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A modified uridine for the synthesis of branched DNA

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Abstract—Branched DNA constructs have found wide application in DNA-based nanotechnology. Several reports describe the generation of branched DNA structures with variable numbers of arms to self-assemble with pre-designed architectures. Branched DNA is generated by using designed rigid crossover DNA molecules as building blocks. Alternatively, branched DNAs can also be generated by using synthetic branch points derived either from nucleoside or non-nucleoside building blocks. Herein, we report the synthesis of modified uridine derivatives as branching monomer for the synthesis of branched DNA and first studies of their self-assembling properties.

1. Introduction

There is a need to expand synthetic approaches for the generation of branched DNA, since their pivotal role in DNA-based nanotechnology and material science.[1] To generate such branched DNA, two basic strategies are followed: either linear oligonucleotides are constructed to result in rigid crossover constructs or covalent DNA branches are generated employing suitably modified branching monomers. Obviously, these branching monomers have to be designed in a way to be suited for automated standard solid phase synthesis. In this direction, a polyhydroxy phosphoramidite was synthesized for generating oligonucleotide dendrimers and these were employed as polylabelled DNA probes and as primer in PCR.[2] A ribonucleoside branching point (adenosine based) was utilized to generate 'Y' and 'V' shaped RNA and DNA with the length of 18 and 21 nucleotides. In this approach the *tert*-butyldimethylsilyl (TBDMS) group was utilized for protection of the additional hydroxyl function orthogonal to 4,4′-dimethoxytrityl (DMTr).[3] In another approach, the levulinyl (Lev) group was used as protecting group orthogonal to DMTr that can easily be deprotected under mild basic or buffered conditions using N_2H_4, under which other base sensitive protective groups remain intact. By using this strategy, Horn and co-workers synthesized a modified cytosine based branching monomer. This branching point was utilized to generate large comb and fork shaped branched DNAs, which were subsequently employed as signal amplifiers in nucleic acid quantification assays.[4] Recently, Müller et al., used the same branching point to generate an artificial hairpin ribozyme, which was shown to have RNA cleavage activity.[5] Interestingly, another kind of branching point was utilized to generate branched nucleic acids by employing 4′-, 3′-, and 5′-modified thymidine derivatives. In these examples TBDMS and tetrahydropyrane (THP) were used as orthogonal protecting groups for DMTr.[6] Recently, von Kiedrowski and co-workers reported a novel class of branched oligonucleotides that were constructed with the aid of a trifunctional linker.[7] We have also reported a modified cytidine-based branching monomer to generate unsymmetrical branched DNA and reported first insights into their property to self-assemble.[8]

Herein, we report the synthesis of novel 2′-hydroxyl modified uridine derivative as branching monomer for the synthesis of branched DNA. First studies of their self-assembling properties are also included.

2. Results and discussion

The synthesis of modified uridine branching monomer is depicted in Scheme 1. The starting precursor **1** was synthesized according to the literature procedures starting from uridine.[9] Brown et al. first synthesized **1** for the generation of DNA signaling probes termed HyBeacons.[9] In this case, the 2′-hydroxyethyl group was protected with fluorenylmethoxycarbonyl (Fmoc), which was deprotected after automated oligonucleotide synthesis on solid support under mild basic conditions and subsequently coupled with a dye phosphoramidite. However, this approach was not yet employed for the synthesis of branched nucleic acids.

Afterward, **1** was coupled with levulinic acid in the presence of 2-chloro-1-methylpyridinium iodide and 1,4-diazabicyclo[2.2.2]octane to give **2**. The tetraisopropyldisiloxane 3′,5′-hydroxy protection group was cleaved by using

