

Synthesis of Formacetal-Linked Dinucleotides to Facilitate dsDNA Bending and Binding to the Homeodomain of Pax6

Marian Pitulescu,^[a] Marcel Grapp,^[b] Ralph Krätzner,^[b] Willhart Knepel,^[b] and Ulf Diederichsen^{*[a]}

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Two formacetal-linked dinucleotides T[^]T and T[^]A were synthesized as phosphoramidite building blocks for solid-phase synthesis. Incorporated in a 29-mer DNA, the oligomers P3_{T[^]T} and P3_{T[^]A} were studied with respect to the binding activity towards the Pax6 homeodomain. Substitution of the negatively charged phosphodiester by a neutral formacetal linker facilitates the bent conformation of double-stranded DNA. The duplex stability was affected more significantly by the T[^]T formacetal modification, whereas destabilization

induced by T[^]A was less pronounced. Based on CD spectroscopy, the T[^]A formacetal-modified oligomer P3_{T[^]A} has mainly B-DNA topology, whereas the P3_{T[^]T} modified oligomer significantly deviated from B-form DNA. The binding affinity of the P3 oligomer towards Pax6 HD was investigated by in vitro EMSA experiments providing even a small increase in binding affinity for the P3_{T[^]T} oligomer. (© Wiley-VCH Verlag GmbH & Co. KGaA, 69451 Weinheim, Germany, 2008)

Introduction

Transcription factors are proteins acting on gene transcription in the cell nucleus. To achieve their activational or inhibitory effects on gene transcription, they bind to specific nucleotide sequences within the promoter region of respective target genes.^[1,2] The transcription factor Pax6 belongs to the family of paired box proteins and plays an important role in α -cell differentiation and α -cell-specific glucagon gene activation.^[3–5] The peptide hormone glucagon is produced in α -cells in the pancreatic islets of Langerhans and acts on the liver to stimulate glycogenolysis and gluconeogenesis.^[6,7] Insulin, the peptide hormone from pancreatic islet β -cells, acts as a functional counterpart of glucagon and directly inhibits glucagon synthesis and secretion.^[4,8–10] In insulin-resistant or -deficient states, glucagon synthesis and secretion becomes disinhibited leading to hyperglucagonemia which contributes to hyperglycemia in type 2 diabetes mellitus, highlighting the role of Pax6 as a potential target for the therapy of type 2 diabetes mellitus.^[7,9,11]

Pax6 is composed of an amino-terminal paired domain (PD) followed by a linker region (L), a homeodomain (HD), and a carboxyl-terminal transactivation domain

(TAD) and binds to specific DNA sequences in the promoter elements G1 and G3 of the rat glucagon gene promoter (Figure 1).^[4] Pax proteins use different combinations of their PD and HD to bind to different promoters and stimulate gene transcription.^[12,13]

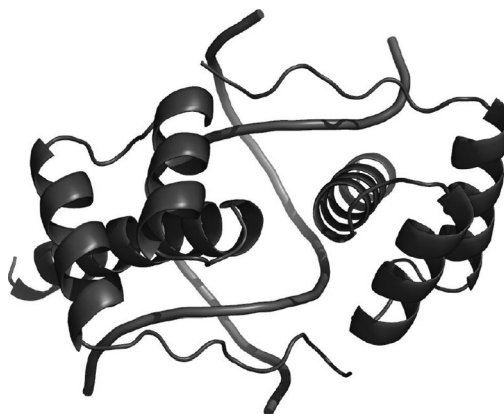


Figure 1. Binding of the HD of the protein paired from *Drosophila* as a dimer to an ideal P3 site TAATCTGATTA.^[16] The Pax6 HD shows high sequence identity with the paired HD, and for the PAX6 HD a similar dimeric binding complex with P3 fragment can be expected. The recognition helix of HD interacts with the specific DNA motif ATTA.

The Pax6 HD belongs to the paired type of homeodomains which were shown to bind to a so-called P3 site consisting of two palindromic ATTA/TAAT motifs separated by three nucleotides.^[14–16]

[a] Institut für Organische und Biomolekulare Chemie, Georg-August-Universität Göttingen, Tammannstr. 2, 37077 Göttingen, Germany
Fax: +49-551-392944
E-mail: udieder@gwdg.de

[b] Institut für Molekulare Pharmakologie, Georg-August-Universität Göttingen, Robert-Koch-Str. 40, 37075 Göttingen, Germany

Therefore, a selective inactivation of the Pax6 HD could be a therapeutically useful approach to reduce glucagon levels in diabetic patients. In this regard, therapeutically active, double-stranded decoy oligonucleotides preventing transcription factors from binding to their target sites in promoter regions of genes are an interesting concept.^[17] A number of chemical modifications has been used to improve the physiological stability of decoy nucleotides, but, to the best of our knowledge, no efforts have been made to stabilize decoy oligonucleotides in a bent conformation that results from binding of certain transcription factors. In this study we investigated the synthesis of methylene acetal linked dinucleotides, their incorporation in oligonucleotides, and the affinity as decoy oligonucleotides to the homeodomain of Pax6. Substitution of a charged phosphodiester by a neutral methylene acetal linkage was expected to facilitate bending of DNA double strands. Based on higher binding affinity, these bent dsDNA are potential decoy oligonucleotides for the Pax6 HD.

Within a large variety of oligonucleotide backbone modifications^[18] the replacement of the negatively charged phosphodiester group by the achiral and neutral formacetal linker results in a double helix that retains a topology similar to B-DNA. The double strand stability is reduced by about 3 °C per modification, and the formacetal backbone has only a slight influence on the backbone conformation.^[19] Furthermore, base pair complementarity by Watson–Crick hydrogen bonding is not affected by this modification. The formacetal linker was already used by He et al. to investigate the thrombin inhibitor activity of modified oligodeoxynucleotides based on charge-charge interactions.^[20] Next to the influence of phosphodiester charge recognition, a missing charge in the DNA backbone might also facilitate bending of the double strand like it is proposed for the neutralization of the phosphate group during the contact with positively charged amino acid residues.^[21] A pre-organization of dsDNA in a bent conformation can be expected replacing the negatively charged phosphate group with a neutral formacetal linker. Therefore, especially biological processes involving recognition of bent oligonucleotides should be affected.

