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Graphical Abstract

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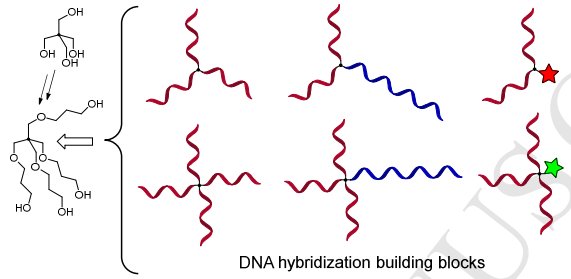

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Tetrahedral DNA conjugates from pentaerythritol-based polyazides

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ABSTRACT

Branching points in DNA nanostructures are usually 3- or 4-way junctions maintained by Watson-Crick non-covalent interactions. However, covalently bound DNA stars could improve the diversity, strength and integrity of DNA nanoscale constructions. We report here the convenient synthesis of three- and four-fold pentaerythritol-based azides and their use for the assembly of branched conjugates containing the same or different oligonucleotides (ODNs) and/or fluorescent dyes by stoichiometry controlled copper (I) catalyzed azide alkyne cycloaddition (CuAAC) functionalization.

1. Introduction

The concept of manipulating DNA by simply programming its strands has been widely used during recent decades with the possibility of designing nanostructures of desired shapes driving a tremendous development of DNA nanotechnology and DNA computing.[1] The structural design of DNA-based logical gates/circuits or dendrimers coupled with various fluorophores becomes more and more complicated, opening up wide opportunities for computation and programming as well as signal amplification for biomedical detection.[1] Diverse and complex three-dimensional DNA structures were assembled and used for capture and controlled release of therapeutic agents.[2]

In this aspect, the necessity of branched building blocks emerges. The approaches to multistrand DNA ligation can be divided into two large subgroups: 1) non-covalent, implying Watson-Crick, Hoogsteen and other weak interactions, and 2) covalent junctions at specific branching point. The first approach, used for the vast majority of DNA nanostructures, relies predominantly on 'immobile' 3- or 4-way Holliday junctions,[3] also referred to as 'Y-shaped' and 'X-shaped' DNA. Much less attention is paid to designing covalent junctions assembling multiple DNA strands at a branching point. Covalently bound oligonucleotides remain together in denaturing conditions. The known approaches to covalent branched DNA conjugates predominantly used non-nucleoside,[4] nucleoside[5] or hybrid[6] branching phosphoramidite reagents or branched supports.[7] Alternatively, nucleoside[8] or non-nucleoside[9] bis-phosphoramidites were used for joining growing oligonucleotide chains. Nevertheless, direct automated synthesis of branched DNA building blocks is complicated by restrictions coming from the chemistry of phosphoramidite synthetic cycle. A post-synthetic approach is another way of assembling branched oligonucleotide structures; e.g. Richert and coworkers coupled protected dinucleoside-3′-phosphonate with adamantane-based branchers.[10] At the same time, the use of azide-alkyne click chemistry,[11] proved to be much more reliable for the preparation of complex and branched oligonucleotide-oligonucleotide conjugates.[12] However, employed organic polyazides were not always 'user friendly'. A planar porphyrin-based tetra-azide[12n] gave low yield of click products and HPLC-inseparable regioisomers of bis-adducts. The *tetrakis*(4-azidophenyl) methane[12o,p] used as 'branching point' has very short phenylene linkers, is hydrophobic and therefore gave no desired tetra-adduct upon 'click' in solution. Moreover, arylazides are potentially photoreactive upon visible light irradiation. To the best of our knowledge, no controllable and facile preparative technique for

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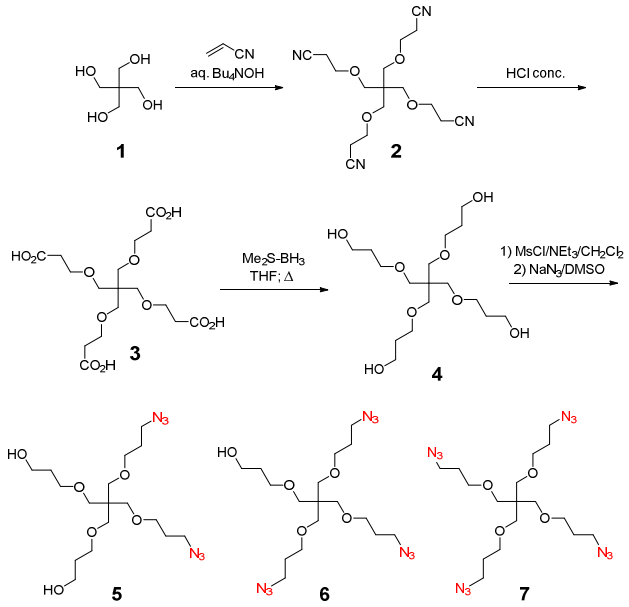
robust connection of different DNA strands to s single branching point has been developed to date.

2. Results and discussion

Herein we report synthesis of pentaerythritol (**1**)-based branching reagents **6** and **7** which carry three and four azido-groups, respectively (Scheme 1). The key tetraol **4** was obtained through the modified three-step synthetic procedure[13] (see Experimental section).[14]



Scheme 1. Synthesis of azide-containing branching reagents.

The alcohol **4** was mesylated in DCM using 3.0 eq. of mesyl chloride and then treated with an excess of sodium azide in DMSO to give mixture of azides **5**, **6** and **7** (Scheme 1). Column chromatography separation on silica gel provided pure compounds **6** (29%) and **7** (26%) together with diazide **5** (8%). In contrast, the excessive mesylation of tetraol **4** followed by nucleophilic substitution[13b] gave tetraazide **7** in 90% yield.[15]

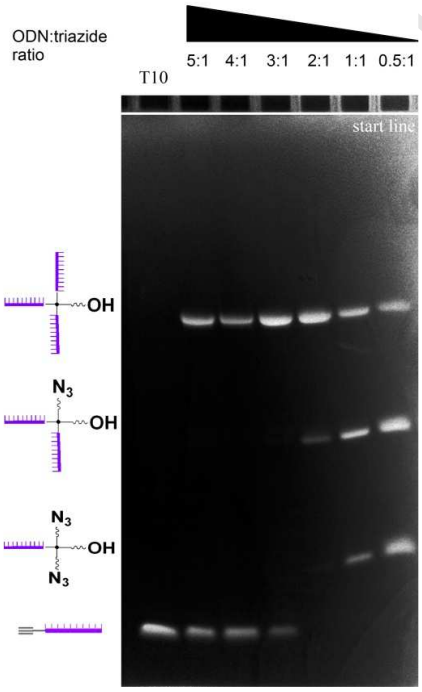


Fig. 1. Conjugation of T_{10} ODN alkyne on triazide **6** in different ratios; 19% PAGE.

