Synthesis of 3´-*S*-(2-Aminoethylthio)-3´-deoxythymidine-5´-triphosphates

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Abstract: 3´-*S*-(2-*N*-(*t*-Butyloxycarbonyl)aminoethylthio)-3´-deoxy-5´-*O*-(4,4´-dimethoxytrityl)thymidine (**3**) was synthesized by treating 5´-*O*-(4,4´-dimethoxytrityl)-2,3´-anhydrothymidine (**2**) with *t*-butyl *N*-(2-mercaptoethyl)carbamate (**1**) and DBU. Compound **3** was further converted to 3´-*S*-(2-*N*-(*t*-butyloxycarbonyl)aminoethylthio)-3´-deoxythymidine-5´-triphosphate (**5**) and 3´-*S*-(2-aminoethylthio)-3´-deoxythymidine-5´-triphosphate (**9**). The latter compound was labeled with a near-IR fluorescent dye.

Key words: fluorescent nucleotides, thioether, triphosphates, chromophores, ring opening

Currently, there is widespread interest in nucleosides, in particular nucleoside-5´-triphosphates that are covalently coupled to fluorophores.[1] Dependent on the application, different positions at the ribose or base moiety within the nucleoside were preferred for introducing the label. Among the base modifications, the exocyclic amino groups[2] or the C-5 position in the pyrimidines[3] and the C-7 or C-8 position in purines[4] are usually the target for labeling. Furthermore, a series of fluorescent base analogues like 2-aminopurine,[5] ethenoadenosine,[6] 2'-deoxyisoinosine,[7] 2-pyrimidinone[8] and polyaromatic hydrogencarbons[9] exists.

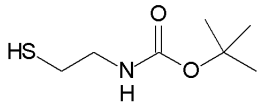
Introducing a probe at the ribose residue of a nucleoside was until now accomplished by conjugating the dye to the 2´-, 3´- or 5´-hydroxyl group through an ester or ether bond.[10] Alternatively, we and others have already reported the syntheses of 3´-amino, amido-, isocyanato- and isothiocyanato- modified 2´,3´-dideoxynucleoside-5´-triphosphates and their labeling with fluorescence dyes.[11] However, Canard et al. have demonstrated that ester- and amide-bonds adjacent to the 3´-carbon of nucleoside-5´-triphosphates may unexpectedly be subjected to enzyme-mediated hydrolysis and loss of the label.[12] These results are in accordance with our investigations on 3´-amido tethered nucleoside triphosphates.[13] In the context of our studies on 3´-modified nucleoside-5´-triphosphates, we decided to alter the type of link into a thioether function which offers a more stable bond against enzymatic degradation. Here, we describe an efficient synthetic route to this novel class of 3´ thioether-thymidine-5´-triphosphates bearing an aminoethyl linker for fluorescent probe coupling. An oxazine dye with spectroscopic properties close to the cyanine dye, CY5 was conjugated.[14]

Introducing the bifunctional linker into the nucleoside afforded an amino protecting group, which is stable under various basic reaction conditions. Different attempts to introduce cysteamine (2-aminoethanthiol) without protection of the amino group failed. We avoided employing amide protective groups since they are critical in the presence of thiolates or enolates. Hence, we chose the butyloxycarbonyl group (Boc) which is stable towards most harsh nucleophilic and basic aqueous or non-aqueous reaction conditions and which can easily be removed with trifluoroacetic acid.

5´-*O*-(4,4´-Dimethoxytrityl)-2,3´-anhydrothymidine (**2**) was obtained as described before.[15] Zuckermann described the introduction of alkane dithiols of the type HS-$(CH_2)_n$-SH into 5´-*O*-(4,4´-dimethoxytrityl)-2,3´-anhydrothymidine (**2**) employing sodium ethoxide in ethanol as the basic medium.[16] The reaction mixture was maintained at 75 °C for 2.5 hours. Although the educt was completely converted to only one major UV-absorbing product as indicated by thin layer chromatography, the yield was only 58%. We also achieved this yield with hexane-1,6-dithiol as nucleophile. We could not observe a considerable loss of product during the work up and isolation procedure. Therefore, a great amount of the nucleobase seems to degrade under these reaction conditions. However, in our studies, application of this method with *t*-butyl *N*-(2-mercaptoethyl)carbamate (**1**) as nucleophile only resulted in starting material.

