SYNTHESIS OF 3′-*O*-(2-CYANOETHYL)-2′-DEOXYTHYMIDINE-5′-PHOSPHATE AS A MODEL COMPOUND FOR EVALUATION OF CYANOETHYL CLEAVAGE

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An essential and challenging task during the development of our sequencing-by-synthesis (SBS) technique is the evaluation of efficient cyanoethyl (CE) cleavage conditions. For this purpose 3′-*O*-(2-cyanoethyl)-2′-deoxythymidine-5′-phosphate as a model compound as well as a short DNA oligomer bearing the CE function as terminal group were synthesized and used for various deprotection experiments. As it is already known for 2′-*O*-CE-protected RNA oligonucleotides, the CE function can be cleaved with tetrabutylammonium fluoride (TBAF) in THF. Indeed, by using 3′-*O*-(2-cyanoethyl)-2′-deoxythymidine-5′-phosphate as a simple model compound for cleavage tests, we found out that the 3′-*O*-CE function is quantitatively cleaved with 1 M TBAF in THF. However, the CE group is also cleaved by other small bases like hydroxy groups under alkaline conditions. The CE cleavage with TBAF in THF gives the fastest and quantitative removal of the CE group under mild conditions for our sequencing-by-synthesis (SBS) application. The efficient removal of the 3′-CE group is crucial for the proof-of-principle of our SBS approach using dye-labeled 3′-CE-blocked dNTPs, which is currently under investigation. Herein we describe the application of 3′-*O*-(2-cyanoethyl)-2′-deoxythymidine-5′-phosphate as model compound for the development of reversible terminators for the SBS technique. Furthermore we suggest that nucleoside phosphates bearing any removable 3′-modification might be suitable model compounds for cleavage studies in a heterogeneous environment comparable to an oligonucleotide/ aprotic solvent system.

Keywords: DNA; Sequencing-by-synthesis; Polymerase incorporation; Phosphorylations; Cyanoethyl protecting group; 3′-*O*-Modified nucleotides; Cleavage reactions; Oligonucleotides; Phosphoramidites; Nucleoside triphosphates.

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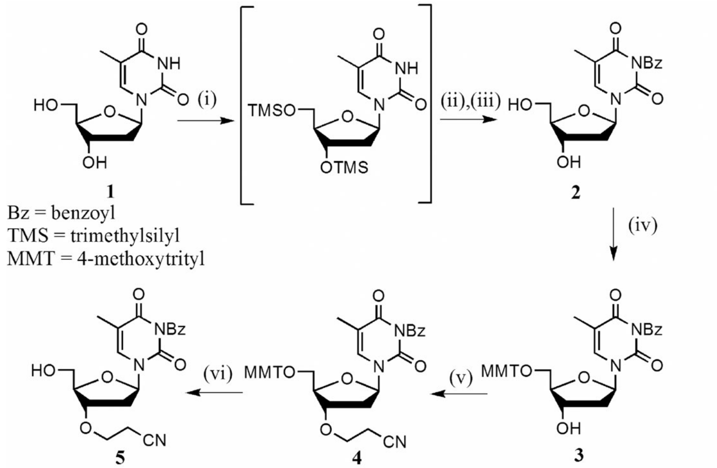
A new approach for high-throughput sequencing is the array-based sequencing-by-synthesis (SBS) technique[1]. This technique does not require gel electrophoresis and affords a direct read-out of the base sequence via fluorescent signal detection. The challenging part of developing this sequencing method is the evolution of a highly tolerant polymerase that incorporates reversible terminators into a DNA-template; another requirement is the synthesis of four reversible terminators[2]. We found out that certain polymerases accept and incorporate cyanoethyl (CE)-bearing nucleotides like 3′-*O*-(2-cyanoethyl)-2′-deoxythymidine-5′-triphosphate (3′-*O*-CE-dTTP); therefore, this 3′-modified nucleotide is a good candidate for a potential reversible terminator. Besides the demanded terminating properties, the 3′-*O*-CE group also has to be quantitatively removable to enable the next incorporation step. For the evaluation of the mildest deprotection conditions, a simple model compound 3′-*O*-(2-cyanoethyl)-2′-deoxythymidine-5′-phosphate was synthesized first and used for several deprotection experiments. Due to its high polarity and solubility in water, the monophosphate simulates well the behavior of small oligodeoxynucleotides (ODNs). It also forms a heterogeneous mixture in aprotic organic solvents, which leads to different deprotection kinetics as in solution. Based on the results from the monomer, we applied the optimized deblocking conditions to a short DNA oligomer (8mer) bearing the CE function on the 3′-terminus.

CHEMISTRY

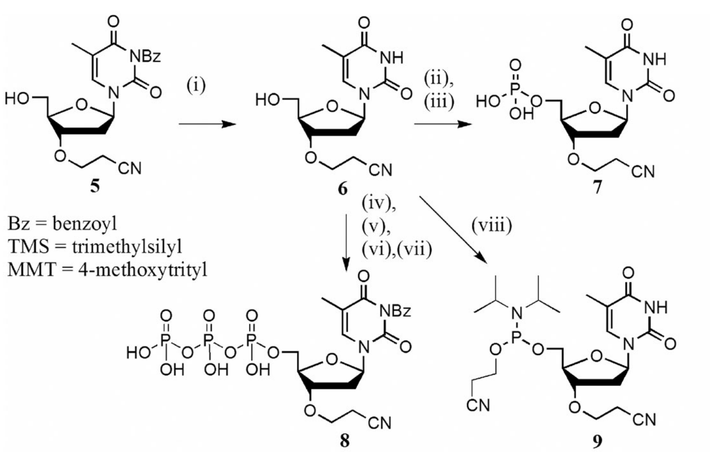
We established a simple synthetic procedure for the preparation of the model compound. The synthesis starts with a one-flask transient protection of both hydroxy groups in the pentose moiety of 2′-deoxythymidine (Scheme 1). Following the transient-protection procedure known from ref.[3], 2.5 equiv. chlorotrimethylsilane is added to **1** in pyridine to protect both OH groups. After 30 min the mixture is treated with 1.1 equiv. benzoyl chloride for selective benzoylation of N^3.

During the aqueous workup, both trimethylsilyl protecting groups are quantitatively removed with trifluoroacetic acid and the N^3-protected nucleoside **2** is obtained in an excellent yield of 83% after purification. The introduction of the trityl group at the 5′-end using standard conditions[4] leads to N^3-benzoyl-2′-deoxy-5′-*O*-(4-methoxytrityl)-thymidine **3** in 78% yield. Then the protected nucleoside **3** undergoes a Michael addition reaction for the introduction of the CE function at the 3′-end according to Sekine et al.[5], which gives the desired nucleoside **4** in 70% yield. The



(i) TMSCl, pyridine, 30 min, rt; (ii) BzCl, pyridine, 1 h, rt; (iii) trifluoroacetic acid, methylene chloride/MeOH 1/1, 30 min, rt; (iv) MMTCl, pyridine, 24 h, rt; (v) Cs_2CO_3, acrylonitrile, *t*-BuOH, 3 h, rt; (vi) TsOH, methylene chloride/EtOH 1/1, 2 h, rt

SCHEME 1

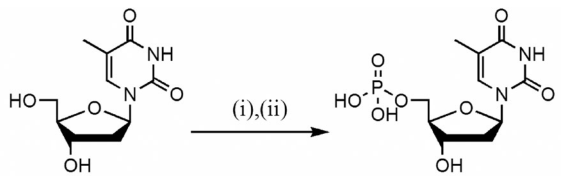


(i) aq. NH_3/MeOH, 1.5 h, rt; (ii) $POCl_3$, trimethyl phosphate, 6 h, 0 °C; (iii) $NaClO_4$, acetone, MeOH, 4 °C; (iv) 1 M 2-chloro-4H-1,3,2-benzodioxaphosphorinan-4-one in dry 1,4-dioxane, dry pyridine, dry DMF, 20 min, rt; (v) 0.5 M $(Bu_3NH)_2H_2P_2O_7$, dry DMF, Bu_3N, 45 min, rt; (vi) 1% I_2 in pyridine/H_2O 98 : 2, 30 min, rt; (vii) 32% aq. NH_3; (viii) dichloromethane, (2-cyanoethyl)di(N,N-diisopropylamino)phosphine, 4,5-dicyanoimidazole, 4 h, rt

SCHEME 2

trityl protecting group of compound **4** is removed quantitatively using *p*-toluenesulfonic acid. After aqueous workup and purification by flash column chromatography, nucleoside **5** is obtained. This compound is dissolved in methanol and treated with an excess of concentrated aqueous ammonia (Scheme 2). Stirring at room temperature under these conditions gives 96% of N^3-deprotected compound **6** without any side products. The CE function is stable during both deprotection steps as long as the cleavage of the benzoyl group in ammonia is not carried out at elevated temperatures.

