 bearing the 7-ethynylated 7-deaza-8-azaguanine nucleoside **1** and bis-azide **4** as starting material to afford the mono-functionalized “click” intermediate ODN-14 (Scheme 5). The ratio of **4** over ODN-8 was 15:1 and NaHCO<sub>3</sub> was used as catalyst (“first click”). In the “second click” reaction, azidomethylbenzyl ODN-14 and dendronized ODN-13 yielded branched oligonucleotide \*BR-3 employing benzoic acid as catalyst (Figure S4a–d, Supporting Information). Route 2 utilized tripropargylated ODN-13 as starting material together with a 30-fold excess of bis-azide **4** resulting in the formation of the double-functionalized dendron ODN-17 (NaHCO<sub>3</sub>, “first

**Scheme 3. Illustration of the Second Concept for the Construction of Branched DNA Structures: Combined “Stepwise Click” and “Double Click” Approach for the Construction of Branched DNA Structures via Two Different Routes**



**Scheme 4.** Synthesis of the Branched Y-Shaped Oligonucleotide BR-1 by “Double Click” Chemistry Using ODN-5 Containing a Tripropargylamine Side Chain and 5'-Azido-Labeled ODN-3



It is widely accepted that the click reaction proceeds with high efficiency;<sup>30</sup> thus, it is predestinated to be employed in the construction of dendronic oligonucleotides. Nevertheless, by serendipity, we observed formation of a side product (BR-3b) during the click reaction of dendron ODN-13 with an additional mass of +3965 Da compared to the main product (LC-ESI-TOF). This compound resulted from an incomplete “double click” reaction of ODN-13 with azide 4 yielding a monofunctionalized adduct with one azido group and one free triple bond. A “bis-click” reaction occurring during the “first click” reaction leads to formation of BR-3a, while the “second click” of BR-3a and two ODN-8 entities affords the four-armed branched BR-3b.

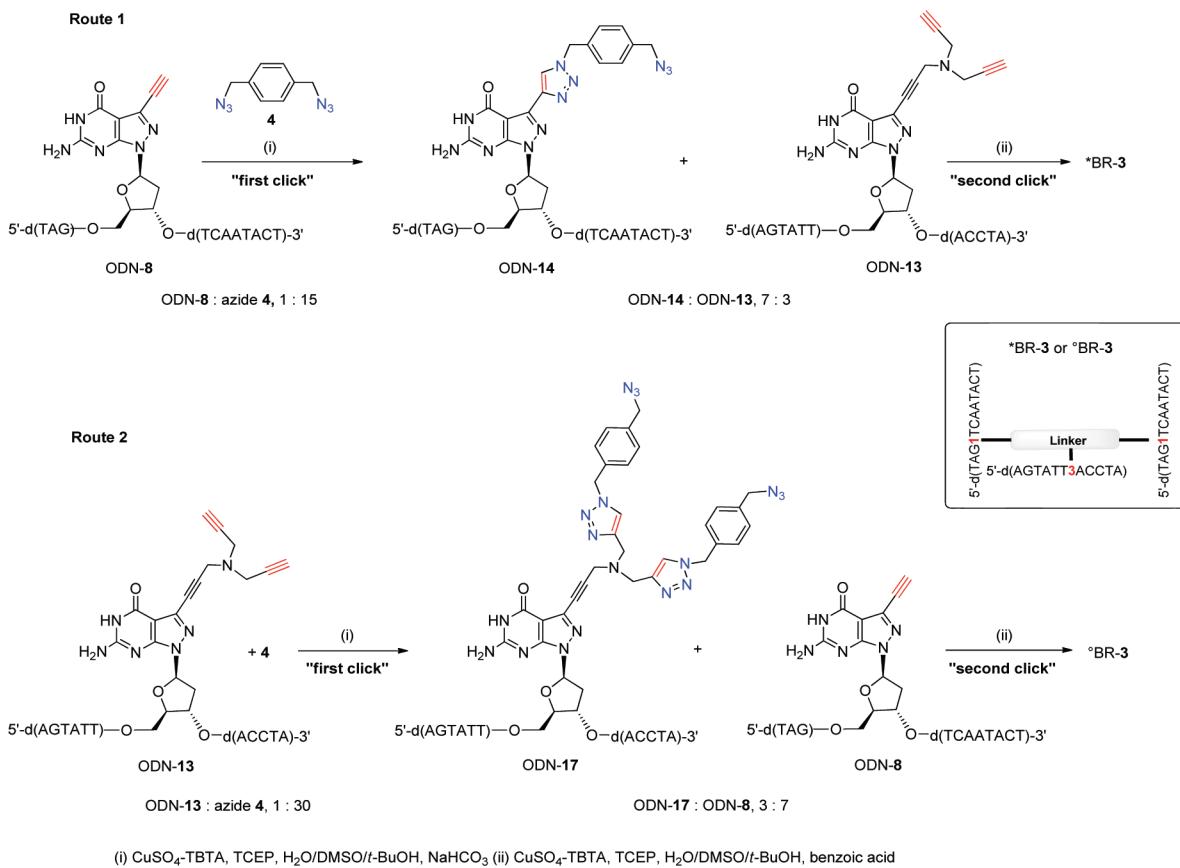
However, functionalization of only one triple bond ( $\rightarrow$  compound 12, Scheme 1) was not observed on the nucleoside level nor in many other oligonucleotide examples. We assume that, during the reaction illustrated in Scheme 6, the amount of Cu(I) present in the reaction mixture was not sufficient to drive the reaction to completeness, probably resulting from the oxidation of Cu(I) to Cu(II) during the reaction.