Results and Discussion

The Pax6 HD preferentially binds to ATTA DNA motifs recognizing dsDNA and bending the DNA at this position.^[16] Therefore, formacetal-linked T-T and T-A dinucleotides **1** (T[^]T) and **2** (T[^]A) were synthesized (Figure 2).^[22–24] By incorporation of these dinucleotides in oligonucleotides the binding affinities of dsDNA with a preformed bent conformation towards the Pax6 HD were investigated. The dinucleotide building blocks were introduced by solid-phase oligonucleotide synthesis requiring phosphoramidites at the 3'-terminal end and DMT protection of the primary 5'-OH.

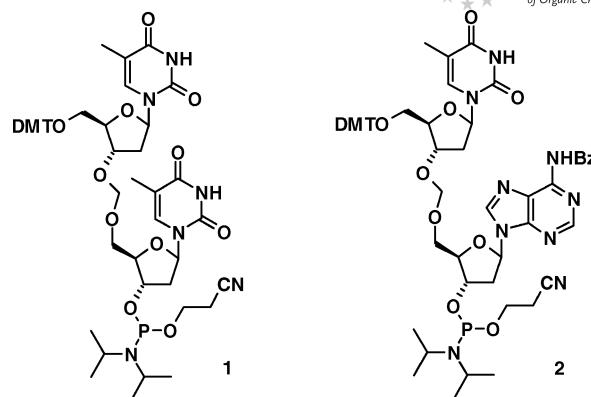


Figure 2. Formacetal-linked dinucleotide building blocks T[^]T (**1**) and T[^]A (**2**) for oligonucleotide solid-phase synthesis.

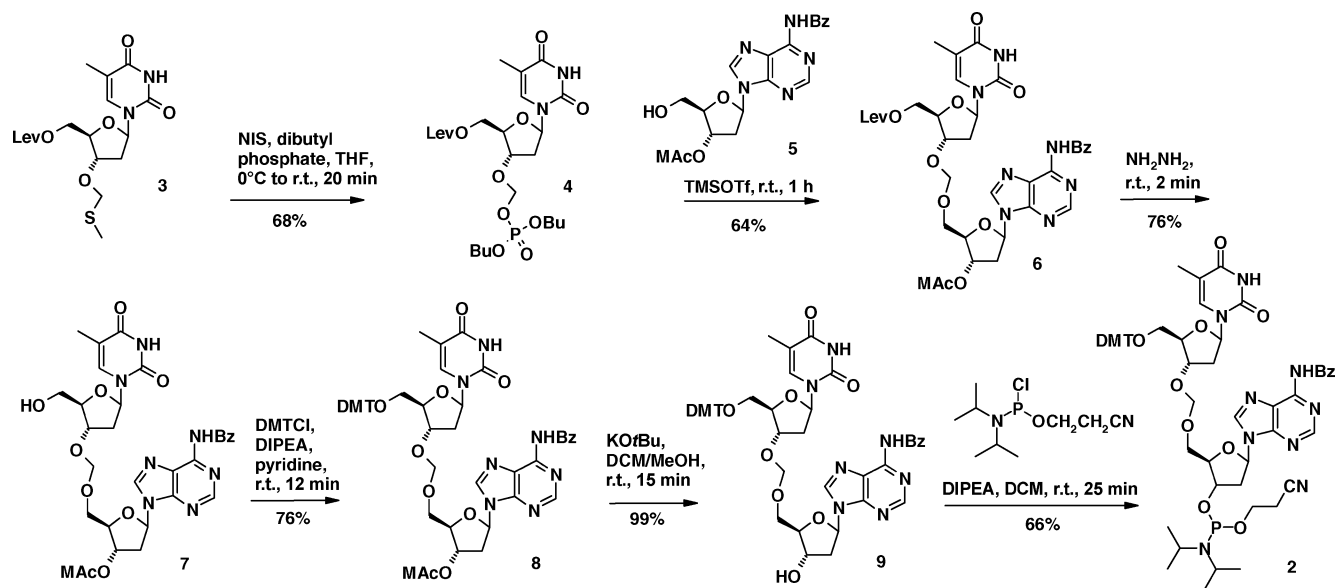
Synthesis of Formacetal-Linked Dinucleotides

The synthesis of the formacetal-linked dinucleotide (T[^]A) **2** was based on the methylthiomethyl nucleoside **3** and 2'-deoxyadenosine **5** (Scheme 1). Both have been prepared according to literature procedures.^[23,24] The phosphate group in the thymine nucleotide was introduced with *N*-iodosuccinimide (NIS) and dibutyl phosphate providing the phosphotriester **4** as electrophile for dimer formation. Connecting the two nucleosides **4** and **5** by acetalization was described to be promoted by TMSOTf.^[24] By using reaction conditions optimized with respect to reaction time and addition of TMSOTf in small portions, the formacetal T[^]A dimer **6** was provided in 64% yield.

Exchange of the protecting group of the primary 5'-OH group from the levulinic ester to the DMT protection, which is cleavable under acidic conditions as it is required for solid-phase synthesis, was achieved with hydrazine hydrate followed by tritylation of dinucleotide **7** with dimethoxytrityl chloride and DIPEA. The dinucleotide **9** was obtained quantitatively from derivative **8** by removal of the methoxyacetyl protecting group with potassium *tert*-butoxide. Finally, phosphitylation of the secondary 3'-OH group with chloro(2-cyanoethoxy)(diisopropylamino)phosphane in the presence of DIPEA gave the phosphoramidite **2** in excellent quality after chromatography on silica gel.

The formacetal-linked dinucleotide **1** with two thymine recognition units (T[^]T) was synthesized according to a procedure of van Boom^[23] by generating the phosphoramidite in analogy to the preparation of the T[^]A dinucleotide **2** as described in Scheme 1.

The formacetal-linked dinucleotide phosphoramidites **1** and **2** were incorporated in DNA sequences by standard protocols used for automated solid-phase oligonucleotide synthesis.^[25] The sequence was oriented on the Pax6 HD binding P3 oligonucleotide 5'-GATCCCTGAGAA-TAATCTGATTACTGTA^{3'} together with its complementary strand 5'-GATCTACAGTAATCAGATTATTC TCAGG^{3'} (**P3**) providing a four-nucleotide overhang on both ends.^[16] The formacetal modifications were introduced



Scheme 1. Synthesis of the dinucleotide T^A as phosphoramidite **2** ready to use in solid-phase oligonucleotide synthesis.

as indicated: 5'¹GATCCCTGAGAAT[^]AATCTGATTAC-TGTA3' (**P3_{T^A}**) and 5'¹GATCTACAGTAATCAGAT[^]T-ATTCTCAGG3' (**P3_{T^T}**).