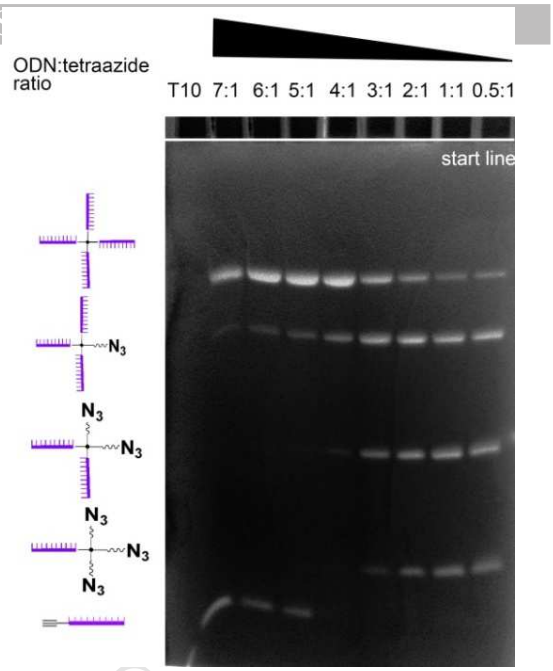


Fig. 2. Conjugation of T_{10} ODN alkyne on tetraazide **7** in different ratios; 19% PAGE.

Azides **6** and **7** easily undergo CuAAC click reaction with alkyne-modified oligonucleotides in aqueous solution. The stoichiometry of the resulting building blocks can be tuned by varying the excess of ODNs in click-reaction with bubsequent HPLC or electrophoretic separation. In this paper we demonstrate the unlimited potential of 'asymmetric' building block fabrication using two different oligonucleotides and fluorescent dyes.

First, click conditions were studied using T_{10} as a model oligonucleotide (ODN). Conjugation of alkyne-modified oligonucleotide to azide branching points resulted in a pool of products. The CuAAC reactions with various excesses of ODN were carried out for both branching points and analyzed in 19% PAGE (Figures 1, 2).

The products migrate according to the number of oligonucleotides attached to the branched backbone: the more oligo units the block contains, the more slowly it moves in the gel. As seen from gel images, the tris or tetrakis product is formed at any ODN:'branching point' ratio. Tri-product from triazide **6** dominates in the reaction mixture starting from 2:1 ratio and becomes the only product at ratio 3:1 and higher. Tetra-product from tetraazide **7** becomes the major product at 4:1 ratio. However, trace amounts of tri-product are always present, even at higher excesses of ODN.

The isolated yields of products from triazide **6** (2:1 ratio) after elution from the gel were following: 12% tri-product, 11% di-product, 5% mono-product, and for tetraazide **7** (3:1 ratio): 8% tetra product, 11% tri-product, 9% di-product, 3% mono-product. Since the yields of elution from the gel vary depending on fragment length from <30% up to >90%,[16] the majority of every product is lost on this step. We suggest that, an electroelution technique providing high oligonucleotide recovery (>85%)[16b] should be used for preparative purposes.

Partial conjugation of ODNs on branching points is more attractive than full attachment of ODNs on all azido groups available. The possibility of further modification of residual azido-groups then leads to fabrication of 'asymmetric' blocks.

By lowering the ODN:polyazide ratio from 5 to 2 for **6** and from 7 to 3 for **7**, the formation of products with one or more intact azido-groups is achieved. The separation of reaction mixture can be achieved using HPLC (Figures 3 and 4) with significantly higher isolated yields than in PAGE procedure: 32% tri-product, 52% di-product, 16% mono-product from triazide **6** (2:1 ratio) and 25% tetra-product, 42% tri-product, 28% di-product, 5% mono-product from tetraazide **7** (3:1 ratio). Each HPLC peak and PAGE band were isolated and their compositions were confirmed by LC-MS (see Supporting Information). The procedure is well suitable for oligonucleotides of any sequence (e.g., see Supporting Information for the mass spectrum of conjugate containing three 21-mers D11 and one azido group).

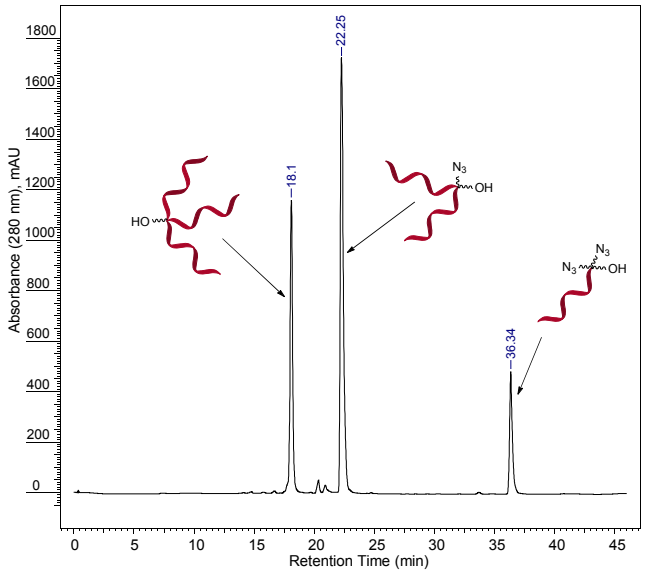


Fig. 3. HPLC profile of partial functionalization of triazide branching point **6** with T_{10} ODN in ratio 1:2.