Following route 1 or 2 shown in Scheme 5 and using a large excess of 4, a series of branched oligonucleotides (BR-3–BR-8) was synthesized. The linker within the oligonucleotide chains were changed in length and position. The branched oligonucleotides show the following structural features: (i) one branched linker was combined with two short linkers or (ii) one branched linker was combined with two long linkers. The linking positions were placed at central or terminal positions of the oligonucleotides. Purification of the crude

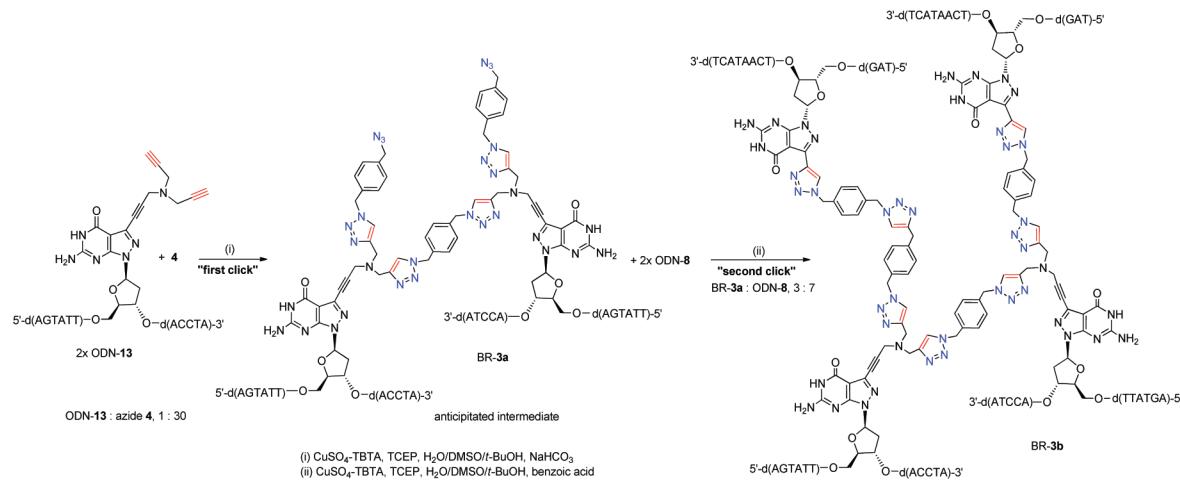
products was performed by reversed-phase HPLC (Figures S5–6, Supporting Information), and LC-ESI-TOF or MALDI-TOF mass spectrometry were used for characterization (Table 2). The structures of the branched oligonucleotides BR-3–BR-8 as well as the alkynylated starting oligonucleotides (ODN-8–ODN-13, ODN-18<sup>53</sup>) and the azido-functionalized intermediates ODN-14–ODN-17 and ODN-19 are shown in Table 2.

