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New Branched DNA Constructs

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Abstract: The Watson–Crick base pairing of DNA is an advantageous phenomenon that can be exploited when using DNA as a scaffold for directed self-organization of nanometer-sized objects. Several reports have appeared in the literature that describe the generation of branched DNA (bDNA) with variable numbers of arms that self-assembles into predesigned architectures. These bDNA units are generated by using cleverly designed rigid crossover DNA molecules. Alternatively, bDNA can be generated by using synthetic branch points derived from either nucleoside or non-nucleoside building blocks. Branched DNA has scarcely been explored for use in nanotechnology or from self-assembling perspectives. Herein, we wish to report our results for the synthesis, characterization, and assembling properties of asymmetrical bDNA molecules that are able to generate linear and circular bDNA constructs. Our strategy for the generation of bDNA is based on a branching point that makes use of a novel protecting-group strategy. The bDNA units were generated by means of automated DNA synthesis methods and were used to generate novel objects by employing chemical and biological techniques. The entities generated might be useful building blocks for DNA-based nanobiotechnology.

Keywords: DNA · ligation · nanobiotechnology · oligonucleotides · self-assembly

Introduction

DNA is the fundamental genetic material used to store and pass information from one generation to the next. The Watson–Crick base pairing of DNA is an advantageous phenomenon that can be exploited when using DNA as a scaffold for directed self-organization of nanometer-sized objects.[1–10] In an early report, Seeman proposed ordered arrays of DNA with branched DNA (bDNA) building blocks, which naturally exist in cellular DNA metabolism.[11] Subsequently, several reports have appeared in the literature that describe the generation of bDNA units with variable numbers of arms that self-assemble into predesigned architectures.[12–16] These bDNA molecules are generated by using cleverly designed rigid crossover DNA molecules. Alternatively, bDNA can be generated by using synthetic branch points derived from either nucleoside or non-nucleoside building blocks. As a result of this, several branch points have been reported in the literature to generate bDNA for several applications.[17–26] Additionally, bDNA can be generated by the covalent cross-linking of two DNA strands through disulfide linkages.[27–28] However, bDNA has scarcely been explored for use in nanotechnology or from self-assembling perspectives. Herein, we wish to report our results for the synthesis, characterization, and assembling properties of asymmetrical bDNA molecules that are able to generate linear and circular bDNA constructs. We employed a combination of chemical and enzymatic approaches to generate these objects, which represent a novel class of bDNA constructs.

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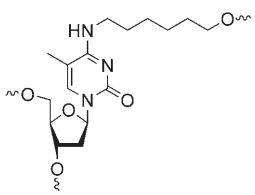
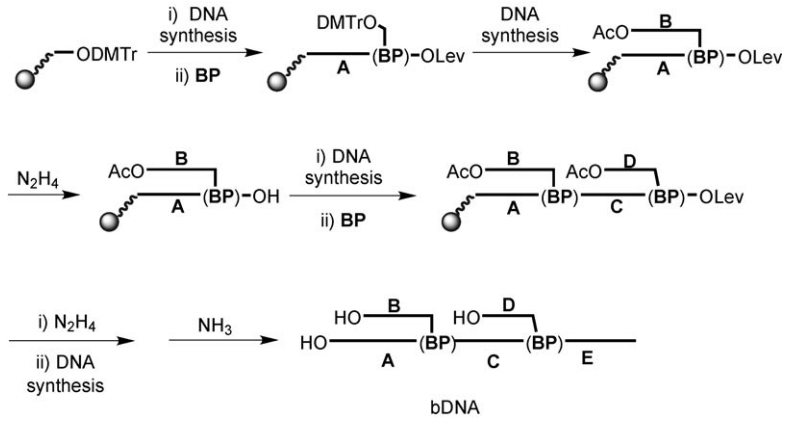
Results and Discussion

Synthesis of the branching point and bDNA: We envisaged the synthesis of asymmetric bDNA constructs by using the strategy outlined in Scheme 1. To use this method, one requires a protecting-group strategy at the branching point (**BP**)

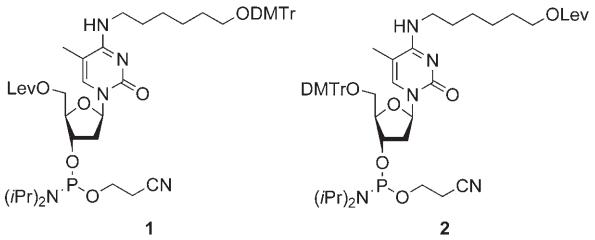


BP



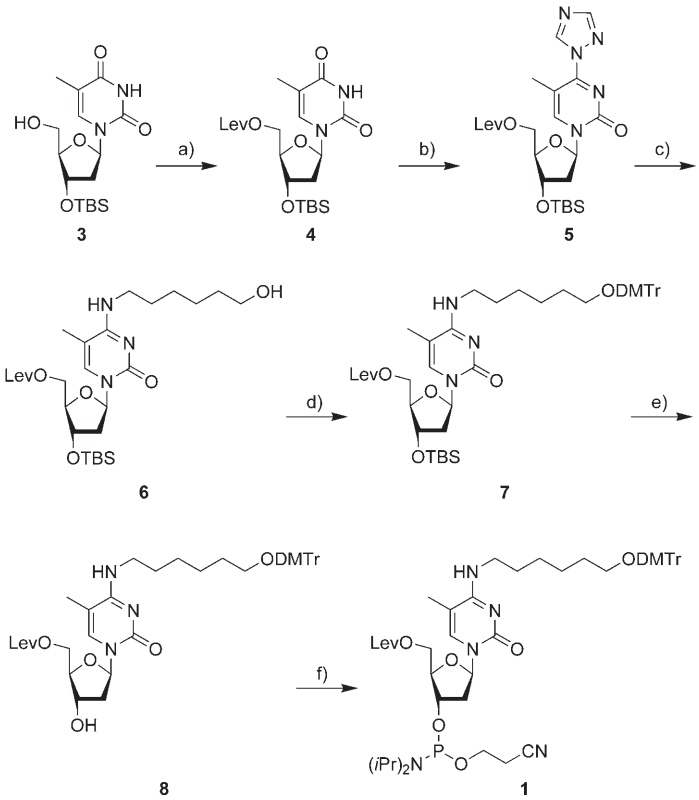
Scheme 1. General synthetic strategy for asymmetrical bDNA. **BP** = branching point, **A**–**E** = synthesized DNA strands.