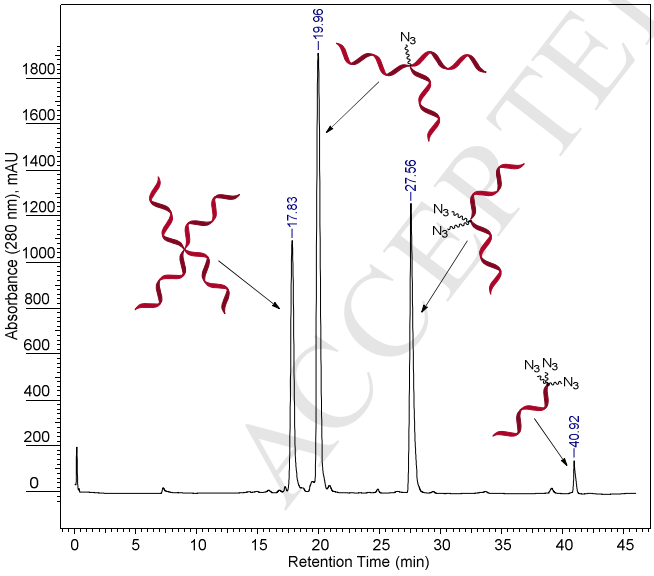
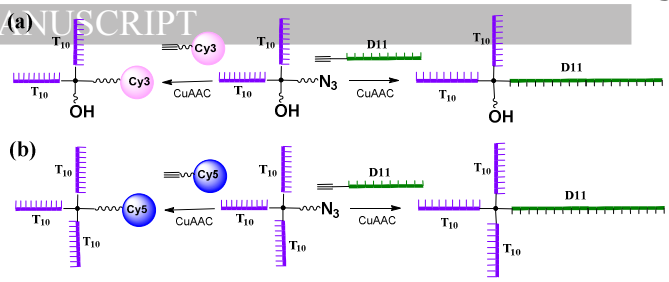


Fig. 4. HPLC profile of partial functionalization of tetraazide branching point **7** with T_{10} ODN in ratio 1:3.

The presence of loose azido-group(s) opens up wide opportunities for the synthesis of 'asymmetric' building blocks based on the branched azide structure (Scheme 2). The model experiments showing that azido-groups are capable of further conjugation after first CuAAC reaction and HPLC isolation were performed with fluorescent Cy5 and Cy3 alkyne derivatives. The reaction gave high yields of dye conjugates.



Scheme 2. Further modification of partially functionalized building blocks based on triazide **6** (a) and tetraazide **7** (b): preparation of [2+1] and [3+1] conjugates.

Similar experiments were performed for attachment of alkyne-modified ODN with different sequences to intact azido groups of branched T_{10} blocks. The blocks carrying a single intact azido group reacted with 3-fold excess of 5′-alkyne-modified oligonucleotide GGTCGCTTATCTGCACTCGGA (D11) in the same conditions as in synthesis of initial blocks (Scheme 2). The study of reaction products was carried out in 15% PAGE (Figure 5). The formation of the 'asymmetric' block is proved by the appearance of a new band migrating more slowly than all others.

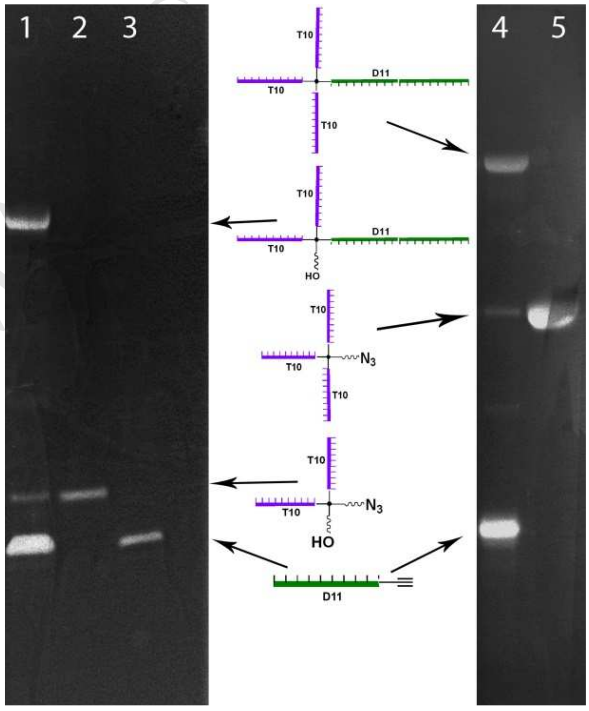


Fig. 5. Fabrication of asymmetric blocks by conjugation of D11 ODN on intact azide groups of T_{10} blocks studied in 15% PAGE: click of 21-mer ODN D11 on azide group of di-product from triazide **6** (1), initial di-product (2), initial D11 (3), click of D11 on azide group of tri-product from tetraazide **7** (4), initial tri-product (5).

All blocks including the resulting asymmetric and initial ones can be well separated in gel and purified for further use. The estimated yields of asymmetric blocks are 29% for di-product from triazide **6** and 9% for tri-product from tetraazide **7** after elution from the gel.

The conjugates have a tetrahedral branching point and rather long and flexible linkers making oligonucleotides well accessible for hybridization. Our preliminary data evidences that building blocks comprised of four, three, or two ODNs covalently joined to one branching point are capable of self-assembly into simple discrete structures and can act as PCR primers for the synthesis of long branched DNA (the results will be reported elsewhere).

The ability to control stoichiometry of building block opens up great perspectives for 'asymmetric' building blocks synthesis and assembly into diverse functional nanoconstructions. The intermediate azide-containing derivatives, as well as cyanine dye-labelled products, can be easily isolated and purified by HPLC. In contrast, PAGE is the method of choice for isolation of oligonucleotide–oligonucleotide conjugates, e.g. [2+1] and [3+1].

3. Conclusions

To conclude, we report the synthesis of two polyazide 'branching points' **6** and **7** from pentaerythritol and the approach to fabrication of branched building blocks comprised from one to four oligonucleotides by means of CuAAC reaction. 'Asymmetric' [2+1] and [3+1] conjugates were prepared via further modification of products isolated from partial functionalization by conjugating a different oligonucleotide or Cy3 and Cy5 dye alkynes on residual azido-groups. The products are easily isolated using HPLC or gel electrophoresis. The combined blocks reported herein could appear useful for rapidly developing DNA nanotechnology as building blocks for nanoscale objects self-assembly, staples for DNA origami etc.