**Characterization of Branched Oligonucleotides by Ion-Exchange Chromatography and PAGE.** The purified (RP-18 HPLC chromatography) branched oligonucleotides were characterized by ion-exchange chromatography and denaturing polyacrylamide gel electrophoresis (PAGE). As it cannot be excluded that an individual peak isolated during RP-18 chromatography contains more than one compound, we used ion-exchange chromatography to distinguish between compounds by their different number of negative charges and to proof their purity. Oligonucleotides ODN-8, ODN-13, and ODN-14 (starting materials), as well as the branched oligonucleotide BR-3, were characterized by this method as demonstrated in Figure 3. Other examples are shown in the Supporting Information (Figure S7). Figure 3a,b displays the elution profiles of the starting materials, and Figure 3c shows the elution profile of the branched oligonucleotide BR-3. From these profiles, it is apparent that BR-3 (36 negative charges) migrates significantly more slowly than the starting materials ODN-8, ODN-13, and ODN-14 (12 negative charges). An ion-exchange profile of an artificial mixture of all three components is shown in Figure 3d. In this experiment, the complementary oligonucleotides ODN-13 and ODN-14 form a duplex. Only the excess of dendritic ODN-13 gives a separate peak. The duplex ODN-13·ODN-14 migrates between ODN-13 and BR-3.

**Scheme 5. Formation of Branched Oligonucleotides by a Combined “Stepwise Click” and “Double Click” Approach Using ODN-8 (Short Linker) or Dendronic ODN-13 (Branched Linker) in the “First Click” and ODN-14 (Short Linker) or the Dendronic ODN-17 (Branched Linker) in the “Second Click” Reaction**