that enables selective manipulations to be carried out to elongate branch **B** without affecting the extension of linear strand **A**. Phosphoramidite building block **1** was found to fulfill these criteria and was used in the studies reported herein. This is different to the well-known commercially available N^4-(6-hydroxyhexyl)-5-methyl-2′-deoxycytidine building block (**2**) that exhibits an inverted protecting-group strategy with respect to N^4-(6-hydroxyhexyl)- and 5′-OH-protection.



We developed a straightforward synthetic strategy for desired phosphoramidate **1**, as shown in Scheme 2. Our synthesis starts with 3′-*O*-TBS-protected thymidine **3** that was treated with 2-chloro-1-methylpyridinium iodide in the presence of 1,4-diazabicyclo[2.2.2]octane (DABCO) and levulinic acid to yield compound **4**. Thymidine analogue **4** was subsequently treated with 1,2,4-triazole in the presence of $POCl_3$ to give triazole derivative **5**, which was then converted into 2′-deoxycytosine derivative **6** after treatment with 6-aminohexanol. This compound was treated with 4,4′-dimethoxytriphenylmethylchloride (DMTr-Cl) in the presence of (*N*,*N*-dimethylamino)pyridine (DMAP) to give fully protected intermediate **7**. The 3′-*O*-TBS group was then removed by using a mixture of AcOH and tetrabutyl ammonium fluoride (TBAF) to give compound **8,** which was converted into target phosphoramidite **1** by reacting **8** with *O*-(2-cyanoethyl)-*N*,*N*′-diisopropylchlorophoramidite in presence of *N*,*N*-diisopropylethyl amine (DIPEA).

The general synthesis for the generation of bDNA outlined in Scheme 1 shows that after insertion of branched monomer **1**, synthesis was continued to form segment **B** as the side arm. The DMTr group of the last incorporated monomer was deprotected by using standard automated DNA synthetic procedures, and was subsequently acetylated by using the capping protocol of the synthesizer. The levulinyl group on the 5′-OH position of the branch point was cleaved by treatment of the controlled pore glass (CPG) loaded column with hydrazine (0.5 M) in a mixture of pyridine/acetic acid (1:1). Synthesis was continued to extend the branch to form strand **C**. From the detection of the DMTr cation, the yield of every coupling step was found to be >98%. Remarkably, the depicted synthetic route towards bDNA is compatible with conventional and inverse amidites (5′-CE phosphoramidites, CE = cyanoethyl). Figure 1 depicts the bDNA molecules that were synthesized and investigated in this study.



Scheme 2. Synthesis of phosphoramidite **1**: a) 2-chloro-1-methylpyridinium iodide, DABCO, levulinic acid, CH_3CN/dioxane, 90%; b) 1,2,4-triazole, NEt_3, $POCl_3$, CH_3CN, 100%; c) 6-aminohexanol, CH_3CN, 88%; d) DMTr-Cl, DMAP, pyridine, 88%; e) AcOH/TBAF, THF, 90%; f) DIPEA, *O*-(2-cyanoethyl)-*N*,*N*′-diisopropylchlorophoramidite, CH_2Cl_2, 97%. (TBS = *tert*-butyldimethylsilyl)

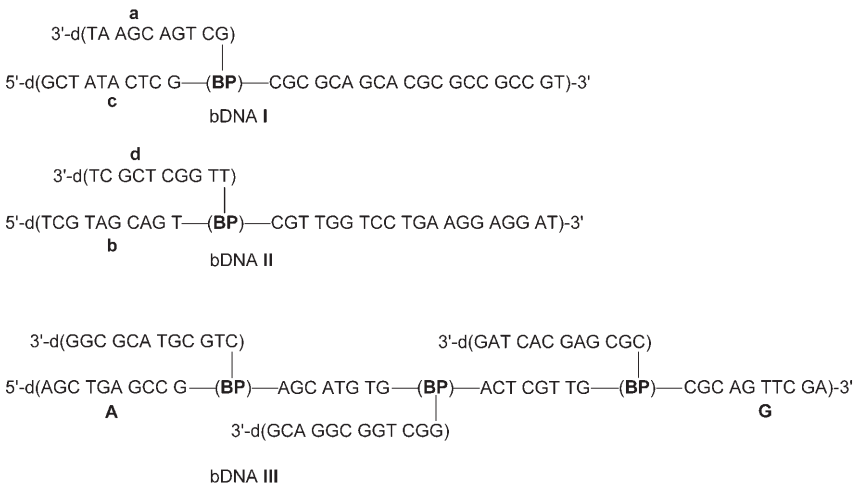


Figure 1. Representations of the bDNA molecules employed in this study; bDNA **I** = 41-mer, bDNA **II** = 41-mer, and bDNA **III** = 75-mer.