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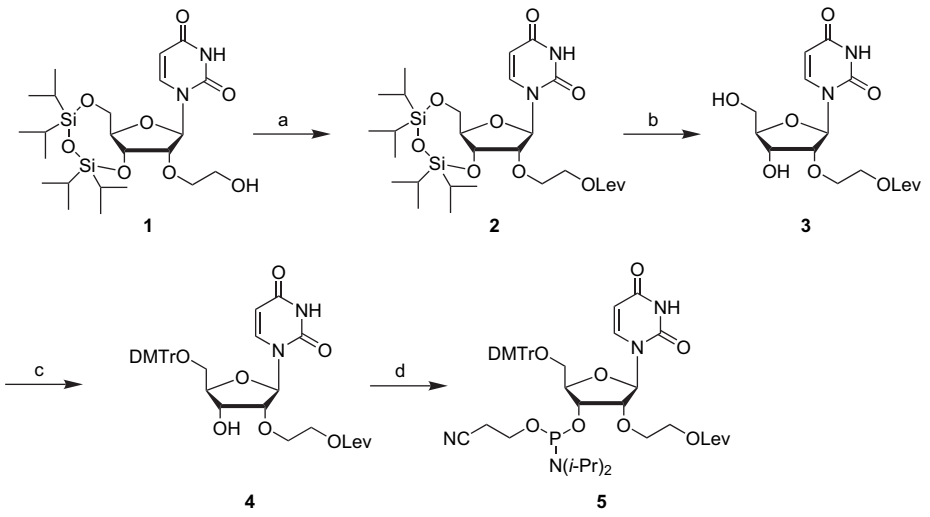


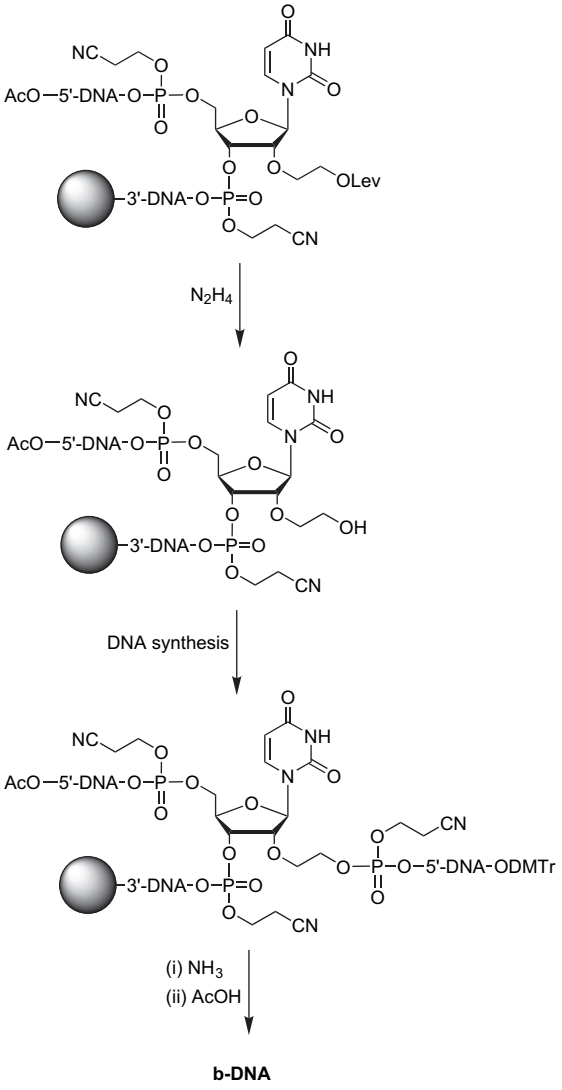
Scheme 1. Synthesis of uridine-based branching monomer. Reactions and conditions: (a) 2-chloro-1-methylpyridinium iodide, 1,4-diazabicyclo[2.2.2]octane, levulinic acid, CH_3CN/dioxane, 15 h, 98%; (b) TBAF/AcOH, THF, 30 min, 95%; (c) 4,4′-dimethoxytriphenylmethyl chloride, DMAP, pyridine, 9 h, 80%; (d) *N*,*N*-diisopropylethylamine, 2-cyanoethoxy-*N*,*N*-diisopropylaminochlorophosphine, CH_2Cl_2, 3 h, 77%.

tetrabutylammonium fluoride (TBAF) in the presence of acetic acid to yield compound **3**. Diol **3** was subsequently treated with DMTr chloride in the presence of a catalytic amount of 4-dimethyaminopyridine (DMAP) to give 5′-*O*-(4,4′-dimethoxytriphenylmethyl)-2′-*O*-(2-levulinyl-hydroxyethyl)-uridine (**4**) in 80% yield. Phosphitylation by treatment with 2-cyanoethoxy-*N*,*N*-diisopropylaminochlorophosphine yielded the corresponding phosphoramidite **5**.