Double-Strand Stabilities and Secondary Structures

First evidence for the influence of the formacetal modifications was obtained by determining double-strand stabilities using temperature-dependent UV spectroscopy (Figure 3). Compared to the native **P3** double strand ($T_m = 61\text{ }^{\circ}\text{C}$, 1.8 μmol , 10 mM NaH₂PO₄, pH = 7, 100 mM NaCl) the modified double strands **P3_{T^A}** ($T_m = 58\text{ }^{\circ}\text{C}$, 1.8 μmol , 10 mM NaH₂PO₄, pH = 7, 100 mM NaCl) and **P3_{T^T}** ($T_m = 56\text{ }^{\circ}\text{C}$, 1.8 μmol , 10 mM NaH₂PO₄, pH = 7, 100 mM NaCl) provided significantly lower stabilities. The replacement of a negatively charged phosphodiester group by the neutral formacetal linker seems to interfere with the double-helix topology leading to reduced stability as it is known already from other formacetal-linked DNA oligomers.^[19] The destabilization induced by the T^T ($\Delta T = 5\text{ }^{\circ}\text{C}$) modification is considerably higher than the respective effect based on the T^A formacetal dinucleotide ($\Delta T = 3\text{ }^{\circ}\text{C}$). This points to a more pronounced distortion of nucleobase pairing or stacking as it would result from a stronger bent double helix.

Conformational distortion of the B-DNA double helix should be also detectable by circular dichroism (CD) spectroscopy. The CD spectra recorded for the 29-mer **P3_{T^A}** provided a typical B-DNA profile, almost identical with the native DNA oligomer **P3** (Figure 4). The **P3_{T^A}** oligomer exhibited a positive Cotton effect at 286 nm and a negative one at 242 nm. A shift from the B-DNA conformation was registered in case of the **P3_{T^T}** oligomer (Figure 4). The CD spectrum shows a maximum at 288 nm and a very weak negative Cotton effect at 236 nm.

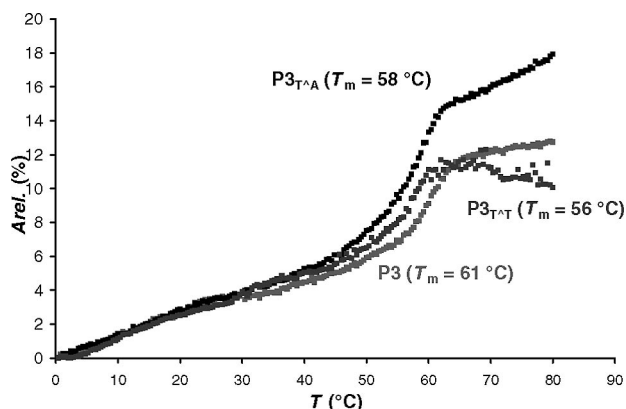


Figure 3. Temperature-dependent UV measurements of **P3**, **P3_{T^A}** and **P3_{T^T}** measured in 10 mM phosphate buffer, pH = 7.0, 260 nm.

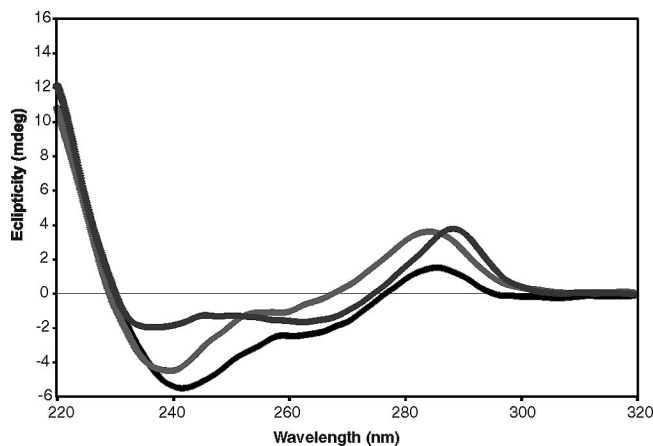


Figure 4. CD spectra of **P3_{T^T}** and **P3_{T^A}** compared to the native **P3** oligomer, measured in 10 mM phosphate buffer, pH = 7.0.

Electrophoretic Mobility Shift Assay (EMSA)

The oligonucleotides containing the modified T[^]T and T[^]A dimers were annealed with their complementary strands. The binding affinity of modified DNA oligomers **P3**_{T[^]A} and **P3**_{T[^]T} was tested towards glutathione S-transferase (GST)-Pax6 HD and GST produced in *E. coli* DH5a cells by performing an EMSA experiment. The dimeric oligonucleotide **P3** was included as a native control for Pax6 HD binding. As described, strong binding of GST-Pax6 HD to **P3** was achieved. GST-Pax6 HD was further found to bind at least as strong to **P3**_{T[^]T} as to the native **P3** oligonucleotide. None or very weak binding was measured for **P3**_{T[^]A} incubated with GST-Pax6 HD. As a control experiment, complex formation was not observed between the **P3** oligomers and GST only (Figure 5, Lanes 7–9). Statistical evaluation of four independent EMSA experiments showed a slightly, but not significantly stronger binding of GST-Pax6 HD to **P3**_{T[^]T} compared to **P3**. The modification **P3**_{T[^]A} caused a decrease in binding affinity by more than 85% compared to **P3**_{T[^]T} (Figure 5, right).

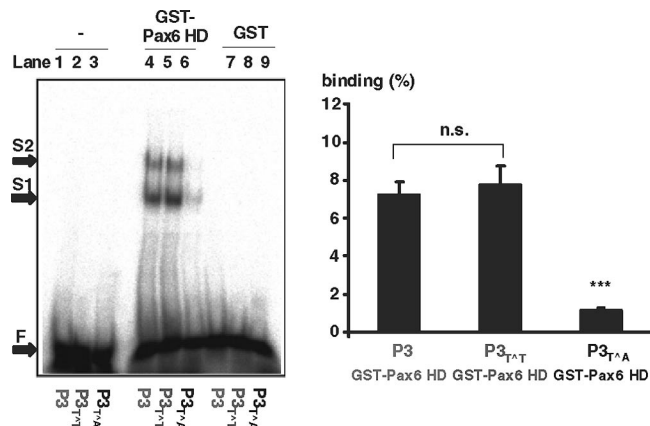


Figure 5. Binding of the Pax6 HD to the modified **P3** sites. Left: electrophoretic mobility shift assay of the GST-Pax6 HD fusion protein with the modified oligonucleotides. The labeled oligonucleotides **P3**, **P3**_{T[^]T} and **P3**_{T[^]A} were incubated with 250 ng of GST-Pax6 HD. GST was incubated with the indicated oligonucleotides as a specific control (Lanes 7–9). F = free probe; S1 and S2 = shifted probes; the two bands appearing for shifted probes were interpreted as homodimeric (S2) and monomeric (S1) binding complexes of HD with the probe.^[16] Sequences of the oligonucleotides used in the gel retardation analysis are shown in the Exp. Sect. Right: Densitometric measurement of the percentage of binding of the indicated probes by the Pax6 HD using the PhosphorImager. Values are means ($\pm$ SEM) of four independent experiments statistically evaluated by Student's *t* test ***, *p* < 0.001.