To generate novel noncovalent and covalent bDNA constructs, we synthesized two different bDNA molecules with a single branch point (41-mer, **I** and **II**), and a third one with three branch points (75-mer **III**). In bDNA **I**, the **a** branch is designed with a partial EcoRI restriction sequence (5′-GAAT-3′), and in bDNA **II** the **b** branch ends up with the remaining EcoRI restriction sequence (5′-TC-3′). The bDNA molecules were characterized by using ESI mass spectrometry, and the purity was checked by means of analytical polyacrylamide gel electrophoresis (PAGE) with ^{32}P-labeled bDNA.

Generation of noncovalent self-assemblies of bDNAs: Initially we envisaged generating noncovalent self-assemblies of bDNA **I** and **II** in the presence of small templating oligonucleotide splints. For this purpose, three splints (20-mer; **ab**, **cd**, and **3′–3′**) were used as linking strands. Splint **ab** represents the complementary sequence to branch **a** of bDNA **I** and branch **b** of bDNA **II**. Splint **cd** represents the complementary sequence to branch **c** of bDNA **I** and branch **d** of bDNA **II**. The **3′–3′** splint is complementary to both of the 3′ branches of bDNA **I** and **II**. This splint was synthesized with the aid of 3′– and 5′-CE phosphoramidites.

Next, we investigated the self-assembling properties of the bDNA molecules. To follow the predicted processes, **I** was labeled with ^{32}P at its 5′-OH position by employing standard procedures. Formation of the noncovalent constructs composed of **I** and **II** was monitored by means of native PAGE analysis. First, we investigated the ability of the **ab** splint to hybridize with complementary sites in **I** and **II** to connect both bDNA molecules through self-assembly. Indeed, we identified significantly lower electrophoretic mobility of **I** when it was incubated with **II** and the **ab** splint (see Figure 2, lane 2).

The newly formed product was generated exclusively and in high yields, as determined by quantification of the PAGE analysis results by means of phosphorimaging. When two splints (**ab** and **cd**) were used, a product of similar mobility was formed in high yields (see Figure 2, lane 3). Employment of the **3′–3′** splint resulted in a product that migrated slightly differently to that described previously (see Figure 2, lane 4). In the same lane another new product appeared, which might be a 61-mer that results from annealing bDNA **I** and the **3′–3′** splint. Interestingly, annealing bDNA **I** and bDNA **II** in the presence of all three splints resulted in a new product (Figure 2, lane 5), which has the slowest electrophoretic mobility observed in this series compared with other

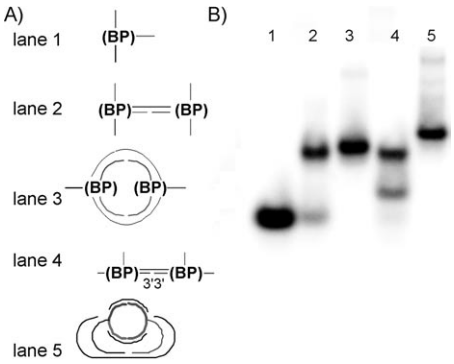


Figure 2. Self-assembly of bDNA molecules **I** and **II**. A) Depiction of the proposed structures. B) Native PAGE analysis of self-assembled bDNAs. Lane 1 = bDNA **I**; lane 2 = bDNA **I**+**II**+**ab** splint; lane 3 = bDNA **I**+**II**+**ab** and **cd** splints; lane 4 = bDNA **I**+**II**+**3′–3′** splint; and lane 5 = bDNA **I**+**II**+all three splints.

annealed products. This might be a result of the intramolecular annealing of two 3′-ends in **I** and **II** with the **3′–3′** splint to lead to a compact double circular structure. We then investigated the thermal stability of the duplexes formed between bDNA **I** and **II** in the presence of splint oligonucleotides **ab**, **cd**, and **3′–3′** by measuring the melting temperatures. In all cases, we have observed curves with single transitions that indicate melting temperatures ranging from 50 to 52 °C (data not shown). This study further indicated that stable constructs were formed. Circular dichroism (CD) studies were performed to determine the conformations of the self-assembled bDNA. Three CD curves were generated with bDNA **I** and **II** in the presence of i) an **ab** splint; ii) **ab** and **cd** splints; and iii) **ab**, **cd**, and **3′–3′** splints. In all three cases, characteristic signatures for the B form of DNA were observed (data not shown).

Enzymatic generation of novel bDNAs constructs: The remarkable thermal stability of the duplexes generated in the presence of bridging oligonucleotides prompted us to syn-

thesize covalently linked, novel bDNA structures by using enzymatic approaches. Previously, ligation of oligonucleotides was employed for the synthesis of circular DNA molecules.[29–32] By using this methodology, nanocircles containing 36–54 nucleotides were synthesized.[29]

However, this methodology has not yet been employed for the generation of novel bDNA structures. For this purpose, bDNA **I** was 5′-O-phosphorylated with ^{32}P by T4 polynucleotide kinase and thus, radioactively labeled, whereas **II** was nonradioactively phosphorylated. In the first experiment, both radioactively labeled bDNA **I** and phosphorylated bDNA **II** were ligated with T4 DNA ligase in the presence of the **ab** splint. Denaturating PAGE indicates that the new covalently linked assembly (bDNA **IV**) is formed in high yields (Figure 3B, section a). Under similar conditions,