4. Experimental section

4.1. General methods

DMSO was used freshly distilled from CaH_2 under reduced pressure. Dichloromethane, chloroform and acetonitrile were used freshly distilled from CaH_2. All other solvents (hexane, ethyl acetate, ethanol, acetone) were purified by distillation. Tetrahydrofuran was absolutized by distillation with sodium-benzophenone ketyl. Pentaerythritol (98%) was purchased from Sigma-Aldrich; methanesulfonyl chloride and tetrabutylammonium hydroxide (40% aq.) were from Fluka. 500 MHz ^{1}H and 125.7 MHz ^{13}C NMR spectra were recorded on a Bruker DRX-500 or Bruker Avance 500 spectrometers and referenced to DMSO-d_6 (2.50 ppm for ^{1}H and 39.5 ppm for ^{13}C) or $CDCl_3$ (7.26 ppm for ^{1}H and 77.16 ppm for ^{13}C). ^{1}H NMR coupling constants are reported in hertz (Hz) and refer to apparent multiplicities. IR spectra were recorded on a Bruker ALPHA FT-IR spectrometer. Samples were measured either as KBr pellets or as thin films between KBr plates. Electrospray ionization high resolution mass spectra (ESI HRMS) of low molecular weight compounds were recorded using Thermo Scientific Orbitrap Exactive mass spectrometer in positive ion mode. Analytical thin layer chromatography was performed on Kieselgel 60 F_{254} precoated aluminum plates (Merck); spots were visualized with "chromic acid". Silica gel column chromatography was performed using Merck Kieselgel 60 0.040–0.063 mm. HPLC was carried out on Agilent 1100 instrument using Sunfire C18 column 4.6×250 mm, linear gradient from 0 to 40% MeCN in gradient of NH_4OAc from 0.05M to 0.03M for 24min, linear gradient from 40 to 80% MeCN in gradient of NH_4OAc from 0.03 M to 0.01 M for 3 min, linear gradient from 0.01 M to 0 of NH_4OAc in 80% MeCN, linear gradient from 80 to 100% MeCN. Oligonucleotides were assembled in an ABI 3400 DNA synthesizer by the phosphoramidite method according to the manufacturer's recommendations. Protected 2′-deoxyribonucleoside 3′-phosphoramidites, Unylinker-CPG (500Å) and *S*-ethylthio-1*H*-tetrazole were purchased from ChemGenes; 5′-alkyne phosphoramidite, Cy3 and Cy5 alkynes were from Lumiprobe LLC. Oligonucleotides were cleaved from the support and deprotected using AMA – 1:1 (v/v) conc. aq. ammonia and 40% aq. methylamine for 2 h at room temperature. ESI-MS spectra for oligonucleotide building blocks were recorded using Bruker Maxis Impact q-TOF system as prescribed.

4.2. Synthetic procedures[13,14]

*4.2.1. 5,5-Bis(4-cyano-2-oxabutyl)-1,9-dicyano-3,7-dioxanonane (**2**)*

Pentaerythritol **1** (20.0 g, 147 mmol) was suspended in water (150 mL) under vigorous stirring; after 15 min, acrylonitrile (58.5 mL, 46.7 g, 882 mmol) and 40% tetrabutylammonium hydroxide (4.4 mL) were added and the stirring was continued for 24 h. A two-phase liquid reaction mixture was obtained. The crude oily product (lower phase) was separated, and the upper phase was extracted with ethyl acetate (3×150 mL). Ethyl acetate extracts and oily product were combined, washed with water (3×300 mL), brine (100 mL), dried over Na_2SO_4 and evaporated. The resulting oil was coevaporated with DCM (100 mL), diluted with an equal volume of 96% ethanol and cooled at -20°C. The crystalline precipitate was collected. Evaporation of mother liquor and recrystallization of residual oil from cold 96% ethanol gave a second crop of crystals. Desiccation afforded product as colorless crystals (33.41 g; 96 mmol; 65% yield), mp 44–45°C (EtOH), lit.[13a] mp 44–46°C. ^{1}H NMR (500 MHz; DMSO-d_6): δ 3.58 (t; 8H; *J* 5.9 Hz), 3.39 (s, 8H), 2.74 (t, 8H, *J* 5.9 Hz); ^{13}C NMR (500 MHz; DMSO-d_6) δ 119.26, 68.42, 65.63, 45.21, 17.97. IR (KBr) ν_{max} 3501, 2975, 2914, 2876, 2251, 1495, 1370, 1267, 1173, 1108, 853 cm^{-1}.

*4.2.2. 6,6-Bis(4-carboxy-2-oxabutyl)-4,8-dioxaundecane-1,11-dicarboxylic acid (**3**)*

The mixture of 5,5-bis(4-cyano-2-oxabutyl)-1,9-dicyano-3,7-dioxanonane **2** (50 g, 143.5 mmol) and 36% hydrochloric acid (130 mL) was stirred and heated to 80°C in an oil bath. After 30 min white solid starts to precipitate, and after 3 h of heating the reaction mixture was cooled initially in cold water, and then in a refrigerator (-20°C). The precipitate of NH_4Cl was filtered and washed with acetone (2×50 mL). The filtrate was evaporated in vacuum to give a mixture of solid and oily compounds. A portion of acetone (200 mL) was added to dissolve crude tetracarboxylic acid **5**, and white precipitate (the residual NH_4Cl) was removed by filtration. The filtrate was evaporated in vacuum to give crude **5** which was recrystallized from dry acetonitrile (70 mL). After removing of the obtained crystals the filtrate was evaporated and the residue was repeatedly recrystallized from acetonitrile. (The product crystallizes from acetonitrile very slowly therefore the solution was left at -20°C overnight.) Two portions of solid products were combined to one and desiccation of it in vacuum of oil pump afforded product as white crystals (58.0 g; 137 mmol; 95% yield), mp 99–101°C (MeCN), lit.[13a] mp 104–106°C, lit.[13b] mp 107–109°C. ^{1}H NMR (500 MHz; DMSO-d_6) δ 12.08 (br. s, 4H), 3.53 (t, 8H; *J* 6.3 Hz), 3.24 (s, 8H), 2.4 (t, 8H, *J* 6.3 Hz); ^{13}C NMR (500 MHz; DMSO-d_6) δ 172.63, 69.03, 66.75, 45.10, 34.68.