Monophosphate **7** is prepared by a variation of Yoshikawa's method[6] using phosphorus oxychloride in trimethyl phosphate according to El-Tayeb's protocol[7]. The workup procedure of compound **7** is time-demanding due to product purification by preparative RP-HPLC. The 5′-hydroxy group is phosphorylated selectively in a moderate yield of 52% after purification. Spectral analysis by ^{31}P NMR shows the singlet signal of the desired compound. All signals of the sugar moiety and base could be assigned by ^{1}H NMR. Monophosphate **7** is precipitated as sodium salt after purification by RP-HPLC to give a colorless crystalline product with defined counterions. The triphosphate used for the incorporation tests, 3′-*O*-(2-cyanoethyl)thymidine-5′-*O*-triphosphate **8** (see Scheme 2) was prepared according to the protocol of Ludwig and Eckstein[8]. However, the purification of triphosphate **8** appeared to be time-demanding, too: Crude separation from corresponding mono- and diphosphate was achieved by ion-exchange chromatography (FPLC), further purification of the triphosphate-containing fraction was done by RP-HPLC. Triphosphate **8** was obtained in 12% yield only, but in high purity as it is needed for the polymerase acceptance tests. As a reference substance for the deprotected product, 2′-deoxythymidine-5′-phosphate **10** was prepared in the same way as compound **7** (Scheme 3). It is accessible from 2′-deoxythymidine in a moderate yield of 45% after purification by preparative RP-HPLC with subsequent precipita-



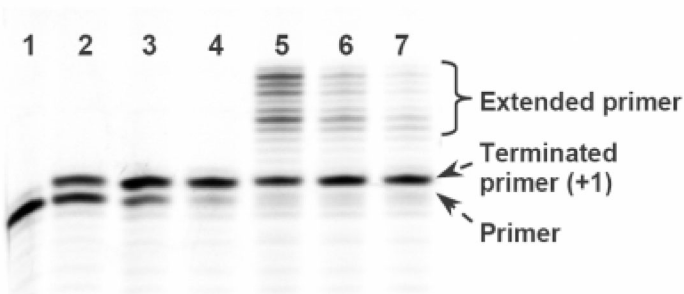
(i) $POCl_3$, $(MeO)_3PO$, 6 h, -4 - 0 °C; (ii) $NaClO_4$, acetone, MeOH 4 °C, 45%

SCHEME 3

tion as sodium salt. For the incorporation into the DNA 8mer used for the CE deprotection test, 3′-*O*-(2-cyanoethyl)-5′-[(2-cyanoethyl)-(*N*,*N*-diisopropyl)]-2′-deoxy-phosphoramidite-thymidine had to be prepared. Therefore compound **6** was converted into its corresponding phosphoramidite **9** in 85% yield, purified on a short silica gel column under argon pressure and subjected to inverse ODN synthesis of the DNA 8mer with 3′-CE-terminus.

Polymerase Incorporation of 3′-O-(2-Cyanoethyl)thymidine-5′-O-triphosphate

Triphosphate **8** was found to be efficiently incorporated into DNA substrate, demonstrating good terminating properties. Figure 1 illustrates acceptance of **8** by a previously selected polymerase[1b]. The DNA primer competent for multiple incorporations of dTTP was extended only by a single residue during polymerization reaction employing triphosphate **8** in a time-course mode (lanes 2–4). In parallel, the resulting products were challenged by addition of dTTP and the reactions were allowed to proceed further (lanes 5–7). The product of primer extension by **8** (marked as +1) was found to be resistant to further extension (see arrows in the right part of Fig. 1), while the unreacted primer was extended further (top part of Fig. 1, lanes 5–7). This observation suggests that the incorporated modified nucleotide serves as an efficient polymerization terminator.



-	5	15	60	5	15	60	3'-*O*-CE-dTTP incorporation time, min
	-			+			Further extension using dTTP

FIG. 1

Enzymatic incorporation of 3′-*O*-(2-cyanoethyl)thymidine-5′-*O*-triphosphate into DNA substrate and its terminating properties

Cleavage Experiments

The synthesized monophosphates **7** and **10** were used as reference materials for the following cleavage experiments. The chromatogram of both the starting material (model compound) and the deprotected monophosphate are shown in Fig. 2, while the commercially available phosphate **10** was coinjected for calibration of the method (see chromatogram in the background of Fig. 2).

Figure 2 shows that the model compound after CE cleavage, which is assumed to be 2′-deoxythymidine-5′-phosphate, is well separable from the starting material **7** by the RP-HPLC method we have developed. The deprotected nucleotide elutes significantly (at 7.78 min) earlier from the column than the CE-protected nucleotide (deprotection time 10.88 min). This separation method was also used for the analysis of the cleavage experiment probes. All cleavage experiments done with the model compound (except experiment No. 2, Table I) were carried out under dry conditions in argon atmosphere. Each cleavage test was performed by one standard procedure described in the Experimental.

The results of the first CE cleavage experiments using the model compound 3′-*O*-(2-cyanoethyl)-2′-deoxythymidine-5′-phosphate are shown in Table I. At the beginning, we tried to find an appropriate cleaving agent efficient enough for quantitative CE removal (see Table I).

The temperature for the first four experiments listed in Table I was kept constant at 60 °C. We assume the CE cleavage following a β-elimination

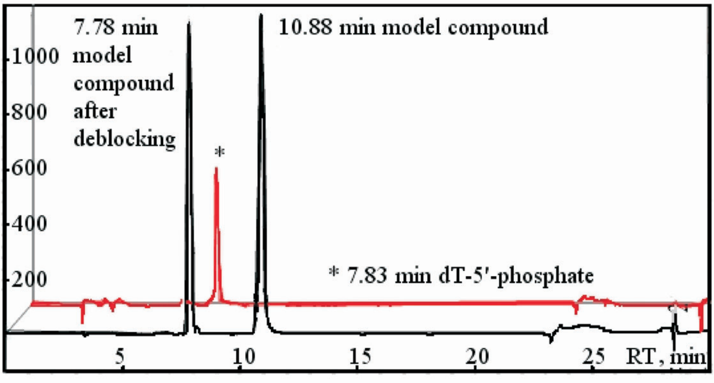


FIG. 2
RP-HPLC chromatogram of model compound **7** and deprotected monophosphate **10**

TABLE I
Cyanoethyl cleavage tests with variation of cleaving agent, reaction temperature, solvents and amount of cleaving agent