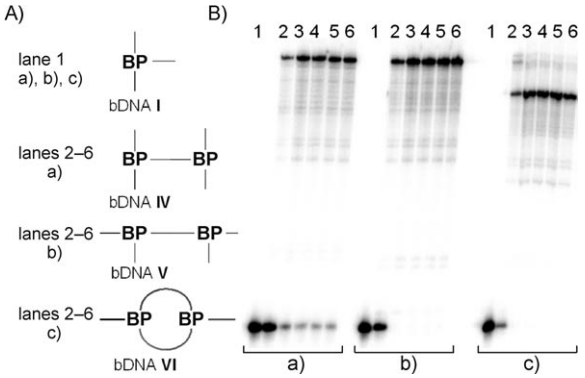


Figure 3. Denaturing PAGE analysis of bDNA ligation. A) Depiction of proposed structures. B) Denaturating PAGE analysis of bDNA objects: a) lane 1 = bDNA **I**; lanes 2–6 = ligation with bDNA **II** for 5 min, 1 h, 3 h, 5 h, and 7 h, respectively, by using the **ab** splint; b) lane 1 = bDNA **I;** lanes 2–6 = ligation with bDNA **II** for 5 min, 1 h, 3 h, 5 h, and 7 h, respectively, by using the **cd** splint; c): lane 1 = bDNA **I**; lanes 2–6 = ligation with bDNA **II** for 5 min, 1 h, 3 h, 5 h, and 7 h, respectively, by using the **ab** and **cd** splint.

but in the presence of the **cd** splint, ligation resulted in the expected product, which has the same length as that obtained by using the **ab** splint (Figure 3B, section b). To synthesize a circular 42-mer bDNA unit, bDNA molecules **I** and **II** were ligated in the presence of the **ab** and **cd** splints. An entirely new product was obtained (bDNA **VI**) that exhibits greater electrophoretic mobility than the former products (Figure 3B, section c). Interestingly, in all cases exclusively one ligation product was formed after a short reaction time. This result reveals that the depicted approach is suitable to generate linear bDNA molecules and circular bDNA molecules that contain two branches.

The formation of the desired linear and circular bDNA molecules was confirmed by treating the ligated products with the restriction endonuclease enzyme EcoRI. Linear ligated bDNA **IV** was incubated with EcoRI, which converted **IV** into a new bDNA structure that is shorter than bDNA **I** (Figure 4, lane 4). This shorter bDNA fragment resulted from the nicking of the restriction sequence present

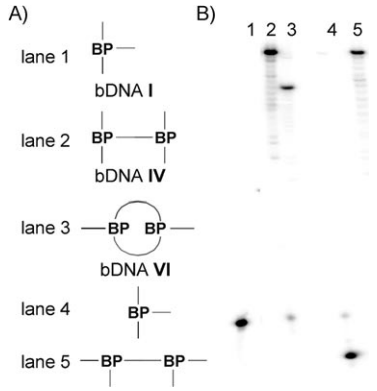


Figure 4. EcoRI digestion of linear and circular bDNA. A) Depiction of the proposed structures. B) Denaturating PAGE analysis. Lane 1 = bDNA **I**, lane 2 = bDNA **IV**, lane 3 = bDNA **VI,** lane 4 = EcoRI digestion of linear bDNA **IV**, and lane 5 = EcoRI digestion of circular bDNA **VI**.

in the **a** branch of bDNA **I**. Interestingly, EcoRI digestion of circular ligated bDNA **VI**, which resulted from the ligation of bDNA **I** and bDNA **II** in the presence of two splints, gave exactly the same length of linear ligated bDNA **IV** (Figure 4, lane 5). This occurred as a consequence of a single nick in the circular portion of bDNA **IV** and resulted in a structural change, namely, from the circular to the linear form.

Then we attempted to generate circular bDNA constructs containing three branches. Thus, we synthesized bDNA **III** with three branch points, in which all three branches (**B**, **D**, and **F**) are fixed as side arms at the N^4-(6-hydroxyhexyl) group of the branch point and contain 3′-ends. Branches **A** and **G** of bDNA **III** were intramolecularly ligated with T4 DNA ligase, in the presence of 20-mer splint **AG** that is complementary to the **A** and **G** strands. As shown in Figure 5, the conversion of linear bDNA **III** to the 43-mer circular form resulted in one product with a slower electrophoretic mobility than the linear one.

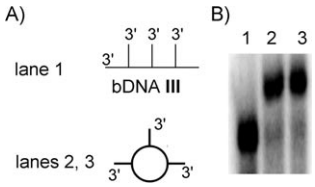


Figure 5. Synthesizing circular bDNA from bDNA **III**. A) Depiction of the proposed structures. B) Denaturating PAGE analysis. Lane 1 = bDNA **III**, lanes 2 and 3 = ligation in presence of **AG** splint for 1 and 4 h, respectively.

The CD spectrum of the duplex, which resulted from bDNA **III** and splint **AG,** showed the characteristic profile of the B form DNA conformation (data not shown). The thermal denaturing curve was generated in the presence of splint **AG** and the melting temperature was found to be 59.4 °C. This indicates the remarkable stability of the

duplex, which eventually led to the formation of circular bDNA.

Conclusion

In conclusion, we have demonstrated the synthesis of novel asymmetric bDNA. Herein, the strategy depicted is based on the employment of a nucleotide analogue that functions as a branching point. By changing the protecting-group strategy in comparison with known branching nucleotides we were able to synthesize novel noncovalent and covalent bDNA constructs that might be useful for DNA-based nanobiotechnology.

Experimental Section

General: All synthetic reactions were performed under an inert atmosphere. Dry solvents were purchased from Fluka and were stored over molecular sieves and used without further purification. Elemental analysis was carried out by the microanalysis facility of the University of Konstanz. NMR spectra were recorded by using Bruker AC 250 Cryospec (^{1}H: 250 MHz), Jeol JNA-LA-400 (^{1}H: 400 MHz), and Bruker DRX 600 (^{1}H: 600 MHz) spectrometers. Chemical shifts are given in parts per million and tetramethyl silane was used as the external standard. Electrospray ionization ion trap (ESI-IT) mass spectra were recorded by using a Bruker Daltonics esquire 3000+ instrument in positive or negative mode with a flow rate of 3 μL min^{-1}. DNA oligonucleotide synthesis was carried out by using an Applied-Biosystems 392 DNA/RNA synthesizer. Reverse-phase HPLC was performed by using a Prominence HPLC (Shimadzu) instrument equipped with a Nucleosil-100-5 C18 column (250× 4 mm, Macherey-Nagel). A binary gradient system (triethyl ammonium acetate buffer (0.1 M, pH 7.0)/CH_3CN, 25 °C) was used.