The synthetic strategy of branched DNA using **5** is shown in Scheme 2.

After insertion of the branching monomer by usage of **5**, DNA synthesis was continued to yield the 5′-*O*-DNA segment. The 5′-*O*-terminal DMTr was cleaved on the DNA synthesizer and the 5′-hydroxyl group subsequently acetylated by using the standard capping conditions. Then, the levulinyl protection group at the 2′-*O*-hydroxyethyl group was removed by using 0.5 M N_2H_4 (10 mL of 1:1 AcOH/pyridine). Subsequently, the DNA synthesis was continued to yield the respective DNA segment starting from the 2′-*O*-ethoxy group of the branching monomer. According to the DMTr deprotection, the insertion yield of the modified uridine branch monomer is >98%. For the synthesis of unmodified nucleotides standard β-cyanoethyl phosphoramidites were employed and gave the best yields. Interestingly, usage of commercial phosphoramidites that bear acetyl groups to protect nucleobase functionalities resulted in very poor yield of desired branched DNA. This is probably due to the incompatibility of acetyl protection with the conditions required for levulinyl deprotection.

To investigate the self-assembling properties of branched DNAs, we have synthesized two different branched DNAs, b-DNA **A** and b-DNA **B** (46-mer) employing **5** and the strategy is depicted in Scheme 2. Each of the branches is composed of 15-mer oligonucleotides (Fig. 1). All branches are designed in such a way that the end segment (10-mer) of each branch of b-DNA **A** is complementary to the 10-mer end segment of b-DNA **B** branches. Thus, the 10-mer



Scheme 2. Synthesis of b-DNA using **5**.

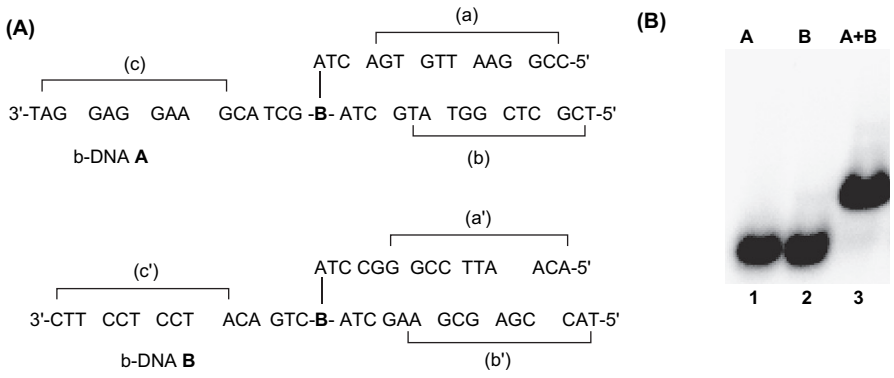


Figure 1. Synthesized b-DNA and their self-assembly. (A) b-DNA synthesized employing **5**; (B) 8% native PAGE analysis of radioactively labeled b-DNA. Lane 1: b-DNA **A**; lane 2: b-DNA **A**; lane 3: b-DNA **A**+b-DNA **B**.

end segments (a, b, c) of b-DNA are complementary to the 10-mer end segments of b-DNA **B** (a′, b′, c′).

In order to gain first insights into the non-covalent self-assembling properties of these branched DNA, both b-DNAs were ^{32}P-labeled by using T4 polynucleotide kinase under standard conditions. Afterward, **A** and **B** were mixed in 1:1 ratio and heated up to 95 °C for 5 min and slowly cooled to 4 °C (0.1 °C/min). The samples were then analyzed by native polyacrylamide gel electrophoresis (see Fig. 1B). The exclusive formation of a slower migrating reaction product was observed. As judged from the migration mobility of the reaction products, the chosen set-up resulted in the formation of dimeric structures rather than the formation of any higher order structures.

3. Conclusions

In conclusion, we have shown the synthesis of branched DNAs using a novel 2′-*O*-modified uridine branching monomer and investigated first self-assembling properties of the derived branched DNA oligonucleotides.