The homeodomain of the *Drosophila* paired protein has been shown to bind as a dimer to a P3-consensus site.^[14] The highly homologous Pax6 HD shows also strong binding affinity to the P3-consensus site. As a consequence of chemical modification, binding to the double-stranded oligonucleotide **P3**_{T[^]A} was mainly lost in EMSA experiments, while binding to **P3**_{T[^]T} was not affected. The TA step seems to be highly involved in the helix recognition by the Pax6 HD, and any change in the DNA linker structure lowers the binding affinity also from the P3-consensus site. Absence of

the phosphate with its negative charge plays a major role at the TT step, where a stronger binding of Pax6 to DNA was observed. The binding properties of the modified DNA oligomer were slightly improved with the neutral T[^]T formacetal linker better accommodating the interaction with the Pax6 HD.

Conclusions

Two modified dinucleotides T[^]T and T[^]A containing one formacetal linker instead of the native phosphodiester were synthesized and incorporated in a DNA 29-mer. The binding activities of the modified DNA oligomers were tested in vitro towards GST-Pax6 HD. The protein-binding activity was slightly increased only in the case of the T[^]T modified oligomer, whereas the oligomer with the formacetal linker at the TA step emerged as weak binder. These results might be in accordance with a pre-organization of the DNA in a bent conformation induced by a neutral formacetal linker (T[^]T modification). On the other hand, protein binding of double-stranded DNA could be significantly diminished in case the negative phosphate linkage is of importance for recognition by electrostatic interactions (T[^]A modification).

Experimental Section

Materials and Methods: Dry reactions were carried out under a dry argon using an appropriate Schlenk technique. The chemicals were used as supplied by Fluka, Sigma–Aldrich or Lancaster. Methoxyacetic anhydride was synthesized according to a literature procedure.^[24] The dry solvents were purchased from commercial sources, except dry pyridine, which was dried with CaC₂ and distilled prior to use. ¹H, ¹³C and ³¹P NMR spectra were recorded at room temperature with Bruker AMX 300 or Varian Inova 600 spectrometers. Chemical shifts are given relative to TMS, as internal reference for ¹H NMR as well as ¹³C NMR spectroscopy and to external 85% H₃PO₄ in D₂O for ³¹P NMR spectroscopy. ESI-MS and HRMS data were recorded with LCQ and TSQ 7000 instruments from Finnigan and APEX-Q IV 7T, respectively. CD spectra were recorded with a JASCO J-810 spectrometer equipped with a JASCO ETC-505S/PTC-423S temperature controller. Temperature-dependent UV spectra were measured with a JASCO V-550 UV/Vis spectrometer equipped with a JASCO ETC-505S/ETC-505T temperature controller.

Pax6 Homeodomain Glutathione S-Transferase Fusion Protein Preparation: The procaryotic expression vector for GST-Pax6 HD encoding the Pax6 homeodomain was described before^[26] and kindly provided by Prof. Dr. I. Mikkola (University of Tromsø, Norway). *E. coli* DH5a cells with this expression vector were grown at 26 °C and induced, after reaching OD₆₀₀ = 0.8, with 1 mM IPTG for 1.5 h. Cells were harvested and suspended (10 mL/1 L culture) in phosphate buffer, and the protease inhibitors PMSF (1 mM) and DTT (1 mM) were added. The resuspended cells were frozen at –80 °C and lysed by thawing on ice for 2–3 h followed by sonication for 3 min. The GST-Pax6 fusion protein was purified from the bacterial extract using glutathione-agarose beads (Sigma, Munich, Germany). The protein was eluted using 100 mM glutathione in phosphate-buffered saline (pH = 7.2; containing PMSF and DTT,

each 1 mM) and dialyzed against phosphate-buffered saline (containing 0.25 mM PMSF) at 4 °C.

Electrophoretic Mobility Shift Assay: Synthetic complementary oligonucleotides with 5'-GATC overhangs were annealed and labeled by a fill-in reaction with [α - 32 P]dCTP (Amersham Biosciences, Braunschweig, Germany) using the Klenow fragment of DNA polymerase (Fermantas, Munich, Germany). Bacterially expressed recombinant fusion proteins (ca. 250 ng) were incubated with 0.2 μ L of poly(dI-dC)-(dI-dC) (Sigma) as nonspecific competitor in 20 μ L (total volume) of 20 mM HEPES, pH = 7.9, 1 mM EDTA, 0.5 mM DTT, 140 mM KCl and 10% glycerol for 10 min on ice. The assay was then performed as described.^[27]

Oligonucleotides: Incorporation of the phosphoramidite derivatives **1** and **2** in the respective DNA oligomers was performed by IBA GmbH, Göttingen, Germany, using a standard protocol for solid-phase chemistry based on phosphoramidites.^[25] The following oligomers were synthesized with 0.1 nmol/ μ L concentration with the respective formacetal modification indicated in bold letters: 5'-GATCCCTGAGAATAATCTGATTACTGTA^{3'} (**P3**): ESI-MS: m/z = 8651.54 [M - 4 H + 3 Na]⁺, 8673.52 [M - 5 H + 4 Na]⁺; 5'-GATCTACAGTAATCAGATTATTCTCAGG^{3'} (**P3** complementary strand): ESI-MS: m/z = 8651.54 [M - 4 H + 3 Na]⁺, 8673.52 [M - 5 H + 4 Na]⁺; 5'-GATCCCTGAGAAT[^]AATCTGATTACTGTA^{3'} (**P3-T^A**): ESI-MS: m/z = 8601.54 [M - 4 H + 3 Na]⁺, 8623.58 [M - 5 H + 4 Na]⁺; 5'-GATCTACAGTAATCAGAT[^]TATTCTCAGG^{3'} (**P3-T^G**): ESI-MS: m/z = 8601.53 [M - 4 H + 3 Na]⁺, 8623.51 [M - 5 H + 4 Na]⁺.