*4.2.3. 6,6-Bis(5-hydroxy-2-oxapentyl)-4,8-dioxaundecane-1,11-diol (**4**)*

An oven dried 1 L three-necked round-bottomed flask, equipped with overhead stirrer, condenser (with $CaCl_2$-tube) and septum was purged with argon and charged with absolute THF (200 mL) and BMS (borane dimethylsulfide) (61.5 mL; 648.6 mmol; d 0.801 g/mL). The obtained solution was heated to 50°C in an oil bath and stirred. The septum was exchanged with the 500 mL pressure-equalizing dropping funnel, which was charged with a solution of 6,6-bis(4-carboxy-2-oxabutyl)-4,8-dioxaundecane-1,11-dicarboxylic acid **3** (55 g; 129.7 mmol) in absolute THF (400 mL). The solution of acid was added dropwise to the stirred solution of BMS. After the addition was

complete, the reaction mixture was stirred and heated for 1 h and then cooled. Aqueous NaOH solution (25%; 150 mL) was added dropwise to the vigorously stirred reaction mixture. Organic layer was removed and the residual crude product was extracted from alkaline solution with THF (2×100 mL). Organic layers were combined, dried with Na_2SO_4 and the solvent was evaporated in vacuum (*STENCH*!). The obtained crude product was chromatographed on silica gel in $CHCl_3$–EtOH (gradient 10%→20% EtOH). The composition of fractions was controlled by TLC (20% EtOH in $CHCl_3$). The fractions containing product were combined and evaporated to give pure tetraol **4** (32.02 g; 86.9 mmol; 67% yield) as a colorless oil, lit.[13b] colorless viscous liquid. ^{1}H NMR (500 MHz; DMSO-d_6) δ 4.37 (t, 4H, *J* 5.1 Hz), 3.40–3.46 (m, 8H), 3.35–3.40 (m; 8H), 3.25 (s, 8H), 1.61 (q, 8H, *J* 6.4 Hz); ^{13}C NMR (500 MHz; DMSO-d_6) δ 69.19, 67.93, 57.95, 45.00, 32.63. IR (neat) ν_{max} 3356, 2944, 2871, 1672, 1485, 1422, 1372, 1300, 1110, 644 cm^{-1}. ESI HRMS m/z 369.2477 $[M+H]^+$, 391.2295 $[M+Na]^+$ (calcd for $C_{17}H_{37}O_8^+$, 369.2483; $C_{17}H_{36}O_8Na^+$, 391.2302).

*4.2.4. 6,6-Bis(5-azido-2-oxapentyl)-4,8-dioxa-undecane-1,11-diol (**5**) and 6,6-bis(5-azido-2-oxapentyl)-4,8-dioxa-11-azidoundecane-1-ol (**6**)*

Mesyl chloride (3.73 g, 32.7 mmol) was added dropwise to a mixture of 6,6-bis(5-hydroxy-2-oxapentyl)-4,8-dioxaundecane-1,11-diol **4** (4.00 g, 10.9 mmol) and triethylamine (5.31 mL, 38.2 mmol, d 0.726 g/mL) in dry DCM (50 mL). The reaction was controlled by TLC (10% EtOH in $CHCl_3$, starting alcohol R_f 0.33). After the con-sumption of all starting material reaction mixture was washed with distilled water (2×50 mL) and dried over Na_2SO_4. Evaporation of the solvent in vacuum gave yellowish oily liquid. It was dissolved in dry DMSO (30 mL) and sodium azide (7.00 g; 108 mmol) was added under magnetic stirring. Two days later TLC (50% EtOAc in hexane; triazide R_f 0.5) showed that reaction was complete. Water (30 mL) was added and the mixture of products was extracted from water with EtOAc (3×100 mL); organic fractions were combined, washed with distilled water (5×100 mL) and dried over Na_2SO_4. After the evaporation of the solvent in vacuum, the residue was chromatographed on silica gel (EtOAc in hexane, gradient of EtOAc from 20 to 45%). The desired compound **6** was obtained as a colorless liquid, yield 1.38 g (3.1 mmol, 28.6%). ^{1}H NMR (500 MHz; $CDCl_3$) δ 3.73–3.81 (m, 2H), 3.59 (t, 2H, *J* 5.5 Hz), 3.46 (t, 6H; *J* 5.9 Hz), 3.32–3.43 (m, 14H), 2.58 (br. s, 1H), 1.77–1.89 (m, 8H); ^{13}C NMR (500 MHz; $CDCl_3$) δ 71.46, 71.00, 70.18, 68.09, 62.55, 48.61, 45.27, 31.93, 29.11. IR (neat) ν_{max} 3449, 2929, 2871, 2097, 1718, 1459, 1300, 1263, 1111, 944 cm^{-1}. ESI HRMS m/z 444.2673 $[M+H]^+$, 466.2423 $[M+Na]^+$ (calcd for $C_{17}H_{34}N_9O_5^+$, 444.2677; $C_{17}H_{33}N_9O_5Na^+$, 466.2497). Byproducts tetraazide **7** (1.318 g; 2.8 mmol; 25.8% yield) and diazide **5** (0.36 g; 0.9 mmol; 8% yield) were also isolated as colorless liquids. (*CAUTION*! Azides are potentially explosive upon heating and impact,[17] especially compounds having (C+O)/N ratio <3; thus compounds **6** and **7** should be treated carefully). NMR data for diazide **5**: ^{1}H NMR (500 MHz; $CDCl_3$) δ 3.74 (t, 4H, *J* 5.3 Hz), 3.58 (t, 4H, *J* 5.5 Hz), 3.46 (t, 4H, *J* 5.9 Hz), 3.31–3.42 (m, 12H), 3.05 (s, 2H), 1.74–1.89 (m, 8H); ^{13}C NMR (500 MHz; $CDCl_3$) δ 71.10, 70.87, 70.35, 68.17, 61.94, 48.64, 45.11, 31.89, 29.11. IR (neat) ν_{max} 3419, 2929, 2871, 2097, 1372, 1301, 1263, 1111, 942 cm^{-1}. ESI HRMS m/z 419.2604 $[M+H]^+$, 441.2423 $[M+Na]^+$ (calcd for $C_{17}H_{35}N_6O_6^+$, 419.2613; $C_{17}H_{34}N_6O_6Na^+$, 441.2432).