5′-*O*-Levulinyl-3′-*O*-*tert*-butyldimethylsilylthymidine (4): A solution of 3′-*O*-(*tert*-butyldimethylsilyl)thymidine (**3**) (5.53 g, 15.5 mmol, 1.0 equiv) in dioxane (100 mL) was added to a suspension of 2-chloro-1-methylpyridinium iodide (7.9 g, 31 mmol, 2.0 equiv) in CH_3CN (100 mL). Subsequently, a solution of levulinic acid (7.2 g, 62 mmol, 4.0 equiv) and DABCO (8.7 g, 77 mmol, 5.0 equiv) in dioxane (100 mL) was added dropwise to the reaction mixture over a period of 1 h at RT. Stirring was continued until the starting material had disappeared (7 h), as determined by TLC, before the solvent was evaporated under reduced pressure. EtOAc was added to the resulting residue, and the solution was washed with aqueous $NaHCO_3$ (80%) and with brine. The organic layer was dried over anhydrous magnesium sulfate and concentrated under reduced pressure. The crude compound was purified by means of flash column chromatography on silica gel (6% acetone in CH_2Cl_2) to give compound **4** (6.5 g, 13.9 mmol, 90%). R_f = 0.25 (6% acetone in CH_2Cl_2); ^{1}H NMR ($CDCl_3$, 250 MHz): δ = 9.35 (s, 1H; H-N), 7.27 (s, 1H; H-6), 6.21 (t, J = 6.5 Hz, 1H; H-1′), 4.33–4.12 (m, 3H; H-3′, H-5′), 3.97 (q, J = 3.8 Hz, 1H; H-4′), 2.23 (m, 2H; CH_2-Lev), 2.55–2.50 (m, 2H; CH_2-Lev), 2.23 (ddd, J = 3.8, 6.3, 10.3 Hz, 1H; H-2′), 2.12 (s, 3H; CH_3-CO), 2.07–1.95 (m, 1H; H-2′), 1.86 (s, 3H; CH_3-5), 0.8 (s, 9H; $(CH_3)_3$C-Si), 0.003 (s, 3H; CH_3-Si), −0.003 ppm (s, 3H; CH_3-Si); ^{13}C NMR ($CDCl_3$, 62.5 MHz): δ = 206.2, 172.3, 163.8, 150.3, 135.2, 111.0, 84.9, 84.6, 71.6, 66.9, 63.3, 40.7, 37.7, 29.6, 27.7, 25.6, 17.8, 12.5, −4.8, −5.03 ppm; ESIMS: m/z: 477.2 $[M+Na]^+$.

Synthesis of 4-(1,2,4-triazol-1-yl)-5′-*O*-levulinyl-3′-*O*-*tert*-butyldimethylsilyl-2′-deoxythymidine (5): $POCl_3$ (4.12 g, 27 mmol, 3.5 equiv) was added dropwise at 0 °C with vigorous stirring to a suspension of 1,2,4-triazole (8.03 g, 116 mmol, 15.0 equiv) in CH_3CN (135 mL). NEt_3 (18 mL, 140 mmol, 18.0 equiv) was then added dropwise over a period of 15 min and was stirred for an additional 30 min at 0 °C. Compound **4** (3.51 g, 7.7 mmol, 1.0 equiv) in CH_3CN (35 mL) was added dropwise over a period of 30 min to the resulting slurry, which was then stirred for 1 h at RT. The reaction mixture was diluted with EtOAc (100 mL) and washed with aqueous $NaHCO_3$ (80%) and with brine. The organic layer was dried over anhydrous magnesium sulfate and concentrated under reduced pressure. The crude compound was purified by means of flash chromatography on silica gel (5–6% acetone in CH_2Cl_2) to give compound **5** in a quantitative yield. R_f = 0.45 (5% acetone in CH_2Cl_2; highly fluorescent when visualized with UV light); ^{1}H NMR ($CDCl_3$, 250 MHz): δ = 8.10 (s, 1H; triazole H-3), 8.07 (s, 1H; triazole H-5), 8.06 (s, 1H; H-6); 6.20 (t, J = 5.8 Hz, 1H; H-1′), 4.40–4.15 (m, 5H; H-5′, H-4′, H-3′), 2.80–2.53 (m, 5H; CH_2CH_2-Lev, H-2′), 2.44 (s, 3H; CH_3-CO), 2.21–2.11 (m, 4H; CH_3-5, H-2′), 0.85 (s, 9H; $(CH_3)_3$C-Si), 0.04 (s, 3H; CH_3-Si), 0.03 ppm (s, 3H; CH_3-Si); ^{13}C NMR ($CDCl_3$, 62.5 MHz): δ = 206.1, 172.3, 158.1, 153.7, 153.3, 146.3, 105.4, 96.0, 87.6, 85.4, 70.8, 62.9, 41.8, 37.7, 29.6, 27.7, 25.6, 17.8, 17.2, −4.8, −5.1 ppm; ESIMS: m/z: 528.2 $[M+Na]^+$.