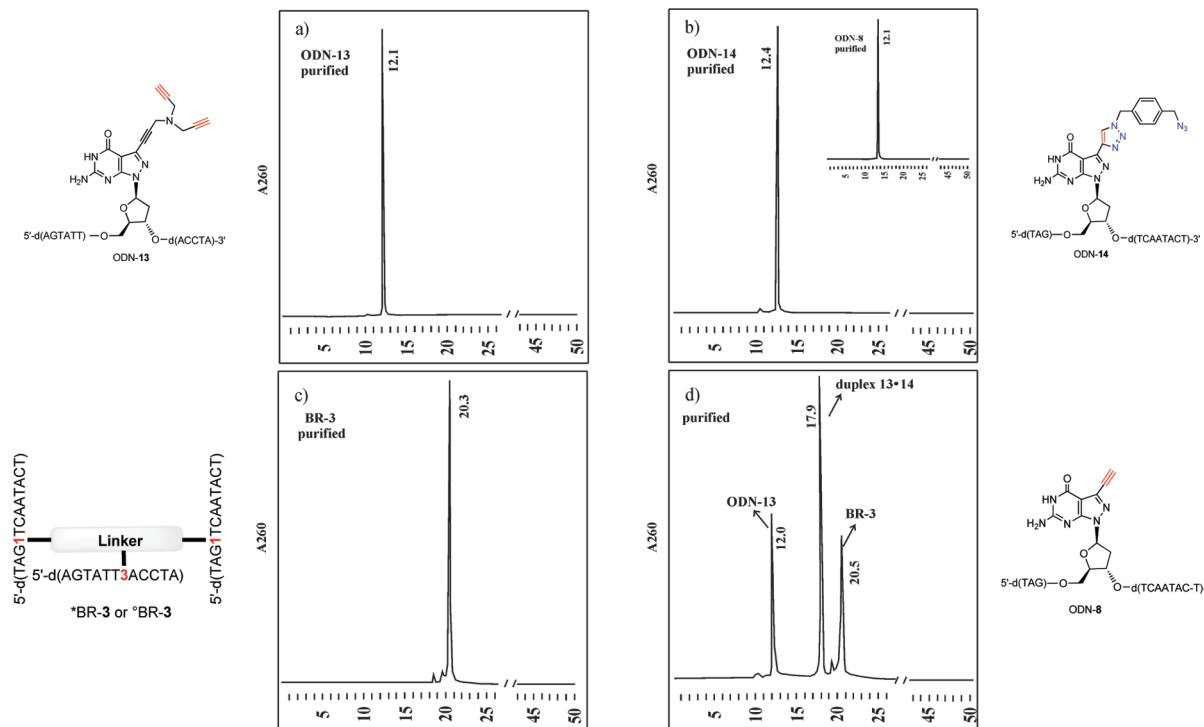


**Scheme 6. “Bis-Click” Reaction of Two ODN-13 Entities during the “First Click” Reaction Followed by the Reaction of Bis-Functionalized BR-3a with Two ODN-8 Entities in the “Second Click” Reaction Affording the Four-Armed Branched BR-3b**

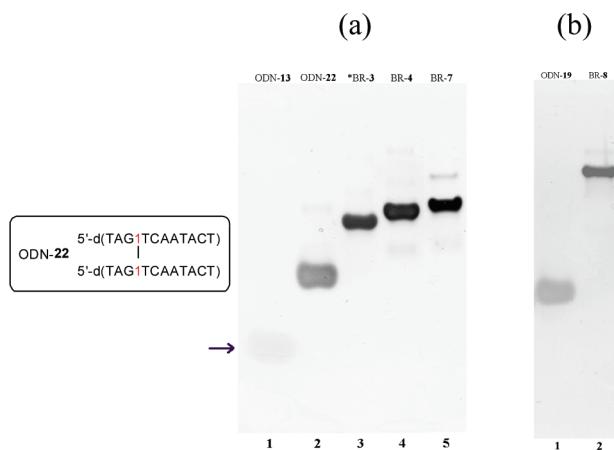


Next, the electrophoretic mobility analysis of nonbranched oligonucleotides utilized as starting materials and branched oligonucleotides obtained after “click reactions” following our first or second concept was performed by denaturing PAGE. Representative examples are shown in Figure 4 (for additional examples, see Figure S8, Supporting Information). Figure 4a depicts the relative mobilities of dendronic ODN-13 (12-mer, lane 1), interstrand cross-linked ODN-22<sup>42</sup> as comparison (24-mer, lane 2) and the branched oligonucleotides \*BR-3 (36-mer,

lane 3), BR-4 (36-mer, lane 4), and BR-7 (38-mer, lane 5). On the basis of the different number of nucleotides (38-mers compared to 36-mers) and the different flexibility or rigidity of the linker arms, BR-7 is migrating slower than \*BR-3 and BR-4 (36-mers each). The slightly different migration velocity of \*BR-3 and BR-4 can be attributed to intramolecular duplex formation of \*BR-3, which is not possible in the case of BR-4 (see Table 3). The PAGE analysis of branched BR-8 (60-mer, line 3), as well as the azidomethylbenzyl-labeled ODN-19



**Figure 3.** Ion-exchange HPLC elution profiles of (a) ODN-13, (b) the starting material ODN-8 and azidomethylbenzyl-labeled ODN-14, (c) the branched oligonucleotide BR-3, (d) an artificial mixture of ODN-13 (excess over complementary ODN-14), ODN-14, and BR-3 on a 4 × 250 mm DNA PA-100 column using the following buffer system: (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. Elution gradient IV: 0–30 min 20–80% D in C with a flow rate of 0.75 mL min<sup>-1</sup>.



**Figure 4.** Denaturing PAGE analysis of oligonucleotides on a 17% polyacrylamide/7 M urea gel. (a) Lane 1, 12-mer ODN-13; lane 2, 24-mer cross-linked ODN-22;<sup>42</sup> lane 3, 36-mer branched \*BR-3; lane 4, 36-mer branched BR-4; and lane 5, 38-mer branched BR-7. (b) Lane 1, 24-mer ODN-19; and lane 2, 60-mer branched BR-8.