Experiment	Reagent, mole % solvent	Temperature °C	Cleavage time min
1	TEA 3 HF (10), acetonitrile	60	no cleavage
2	KOH (40), water	60	ca. 30
3	dicyclohexano-18-crown-6 (0.1), potassium fluoride (2), THF	60	no cleavage
4	1 M TBAF (40), THF	60	6
5	1 M TBAF (40), THF	25	>60
6	1 M TBAF (80), THF	37	60
7	1 M TBAF (40), THF	60	6
8	1 M TBAF (80), THF	66	3
9	1 M TBAF (5), THF	60	>20
10	1 M TBAF (20), THF	60	10
11	1 M TBAF (40), THF/DMF 1:1	60	1.5
12	1 M TBAF (40), THF/DMSO 1:1	60	1
13	1 M TBAF (40), THF/DMF 1:1	40	12
14	1 M TBAF (40), THF/DMSO 1:1	40	3

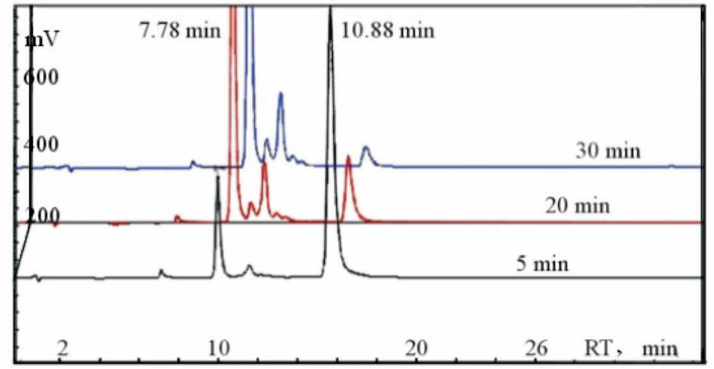


FIG. 3
Stacked RP-HPLC chromatograms illustrating the OH-induced 3′-O-CE cleavage of monophosphate **7**

mechanism; so one might consider removal of the CE group by small bases like hydroxy groups. Experiment No. 2 afforded the assumed results: here, 20 mg of the model compound was dissolved in water and treated with 2 ml of 1 M aqueous KOH at 60 °C. In contrast to the other three experiments, a homogeneous solution was formed due to complete dilution of the nucleotide in water. The samples taken out every minute were quenched in 0.5 M aqueous acetic acid by neutralization, filtered through syringe-filter and subjected to analytical RP-HPLC with autosampler (the same conditions as described for the standard procedure). The stacked chromatograms of experiment No. 2 are shown in Fig. 3, illustrating the time-dependent CE cleavage. The peaks on the right-hand side in the stacked chromatograms (Fig. 3) decrease due to consumption of model compound **7** (retention time 10.88 min), the peaks on the left-hand side increase due to the formation of 2′-deoxythymidine-5′-phosphate **10** as the main product (retention time 7.78 min).

The dependence of the CE removal on alkaline conditions was also confirmed by experiment No. 1 in Table I. The reagent triethylammonium trihydrofluoride in THF is slightly acidic (pH 5), which prevents the CE removal. Figure 4 shows the stacked RP-HPLC chromatograms from experiment No. 1. Not even after 75 min reaction time at 60 °C, cleavage was observed, only the peak with the retention time 10.88 min remained. Experiment No. 3 (Table I) was a single attempt to remove the CE function with potassium fluoride and dicyclohexano-18-crown-6 ether in THF as

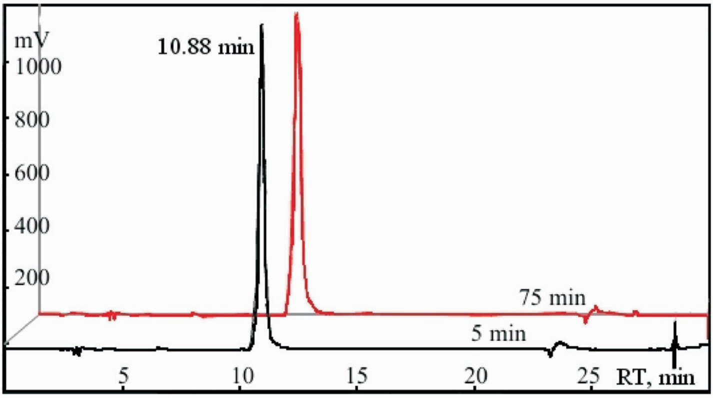


FIG. 4
Stacked RP-HPLC chromatograms of cyanoethyl cleavage experiment under acid conditions

phase transfer catalyst. No cleavage was observed due to the insolubility of both the nucleotide and fluoride reagent in THF, which means that the reaction mixture remained heterogeneous through the whole experiment without activation by the crown ether.

Finally we tried to use the 1 M TBAF/THF standard solution as cleaving agent. At 60 °C and with a forty-fold excess of TBAF (pH 13), the starting material was quantitatively deprotected within 6 min. Therefore, we investigated further optimization of CE cleavage with 1 M TBAF/THF solution as cleaving agent.

In the second stage of cleavage experiments, both reaction temperature and the amount of cleaving agent were varied (see Table I). The next four experiments (Nos 5, 6, 7 and 8 in Table I) showed clearly a temperature-dependent reaction rate. Considering experiments Nos 1 and 3, deprotection of the model compound **7** occurred ten times faster at 60 °C than at 25 °C, although 25 °C (or 37 °C) as reaction temperature was preferable due to biological compatibility. At first sight, experiment No. 8 shows the best and fastest CE cleavage within 3 min. Unfortunately, THF boils at 66 °C, which makes this reaction temperature inapplicable to DNA-chip arrays.

In the third stage, we varied the amount of cleaving agent keeping the reaction temperature constant (see experiments Nos 7, 9 and 10 in Table I). Regarding the model compound, a minimum of 40 equiv. TBAF are needed for quantitative cleavage. Lowering the amount of fluoride from 40 to 5 equiv., the cleavage time was significantly prolonged.

For enhancing the cleavage rate by lowering the reaction temperature, we refined our cleavage model by employing co-solvents. The conditions for the CE cleavage of the model compound with co-solvents are listed in Table I (experiments Nos 11, 12, 13 and 14). As we prefer the reaction temperature 40 °C, we optimized the cleavage rate by addition of DMF or DMSO in the ratio DMSO/THF or DMF/THF of 1:1 (v/v). By comparing the co-solvents regarding the solubility enhancement of the model compound, DMSO showed better results. By treating 3′-*O*-(2-cyanoethyl)-2′-deoxythymidine-5′-phosphate with 40 equiv. TBAF in DMSO/THF 1:1 (experiment No. 14) at 40 °C, the cleavage was four times faster than in DMF/THF 1:1 (experiment No. 13). Increasing the temperature from 40 to 60 °C improved the cleavage rate, as seen from experiments Nos 11 and 12. The stacked chromatograms of both experiments are shown in Figs 5 and 6. The chromatograms in Fig. 5 correspond to experiment No. 13, the ones in Fig. 6 to experiment No. 14.

In both Figs 5 and 6 the chromatogram in the front shows the consumption of model compound **7** after 1 min, the chromatogram in the background shows the consumption after 3 min. In experiment No. 14, monophosphate **7** is completely deprotected after 3 min. In contrast, the CE cleavage was not complete after 3 min in case of experiment No. 13.