*4.2.5. 6,6-Bis(5-azido-2-oxapentyl)-4,8-dioxa-1,11-diazidoundecane (**7**)*

Mesyl chloride (4.23 g, 37 mmol) was added dropwise to the mixture of 6,6-bis(5-hydroxy-2-oxapentyl)-4,8-dioxaundecane-1,11-diol **4** (3.00 g, 8 mmol) and triethylamine (5.29 mL, 38 mmol) in dry DCM (50 mL). The reaction was controlled by TLC (10% EtOH in $CHCl_3$, the intermediate tetramesylate has R_f 0.79). After the consumption of all starting material the reaction mixture was washed with distilled water (2×50 mL) and dried over Na_2SO_4. Evaporation of the solvent in vacuum gave off-white crude solid mesylate. It was dissolved in dry DMSO (30 mL), and sodium azide (7.0 g, 108 mmol) was added under magnetic stirring. After 48 h the reaction mixture was diluted with water (30 mL). Product was extracted from aqueous layer with EtOAc (3×100 mL), organic fractions were combined, washed with distilled water (5×100 mL) and dried over Na_2SO_4. After the evaporation of the solvent in vacuum, residue was chromatographed on silica gel (0 to 2% gradient of EtOH in $CHCl_3$). Slightly yellowish liquid product **7** was obtained as an oil; yield 3.40 g (7.2 mmol, 90%). ^{1}H NMR (500 MHz; $CDCl_3$) δ 3.47 (t, 8H; *J* 5.9 Hz), 3.39–3.35 (m, 16H), 1.83 (q, 8H, *J* 6.3 Hz); ^{13}C NMR (500 MHz; $CDCl_3$) δ 69.81, 68.0, 48.63, 45.52, 29.18. IR (neat) ν_{max} 3334, 2928, 2871, 2802, 2097, 1486, 1460, 1372, 1301, 1263, 1112, 923 cm^{-1}. ESI HRMS m/z 469.2734 $[M+H]^+$, 491.2551 $[M+Na]^+$ (calcd for $C_{17}H_{35}N_6O_6^+$, 469.2742; $C_{17}H_{34}N_6O_6Na^+$, 491.2562).

4.3. Synthesis of branched oligonucleotide conjugates

4.3.1. Conjugation of oligonucleotides on branching points

Three- and four-fold building blocks were synthesized starting from triazide **6** and tetraazide **7** branching points and oligonucleotides with alkyne modifications using copper-catalyzed click chemistry. The structure of alkyne modification is given in the Supporting Information. The full functionalization of branching points with three and four oligonucleotide strands was achieved by mixing 5-fold molar oligonucleotide excess with triazide **6** and 7-fold excess for tetraazide **7**. The aqueous solution of oligonucleotide and DMSO solution of the corresponding azide in ratio chosen were mixed with the addition of triethylammonium acetate buffer (pH 7) till the final concentration 0.2 M, 0.5 mM ascorbic acid and 0.5 mM Cu-TBTA solution in 55% DMSO. The total volume of reaction mixture in 50% DMSO was adjusted to 0.3 mL. The solution was saturated with argon before and after addition of copper complex and left overnight. The products were precipitated with 4-fold excess of acetone with addition of $LiClO_4$ solution till 0.2 M final concentration. Analysis and separation of products were performed using HPLC and polyacrylamide gel electrophoresis (19% for T_{10}-based products and 15% for D11 containing products). The composition and stoichiometry of building blocks was confirmed by electrospray mass-spectrometry.

4.3.2. Conjugation of Cy3, Cy5 and D11 on residual azide group of building blocks

A building block with residual azide group(s) (1 nmol) was mixed with 1.5-fold excess of dye alkyne (or 3-fold excess of D11 oligonucleotide) derivative in the same conditions as described above for building blocks synthesis. The reaction was left overnight and precipitated with addition of 2M $LiClO_4$ solution (200 μL) and 4-fold excess of acetone. The product was dissolved in 40 μL of MilliQ water and analyzed/isolated with reverse phase HPLC (for Cy derivatives) or PAGE (for D11 ODN derivatives). For structures of Cy3 and Cy5 dyes see Supporting Information.

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Supplementary data

Supplementary data associated with this article (HRMS, IR, and NMR spectra of all new compounds, LC/MS data of oligonucleotide conjugates) can be found, in the online version, at

5. References and notes

1. (a) LaBoda, C.; Duschl, H.; Dwyer, C. L. *Acc. Chem. Res.* **2014**, *47*, 1816–1824; (b) Wang, D; Fu, Y.; Yan, J.; Zhao, B.; Dai, B.; Chao, J.; Liu, H.; He, D.; Zhang, Y.; Fan, C.; Song, S. *Anal. Chem.* **2014**, *86*, 1932–1936; (c) Li, T.; Lohmann, F.; Famulok, M. *Nat. Commun.* **2014**, *5*, 4940; (d) Ponomarenko, A. I.; Brylev, V. A.; Nozhevnikova, E. V.; Korshun, V. A. *Curr. Top. Med. Chem.* **2015**, *15*, 1162–1178; (e) Yang, Y. R.; Liu, Y.; Yan, H. *Bioconjugate Chem.* **2015**, *26*, 1381–1395.

2. (a) Cho, Y.; Lee, J. B.; Hong, J. *Sci. Rep.* **2014**, *4*, 4078; (b) Hu, R.; Zhang, X.; Zhao, Z.; Zhu, G.; Chen, T.; Fu, T.; Tan, W. *Angew. Chem. Int. Ed.* **2014**, *53*, 5821–5826; (c) Chao, J.; Liu, H.; Su, S.; Wang, L.; Huang, W.; Fan, C. *Small* **2014**, *10*, 4626–4635; (d) Zhan, P.; Jiang, Q.; Wang, Z.; Li, N.; Yu, H.; Ding, B. *ChemMedChem* **2014**, *9*, 2013–2020.