3'-O-[(Dibutylphosphoryl)oxy]methyl-5'-O-levulinyl-2'-deoxythymidine (4): 5'-O-Levulinyl-3'-O-(methylthiomethyl)-2'-deoxythymidine (**3**) (535 mg, 1.36 mmol) was dissolved in dry DCE (2 mL) and the mixture cooled to 0 °C. A solution of dibutyl phosphate (380 μ L, 2.00 mmol) and NIS (450 mg, 2.00 mmol) in dry THF (2 mL) was added and the mixture stirred at 0 °C for 5 min. The ice bath was removed, and the reaction mixture was stirred at room temperature for additional 20 min. The reaction mixture was diluted with DCM (20 mL) and washed with a mixture of 1 M Na₂S₂O₃ and 0.9 M NaHCO₃ solution (1:1, v/v; 30 mL). The organic phase was dried with MgSO₄, concentrated in vacuo (the water bath temperature maintained at less than 30 °C) and purified by column chromatography (DCM/MeOH, 97:3 $\rightarrow$ 95:5) to yield **4** (485 mg, 0.91 mmol, 68%) as a viscous yellow oil. TLC (EtOAc) R_f = 0.33, ¹H NMR (300 MHz, CD₂Cl₂): δ = 0.91 (dt, ³J = 7.6 Hz, 6 H, 2 $\times$ CH₃, Bu), 1.38 (m, 4 H, 2 $\times$ CH₂, Bu), 1.64 (m, 4 H, 2 $\times$ CH₂, Bu), 1.89 (s, 3 H, CH₃, T), 2.14 (s, 3 H, CH₃, Lev), 2.14–2.24 (m, 1 H, H^{2'/2''}), 2.47 (m, 1 H, H^{2'/2''}), 2.50–2.58 (m, 2 H, CH₂, Lev), 2.68–2.90 (m, 2 H, CH₂, Lev), 4.02 (dq, ³J = 6.3, ³J = 6.8 Hz, 4 H, CH₂, Bu), 4.22–4.66 (m, 4 H, H^{3'}, H^{4'}, H^{5'}, H^{5''}), 5.20 (d, ²J = 11.8 Hz, 2 H, OCH₂O), 6.23 (dd, ³J = 6.1, 7.8 Hz, 1 H, H_{1'}), 7.32 (s, 1 H, H^{6'}), 9.42 (br. s, 1 H, NH) ppm. ¹³C NMR (75 MHz, CD₂Cl₂): δ = 12.6 (CH₃, T), 13.6 (CH₃, Bu), 18.9 (C ^{γ} , Bu), 28.1 (C ^{β} , Lev), 29.8 (CH₃, Lev), 32.4, 32.5 (C ^{β} , Bu), 38.0, 38.1 (C^{2'}, C ^{α} , Lev), 63.9 (C^{5'}), 68.0, 68.0 (C ^{α} , Bu), 79.2 (C^{4'}), 82.5 (C^{3'}), 85.2 (C^{1'}), 91.7, 91.8 (OCH₂O), 111.4 (C⁵), 135.5 (C⁶), 150.7 (C²), 164.2 (C⁴), 172.6, 206.6 (CO, Lev) ppm. ESI-MS: m/z = 585.2 [M + Na]⁺, 1146.8 [2 M + Na]⁺.

6-Benzoyl-3'-O-(methoxyacetyl)-2'-deoxyadenosine (5):^[24] Methoxyacetic anhydride (200 mg, 1.22 mmol) was added to a solution of 6-benzoyl-5'-O-(4,4'-dimethoxytrityl)-2'-deoxyadenosine (400 mg, 0.61 mmol) in dry pyridine (4 mL). After stirring for 2 h, the reaction was quenched with water (1 mL), the mixture concentrated under reduced pressure and co-evaporated with toluene (2 $\times$ 4 mL). The viscous oil was dissolved in CH₃NO₂/MeOH (95:5;

100 mL), and CCl₃COOH (3.20 g, 19.6 mmol) was added. After stirring for 10 min, the red mixture was poured carefully into NaHCO₃ (0.9 M, 200 mL) and extracted with DCM (5 $\times$ 50 mL). The combined organic phases were dried with MgSO₄, concentrated to a small volume, and the residue was subjected to flash chromatography (EtOAc/MeOH, 96:4) to afford **5** (208 mg, 0.48 mmol, 80%) as a colorless hygroscopic foam. TLC (EtOAc/MeOH, 95:5, v/v): R_f = 0.25. ¹H NMR (300 MHz, CDCl₃): δ = 2.48 (m, 1 H, H^{2'/2''}), 3.18 (m, 1 H, H^{2'/2''}), 3.45 (s, 3 H, CH₃, methoxyacetyl), 3.85–3.99 (m, 2 H, H^{5'}, H^{5''}), 4.07 (s, 2 H, CH₂, methoxyacetyl), 4.27 (m, 1 H, H^{4'}), 5.64 (m, 1 H, H^{3'}), 5.85 (br. s, 1 H, OH), 6.34 (dd, ³J = 5.2, 9.8 Hz, 1 H, H^{1'}), 7.45–7.62 (m, 3 H, H^{ar}), 7.99 (m, 2 H, H^{ar}), 8.09 (s, 1 H, H⁸), 8.74 (s, 1 H, H²), 9.17 (br. s, 1 H, NH) ppm. ¹³C NMR (75 MHz, CDCl₃): δ = 37.7 (C^{2'}), 59.5 (OCH₃, methoxyacetyl), 63.0 (C^{5'}), 69.7 (CH₂, methoxyacetyl), 76.7 (C^{3'}), 87.3, 87.4 (C^{1'}, C^{4'}), 124.5 (C⁵), 127.9, 128.9, 133.0, 133.3 (C^{ar}), 142.3 (C⁴), 150.3 (C³), 150.7 (C²), 152.2 (C⁶), 164.6 (CO), 169.6 (CO) ppm. ESI-MS: m/z = 450.1 [M + Na]⁺, 877.1 [2 M + Na]⁺.