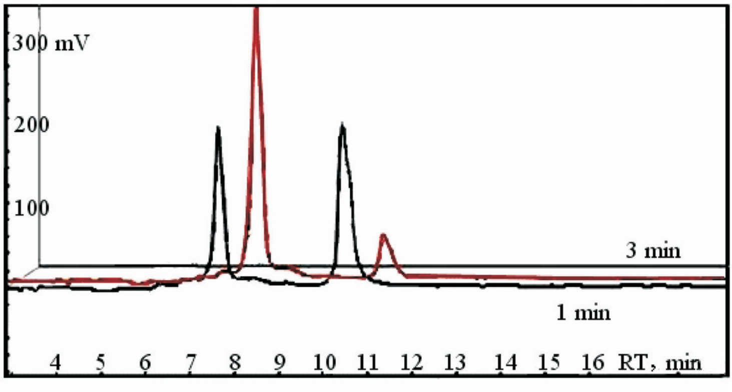


FIG. 5
Stacked RP-HPLC chromatograms for Experiment No. 13 (THF/DMF 1:1)

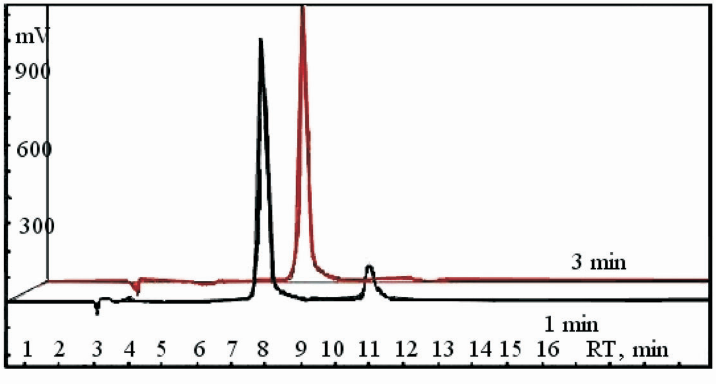


FIG. 6
Stacked RP-HPLC chromatograms for Experiment No. 14 (THF/DMSO 1:1)

Cyanoethyl Cleavage on an Oligomer

For the evaluation of the CE cleavage on an oligodeoxynucleotide (ODN), we selected the sequence of the terminal region of the template oligomer consisting of 8 nucleotides. First we synthesized the corresponding DNA 8mer without CE function as a reference oligomer. After purification on anion exchange HPLC and desalting, the ODN could be obtained in high purity as it is shown in the MALDI-MS spectrum below (Fig. 7).

The modified DNA 8mer consisting of the same sequence like the unmodified ODN was then synthesized bearing the CE function on the 3′-terminus. The resulting oligomer was purified in the same manner like the unmodified reference material giving the pure DNA 8mer ready for the cleavage experiment (Fig. 8).

The modified DNA 8mer was treated with a high excess of TBAF in THF/DMF 2:3 (v/v) at 45 °C for 15 min (see Experimental for details). Figure 9 shows the resulting MALDI-MS spectrum after purification and desalting. The mass of the obtained peak is identical with the one of the unmodified reference ODN which proves the efficient CE cleavage employing TBAF in THF/DMF.

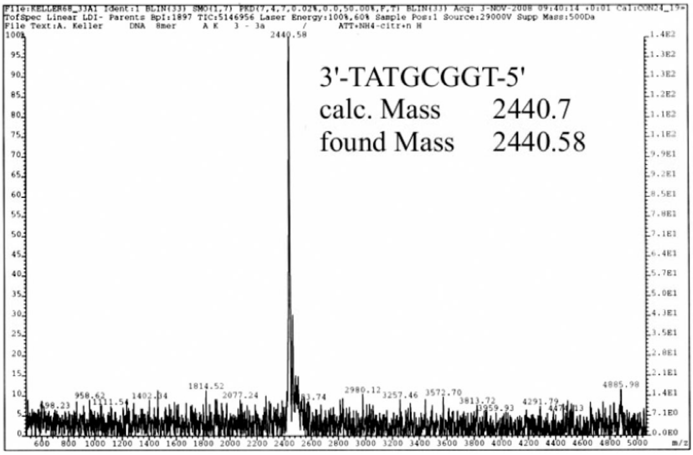


FIG. 7
MALDI-MS spectrum of the unmodified DNA 8mer

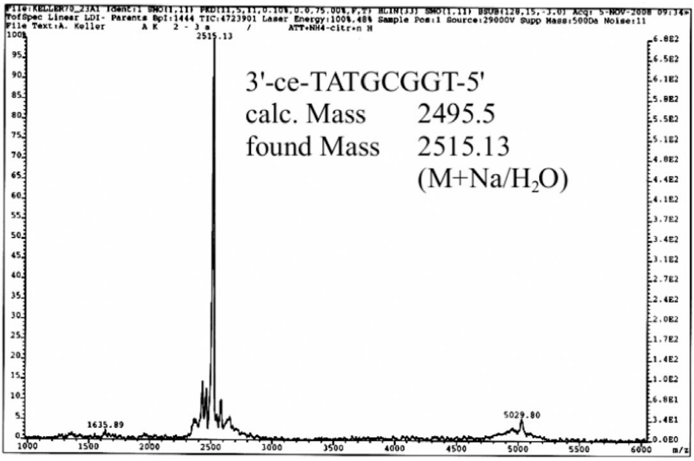


FIG. 8
MALDI-MS spectrum of the modified DNA 8mer with 3′-CE-terminus

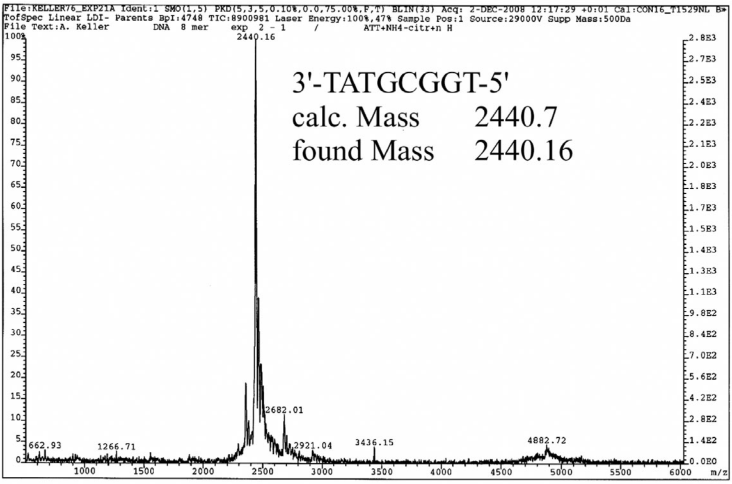


FIG. 9
MALDI-MS spectrum of the modified DNA 8mer after cyanoethyl cleavage

DISCUSSION

TBAF was used by Saneyoshi et al.[9] as a deprotection agent for 2′-*O*-CE-oligonucleotides in RNA synthesis. It can also be used for removal of CE groups in DNA nucleotides like our model compound 3′-*O*-(2-cyanoethyl)-2′-deoxythymidine-5′-phosphate. The CE function is cleaved in an alkaline environment with strong bases like fluoride; the cleavage rate is significantly improved at elevated temperatures, for example at 60 °C. Furthermore the cleavage efficiency depends also on the solvent and TBAF amount. As a result of deprotection tests, the cleavage in the model compound **7** is enhanced by addition of polar solvents like DMF or DMSO to 1 M TBAF/THF standard solution. These cleavage conditions were also applied to a short oligomer with 3′-CE-terminus leading to quantitative removal of the CE function although a significantly higher amount of fluoride is needed than for the monophosphate. We are now at the stage of applying the optimized CE cleavage conditions to dye labeled 3′-*O*-CE-blocked (oligo)nucleotides that are used for our SBS approach which is currently under investigation.