3. (a) Seeman, N. C. *J. Theor. Biol.* **1982**, *99*, 237–247; (b) Kallenbach, N. R.; Ma, R.-I.; Seeman, N. C. *Nature* **1983**, *305*, 829–831.

4. (a) Shchepinov, M. S.; Udalova, I. A.; Bridgman, A. J.; Southern, E. M. *Nucl. Acids Res.* **1997**, *25*, 4447–4454; (b) Scheffler, M.; Dorenbeck, A.; Jordan, S.; Wüstefeld, M.; von Kiedrowski, G. *Angew. Chem. Int. Ed.* **1999**, *38*, 3311–3315; (c) Gothelf, K. V.; Thomsen, A.; Nielsen, M.; Cló, E.; Brown, R. S. *J. Am. Chem. Soc.* **2004**, *126*, 1044–1046; (d) Nielsen, M.; Thomsen, A. H.; Cló, E.; Kirpekar, F.; Gothelf, K. V. *J. Org. Chem.* **2004**, *69*, 2240–2250; (e) Katajisto, J.; Heinonen, P.; Lönnberg, H. *J. Org. Chem.* **2004**, *69*, 7609–7615; (f) Tumpane, J.; Sandin, P.; Kumar, R.; Powers, V. E. C.; Lundberg, E. P.; Gale, N.; Baglioni, P.; Lehn, J.-M.; Albinsson, B.; Lincoln, P.; Wilhelmsson, L.M.; Brown, T.; Nordén, B. *Chem. Phys. Lett.* **2007**, *440*, 125–129; (g) Utagawa, E.; Ohkubo, A.; Sekine, M.; Seio, K. *J. Org. Chem.* **2007**, *72*, 8259–8266; (h) Hannestad, J. K.; Gerrard, S. R.; Brown, T.; Albinsson, B. *Small* **2011**, *7*, 3178–3185; (i) Lundberg, E. P.; Plesa, C.; Wilhelmsson, L.M.; Lincoln, P.; Brown, T.; Nordén, B. *ACS Nano* **2011**, *5*, 7565–7575; (j) Ferreira, R.; Alvira, M.; Aviñó, A.; Gómez-Pinto, I.; González, C.; Gabelica, V.; Eritja, R. *ChemistryOpen* **2012**, *1*, 106–114; (k) Eryazici, I.; Yildirim, I.; Schatz, G. C.; Nguyen, S. T. *J. Am. Chem. Soc.* **2012**, *134*, 7450–7458; (l) Hong, B. J.; Cho, V. Y.; Bleher, R.; Schatz, G. C.; Nguyen, S. T. *J. Am. Chem. Soc.* **2015**, *137*, 13381–13388.

5. (a) Hudson, R. H. E.; Robidoux, S.; Damha, M. J. *Tetrahedron Lett.* **1998**, *39*, 1299–1299; (b) Damha, M. J.; Braich, R. S.; *Tetrahedron Lett.* **1998**, *39*, 3907–3910; (c) Ivanov, S. A.; Volkov, E. M.; Oretskaya, T. S.; Müller, S. *Tetrahedron* **2004**, *60*, 9273–9281; (d) Chandra, M.; Keller, S.; Gloeckner, C.; Bornemann, B.; Marx, A. *Chem. Eur. J.* **2007**, *13*, 3558–3564; (e) Paredes, E.; Zhang, X.; Ghodke, H.; Yadavalli, V. K.; Das, S. R. *ACS Nano* **2013**, *7*, 3953–3961; (f) Katolik, A.; Johnsson, R.; Montemayor, E.; Lackey, J. G.; Hart, P. J.; Damha, M. J. *J. Org. Chem.* **2014**, *79*, 963–975; (g) Ghosh, S.; Greenberg, M. M. *J. Org. Chem.* **2014**, *79*, 5948–5957.

6. (a) Pathak, R.; Marx, A. *Chem. Asian J.* **2011**, *6*, 1450–1455; (b) Bußkamp, H.; Keller, S.; Robotta, M.; Drescher, M.; Marx, A. *Beilstein J. Org. Chem.* **2014**, *10*, 1037–1046.

7. (a) Shi, J.; Bergstrom, D. E. *Angew. Chem. Int. Ed.* **1997**, *36*, 111–113; (b) Grøtli, M.; Eritja, R.; Sproat, B. *Tetrahedron* **1997**, *53*, 11317–11346; (c) Ueno, Y.; Shibata, A.; Matsuda, A.; Kitade, Y. *Bioconjugate Chem.* **2003**, *14*, 684–689; (d) Grimau, M. G.; Iacopino, D.; Aviñó, A.; de la Torre, B. G.; Ongaro, A.; Fitzmaurice, D.; Wessels, J.; Eritja, R. *Helv. Chim. Acta* **2003**, *86*, 2814–2826; (e) Aviñó, A.; Grimau, M. G.; Frieden, M.; Eritja, R. *Helv. Chim. Acta* **2004**, *87*, 303–316; (f) Oliviero, G.; Amato, J.; Borbone, N.; Galeone, A.; Petraccone, L.; Varra, M.; Piccialli, G.; Mayol, L. *Bioconjugate Chem.* **2006**, *17*, 889–898; (g) Meng, M.; Ahlborn, C.; Bauer, M.; Plietzsch, O.; Soomro, S. A.; Singh, A.; Muller, T.; Wenzel, W.; Bräse, S.; Richert, C. *ChemBioChem* **2009**, *10*, 1335–1339; (h) Singh, A.; Tolev, M.; Meng, M.; Klenin, K.; Plietzsch, O.; Schilling, C. I.; Muller, T.; Nieger, M.; Bräse, S.; Wenzel, W.; Richert, C. *Angew. Chem. Int. Ed.* **2011**, *50*, 3227–3231; (i) Griesser, H.; Tolev, M.; Singh, A.; Sabirov, T.; Gerlach, C.; Richert, C. *J. Org. Chem.* **2012**, *77*, 2703–2717.