Synthesis of 5′-*O*-levulinyl-3′-*O*-*tert*-butyldimethylsilyl-N^4-(6-hydroxyhexyl)-5-methyl-2′-deoxycytidine (6): Compound **5** (2.64 g, 5.2 mmol, 1.0 equiv) in CH_3CN (40 mL) was added dropwise at RT to a solution of 6-aminohexanol (5.5 g, 47 mmol, 9.0 equiv) in CH_3CN (200 mL) and the reaction mixture was stirred for 3 h. The reaction mixture was then diluted with EtOAc (300 mL) and washed with aqueous $NaHCO_3$ (80%) and with brine. The organic layer was dried over anhydrous magnesium sulfate and the solvent was removed under reduced pressure. The crude compound was purified by means of flash column chromatography on silica gel (4% MeOH in CH_2Cl_2) to yield compound **6** (2.56 g, 4.6 mmol, 88%). R_f = 0.33 (4% methanol in CH_2Cl_2); ^{1}H NMR ($CDCl_3$, 250 MHz): δ = 7.3 (s, 1H; H-6), 6.29 (t, J = 6.3 Hz, 1H; H-1′), 5.1 (br s, 1H; H-NCH_2), 4.34–4.20 (m, 3H; H-5′, H-3′), 4.00 (q, J = 4.1 Hz, 1H; H-4′), 3.61 (t, J = 6.3 Hz, 2H; CH_2-OH), 3.5 (br q, J = 4.2 Hz, 2H; CH_2-NH), 2.78 (t, J = 6.3 Hz, 2H; CH_2-Lev), 2.61–2.55 (m, 2H; CH_2-Lev), 2.41–2.35 (ddd, J = 4.8, 6.3, 11.3 Hz, 1H; H-2′), 2.18 (s, 3H; CH_3-CO), 2.12–2.01 (m, 1H; H-2′), 1.91 (s, 3H; CH_3-5), 1.65–1.35 (m, 8H; CH_2-N^4-alkyl), 0.85 (s, 9H; $(CH_3)_3$C-Si), 0.04 (s, 3H; CH_3-Si), 0.03 ppm (s, 3H; CH_3-Si); ^{13}C NMR ($CDCl_3$, 62.5 MHz): δ = 206.3, 172.4, 162.9, 156.0, 136.4, 101.6, 85.8, 84.1, 71.4, 63.6, 62.6, 41.4, 40.8, 37.7, 32.4, 29.7, 29.1, 27.7, 26.4, 25.6, 25.2, 17.9, 13.2, −4.7, −5.0 ppm; ESIMS: m/z: 554.3 $[M+1]^+$, 576.3 $[M+Na]^+$; elemental analysis calcd (%) for $C_{27}H_{47}N_3O_7Si$: C 58.56, H 8.55, N 7.59; found: C 59.13, H 7.99, N 6.86.

Synthesis of 5′-*O*-levulinyl-3′-*O*-*tert*-butyldimethylsilyl-N^4-[*O*-(4,4′-dimethoxytrityloxy)hexyl]-5-methyl-2′-deoxycytidine (7): Compound **6** (2.4 g, 4.33 mmol) was co-evaporated twice with dry pyridine (20 mL) and then dissolved in dry pyridine (45 mL). 4,4′-Dimethoxytritylchloride (1.76 g, 5.2 mmol) and a catalytic amount of DMAP (0.05 g) were added to the solution, which was stirred overnight at RT. The reaction mixture was then quenched with dry methanol (0.9 mL, 26 mmol) before it was diluted with EtOAc (100 mL) and washed with aqueous $NaHCO_3$ (80%) and with brine. The organic layer was then dried over anhydrous magnesium sulfate, the solvent was evaporated under reduced pressure, and the crude compound was purified by means of flash column chromatography on silica gel (75–80% EtOAc in petroleum ether, 1% NEt_3) to give compound **7** (3.02 g, 3.5 mmol, 80%). R_f = 0.25 (80% EtOAc in petroleum ether); ^{1}H NMR ($CDCl_3$, 250 MHz): δ = 7.43–7.17 (m, 10H; H-Ar, H-6), 6.79 (d, J = 10.0 Hz, 4H; H-Ar), 6.29 (t, J = 6.3 Hz, 1H; H-1′), 4.83 (t, J = 4.9 Hz, 1H; H-N^4), 4.35–4.20 (m, 3H; H-5′, H-3′), 4.03–3.98 (m, 1H; H-4′), 3.77 (s, 6H; CH_3-O), 3.48 (q, J = 5.9 Hz, 2H; CH_2-NH), 3.01 (t, J = 6.5 Hz, 2H; CH_2-ODMTr), 2.77 (t, J = 6.3 Hz, 2H; CH_2-Lev), 2.63–2.56 (m, 2H; CH_2-Lev), 2.42 (ddd, J = 5.0, 6.5, 11.3 Hz, 1H; H-2′), 2.18 (s, 3H; CH_3-CO), 2.12–2.02 (m, 1H; H-2′), 1.90 (s, 3H; CH_3-5), 1.59–1.30 (m, 8H; CH_2-N^4-alkyl), 0.85 (s, 9H; $(CH_3)_3$C-Si), 0.04 (s, 3H; CH_3-Si), 0.03 ppm (s, 3H, CH_3-Si); ^{13}C NMR ($CDCl_3$, 150 MHz): δ = 209.1, 174.1, 164.7, 159.9, 158.5, 146.9, 137.8, 137.7, 131.1, 129.3, 128.6, 127.6, 113.9, 113.4, 105.2, 87.2, 87.0, 86.3, 73.6, 64.7, 64.3, 55.7, 42.0, 38.6, 31.0, 30.1, 29.6, 28.8, 27.8, 27.3, 26.2, 18.8, 13.4, −4.6, −4.8 ppm; ESIMS: m/z: 856.1 $[M]^+$, 878.4 $[M+Na]^+$; elemental analysis calcd (%) for $C_{48}H_{65}N_3O_9Si$: C 67.34, H 7.65, N 4.91; found: C 67.69, H 7.76, N 4.78.