EXPERIMENTAL

Biological Materials and Methods

Incorporation of 3′-*O*-(2-cyanoethyl)thymidine-5′-*O*-triphosphate **8** was performed in the presence of 1 μM polymerase, 50 μM 3′-*O*-CE-dTTP and 10 nM DNA duplex substrate consisting of the primer radioactively labeled by ^{33}P at 5′-terminus (as indicated by asterisk): 5′-*TGCAGGCATGCAAGCTTGGCGTA-3′ and template oligonucleotide: 5′-AAAAAAAAAAAATACGCCAAGCTTGCATGCCTGCA-3′. Polymerization was performed in 20 μl of the reaction mixture at 37 °C. After the indicated time (see the legend of Fig. 1), an aliquot of the reaction mixture was supplemented with dTTP (up to 50 μM final concentration) and the reaction was allowed to proceed for additional 5 min at the same temperature. The reactions were stopped by adding an equal volume of a STOP solution (Fermentas catalogue, item #K1711: CycleReader™ DNA sequencing kit) containing EDTA, products resolved on 15% 29:1 denaturing (7 M urea) polyacrylamide (PAA) gel run at 50 °C, dried on a Whatman paper sheet and autoradiographed using Fuji phosphorimager screen.

Oligonucleotide Synthesis

The 3′-phosphoramidites for the synthesis (direction 3′ to 5′) of the unmodified DNA 8mer were purchased from Pharmacia Biotech, the 5′-phosphoramidites for the inverse synthesis (direction 5′ to 3′) of the modified DNA 8mer from Glen Research. The oligodeoxynucleotides were synthesized on Expedite Nucleic Acid Synthesis System using 500 Å CPG columns from Applied Biosystems. The sequence of the unmodified reference oligomer is 3′-TATGCGGT-5′, the sequence of the modified oligomer is 3′-CE-TATGCGGT-5′ where CE is cyanoethyl. The deprotection test on the oligomer was performed in a heating block

Thermomixer comfort from Eppendorf. OD measurements were carried out with Hitachi U-1100 Spectrophotometer. The oligomers were purified on anion-exchange HPLC consisting of Dionex DNA Pac © PA-100 column (9 × 250 mm), Jasco intelligent UV-vis detector UV-970, Jasco intelligent HPLC pump PU-980, Jasco ternary gradient unit LG-980-02, Jasco 3-line degasser DG-980-50, and Jasco interface LC-NetII/ADC. The purified oligomers were desalted with PD-10 columns (prepacked with Sephadex™ G-25 M) from GE Healthcare, dried under reduced pressure and characterized by MALDI-TOF mass using 6-aza-2-thiothymine (ATT)/ammonium citrate matrix. The CE cleavage was performed as the following: The modified 8mer (2.4 OD) was dissolved in 330 μl of DMF, then treated with 230 μl (7500 equiv.) of 1 M TBAF in THF and incubated with gentle shaking at 45 °C for 15 min. The reaction was quenched with 1 ml of water, the mixture was freeze-dried and the residue purified on anion-exchange HPLC. After desalting and drying, the residual ODN was characterized by MALDI spectroscopy.

Chemical Synthesis

NMR spectra (^{1}H and ^{13}C) of nucleosides were run in $CDCl_3$ or DMSO-d_6 on a Bruker AM 250 spectrometer operating at 250.132 MHz. NMR spectra (^{1}H, ^{13}C, ^{31}P and two-dimensional spectra) of the monophosphate, triphosphate and phosphoramidite were run in D_2O or acetone-d_6 on a Bruker Avance 400 spectrometer operating at 400.132 MHz (161.984 MHz for coupled ^{31}P NMR, measured against 85% phosphoric acid as external standard). Chemical shifts (δ) are given in ppm downfield from tetramethylsilane and coupling constants (*J*) in Hz. All spectra were measured at 300 K. Peak patterns of each signal are characterized with the following abbreviations: s for singlet, d for doublet, dd for double-doublet, t for triplet, ψt for pseudo-triplet, q for quartet and m for multiplet. Mass spectra were recorded on a Fisons MALDI VG time-of-flight (Tofspec) mass spectrometer in positive or negative mode using ATT matrix or on a Fisons Electrospray (ES) VG Platform II mass spectrometer. FPLC purification of the triphosphate was performed on a Pharmacia ion-exchange FPLC system consisting of LCC-500 Plus controller, two P-500 pumps and one single-path UV Monitor UV-1. The column was packed with DEAE-Sepharose gel DEAE Sephadex A 25 from Pharmacia. The triphosphate sample was loaded on column with peristaltic pump P1, fractions were collected with a fraction collector RediFrac. Further purification of the triphosphate was performed on a semipreparative column LiChroCart 250-10 RP-18e (5 μm) from Merck on the Merck-HPLC system consisting of Merck–Hitachi D-6000 interface, Merck–Hitachi L-4250 UV-vis-detector, Merck–Hitachi L-6220 intelligent pump, Shimadzu PF-535 fluorescence monitor and Merck–Hitachi LC-Organizer. The aqueous fractions containing purified monophosphate and triphosphate were lyophilized on a Christ alpha 2-4 freeze-dryer. HPLC purification of both monophosphates **7** and **10** after synthesis was done on a preparative column Phenomenex Jupiter 4 μ Proteo 90 Å (250 × 15 mm) (at 300 K in Thermotechnic-products Jetstream plus column thermostat) on a Jasco RP-HPLC system consisting of Jasco interface LC-NetII/ADC, Jasco intelligent HPLC pump PU-2080 Plus, Jasco intelligent UV/Vis detector UV-2075 Plus, Jasco ternary gradient unit LG-2080-02, Jasco intelligent sampler AS-950-10, and Jasco 3-line degasser DG-2080-53. The crude monophosphates as well as the samples from the deprotection tests were filtered with syringe filters Spartan 13/0,45 RC (0.45 μm) from Whatman. Elemental analyses were performed on a CHN-O-Rapid analyzer from Foss–Heraeus. Thin layer chromatography was carried out on Silica Gel 60 F_{254}-coated aluminum sheets from Merck. The separated compounds were visualized by UV-absorption at λ = 254 nm (Camag UV lamp). Column chromatography was performed at atmospheric pressure using Silica Gel 60 from Roth, pH values were measured with a pH-meter 761 Calimatic from Knick.

CE Cleavage Tests with the Model Compound

Dry 3′-*O*-(2-cyanoethyl)-2′-deoxythymidine-5′-phosphate **7** (10 or 20 mg, depending on the solvent volume) as sodium salt was suspended in water-free solvent (except experiment No. 2 in Table I) and heated up to the desired reaction temperature with stirring. After keeping the suspension at that temperature for a few minutes, the cleaving reagent was added in one portion. Samples of 100 or 200 μl each were taken out from the reaction chamber after selected time periods and quenched with 500 μl of demineralized water. The probes were transferred after syringe filtration into small vials and subjected to analytical RP-HPLC with autosampler. Both monophosphates **7** and **10** were characterized by their retention times after separation on analytical column (Phenomenex Jupiter 4 m Proteo) using the following eluents: eluent A – 1 M aqueous triethylammonium acetate (TEAA); eluent B – water; eluent C – acetonitrile (ACN); linear gradient 0–30% buffer C during 20 min. Thymidine-5′-monophosphate disodium salt hydrate was purchased from Sigma–Aldrich and coinjected as reference material.