(24-mer, lane 2), is shown in Figure 4b. Apart from its branched structure, BR-8 comprises an additional unique feature, as two of its branches were designed to form hairpin structures.<sup>53</sup>

**Chain Assembly and Base Pairing of Branched Oligonucleotides.** Branched oligonucleotides are expected to form multistranded assemblies upon hybridization with complementary strands. To evaluate the type of assembly, *T<sub>m</sub>* measurements were performed, and the results were summarized in Table 3. Incorporation of the tripropargylamine nucleoside **3** resulted in a duplex with a slightly increased *T<sub>m</sub>* value (**5·7**). The self-complementary 5'-azido-labeled ODN-3 showed a

similar *T<sub>m</sub>* value as the parent unmodified duplex 5'-d[(A-T)<sub>6</sub>]<sub>2</sub>.<sup>54</sup> Next, the assembly of branched oligonucleotides was studied. For branched BR-1 containing two arms with the identical self-complementary 5'-d(A-T)<sub>6</sub> sequence, a *T<sub>m</sub>* value of 35.5 °C is observed. This molecule can only form dA-dT base pairs, but several hybridization patterns are possible, affording different types of assemblies. Some examples are outlined in Figure 5. The intramolecular monomeric motif (I) requires a parallel orientation of the 5'-d(A-T) arms, while in motifs (II) and (III), dimeric and multimeric assemblies with antiparallel chain orientation are shown. We anticipate that an extended network-like assembly is formed, which, however, remains to be proven.

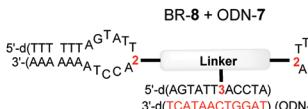
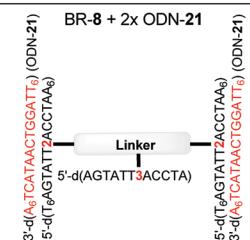
Interestingly, when we added ODN-7 to BR-1, a higher *T<sub>m</sub>* value was obtained (48.5 °C) indicating that ODN-7 hybridized to its complementary arm in BR-1. It was not possible to determine a lower *T<sub>m</sub>* value in the range 32–35 °C. Probably, the lower *T<sub>m</sub>* melting profile was superimposed by the high *T<sub>m</sub>* melting profile.

The branched oligonucleotides BR-6 and BR-7 are closely related to BR-1, but were constructed following our second concept of combined “stepwise click” and “double click” reactions. Also, the 5'-d(A-T)<sub>6</sub> arms of BR-6 and BR-7 can form only dA-dT base pairs. The somewhat lower *T<sub>m</sub>* values of BR-6 (28.5 °C) and BR-7 (26.0 °C) compared to BR-1 might result from the different linker types. Exemplarily, nondenaturing PAGE was performed with BR-7 (Figure 6), indicating that different assemblies are formed consisting of presumably 72 nucleosides (2 molecules) or 108 nucleosides (3 molecules). After addition of ODN-7 to BR-6 or BR-7, *T<sub>m</sub>* values of 37 °C were observed indicating that ODN-7 formed a duplex with the remaining complementary chains of BR-6 or BR-7 as shown in Table 3.

Table 3.  $T_m$  Values of DNA Duplexes, Hairpins, and Branched Oligonucleotide Assemblies