4. Experimental section

4.1. General

All temperatures quoted are uncorrected. All reagents are commercially available and used without further purification. Solvents are purchased over molecular sieves (Fluka) and used directly without further purification unless otherwise noted. All reactions were conducted under rigorous exclusion of air and moisture. NMR spectra were recorded on a Bruker AC 250 Cryospec and Jeol JNA-LA-400 (^{1}H: 250 MHz, ^{13}C: 62.5 MHz, ^{31}P: 160.0 MHz). The solvent signals were used as references and the chemical shifts converted to the TMS scale and are given in parts per million (δ). High resolution electrospray ionization-Fourier transform ion cyclotron mass spectrometry (ESI-FTICR) was recorded on a Bruker Daltonics Apex II in positive mode. DNA oligonucleotides were synthesized on an Applied Biosystems 392 DNA/RNA-synthesizer employing standard phosphoramidite strategy. Flash chromatography: Merck silica gel G60 (230–400 mesh). Thin layer chromatography: Merck precoated plates (silica gel 60 F_{254}). Reversed-phase HPLC was performed on a prominence-line HPLC (Shimadzu) with a Nucleosil-100-5-(250/4)-C18-column from Macherey–Nagel and a binary gradient system (TEAA—buffer (0.1 M), acetonitrile).

4.1.1. 3′,5′-*O*-(1,1,3,3-Tetraisopropyldisiloxane-1,3-diyl)-2′-*O*-(2-levulinyl-hydroxyethyl)-uridine (2). To the stirred solution of **1** (2.00 g, 3.77 mmol) in dry 1,4-dioxane (40 mL), the suspension of 2-chloro-1-methylpyridinium iodide (1.90 g, 7.44 mmol) in dry acetonitrile (20 mL) was added with constant stirring, at room temperature. To this, the mixture of 1,4-diazabicyclo[2.2.2]octane (2.07 g, 18.5 mmol) and levulinic acid (1.71 g, 14.7 mmol) in 1,4-dioxane (40 mL) was added dropwise with constant stirring. Stirring was continued for 9 h at room temperature. Solvent was evaporated under reduced pressure and the residue was dissolved in dichloromethane. The organic layer was washed with 10% sodium bicarbonate followed by brine and dried over anhydrous magnesium sulfate. The crude compound was purified by silica gel column chromatography (55% ethyl acetate in petroleum ether) to give **2** as an oil (2.30 g, 98%). R_f: 0.45 (66% ethyl acetate in petroleum ether). ^{1}H NMR ($CDCl_3$): δ 7.89 (d, J=8.0 Hz, 1H, H-6); 5.75 (s, 1H, H-1′); 5.67 (d, J=8.0 Hz, 1H, 5-H); 3.87–4.29 (m, 9H, H-5′, H-4′, H-3′, H-2′, CH_2CH_2–O); 2.57–2.64 (m, 2H, CH_2-Lev); 2.73–2.78 (m, 2H, CH_2-Lev); 2.18 (s, 3H, CH_3–CO); 1.01, 1.10 (m, 28H, $(CH_3)_2$CH–Si). ^{13}C NMR ($CDCl_3$): δ 206.6, 172.6, 163.6, 149.8, 139.6, 101.4, 89.0, 82.6, 81.6, 69.1, 68.3, 63.7, 59.3, 37.9, 29.8, 27.9, 17.4–16.8 (m, ^{i}Pr*C*); 13.4–12.5 (m, ^{i}Pr*C*). ESI-MS (*m*/*z*) calcd for $C_{28}H_{48}N_2O_{10}Si_2$ $[M+Na]^+$: 651.2745; found: 651.2744.

4.1.2. 2′-*O*-(2-Levulinyl-hydroxyethyl)-uridine (3). To the stirred solution of **2** (2.10 g, 3.33 mmol) in dry THF (30 mL), acetic acid (0.50 g, 8.33 mmol) followed by tetrabutylammonium fluoride (1 M in THF, 8.5 mL, 8.5 mmol) was added dropwise at room temperature. Stirring was continued for 1 h and the solvent was evaporated under reduced pressure. The crude compound was purified by silica gel column chromatography (5% methanol in dichloromethane) to give **3** (1.22 g, 95%) as an oil. R_f: 0.3 (10% methanol in dichloromethane). ^{1}H NMR ($CDCl_3$ and CD_3OD): δ 7.95 (d, J=8.1 Hz, 1H, H-6); 5.76 (d, J=2.7 Hz, 1H, H-1′); 5.62 (d, J=8.1 Hz, 1H, H-5); 4.16–4.21 (m, 2H, H-2′, H-3′); 3.43–3.96 (m, 7H, H-4′, H-5′, CH_2CH_2–O); 2.67–2.72 (m, 2H, CH_2-Lev); 2.47–2.52 (m, 2H, CH_2-Lev); 2.11 (s, 3H, CH_3–CO). ^{13}C NMR ($CDCl_3$ and CD_3OD): δ 207.6, 172.8,