N^3-Benzoyl-2′-deoxythymidine (**2**)

To a solution of 2′-deoxythymidine **1** (6.06 g, 25 mmol), which was rendered anhydrous by repeated coevaporation with dry pyridine in 50 ml of absolute pyridine, ethyldiisopropylamine (21.8 ml, 125 mmol) and chlorotrimethylsilane (8 ml, 62.5 mmol) were added. The mixture was stirred under argon atmosphere at room temperature for ca. 30 min until the starting material was fully consumed. Then benzoyl chloride (4.4 ml, 37.5 mmol) was added and stirring continued for another hour. After consumption of the starting material, the reaction mixture was quenched by addition of 20 g of KH_2PO_4 and 100 ml of ice water. After stirring the mixture for several minutes, colorless crystals precipitated which were collected by filtration, washed with 200 ml of cold water and dried under reduced pressure. The solid was dissolved in 200 ml of methylene chloride/methanol 1:1 and treated with 3.5 ml of trifluoroacetic acid. The mixture was stirred at room temperature for 30 min until all starting material was desilylated, then transferred into a separatory funnel and washed once with 100 ml of aqueous 5% $NaHCO_3$ solution. The aqueous layer was extracted three times with 100 ml of methylene chloride and the combined organic layers were dried over anhydrous $MgSO_4$. After filtration and evaporation to dryness, the crude product was purified by column chromatography using methylene chloride/methanol (5–10% methanol) as eluent to give 7.2 g (83%) of the pure crystalline compound. ^{1}H NMR (400 MHz, DMSO-d_6): 7.97 (dd, 2 H, H_A benzoyl, J = 7.32, 8.42), 7.78 (t, 1 H, H_C benzoyl, J = 7.46), 7.60 (t, 2 H, H_B benzoyl, $J_{B,C}$ = 7.63), 6.15 (ψt, 1 H, H-1′, $J_{1',2'}$ = 6.57), 5.26 (d, 1 H, 3′-OH, $J_{3',OH}$ = 4.29), 5.11 (t, 1 H, 5′-OH, $J_{5',OH}$ = 5.07), 4.29 (m, 1 H, H-3′), 3.81 (m, 1 H, H-4′), 3.62 (m, 2 H, H-5′), 2.24–2.14 (m, 2 H, H-2′), 1.86 (s, 3 H, H-7). ^{13}C NMR (100 MHz, DMSO-d_6): 169.6 (C=O benzoyl), 162.5 (C-4), 149 (C-2), 137.1 (C-6), 135.5 (C_C benzoyl), 131.1 (C-quart benzoyl), 130.3 (C_A benzoyl), 129.5 (C_B benzoyl), 109.3 (C-5), 87.6 (C-4′), 84.5 (C-1′), 70.2 (C-3′), 61.1 (C-5′), 39.6 (C-2′, overlapped with DMSO triplet), 12.2 (C-7). MS (ES), *m/z*: 346.9 $(M + H)^+$.

N^3-Benzoyl-2′-deoxy-5′-*O*-(4-methoxytrityl)thymidine (**3**)

Dry nucleoside **2** (3.46 g, 10 mmol) was dissolved in 40 ml of absolute pyridine under argon atmosphere. 4-Methoxytrityl chloride (4.14 g, 13 mmol) was added in one portion and the yellow solution was stirred at room temperature overnight. The reaction was stopped by addition of 10 ml of methanol and stirred for another 30 min. The solvent was evaporated and the yellow oily residue was diluted with 200 ml of methylene chloride. The organic layer was washed three times with 100 ml of water, dried over anhydrous $MgSO_4$ and concentrated. The crude product was purified by column chromatography with methylene chloride/methanol (2–5%) to give 4.85 g (78%) of the pure product as a pale yellow foam. ^{1}H NMR (400 MHz, $CDCl_3$): 7.93 (m, 2 H, H_A benzoyl), 7.70 (s, 1 H, H6), 7.63 (t, 1 H, H_C benzoyl, J = 7.36), 7.48 (t, 2 H, H_B benzoyl, $J_{B,C}$ = 7.58), 7.44–7.27 (m, 12 H, MMT), 6.87 (d, 2 H, MMT, J = 8.84), 6.38 (dd, 1 H, H-1′, $J_{1',2'}$ = 6.06, 7.33), 5.26 (br d, 1 H, 3′-OH), 4.59 (m, 1 H, H-3′), 4.04 (m, 1 H, H-4′), 3.81 (s, 3 H, MMT-OCH_3), 3.45 (m, 2 H, H-5′), 2.39 (m, 2 H, H-2′), 1.43 (s, 3 H, H-7). ^{13}C NMR (100 MHz, $CDCl_3$): 169.2 (C=O benzoyl), 163.0 (C-4), 159 (C-OCH_3), 149.4 (C-2), 143.9 (C-quart MMT), 135.6 (C-6), 135.1 (C_C benzoyl), 134.9 (MMT), 131.1 (C-quart benzoyl), 130.7 (C_A benzoyl), 130.5 (MMT), 129.3 (C_B benzoyl), 128.5 (MMT), 128.2 (MMT), 127.5 (MMT), 113.5 (MMT), 111.5 (C-5), 87.4 (C-4′), 86.3 (C-quart MMT), 85.1 (C-1′), 72.5 (C-3′), 63.7 (C-5′), 55.4 (OCH_3), 41.3 (C-2′), 12 (C-7). MS (ES), *m/z*: 641.2 $(M + H + Na)^+$. For $C_{37}H_{34}N_2O_7$ (618.24) calculated: 71.83% C, 5.54% H, 4.53% N; found: 71.81% C, 5.59% H, 4.55% N.

N^3-Benzoyl-3′-*O*-(2-cyanoethyl)-2′-deoxy-5′-*O*-(4-methoxytrityl)thymidine (**4**)

In a well-dried Erlenmeyer flask with magnetic stirrer, compound **3** (1.24 g, 2 mmol) was dissolved in freshly distilled acrylonitrile (2.7 ml, 40 mmol) and *tert*-butyl alcohol (5 ml). After a few minutes, cesium carbonate (652 mg, 2 mmol) was added and the pale yellow suspension was vigorously stirred under argon atmosphere at room temperature for about 2.5 h. The insoluble material was removed by filtration through Celite, then the filtrate was concentrated and purified on a silica gel column with methylene chloride/methanol (0–1%) to give 1.23 g (1.84 mmol, 92%) of the product as a pale yellow foam. ^{1}H NMR (400 MHz, $CDCl_3$): 7.93 (dd, 2 H, H_A benzoyl, J = 7.07, $J_{A,B}$ = 8.59), 7.71 (s, 1 H, H-6), 7.64 (t, 1 H, H_C benzoyl, $J_{B,C}$ = 7.58), 7.49 (t, 2 H, H_B benzoyl, $J_{B,C}$ = 7.58), 7.43–7.26 (m, 12 H, MMT), 6.87 (d, 2 H, MMT, J = 8.84), 6.31 (dd, 1 H, H-1′, $J_{1',2'}$ = 5.81, 8.08), 4.24 (m, 1 H, H-3′), 4.15 (q, 1 H, H-4′, $J_{3',4',5'}$ = 2.78), 3.81 (s, 3 H, MMT-OCH_3), 3.63 (t, 2 H, H-8, $J_{8,9}$ = 6.04), 3.54–3.33 (m, 2 H, H-5′), 2.55 (t, 2 H, C-9, $J_{8,9}$ = 6.04), 2.51–2.28 (m, 2 H, H-2′), 1.51 (s, 3 H, H-7). ^{13}C NMR (100 MHz, $CDCl_3$): 169.2 (C=O benzoyl), 162.9 (C-4), 159 (C-OCH_3), 149.4 (C-2), 143.8 (C-quart MMT), 135.4 (C-6), 135.1 (C_C benzoyl), 134.8 (MMT), 131.1 (C-quart benzoyl), 130.6 (C_A benzoyl), 130.5 (MMT), 129.8 (MMT), 129.3 (C_B benzoyl), 128.5 (MMT), 128.3 (MMT), 127.6 (MMT), 117.5 (C-10), 113.5 (MMT), 111.4 (C-5), 87.5 (C-1′), 85.1 (C-4′), 84.1 (C-quart MMT), 80.5 (C-3′), 64.1 (C-8), 63.7 (C-5′), 55.4 (OCH_3), 37.9 (C-2′), 19.1 (C-9), 12 (C-7). MALDI(+)-MS, *m/z*: 694.8 $(M + Na + H)^+$. For $C_{40}H_{37}N_3O_7$ (671.26) calculated: 71.52% C, 5.55% H, 6.26% N; found: 71.24% C, 5.54% H, 6.35% N.