Hybridization of oligonucleotides	$T_m^{a,b}$ [°C]	Hybridization of oligonucleotides	$T_m^{a,b}$ [°C]
5'-d(AGTATTGACCTA) (ODN-6) 3'-d(TCATAACTGGAT) (ODN-7)	49.0	5'-d(A <sup>3</sup> TATTG ACCTA) (ODN-5) 3'-d(TCATAACTGGAT) (ODN-7)	54.0 <sup>44</sup>
		5'-[d(A-T) <sub>6</sub> ] <sup>c</sup>	32.0 <sup>54</sup>
5'-d( <b>5</b> ATATATATATATAT) (ODN-15) 3'-d(TATATATATATA <b>5</b> ) (ODN-15)	28.0	5'-d( <b>6</b> ATATATATATAT) (ODN-16) 3'-d(TATATATATATA <b>6</b> ) (ODN-16)	29.5
<p style="text-align: center;">ODN-3 + ODN-3</p>		<p style="text-align: center;">BR-1</p>	
<p style="text-align: center;">BR-1</p>		<p style="text-align: center;">BR-1 + ODN-7</p>	
<p style="text-align: center;">BR-6</p>		<p style="text-align: center;">BR-6 + ODN-7</p>	
<p style="text-align: center;">BR-7</p>		<p style="text-align: center;">BR-7 + ODN-7</p>	
<p style="text-align: center;">BR-4 + 3x ODN-7</p>		<p style="text-align: center;">BR-5 + 3x ODN-7</p>	
<p style="text-align: center;">BR-3</p>		<p style="text-align: center;">BR-3 + ODN-6</p>	
<p style="text-align: center;">ODN-20</p>		<p style="text-align: center;">ODN-18</p>	
<p style="text-align: center;">ODN-19</p>		<p style="text-align: center;">+ ODN-7</p>	
<p style="text-align: center;">ODN-18 3'-d(A<sub>6</sub>TCATAACTGGATT<sub>6</sub>) (ODN-21)</p>		<p style="text-align: center;">BR-8</p>	
<p style="text-align: center;">ODN-19 3'-d(A<sub>6</sub>TCATAACTGGATT<sub>6</sub>) (ODN-21)</p>		<p style="text-align: center;">BR-8</p>	

Table 3. continued

Hybridization of oligonucleotides	$T_m^{a,b}$ [°C]	Hybridization of oligonucleotides	$T_m^{a,b}$ [°C]
BR-8 + ODN-7 	53.5	BR-8 + 2x ODN-21 	61.5 <sup>e</sup>

<sup>a</sup>Measured at 260 nm in a 1 M NaCl solution containing 100 mM MgCl<sub>2</sub> and 60 mM Na-cacodylate (pH 7.0). <sup>b</sup>The concentration of nonbranched oligonucleotides and branched compounds was 2 μM each. <sup>c</sup>The nonbranched oligonucleotide concentration was 6 μM. <sup>d</sup>For possible hybridization pattern of BR-1, see Figure 5. <sup>e</sup>The nonbranched oligonucleotide concentration was 4 μM. For linker details see Tables 1 and 2.