8. (a) Damha, M. J.; Zabarylo, S.; *Tetrahedron Lett.* **1989**, *30*, 6295–6298; (b) Damha, M. J.; Ganeshan, K.; Hudson, R. H. E.; Zabarylo, S. V. *Nucl. Acids Res.* **1992**, *20*, 6565–6573; (c) Hudson, R. H. E.; Damha, M. J. *J. Am. Chem. Soc.* **1993**, *115*, 2119–2124.

9. (a) Vargas-Baca, I.; Mitra, D.; Zulyniak, H. A.; Banerjee, J.; Sleiman, H. F. *Angew. Chem. Int. Ed.* **2001**, *40*, 4629–4632; (b) Aldaye, F. A.; Sleiman, H. F. *J. Am. Chem. Soc.* **2007**, *129*, 10070–10071.

10. (a) Singh, A.; Tolev, M.; Schilling, C. I.; Bräse, S.; Griesser, H.; Richert, C. *J. Org. Chem.* **2012**, *77*, 2718–2728; (b) Schwenger, A.; Gerlach, C.; Griesser, H.; Richert, C. *J. Org. Chem.* **2014**, *79*, 11558–11566.

11. Reviews on click chemistry on oligonucleotides: (a) Gramlich, P. M. E.; Wirges, C. T.; Manetto, A.; Carell, T. *Angew. Chem. Int. Ed.* **2008**, *47*, 8350–8358; (b) El-Sagheer, A. H.; Brown, T. *Chem. Soc. Rev.* **2010**, *39*, 1388–1405; (c) Ustinov, A. V.; Stepanova, I. A.; Dubnyakova, V. V.; Zatsepin, T. S.; Nozhevnikova, E. V.; Korshun, V. A. *Russ. J. Bioorg. Chem.* **2010**, *36*, 401–445; (d) El-Sagheer, A. H.; Brown, T. *Acc. Chem. Res.* **2012**, *45*, 1258–1267; (e) Efthymiou, T.; Gong, W.; Desaulniers, J.-P. *Molecules* **2012**, *17*, 12665–12703; (f) Kore, A. R.; Charles, I. *Curr. Org. Chem.* **2013**, *17*, 2164–2191; (g) Haque, M. M.; Peng, X. *Sci. China, Chem.* **2014**, *57*, 215–231; (h) Kath-Schorr, S. *Top. Curr. Chem. (Z)* **2016**, *374*, 4.

12. (a) Kumar, R.; El-Sagheer, A.; Tumpane, J.; Lincoln, P.; Wilhelmsson, L. M.; Brown, T. *J. Am. Chem. Soc.* **2007**, *129*, 6859–6864; (b) Lietard, J.; Meyer, A.; Vasseur, J.-J.; Morvan, F. *J. Org. Chem.* **2008**, *73*, 191–200; (c) Xu, Y.; Suzuki, Y.; Komiyama, M. *Angew. Chem. Int. Ed.* **2009**, *48*, 3281–3284; (d) Lundberg, E. P.; El-Sagheer, A. H.; Kocalka, P.; Wilhelmsson, L. M.; Brown, T.; Nordén, B. *Chem. Commun.* **2010**, *46*, 3714–3716; (e) Pujari, S. S.; Xiong, H.; Seela, F. *J. Org. Chem.* **2010**, *75*, 8693–8696; (f) Jacobsen, M. F.; Ravnsbæk, J. B.; Gothelf, K. V. *Org. Biomol. Chem.* **2010**, *8*, 50–52; (g) Saccà, B.; Niemeyer, C. M. *Small* **2011**, *7*, 2887–2898; (h) Xiong, H.; Leonard, P.; Seela, F. *Bioconjugate Chem.* **2012**, *23*, 856–870; (i) Xiong, H.; Seela, F. *Bioconjugate Chem.* **2012**, *23*, 1230–1243; (j) Astakhova, I. K.; Kumar, T. S.; Campbell, M. A.; Ustinov, A. V.; Korshun, V. A.; Wengel, J. *Chem. Commun.* **2013**, *49*, 511–513; (k) Ingale, S. A.; Seela, F. *J. Org. Chem.* **2013**, *78*, 3394–3399; (l) Latorre, A.; Lorca, R.; Zamora, F.; Somoza, Á. *Chem. Commun.* **2013**, *49*, 4950–4952; (m) Fomich, M. A.; Kvach, M. V.; Navakouski, M. J.; Weise, C.; Baranovsky, A. V.; Korshun, V. A.; Shmanai, V. V. *Org. Lett.* **2014**, *16*, 4590–4593; (n) Clavé, G.; Chatelain, G.; Filoramo, A.; Gasparutto, D.; Saint-Pierre, C.; Le Cam, E.; Piétrement, O.; Guérineau, V.; Campidelli, S. *Org. Biomol. Chem.* **2014**, *12*, 2778–2783; (o) Thaner, R. V.; Eryazici, I.; Farha, O. K.; Mirkin, C. A.; Nguyen, S. T. *Chem. Sci.* **2014**, *5*, 1091–1096; (p) Hong, B. J.; Eryazici, I.; Bleher, R.; Thaner, R. V.; Mirkin, C. A.; Nguyen, S. T. *J. Am. Chem. Soc.* **2015**, *137*, 8184–8191.

13. (a) Newkome, G. R.; Weis, C. D. *Org. Prep. Proced. Int.* **1996**, *28*, 242–244; (b) Newkome, G. R.; Mishra, A.; Moorefield, C. N. *J. Org. Chem.* **2002**, *67*, 3957–3960.

14. All attempts to reproduce procedures[13] for **1**→→**4** sequence gave in our hands unsatisfactory results. Therefore, some reaction conditions and isolation procedures were altered.

15. The reaction performed without isolation of the intermediate tetramesylate gave better results.

16. (a) Sambrook, J.; Russel, D.W. *CSH Protocols* **2006**, *67*, 3957; (b) Zhang, C., Papakonstantinou, T. In: Handbook of Nucleic Acid Purification. Ed. by D. Liu. CRC Press, **2009**, chapter 23.

17. Keicher, T., Löbbecke, S. In: Organic Azides: Syntheses and Allications. Ed. by S. Bräse and K. Banert. Wiley, **2010**, 3–27.