Synthesis of 5′-*O*-levulinyl-N^4-[*O*-(4,4′-dimethoxytrityl-6-oxy)hexyl]-5-methyl-2′-deoxycytidine (8): Acetic acid (0.26 mL, 4.5 mmol) was added to a solution of compound **7** (1.95 g, 2.3 mmol) in dry THF (30 mL) before TBAF (1 M in THF, 4.6 mL, 4.5 mmol) was added dropwise with

constant stirring at RT. The reaction mixture was stirred for 7 h, and diluted with EtOAc (100 mL). The organic phase was washed with aqueous $NaHCO_3$ solution (80%) and with brine. It was then dried over anhydrous magnesium sulfate and the solvent was evaporated under reduced pressure. The crude compound was purified by means of flash column chromatography on silica gel (4–5% methanol in CH_2Cl_2, 1% NEt_3) to yield compound **8** (1.67 g, 2.2 mmol, 98%). $R_f = 0.4$ (5% methanol in CH_2Cl_2); m.p. 64–66 °C; ^{1}H NMR ($CDCl_3$, 250 MHz): $\delta = 7.43$–7.17 (m, 10H; H-Ar, 6-H), 6.78 (d, $J = 9.0$ Hz, 4H; H-Ar), 6.35 (t, $J = 6.4$ Hz, 1H; H-1′), 4.83 (t, $J = 6.4$ Hz, 1H; H-N^4), 4.40–4.24 (m, 3H; H-5′, H-3′), 4.14 (q, $J = 5.0$ Hz, 1H; H-4′), 3.76 (s, 6H; CH_3-O), 3.47 (q, $J = 5.0$ Hz, 2H; CH_2-NH), 3.00 (t, $J = 7.5$ Hz, 2H; CH_2-ODMTr), 2.77 (t, $J = 6.0$ Hz, 2H; CH_2-Lev), 2.61–2.52 (m, 3H; CH_2-Lev, H-2′), 2.17 (s, 3H; CH_3-CO), 2.07–1.96 (m, 1H; H-2′), 1.90 (s, 3H; CH_3-5), 1.59–1.32 ppm (m, 8H; CH_2-N^4-alkyl); ^{13}C NMR ($CDCl_3$, 62.5 MHz): $\delta = 206.6$, 172.6, 163.0, 158.2, 156.2, 145.3, 136.6, 129.9, 128.1, 127.6, 126.5, 112.9, 101.8, 85.9, 85.6, 83.8, 71.3, 64.2, 63.2, 55.1, 41.1, 37.8, 29.9, 29.8, 29.2, 27.8, 26.8, 26.1, 13.1 ppm; ESIMS: m/z: 764.4 $[M+Na]^+$; elemental analysis calcd (%) for $C_{42}H_{51}N_3O_9$: C 68.00, H 6.93, N 5.66; found: C 67.6, H 6.92, N 5.50.

Synthesis of N^4-(*O*-dimethoxytrityl-6-oxyhexyl)-5′-*O*-levulinyl-5-methyl-2′-deoxycytidine-3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite (1): DIPEA (0.57 mL, 2.5 mmol) was added dropwise with constant stirring to a solution of **8** (0. 31 g, 0.42 mmol) in dry THF (6 mL) at 0 °C. At the same temperature, *O*-(2-cyanoethyl)-*N*,*N*′-diisopropylchlorophoramidite (0.2 mL, 0.84 mmol) was added dropwise with vigorous stirring. Stirring was continued for 3 h at 0 °C. The reaction was quenched with dry methanol (0.33 mL, 8.2 mmol) and the reaction mixture was diluted with CH_2Cl_2 (25 mL). The organic layer was washed with cold water and with brine. It was then dried over anhydrous magnesium sulfate and the solvent was evaporated under reduced pressure. The crude compound was purified by means of flash column chromatography on silica gel (85% EtOAc in petroleum ether, 2% NEt_3) to yield phosphoramidite **1** as a mixture of diastereomers (0.38 g, 0.4 mmol, 97%). ^{1}H NMR ($CDCl_3$, 250 MHz): $\delta = 7.40$–7.16 (m, 10H; H-Ar, 6-H), 6.75 (d, $J = 7.5$ Hz, 4H; H-Ar), 6.29–6.23 (m, 1H; H-1′), 4.53–4.45 (m, 1H; H-4′), 4.28–4.02 (m, 4H; H-5′, CH_2O-CN), 3.68 (s, 6H; CH_3-O), 3.61–3.34 (m, 3H; H-3′, CH-*i*PrN), 2.97 (t, $J = 6.1$ Hz, 2H; CH_2-ODMTr), 2.82–2.62 (m, 6H; CH_2-Lev, CH_2-NH, CH_2-CN), 2.53–2.48 (m, 3H; CH_2-Lev, H-2′), 2.20–2.14 (m, 1H; H-2′), 2.08 (s, 3H; CH_3-CO), 1.87 (s, 3H; CH_3-5), 1.56–1.51 (m, 4H; CH_2-N^4-alkyl), 1.25–1.14 ppm (m, 16H; $(CH_3)_2$CH-N, CH_2-N^4-alkyl); ^{13}C NMR (CD_3OD, 62.5 MHz): $\delta = 209.1$, 174.0, 164.8, 159.9, 158.5, 146.9, 137.8, 131.2, 129.3, 128.7, 119.6, 114.0, 105.3, 87.0, 64.4, 55.8, 46.7, 44.6, 44.4, 42.1, 40.8, 38.6, 31.1, 30.1, 29.7, 28.9, 27.9, 27.3, 25.0, 24.9, 23.2, 21.0, 20.9, 20.4, 13.4 ppm; ^{31}P NMR (CD_3OD, 160 MHz): $\delta = 149.79$, 149.57 ppm; ESIMS: m/z: 941.5 $[M+Na]^+$; elemental analysis calcd (%) for $C_{15}H_{68}N_5O_{10}P$: C 65.02, H 7.28, N 7.43; found: C 65.3, H 7.32, N 7.50.