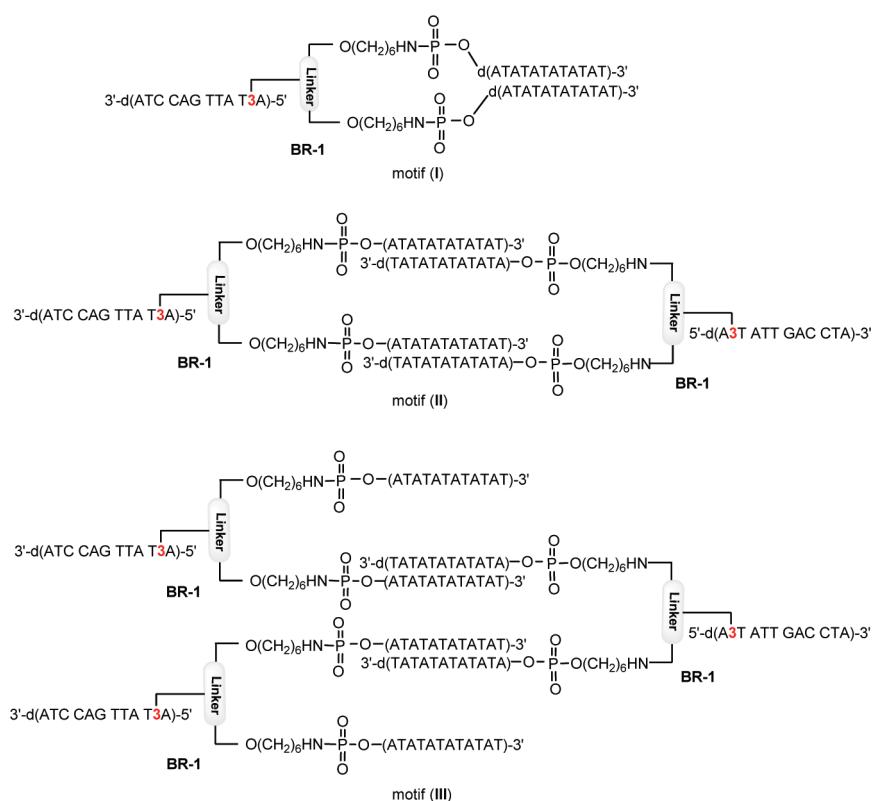


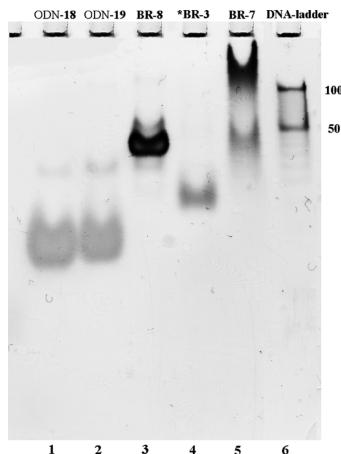
Figure 5. Possible hybridization motifs for the 5'-d(A-T) arms of BR-1: (i) intramolecular parallel duplex formation, (ii) bimolecular antiparallel duplex formation of two BR-1 residues, and (iii) multistranded intermolecular network formation by several BR-1 residues.

The branched oligonucleotides BR-4 and BR-5 were composed of noncomplementary 12-mer sequences. By adding complementary ODN-7, each arm of BR-4 and BR-5 is supposed to form a duplex. However,  $T_m$  values of about 40 °C were detected, while the closely related parent duplex 6·7 shows a  $T_m$  value of 49.0 °C. Apparently, duplex stability is reduced by the linker units situated at central positions of each duplex. This finding correlates with the observation that the destabilization is less pronounced for the long linker in BR-5 ( $\Delta T_m = -4.5$  °C) compared to the short linker in BR-4 ( $\Delta T_m = -11.0$  °C).

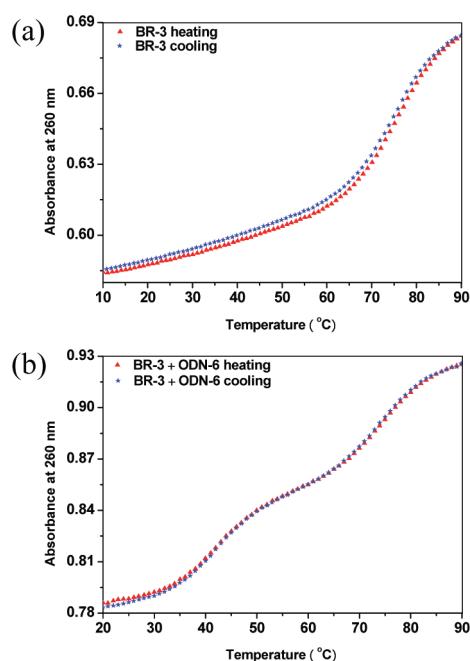
In contrast, branched oligonucleotide BR-3 forms a highly stable intramolecular duplex ( $T_m = 74.0$  °C; for melting profile, see Figure 7a). Pronounced duplex stabilization caused by cross-linking has previously been reported.<sup>41,42,55–57</sup> Native

PAGE supported that no intermolecular network formation took place (Figure 6, lane 4). When ODN-6 was added to BR-3, a biphasic melting was observed as shown in Figure 7b. Now, two duplexes are formed. The higher  $T_m$  value (74.0 °C) can be attributed to the cross-linked duplex, while the newly formed duplex gives the lower  $T_m$  value of 39.5 °C.

The branched oligonucleotide BR-8 contains an additional unique feature, as two of its branches were designed to form a hairpin structure.<sup>53</sup> Alkynylated ODN-18<sup>53</sup> and azido-functionalized ODN-19 as well as the parent unmodified ODN-20 form hairpin structures with  $T_m$  values in the range 57–59 °C (Table 3 and Table S3, Supporting Information). The addition of ODN-7 being complementary to the loop region of ODN-18–ODN-20 did not impair the hairpin structures as demonstrated by the unchanged  $T_m$  values. However, the hairpins were



**Figure 6.** Nondenaturing PAGE analysis of oligonucleotides on a 15% polyacrylamide gel: lane 1, 24-mer ODN-18;<sup>53</sup> lane 2, azido-functionalized 24-mer ODN-19; lane 3, 60-mer branched BR-8; lane 4, 36-mer branched \*BR-3; lane 5, 38-mer branched BR-7; and lane 6, 50-mer and 100-mer DNA ladder.