5'-O-Levulinyl-3'-O-(methoxyacetyl)-T^AB^z (6): To a freshly prepared solution of nucleoside **4** (400 mg, 0.73 mmol) and adenosine derivative **5** (155 mg, 0.36 mmol) in dry DCE (4 mL), TMSOTf (5 $\times$ 33.0 μ L, 0.92 mmol) was added in small portions within 50 min. After the last addition of TMSOTf, the mixture was stirred for additional 10 min and the reaction quenched with Et₃N (0.50 mL). The reaction mixture was diluted with DCM (30 mL) and washed with 0.9 M NaHCO₃ (2 $\times$ 30 mL). The organic phase was dried with MgSO₄, concentrated close to dryness and purified by silica gel column chromatography (EtOAc/MeOH, 96:4 $\rightarrow$ 92:8) to afford **6** (180 mg, 0.23 mmol, 64%, based on the acceptor) as a colorless foam. TLC (EtOAc/MeOH, 9:1, v/v): R_f = 0.32. ¹H NMR (300 MHz, CD₂Cl₂): δ = 1.83 (s, 3 H, CH₃, T), 2.10 (s, 3 H, CH₃, Lev), 2.12 (m, 1 H, H^{2'/2''}, dT), 2.35–2.45 (m, 1 H, H^{2'/2''}, dT), 2.49–2.57 (m, 2 H, CH₂, Lev), 2.60–2.70 (m, 1 H, H^{2'/2''}, dA), 2.73 (m, 2 H, CH₂, Lev), 2.93–3.04 (m, 1 H, H^{2'/2''}, dA), 3.43 (s, 3 H, CH₃, methoxyacetyl), 3.87 (m, 2 H, H^{5'/5''}, dA), 4.09 (s, 2 H, CH₂, methoxyacetyl), 4.11–4.23 (m, 3 H, H^{4'}, dT, H^{5'/5''}, dT), 4.24–4.40 (m, 2 H, H^{3'}, dT, H^{4'}, dA), 4.79 (d, ²J = 7.2 Hz, 2 H, OCH₂O), 5.56 (m, 1 H, H^{3'}, dA), 6.12 (t, ³J = 6.5 Hz, 1 H, H^{1'}, dT), 6.56 (dd, ³J = 6.4, 7.9 Hz, 1 H, H^{1'}, dA), 7.29 (s, 1 H, H⁶, T), 7.45–8.05 (m, 5 H, H^{ar}), 8.37 (s, 1 H, H⁸), 8.72 (s, 1 H, H²), 9.63 (s, 1 H, NH), 9.71 (br. s, 1 H, NH) ppm. ¹³C NMR (125 MHz, CD₂Cl₂): δ = 12.5 (CH₃, T), 28.1 (C ^{β} , Lev), 38.0, 38.2, 38.3 (2 $\times$ C^{2'}, dT, dA, C ^{α} , Lev), 59.5 (CH₃, methoxyacetyl), 64.0 (C^{5'}, dT), 68.4 (C^{5'}, dA), 69.9 (CH₂, methoxyacetyl), 75.5 (C^{3'}, dA), 77.6 (C^{3'}, dT), 82.8 (C^{4'}, dT), 84.3 (C^{4'}, dA), 84.5 (C^{1'}, dA), 85.4 (C^{1'}, dT), 95.6 (OCH₂O), 111.2 (C⁵, dT), 128.5, 128.9, 132.9 (C^{ar}), 135.5 (C⁶, dT), 141.8 (C⁸, dA), 150.1 (C⁴, dA), 150.6 (C⁶, dA), 152.2 (C², dT), 152.7 (C², dA), 164.2 (C⁴, dT), 170.2 (CO, methoxyacetyl), 172.8 (COCH₂, Lev), 206.8 (COCH₃, Lev) ppm. ESI-MS: m/z = 780.2 [M + H]⁺, 802.3 [M + Na]⁺.

3'-O-(Methoxyacetyl)-T^AB^z (7): To a solution of **6** (180 mg, 0.23 mmol) in pyridine/ethyl acetate (4:1, v/v; 3 mL), a freshly prepared hydrazine hydrate solution in pyridine/acetic acid (3:2, v/v; 1 M; 3 mL) was added. After 2 min of stirring, DCM was added and the reaction mixture washed with water (10 mL) and NaHCO₃ (0.9 M, 10 mL). The organic layer was dried with MgSO₄, concentrated in vacuo and subjected to column chromatography (EtOAc/MeOH, 9:1) to afford **7** (120 mg, 0.175 mmol, 76%) as a colorless foam. TLC (EtOAc/MeOH, 9:1, v/v): R_f = 0.16. ¹H NMR (600 MHz, CD₂Cl₂): δ = 1.74 (s, 3 H, CH₃, T), 2.07–2.27 (m, 2 H, H^{2'}, H^{2''}, dT), 2.67 (ddd, ³J = 2.7, 6.4, ²J = 14.4 Hz, 1 H, H^{2'/2''}, dA), 2.98 (m, 1 H, H^{2'/2''}, dA), 3.42 (s, 3 H, CH₃, methoxyacetyl),

3.69–3.89 (m, 4 H, $2\times H^{5'/5''}$, dT, dA), 4.00 (m, 1 H, $H^{4'}$, dT), 4.34 (m, 1 H, $H^{4'}$, dA), 4.37 (m, 1 H, $H^{3'}$, dT), 4.77 (s, 2 H, OCH_2O), 5.58 (m, 1 H, $H^{3'}$, dA), 6.08 (dd, $^3J = 6.4$, 7.6 Hz, $H^{1'}$, dT), 6.58 (t, $^3J = 7.0$ Hz, 1 H, $H^{1'}$, dA), 7.45–7.52 (m, 3 H, H^{ar}), 7.56 (m, 1 H, H^6), 8.01 (d, $^3J = 7.2$ Hz, 2 H, H^{ar}), 8.47 (s, 1 H, H^8), 8.71 (s, 1 H, H^2), 9.91 (br. s, 1 H, NH), 10.45 (br. s, 1 H, NH) ppm. ^{13}C NMR (150 MHz, CD_2Cl_2): $\delta = 12.4$ (CH_3 , T), 38.0, 38.3, 39.0 ($C^{2'}$, dA, dT), 59.5 (CH_3 , methoxyacetyl), 62.3, 62.4, 68.1 ($C^{5'}$, dA, dT), 69.9 (CH_2 , methoxyacetyl), 75.6 ($C^{3'}$, dA), 76.9 ($C^{3'}$, dT), 84.4, 84.5, 84.6 ($C^{1'}$, $C^{4'}$, dA), 85.5, 85.9 ($C^{1'}$, $C^{4'}$, dT), 94.6 (OCH_2O), 111.0 (C^5 , dT), 128.5, 128.6, 128.8, 128.8 (C^{ar}), 132.9 (C^6 , dT), 136.7 (C^{ar}), 142.0 (C^8 , dA), 150.1 (C^4 , dA), 151.0 (C^6 , dA), 151.0 (C^2 , dT), 152.6 (C^2 , dA), 164.6 (C^4 , dT), 170.2 (CO, methoxyacetyl) ppm. ESI-MS: $m/z = 704.3$ [$M + Na$] $^+$, 1314.2 [$2M + Na$] $^+$.