General procedure for the synthesis of bDNA: The synthesis of bDNA oligonucleotides was performed by using an Applied Biosystems 392 DNA synthesizer with a 3′-CPG support (1000 Å) and commercially available 3′-*O*-2-CE phosphoramidites on either a 0.2 or 1.0 μmol scale. After insertion of branch point **1**, DNA synthesis was continued and standard coupling conditions were utilized in the case of the 3′- and 5′-CE phosphoramidites, whereas for the insertion of branch point **1**, the coupling times were extended to 10 min by using **1** (0.12 M) in CH_3CN. The extension of each branch was terminated by first cleaving the respective DMTr group and subsequently passing capping mixtures A (acetic anhydride/pyridine/THF) and B (*N*-methylimdidazole/pyridine/THF) over the solid support for 3×15 s. The automated synthesis was then temporarily interrupted and the column was detached from the synthesizer. The levulinyl group was deprotected manually by using the deprotection solution (hydrazine (0.5 M) in pyridine/acetic acid = 1:1) with the aid of syringes for 55 min. Afterwards the column was thoroughly washed with CH_3CN (30 mL) and with CH_2Cl_2 (30 mL). The column was then reinstalled and the synthesis was continued from the branch point. At the end of the synthesis the DMTr group was retained ("trityl ON"), which allowed failed sequences to be removed by means of reverse-phase HPLC with a binary gradient of CH_3CN in triethylammonium acetate buffer (pH 7.0). The desired bDNA containing the DMTr group was collected, deprotected by using 80% AcOH, purified by using a preparative polyacrylamide gel, and characterized by using ESIMS. The sequences of the splints are as follows: **ab** = 5′-d(ACT GCT ACG AAT TCG TCA GC), **cd** = 5′-d(CGA GTA TAG CAG CGA GCC AA)-3′, **3′–3′** = 3′-d(CGC GGC GGC A)-5′-5′-(ATC CTC CTT C)-3′, and **AG** = 5′-d(CGG CTC AGC TTC GAA CTG CG)-3′.

ESI measurements: The purified bDNA molecules and oligonucleotide templates (50–100 pmol) were dissolved in a solution of 2-propanol (20%) containing NEt_3 (1%).[33] The mass measurements were carried out by using an Esquire 3000+ (Bruker) instrument and nitrogen was used as the nebulizing gas (12 psi) at 300 °C. The samples were injected into the system with the aid of a syringe pump (180 μL h^{-1}).

Radioactive phosphorylation of bDNAs: bDNA (10 pmol) was dissolved in a solution (46 μL, pH 7.6, 25 °C) containing Tris-HCl (79.5 mM), magnesium chloride (11.4 mM), dithiothreitol (5.7 mM), and γ-^{32}P-ATP (4 μL, 2.0 μM). Polynucleotide kinase (2 μL, 10 U μL^{-1}) was added to this solution and it was incubated at 37 °C for 1 h. The reaction was stopped by heating the solution to 95 °C for 5 min, followed by purification by using a G-25 column.

Ligation: Radioactively phosphorylated and nonradioactively phosphorylated bDNA strands (5 pmol) were combined with oligonucleotide templates (10–15 pmol) in ligation buffer (50 μL contains the following final concentrations: Tris-HCl (50 mM), $MgCl_2$ (10 mM), ATP (1 mM), dithiothreitol (10 mM), and BSA (1.25 μg)) and heated up to 95 °C for 3 min and cooled to 4 °C (0.1 °C per 3 s) in a thermocycler. T4 DNA ligase (1 μL, 1 U μL^{-1}) was added to the solution and incubated for 37 °C for 8 h. The reaction was stopped by heating the solution at 65 °C for 25 min.

Denaturing polyacrylamide gel electrophoresis: Samples were loaded onto 8% acrylamide gel (bisacrylamide/acrylamide 29:1) prepared in 1X TBE buffer (Tris-HCl (89 mM), pH 8, boric acid (89 mM), EDTA (2 mM), and urea (8.3 M)). Electrophoresis was carried out at constant 110 W and approximately 2000 V, at constant temperature (45 °C), and by using 1X TBE without urea as the running buffer.

Native polyacrylamide gel electrophoresis: Equimolar amounts of bDNA (radioactively phosphorylated and nonradioactively phosphorylated) and templated oligonucleotides were mixed in ligation buffer and annealed as mentioned in the ligation experiment. The gel was prepared with 8% acrylamide (acrylamide/bisacrylamide 29:1) in a buffer containing Tris-HCl (70 mM, pH 8), boric acid (70 mM), EDTA (1.5 mM), and magnesium acetate (12.5 mM). The samples were loaded onto the gel with an agarose loading buffer (glycerol (50%) with xylene cyanol (0.3%) and bromophenol blue (0.3%) as tracking dyes).

EcoRI digestion of ligated bDNA: EcoR1 buffer (1 μL; Tris-HCl (100 mM), NaCl (50 mM), and $MgCl_2$ (10 mM)) was added to the ligated bDNA (10 μL of 0.125 μM) and the solution was heated to 94 °C then cooled to 4 °C (0.1 °C per 3 s). EcoRI (1 μL, NEB, 1 U μL^{-1}) was then added and incubated at 37 °C for 1 h. The reaction was stopped by heating the solution to 65 °C for 25 min and was analyzed by denaturing PAGE.

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