**Figure 7.** Melting profiles obtained from heating (red triangles) and cooling (blue stars) experiments monitored at 260 nm in 1 M NaCl, 100 mM MgCl<sub>2</sub>, and 60 mM Na-cacodylate, pH 7.0, with 2 μM oligonucleotide concentration for (a) BR-3 and (b) with 2 μM + 2 μM single-strand concentration for BR-3 and ODN-6.

opened upon addition of the fully complementary oligonucleotide ODN-21 ( $T_m$  values around 62–64 °C). Accordingly, results were obtained for the branched BR-8 (56.0 °C) and for the combination of BR-8 with ODN-7 (53.5 °C) and ODN-21 (61.5 °C). The  $T_m$  value of BR-8 with ODN-7 (53.5 °C) is somewhat lower than for the individual BR-8 (56.0 °C), which can be attributed to the overlap of a lower melting profile of the duplex part and the higher melting profile of the hairpins.

As shown in Figure 6, ODN-18,<sup>53</sup> ODN-19, and the branched product BR-8 were also analyzed by nondenaturing PAGE. As expected, only single bands were obtained, demonstrating that no higher aggregates were formed in all three cases.

This study demonstrates the versatility of the combined “stepwise and double click” chemistry approach to construct well-defined branched oligonucleotides including dendronized molecules. This protocol is applicable to most different sequence motifs and was herein exemplified for self-complementary and nonself-complementary DNA chains as well as for hairpin forming oligonucleotides.

## CONCLUSION AND OUTLOOK

Dendronized oligonucleotides decorated with 7-tripropargylamine side chains carrying two terminal triple bonds were synthesized. Further functionalization with bis-azides yielded conjugates with two terminal azido groups. Both types of dendronized oligonucleotides were applied to the Huisgen-Meldal-Sharpless cycloaddition using “stepwise and double click” chemistry thereby affording hyperbranched (Y-shaped) three-armed DNA. Annealing of hyperbranched DNAs with complementary oligonucleotides resulted in supramolecular assemblies. The CuAAC chemistry used in this study shows the necessary efficiency to make dendronized DNA accessible which would be extremely difficult to be synthesized by other means. As tripropargylated phosphoramidites of all four building blocks of DNA have been reported,<sup>43–46</sup> a variety of branched oligonucleotides differing in linking position, linking number, or base pairing are now available. Dendronized DNA gives rise to a number of interesting properties which cannot be realized by linear biopolymers. Dendronized DNA can be utilized to stabilize hydrogels after cross-linking alkynyl chain with bifunctional azides. Dendronized DNA can be used for the signal amplification in DNA detection and quantification.<sup>12,58</sup> The incorporation of photoresponsive units<sup>59</sup> in dendronized DNA offers the possibility of creating light-sensitive DNA devices. Consequently, the concept of a combined “stepwise and double click” chemistry followed by selective hybridization expands the application of DNA conjugates to DNA diagnostics, delivery, and nanobiotechnology.

## ASSOCIATED CONTENT

### Supporting Information

<sup>13</sup>C NMR chemical shifts, <sup>1</sup>H–<sup>13</sup>C NMR coupling constants, synthetic procedure for **13**, HPLC purification profiles of nonbranched and branched oligonucleotides, ion-exchange HPLC profiles of nonbranched and branched oligonucleotides, denaturing PAGE analysis of oligonucleotides, additional  $T_m$  values of hairpin forming oligonucleotides, melting profiles, <sup>1</sup>H NMR, <sup>13</sup>C NMR, DEPT-135 spectra, and <sup>1</sup>H–<sup>13</sup>C-gated decoupled NMR spectra of the new compounds. 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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