5'-O-(4,4'-Dimethoxytrityl)-3'-O-(methoxyacetyl)-T A Bz (8): Compound **7** (100 mg, 0.15 mmol) was dried in high vacuum overnight and dissolved in dry pyridine (3 mL). DIPEA (166 μ L, 0.96 mmol) and DMTCl (224 mg, 0.66 mmol) were added, and the reaction mixture was stirred at room temperature for 12 h. MeOH (500 μ L) was added, and the reaction mixture was stirred for additional 5 min to neutralize the remaining trityl chloride. Pyridine was removed under reduced pressure by co-evaporation with toluene (2×10 mL) and the remaining oil dissolved in DCM (20 mL). The organic layer was washed with $NaHCO_3$ (0.9 M, 2×20 mL) and the DCM layer dried with $MgSO_4$. Silica gel chromatography (EtOAc/MeOH, 95:5) afforded **8** (110 mg, 0.11 mmol, 76%) as a colorless solid. TLC (EtOAc/MeOH, 95:5, v/v): $R_f = 0.27$. 1H NMR (300 MHz, CD_2Cl_2): $\delta = 1.41$ (br. s, 3 H, CH_3 , T), 2.21 (m, 1 H, $H^{2'/2''}$, dT), 2.45 (ddd, $^3J = 2.4$, 5.7, $^2J = 13.6$ Hz, 1 H, $H^{2'/2''}$, dT), 2.61 (ddd, $^3J = 2.4$, 6.3, $^2J = 14.5$ Hz, 1 H, $H^{2'/2''}$, dA), 2.90 (m, 1 H, $H^{2'/2''}$, dA), 3.30 (dd, $^3J = 3.0$, $^2J = 10.8$ Hz, 1 H, $H^{5'/5''}$, dT), 3.37 (dd, $^3J = 3.3$, $^2J = 10.2$ Hz, 1 H, $H^{5'/5''}$, dT), 3.42 (s, 2 H, CH_2 , methoxyacetyl), 3.73 (s, 6 H, OCH_3 , DMT), 3.82 (dd, $^3J = 3.6$, $^2J = 11.1$ Hz, 1 H, $H^{5'/5''}$, dA), 3.87 (dd, $^3J = 3.9$, $^2J = 10.8$ Hz, 1 H, $H^{5'/5''}$, dA), 4.07 (s, 2 H, CH_2 , methoxyacetyl), 4.09 (m, 1 H, $H^{4'}$, dT), 4.30 (q, $^3J = 3.3$ Hz, 1 H, $H^{4'}$, dA), 4.41 (m, 1 H, $H^{3'}$, dT), 4.78 (d, $^2J = 7.5$ Hz, 2 H, OCH_2O), 5.45 (dt, $^3J = 2.1$ Hz, 1 H, $H^{3'}$, dA), 6.22 (dd, $^3J = 5.8$, 7.9 Hz, 1 H, $H^{1'}$, dT), 6.54 (dd, $^3J = 6.5$, 8.3 Hz, 1 H, $H^{1'}$, dA), 6.79–6.86, 7.24–7.53 (m, 16 H, H^{ar}), 7.59 (s, 1 H, H^6), 7.99 (m, 2 H, H^{ar}), 8.31 (s, 1 H, H^8), 8.71 (s, 1 H, H^2), 9.54 (s, 1 H, NH), 9.81 (br. s, 1 H, NH) ppm. ^{13}C NMR (150 MHz, CD_2Cl_2): $\delta = 11.9$, 11.9 (CH_3 , T), 38.2, 38.7 ($C^{2'}$, dT, dA), 55.5 (OCH_3 , DMT), 59.5 (OCH_3 , methoxyacetyl), 63.8 ($C^{5'}$, dT), 68.5 ($C^{5'}$, dA), 69.9 (CH_2 , methoxyacetyl), 75.6 ($C^{3'}$, dA), 78.6 ($C^{3'}$, dT), 84.3, 84.5, 84.6, 85.0 ($C^{1'}$, $C^{4'}$), 87.1 (C^q , DMT), 95.6 (OCH_2O), 111.2 (C^5 , dT), 113.5, 127.27–130.3 (C^{ar}), 132.8 (C^6 , dT), 135.7, 135.8 (C^{ar}), 141.7 (C^8 , dA), 144.9 (C^4 , dA), 150.7 (C^6 , dA), 152.6 (C^2 , dT), 159.0, 159.1 (C^{ar}), 164.3 (C^4 , dT), 170.1 (CO, methoxyacetyl) ppm. ESI-MS: $m/z = 1006.4$ [$M + Na$] $^+$, 1989.2 [$2M + Na$] $^+$.

5'-O-(4,4'-Dimethoxytrityl)-T A Bz (9): Compound **8** (108 mg, 0.11 mmol) was dissolved in DCM/MeOH (1:1, v/v; 3 mL) and potassium *tert*-butoxide (25.0 mg, 0.22 mmol) added. After 15 min of stirring, the reaction mixture was diluted with DCM (25 mL), washed with water (2×20 mL), brine (20 mL), and dried with $MgSO_4$. The organic layer was co-evaporated in vacuo and the residue purified by column chromatography (EtOAc) to provide **9** (100 mg, 0.11 mmol, 99%) as a colorless foam. TLC (EtOAc): $R_f = 0.50$. 1H NMR (600 MHz, CD_2Cl_2): $\delta = 1.43$ (s, 3 H, CH_3 , T), 2.21 (m, 1 H, $H^{2'/2''}$, dT), 2.51 (m, 2 H, $H^{2'/2''}$, dT, dA), 2.73 (m, 1 H, $H^{2'/2''}$, dA), 3.28 (dd, $^3J = 3.0$, $^2J = 10.8$ Hz, 1 H, $H^{5'/5''}$, dT), 3.36 (dd, $^3J = 2.5$, $^2J = 10.4$ Hz, 1 H, $H^{5'/5''}$, dT), 3.67–3.77 (m, 7 H, OCH_3 , DMT, $H^{5'/5''}$, dA), 3.83 (dd, $^3J = 3.2$, $^2J = 10.9$ Hz, 1