164.1, 150.4, 138.9, 101.8, 88.3, 84.3, 82.3, 68.6, 68.1, 63.3, 60.0, 37.7, 27.7, 23.7. ESI-MS (*m/z*) calcd for $C_{16}H_{22}N_2O_9$ $[M+Na]^+$: 409.1223; found 409.1225.

4.1.3. 5′-*O*-(4,4-Dimethoxytrityl)-2′-*O*-(2-levulinyl-hydroxyethyl)-uridine (4). Compound **3** (1.20 g, 3.11 mmol) was coevaporated twice with dry pyridine and re-dissolved in dry pyridine (30 mL). To this DMAP (5 mg, 0.04 mmol) was added at 0 °C. Then DMTrCl (1.25 g, 3.69 mmol) in dry pyridine (10 mL) was added dropwise at the same temperature over the period of 1 h. Stirring was continued at room temperature over night. After this time, methanol (5 mL) was added to quench the reaction. The solvent was evaporated under reduced pressure and the remainder coevaporated twice with toluene. The crude compound was purified by silica gel column chromatography (70–80% of ethyl acetate in petroleum ether containing 1% of triethylamine) to give compound **4** (1.70 g, 80%) as white crystals. R_f: 0.2 (80% of ethyl acetate in petroleum ether containing 1% triethylamine). ^{1}H NMR (CD_3OD): δ 8.01 (d, J=8.1 Hz, 1H, H-6); 7.23–7.36 (m, 9H, Ar-H); 6.82 (d, J=8.7 Hz, 4H, Ar-H); 5.84 (d, J=2.2 Hz, 1H, H-1′); 5.12 (d, J=8.1 Hz, 1H, H-5); 4.39–4.44 (dd, J=5.2 Hz, J=7.7 Hz, 1H, H-3′); 4.20–4.24 (m, 2H, H-4′, –CH_2O–); 3.84–4.04 (m, 4H, H-5′, H-2′, –CH_2O); 3.44–3.45 (m, 2H, –OCH_2–); 2.72 (t, J=6.0 Hz, 2H, CH_2-Lev); 2.46–2.51 (m, 2H, CH_2-Lev); 2.10 (s, 3H, –$COCH_3$). ^{13}C NMR (CD_3OD): δ 209.6, 174.5, 166.2, 160.3, 152.0, 146.0, 136.9, 136.6, 131.6, 131.5, 114.3, 102.3, 88.2, 84.2, 83.9, 69.9, 64.7, 62.9, 55.8, 38.7, 29.7, 28.9. ESI-MS (*m/z*) calcd for $C_{37}H_{40}N_2O_{11}$ $[M+Na]^+$: 711.2530; found: 711.2527.