N^3-Benzoyl-3′-*O*-(2-cyanoethyl)-2′-deoxythymidine (**5**)

Compound **4** (671 mg, 1 mmol) was dissolved in a solution of 10% *p*-toluenesulfonic acid in methylene chloride/ethanol 1:1. The reaction mixture was stirred at room temperature for

2 h until all the starting material was consumed. Subsequently 100 ml of aqueous saturated $NaHCO_3$ solution was added and the organic layer separated. The aqueous layer was extracted three times with 100 ml of methylene chloride, the combined organic fractions were dried over anhydrous $MgSO_4$ and concentrated. The resulting colorless oil was purified by column chromatography with methylene chloride/methanol 95:5 (v/v) to give 399 mg (1 mmol, 100%) of the pure product. ^{1}H NMR (250 MHz, DMSO-d_6): 8.0–7.56 (m, 6 H, H-6, H-benzoyl), 6.11 (t, 1 H, H-1′), 5.20 (t, 1 H, 3′-OH), 4.19 (m, 1 H, H-3′), 3.97 (m, 1 H, H-4′), 3.64 (t, 2 H, H-8, $J_{8,9}$ = 6.04), 3.63 (m, 2 H, H-5′), 2.78 (t, 2 H, H-9, $J_{8,9}$ = 6.04), 2.35–2.28 (m, 2 H, H-2′), 1.87 (s, 3 H, H-7). ^{13}C NMR (250 MHz, DMSO-d_6): 169.6 (C=O benzoyl), 162.5 (C-4), 149.0 (C-2), 137.0 (C-6), 135.5 (C_C benzoyl), 131.1 (C-quart benzoyl), 130.4 (C_A benzoyl), 129.5 (C_B benzoyl), 119.2 (C-10), 109.5 (C-5), 84.9 (C-1′), 84.5 (C-4′), 70.2 (C-3′), 63.5 (C-8), 61.4 (C-5′), 36.5 (C-2′), 18.3 (C-9), 12.2 (C-7). MS (ES), *m/z*: 399.9 (M + H)$^+$. For $C_{20}H_{21}N_3O_6$ (399.14) calculated: 60.14% C, 5.30% H, 10.52% N; found: 60.39% C, 5.47% H, 10.66% N.

3′-*O*-(2-Cyanoethyl)-2′-deoxythymidine (**6**)

Nucleoside **5** (300 mg, 0.75 mmol) was dissolved in 10 ml of methanol and cooled down to 0 °C. NH_4OH (3 ml, 27%) was added dropwise and the mixture was stirred at 0 °C for 30 min, then at room temperature for 1.5 h. Afterwards the reaction mixture was concentrated and purified on a silica gel column with methylene chloride/methanol 95:5 (v/v) to give 212 mg (0.71 mmol, 96%) of the product as colorless crystals. ^{1}H NMR (250 MHz, DMSO-d_6): 11.31 (br s, 1 H, H-3), 7.68 (s, 1 H, H-6), 6.12 (ψt, 1 H, H-1′), 5.12 (m, 1 H, 5′-OH, $J_{5',OH}$ = 5.38), 4.14 (m, 1 H, H-3′), 3.92 (m, 1 H, H-4′), 3.63 (t, 2 H, H-8, $J_{8,9}$ = 6.04), 3.58 (m, 2 H, H-5′), 2.78 (t, 2 H, H-9, $J_{8,9}$ = 6.04), 2.28–2.09 (m, 2 H, H-2′), 1.77 (s, 3 H, H-7). ^{13}C NMR 250 MHz, DMSO-d_6): 163.7 (C-4), 150.5 (C-2), 136.0 (C-6), 119.2 (C-10), 109.6 (C-5), 84.5 (C-1′), 83.7 (C-4′), 79.4 (C-3′), 63.5 (C-8), 61.4 (C-5′), 36.2 (C-2′), 18.3 (C-9), 12.3 (C-7). MS (ES), *m/z*: 318.0 (M + Na + H)$^+$. For $C_{13}H_{17}N_3O_5$ (295.12) calculated: 52.88% C, 5.80% H, 14.23% N; found: 53.07% C, 5.91% H, 14.23% N.

3′-*O*-(2-Cyanoethyl)-2′-deoxythymidine-5′-phosphate (**7**)

Compound **6** (385 mg, 1.3 mmol) was dissolved in 10 ml of dry trimethyl phosphate and cooled down to 0 °C. The solution was treated with 1,8-bis(dimethylamino)naphthalene (418 mg, 1.95 mmol) and, after stirring for 5 min, phosphorus oxychloride (0.16 ml, 1.7 mmol) was added dropwise on cooling. The pale yellow clear solution was stirred at 0 °C for 3 h, subsequently quenched by addition of 20 ml of cold triethylammonium hydrogencarbonate buffer (pH 7) and stirred for 1 h. The aqueous layer was extracted five times with 100 ml of *tert*-butyl methyl ether for removal of the proton sponge and trimethyl phosphate. The aqueous layer was lyophilized and purified by RP-HPLC with 0.1 M triethylammoniun acetate buffer/acetonitrile as eluent. The crude product was obtained as colorless glassy oil which was analyzed by NMR and mass spectrometry then. For easier handling and storage of the product, it was precipitated as a sodium salt. The resulting 250 mg (0.66 mmol) of monophosphate was dried by freeze-drying over three days. The nucleotide was dissolved under argon atmosphere at 4 °C in 6.6 ml of absolute methanol to give a water-free methanolic 0.1 M monophosphate solution. A freshly prepared anhydrous sodium perchlorate solution (made from 1.21 g (9.9 mmol) of sodium perchlorate in 33 ml of absolute acetone) was added dropwise at 4 °C and the sodium salt of the nucleoside precipitated as colorless crystals. The crystals were centrifuged (4 °C, 5 min, 12 000 rpm), the supernatant was removed and the solid dried under reduced pressure yielding 267 mg (52%) of the product as a Na^+-salt. ^1H NMR (400 MHz, D_2O): 7.69 (s, 1 H, H-6), 6.22 (ψt, 1 H, H-1′), 4.31 (m, 1 H, H-3′), 4.22 (m, 1 H, H-4′), 3.95 (m, 2 H, H-5′), 3.72 (t, 2 H, H-8, $J_{8,9}$ = 6.04), 2.71 (t, 2 H, H-9, $J_{8,9}$ = 6.04), 2.39–2.23 (m, 2 H, H-2′), 1.81 (s, 3 H, H-7). ^{13}C NMR (100 MHz, D_2O): 166.3 (C-4), 151.5 (C-2), 137.2 (C-6), 119.6 (C-10), 111.6 (C-5), 85.0 (C-1′), 83.5 (C-4′), 80.0 (C-3′), 64.9 (C-8), 63.7 (C-5′), 36.2 (C-2′), 18.3 (C-9), 11.5 (C-7). ^{31}P NMR (161.98 MHz, D_2O): 2.11 (s, P-α). MS (ES), *m/z*: 374.0 $(M - H)^-$.