H, $H^{5'/5''}$, dA), 4.08 (m, 1 H, $H^{4'}$, dT), 4.15 (m, 1 H, $H^{4'}$, dA), 4.38 (m, 1 H, $H^{3'}$, dT), 4.57 (q, $^3J = 4.7$ Hz, 1 H, $H^{3'}$, dA), 4.72 (m, 2 H, OCH_2O), 6.22 (m, 1 H, $H^{1'}$, dT), 6.50 (t, $^3J = 6.4$ Hz, 1 H, $H^{1'}$, dA), 6.79–6.85 (m, 4 H, H^{ar}), 7.24–7.46 (m, 12 H, H^{ar}), 7.51 (s, 1 H, H^6), 7.98 (m, 2 H, H^{ar}), 8.32 (s, 1 H, H^8), 8.69 (s, 1 H, H^2), 9.88 (br. s, 1 H, NH), 10.82 (br. s, 1 H, NH) ppm. ^{13}C NMR (150 MHz, CD_2Cl_2): $\delta = 11.9$ (CH_3 , T), 38.8 ($C^{2'}$, dT), 40.6 ($C^{2'}$, dA), 55.5 (OCH_3 , DMT), 63.8 ($C^{5'}$, dT), 68.6 ($C^{5'}$, dA), 72.0 ($C^{3'}$, dA), 78.8 ($C^{3'}$, dT), 84.6, 84.8, 85.2, 86.2 ($C^{1'}$, $C^{4'}$, dT, dA), 87.1 (C^q , DMT), 95.6 (OCH_2O), 111.4 (C^5 , dT), 113.5, 127.3, 128.2, 128.3, 128.5, 128.6, 128.7, 128.8, 130.3 (C^{ar}), 132.8 (C^6 , dT), 135.6, 135.7, 135.8 (C^{ar}), 142.2 (C^8 , dA), 144.9 (C^4 , dA), 151.2 (C^6 , dA), 152.4 (C^2 , dA), 159.0, 159.0 (C^{ar}), 164.7 (C^4 , dT) ppm. ESI-MS: $m/z = 934.3$ [$M + Na$] $^+$, 1846.2 [$2M + Na$] $^+$.

3'-O-[(2-Cyanoethyl)(diisopropylamino)phosphanyl]-5'-O-(4,4'-dimethoxytrityl)-T A Bz (2): Compound **9** (100 mg, 0.11 mmol) was dissolved in dry DCM (1.50 mL). DIPEA (76.0 μ L, 0.44 mmol), DMAP (2.60 mg, 22.0 μ mol), and chloro(2-cyanoethoxy)(diisopropylamino)phosphane (36.5 μ L, 0.16 mmol) were carefully added to the reaction mixture. After stirring for 25 min, the reaction mixture was diluted with DCM (10 mL), washed with brine (20 mL), and 0.9 M $NaHCO_3$ (10 mL). The organic layer was dried with $MgSO_4$ and concentrated in vacuo. The residue was purified by column chromatography (EtOAc/MeOH, 95:5) to yield **2** (80 mg, 72.0 μ mol, 66%) as a colorless foam. TLC (EtOAc/MeOH, 95:5, v/v): $R_f = 0.51$. 1H NMR (600 MHz, CD_2Cl_2): $\delta = 1.19$ [m, 12 H, $2\times CH(CH_3)_2$], 1.40 (s, 3 H, CH_3 , T), 2.19 (m, 1 H, $H^{2'/2''}$, dT), 2.43 (m, 1 H, $H^{2'/2''}$, dT), 2.62 (t, $^3J = 6.2$ Hz, 2 H, CH_2CN), 2.67 (m, 1 H, $H^{2'/2''}$, dA), 2.85 (m, 1 H, $H^{2'/2''}$, dA), 3.29 (m, 2 H, $H^{5'}$, $H^{5''}$, dT), 3.63 [m, 2 H, $2\times NCH(CH_3)_2$], 3.70–3.94 (m, 10 H, $2\times OCH_3$, DMT, OCH_2CH_2CN , $H^{5'}$, $H^{5''}$, dA), 4.04 (m, 1 H, $H^{4'}$, dT), 4.29 (dq, $^3J = 4.1$ Hz, 1 H, $H^{4'}$, dA), 4.39 (m, 1 H, $H^{3'}$, dT), 4.74 (m, 3 H, OCH_2O , $H^{3'}$, dA), 6.21 (m, 1 H, $H^{1'}$, dT), 6.49 (dt, $^3J = 6.3$ Hz, 1 H, $H^{1'}$, dA), 6.81 (d, $^3J = 8.2$ Hz, 4 H, H^{ar}), 7.16–7.60 (m, 13 H, H^{ar} , H^6), 7.98 (d, $^3J = 7.7$ Hz, 2 H, H^{ar}), 8.25 (s, 1 H, H^8), 8.70 (s, 1 H, H^2), 9.46 (br. s, 2 H, $2\times NH$) ppm. ^{13}C NMR (150 MHz, CD_2Cl_2): $\delta = 11.9$ (CH_3 , T), 20.6, 20.7, 20.7, 20.7 (CH_2CN), 24.6, 24.6, 24.7, 24.7 [$CH(CH_3)_2$], 38.7, 38.8 ($C^{2'}$, dT), 39.8, 39.8 ($C^{2'}$, dA), 43.5, 43.5, 43.6, 43.6 [$CH(CH_3)_2$], 55.5 (OCH_3 , DMT), 58.4, 58.5, 58.6, 58.7 (OCH_2CH_2CN), 63.8 ($C^{5'}$, dT), 68.2, 68.4 ($C^{5'}$, dA), 73.6, 73.7, 73.8, 73.9 ($C^{3'}$, dA), 78.6, 78.7 ($C^{3'}$, dT), 84.4, 84.5 ($C^{4'}$, dT), 84.7, 84.7 ($C^{1'}$, dA), 84.9, 84.9 ($C^{1'}$, dT), 85.3, 85.4, 85.6, 85.6 ($C^{4'}$, dA), 87.1 (C^q , DMT), 95.7, 95.8 (OCH_2O), 111.1 (C^5 , dT), 113.5 (C^{ar}), 117.4, 118.1 (CN), 123.9, 127.3, 128.2, 128.3, 128.4, 128.9, 130.3, 130.3, 132.8 (C^{ar}), 135.7, 135.8 (C^6 , dT), 142.0, 142.0 (C^8 , dA), 144.9 (C^4 , dA), 150.6 (C^6 , dA), 152.5 (C^2 , dA), 159.0 (C^{ar}), 164.1 (C^4 , dT) ppm. ^{31}P NMR (121 MHz, CD_2Cl_2): $\delta = 149.7$, 149.8 ppm. ESI-MS: $m/z = 1134.4$ [$M + Na$] $^+$.

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