4.1.4. 5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-(2-levulinyl-hydroxyethyl)-uridine-3′-*O*-(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite (5). To the stirred solution of **4** (0.43 g, 0.62 mmol) in dichloromethane (8 mL), at 0 °C *N*,*N*-diisopropyl-*N*-ethylamine (0.53 mL, 3.10 mmol) was added dropwise. Subsequently *N*,*N*-diisopropylethylamine and 2-cyanoethoxy-*N*,*N*-diisopropylaminochlorophosphine (0.28 mL, 1.25 mmol) were added dropwise at the same temperature. Stirring was continued for 4 h at room temperature, and then dry methanol (0.5 mL, 20 mmol) was added to quench the reaction. The reaction mixture was diluted with dichloromethane and washed with cold water followed by brine solution. The organic layer was dried over anhydrous magnesium sulfate and the solvent was evaporated under reduced pressure. The crude compound was purified by silica gel column chromatography (80% ethyl acetate in petroleum ether containing 1% of triethylamine) to give **5** (0.43 g, 77%) as an oil. R_f: 0.66 (80% of ethyl acetate in petroleum ether containing 1% triethylamine). ^{1}H NMR (CD_3OD): δ 8.10 (d, J=8.0 Hz, H-6); 7.20–7.44 (m, 9H, Ar-H); 6.83 (d, J=8.7 Hz, 4H, Ar-H); 5.86 (d, J=1.5 Hz, 1H, H-1′); 5.16 (d, J=8.0 Hz, 1H, H-5); 3.72–4.22 (m, 10H); 3.72 (s, 6H, –OCH_3); 3.50–3.58 (m, 2H); 2.68–2.83 (m, 5H); 2.46–2.55 (m, 2H, CH_2-Lev); 2.09 (s, 3H, –CH_3CO); 1.10–1.26 (m, 12H, H-^{i}Pr). ^{13}C NMR (CD_3OD): δ 209.2, 174.2, 166.1, 160.2, 151.9, 145.9, 136.5, 131.5, 129.5, 1290.0, 128.9, 128.2, 114.2, 102.0, 88.1, 83.6, 83.5, 82.5, 71.0, 69.6, 64.9, 62.1, 61.4, 60.2, 55.8, 46.6, 38.6, 29.8, 28.9, 25.3, 23.2, 20.4. ^{31}P NMR (CD_3OD): δ 151.34, 150.00. ESI-MS (*m/z*) calcd for $C_{46}H_{57}N_4O_{12}P$ $[M+Na]^+$: 911.3609; found: 911.3604.

4.2. General procedure for the synthesis of branched DNA

The synthesis of branched DNA oligonucleotides was performed on an Applied Biosystems 392 DNA synthesizer, by using 3′-CPG support (1000 Å) and commercially available 3′-*O*-2-(cyanoethyl)-phosphoramidites on 0.2 μmol scale. After insertion of **5**, DNA synthesis was continued and standard coupling conditions were utilized in case of standard phosphoramidites, whereas for the insertion of branch point **5**, the coupling times were extended to 10 min using 0.12 M of **5** in acetonitrile and it was coupled twice without capping step after the first coupling step in the synthetic cycle. The extension of the branch was terminated by first cleaving of the respective DMTr group and subsequently passing the capping mixture A (acetic anhydride/pyridine/tetrahydrofuran) and B (*N*-methylimidazole/pyridine/tetrahydrofuran) for 3×15 s over the solid support. Then the automated synthesis was temporarily interrupted and the column was detached from the synthesizer. The levulinyl group was deprotected manually with the aid of syringes; by utilizing 10 mL of the deprotection solution (0.5 M of hydrazine in 1:1 mixture of pyridine and acetic acid) for 55 min. Afterward the column was thoroughly washed with acetonitrile (30 mL) followed by CH_2Cl_2 (30 mL). Then the column was 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 to purify from failure sequences by RP-HPLC with a binary gradient of acetonitrile in triethylammonium acetate buffer (pH 7.0). The desired branched DNA with DMTr group was collected, deprotected by using 80% AcOH, followed by purification over preparative polyacrylamide gel and characterization by ESI-MS.

4.3. Radioactive labeling of branched DNA

b-DNA of 10 pmol was dissolved in a solution (46 μL, 25 °C) containing Tris–HCl (79.5 mM, pH 7.6), magnesium chloride (11.4 mM), dithiothreitol (5.7 mM), and 4 μL of γ-^{32}P-ATP (2.0 μM). To this T4 polynucleotide kinase (2 μL, 10 U/μL) was added and incubated at 37 °C for 1 h. The reaction was stopped by heating the solution to 95 °C for 5 min, followed by purification with a G-25 column.

4.4. Self-assembly and native polyacrylamide gel electrophoresis

Equimolar molar amounts of b-DNA **A** and b-DNA **B** (radio actively phosphorylated and non-phosphorylated) were mixed in a buffer containing, Tris–HCl (50 mM), $MgCl_2$ (10 mM), and DTT (10 mM). The gel was prepared with 8% acrylamide (29:1, acrylamide/bisacrylamide) 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 on to gel suspended in loading buffer (50% of glycerol, 0.3% of xylene cyanol, and bromophenol blue as tracking dyes). The gel was run for 14 h, 4 °C, and 30 V.

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