3′-*O*-(2-Cyanoethyl)-2′-deoxythymidine-5′-triphosphate (**8**)

Compound **7** (55 mg, 0.19 mmol) was coevaporated twice with dry pyridine (3 ml) and dried under vacuum overnight. The flask was filled with argon and closed with a septum. The following steps were carried out under slightly positive argon pressure. The nucleoside was dissolved in absolute pyridine (190 µl) and anhydrous dioxane (570 µl) and then treated with 190 µl (0.19 mmol) of a freshly prepared 1 M solution of 2-chloro-4*H*-1,3,2-benzodioxaphosphorinan-4-one in anhydrous dioxane. After 10 min, a mixture of 570 µl (0.28 mmol) of 0.5 M solution of bis(tributylammonium) dihydrogen diphosphate in anhydrous DMF and 190 µl (0.80 mmol, 4.3 equiv.) of tributylamine was quickly added. The reaction mixture was stirred for 10 min and subsequently treated with 3.8 ml of a 1% iodine solution in pyridine/water 98:2 (v/v). After stirring at room temperature for 15 min, 5% aqueous $NaHSO_3$ solution was added until the brown reaction mixture turned light yellow due to iodine reduction. The solution was concentrated under reduced pressure, the residue dissolved again in 20 ml of water and left at room temperature for 30 min. The mixture was treated with 38 ml of concentrated ammonia and stirred at room temperature for 60 min. The solvent was evaporated, the solid (330 mg) dissolved then in ca. 12 ml of water, filtered through a 0.2 µm syringe filter and purified in four portions on a DEAE-Sepharose fast-flow column (1.6 cm diameter, 15 cm length) at 4 °C (flow rate 4 ml/min, gradient 0–500 ml 100% buffer A in 0.05 M TEAB, 500–650 ml 50% buffer A and 50% buffer B in 1 M TEAB). The product eluted with 0.17 M triethylammonium hydrogencarbonate (TEAB) buffer; 65 mg of the obtained product was further purified by semipreparative HPLC. HPLC-purified 28.3 mg of triphosphate (12%) were isolated after both purification steps. ^1H NMR (300 MHz, D_2O): 7.77 (d, 1 H, H-6), 6.34 (dd, 1 H, H-1′, $J_{1',2'}$ = 5.8), 4.50 (d, 1 H, H-3′), 4.36 (s, 1 H, H-4′), 4.21 (m, 2 H, 2 H-5′), 3.85 (t, 2 H, 2 H-8, $J_{8,9}$ = 6.0), 2.82 (t, 2 H, H-9, $J_{8,9}$ = 6.0), 2.49–2.35 (m, 2 H, H-2′), 1.93 (d, 3 H, H-7). ^{31}P NMR (161.98 MHz, D_2O): –10.9 (s, P-γ), –11.7 (d, P-α), –23.2 (s, P-β). MALDI(–)-MS, *m/z*: 533.95 $(M - 2 H)^-$.

3′-*O*-(2-Cyanoethyl)-5′-[(2-cyanoethyl)-(*N*,*N*-diisopropyl)]-2′-deoxyphosphoramidite-thymidine (**9**)

Dried compound **6** (236 mg, 0.8 mmol) was dissolved under argon in 8 ml of absolute dichloromethane and treated with (2-cyanoethyl)-di(*N*,*N*-diisopropyl)phosphine (381 µl, 1.2 mmol) and 4,5-dicyanoimidazole (110 mg, 0.92 mmol). The mixture was stirred at room temperature for 4 h, then diluted with 20 ml of dichloromethane and washed once with 25 ml of saturated $NaHCO_3$ solution. The organic layer was separated, dried over Na_2SO_4, filtered and concentrated. The crude product was purified on a short silica gel column under argon pressure. The pure product was obtained in 337 mg (85%) yield as an isomeric mixture and stored at –20 °C under argon. ^{1}H NMR (400 MHz, acetone-d_6): 9.88 (br s, 2 H, NH), 7.69 (s, 1 H, H-6), 7.56 (s, 1 H, H-6), 6.31 (dd, 1 H, H-1′, $J_{1',2'}$ = 5.83, 8.38), 6.25 (dd, 1 H, H-1′, $J_{1',2'}$ = 5.60, 8.60), 4.36 (m, 1 H, H-3′), 4.32 (m, 1 H, H-3′), 4.21 (m, 2 H, H-4′), 4.0–3.63 (m, 10 H, H-5′, H-isopropyl phosphine, phosphine-O-CH_2), 3.91 (t, 2 H, 2 H-8, $J_{8,9}$ = 6.10), 3.81 (t, 2 H, 2 H-8, $J_{8,9}$ = 6.10), 2.80 (t, 2 H, CH_2-CN phosphine, $J_{1,2}$ = 5.81), 2.77 (t, 2 H, H-9, $J_{8,9}$ = 6.06), 2.41–2.26 (m, 4 H, H-2′), 1.87 (s, 3 H, H-7), 1.86 (s, 3 H, H-7), 1.28–0.99 (m, 24 H, H-isopropyl phosphine). ^{13}C NMR (100 MHz, acetone-d_6): 163.2 (C-4), 150.2 (C-2), 135.4 (C-6), 135.2 (C-6), 118.2 (CN, phosphine), 118 (C-10), 110 (C-5), 84.6 (C-1′), 84.3 (C-1′), 83.5 (C-4′), 80.1 (C-3′), 63.8 (C-8), 63.2 (C-8), 61.4 (C-5′), 58.4 (O-CH_2, phosphine), 42.7 (C-isopropyl, phosphine), 36.6 (C-2′), 23.9 (C-isopropyl, phosphine), 19.8 (CH_2, phosphine), 18.0 (C-9), 11.5 (C-7). ^{31}P NMR (75 MHz, acetone-d_6): 148.8 (s, P-α), 148.2 (s, P-α). ESI(–)-MS, *m/z*: 494.4 $(M - H)^-$.

2′-Deoxythymidine-5′-phosphate (**10**)

2′-Deoxythymidine **1** (315 mg, 1.3 mmol) was dissolved in 10 ml of absolute trimethyl phosphate under argon atmosphere and cooled down to –4 °C. At that temperature, phosphorus oxychloride (0.25 ml, 2.6 mmol) was added dropwise and the reaction mixture was stirred at –4 °C for 6 h. The reaction was stopped by addition of 20 ml of TEAB buffer (pH 7) and stirring continued for 30 min. The aqueous layer was extracted five times with 100 ml of *tert*-butyl methyl ether, lyophilized and purified on RP-HPLC with 0.05 M triethylammonium acetate buffer/acetonitrile as eluent. The crude product was obtained as a colorless glassy oil which could be analyzed by NMR and mass spectrometry. For facile handling and storage of the product, it was precipitated as a sodium salt. The resulting 190 mg (0.59 mmol) of monophosphate was dried by freeze-drying over three days. The nucleotide was dissolved under argon atmosphere at 4 °C in 5.9 ml of absolute methanol to give a water-free methanolic 0.1 M monophosphate solution. A freshly prepared anhydrous sodium perchlorate solution (1.08 g (8.8 mmol) sodium perchlorate in 29.5 ml of absolute acetone) was added dropwise at 4 °C and the sodium salt of the nucleotide was precipitated. The crystals were centrifuged (5 min, 4 °C, 12 000 rpm), the supernatant was removed and the colorless solid was lyophilized to give 203 mg (45%) of the crystalline product. ^{1}H NMR (400 MHz, D_2O): 7.58 (s, 1 H, H-6), 6.15 (ψt, 1 H, H-1′), 4.37 (m, 1 H, H-3′), 3.97 (m, 1 H, H-4′), 3.87 (m, 2 H, H-5′), 2.16 (m, 2 H, H-2′), 1.71 (s, 3H, H-7). ^{13}C NMR (100 MHz, D_2O): 166.4 (C-4), 151.5 (C-2), 137.3 (C-6), 111.5 (C-5), 85.4 (C-1′), 84.9 (C-4′), 71.0 (C-3′), 64.6 (C-5′), 38.6 (C-2′), 11.5 (C-7). ^{31}P NMR (161.98 MHz, D_2O): 0.66 (s, P-α). MALDI(–)-MS, *m/z*: 373.8 $(M - 2 H - Na)^-$.

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