Bera et al. demonstrated that introducing mercaptoethanol by treating 5´-*O*-trityl-2,3´-anhydrothymidine with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in DMF for 7 h at 60 °C or with 1,1,3,3-tetramethylguanidine resulted in a very low yield of the corresponding 3´-thioether nucleoside.[17]

We have found that the crucial parameters for opening anhydrothymidine with thiols are the reaction temperature and the choice of the basic medium. At 50 °C, no product was observed no matter what kind of base was used. Only when the reaction temperature was raised to 100 °C and DBU was utilized as base, did 5´-*O*-(4,4´-dimethoxytrityl)-2,3´-anhydrothymidine (**2**) readily react with *t*-butyl *N*-(2-mercaptoethyl)carbamate (**1**)**,** within 24 hours, to produce the desired thioether 3´-*S*-(2-*N*-(*t*-butyloxycarbonyl)aminoethylthio)-3´-deoxy-5´-*O*-(4,4´-dimethoxytrityl)thymidine (**3**) in 80% yield.



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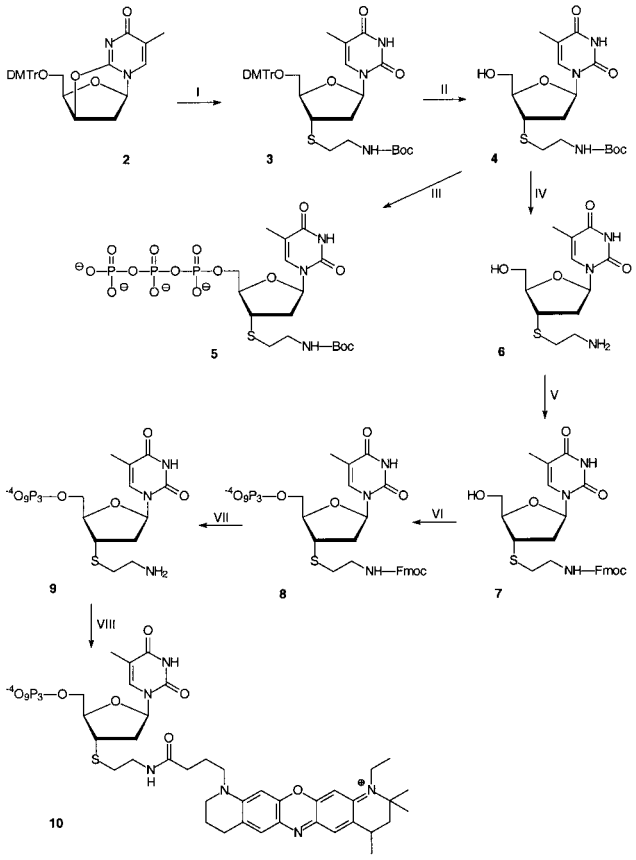
Compound **3** was detritylated in 80% aqueous acetic acid at room temperature. Synthesis of 3´-*S*-(2-*N*-(*t*-butyloxycarbonyl)aminoethylthio)-3´-deoxythymidine-5´-triphosphate (**5**) was performed according to the procedure of Ludwig and Eckstein.[18] The purification was done by FPLC on DEAE-Sephadex with a gradient of 0.05-1M triethylammonium bicarbonate pH 7.5. It is known that repeated co-evaporation or freeze drying of the collected triphosphate fractions with ethanol until weight constancy should remove triethylammonium bicarbonate completely. However, the yield determined by optical density measurements is considerably lower than by weighing the dried product. The difference in weight is due to the presence of further buffer. For obtaining the pure product, a small amount was therefore purified by analytical anion exchange HPLC followed by reverse phase HPLC. All triphosphate yields were determined by their optical density in water.

Compound **5** proved to be inappropriate for dye-labeling experiments, since the triphosphate unit was not stable under deprotection conditions. With similar nucleotides, it has been shown that treatment with concentrated trifluoroacetic acid for 2 min, instant freezing with liquid nitrogen and quenching with a great excess of diethyl ether readily removed the Boc group without destroying the triphosphate.[11,19] However, in our studies, no nucleotide could be isolated with compound **5** utilizing these reaction conditions. Hence, we deprotected at the nucleoside level. Treatment of 3´-*S*-(2-*N*-(*t*-butyloxycarbonyl)aminoethylthio)-3´-deoxythymidine (**4**) with concentrated trifluoroacetic acid within 2 min gave **6** in 79% yield. It should be mentioned that removal of both protecting groups of 3´-*S*-(2-*N*-(*t*-butyloxycarbonyl)aminoethylthio)-3´-deoxy-5´-*O*-(4,4´-dimethoxytrityl)thymidine (**3**) with trifluoroacetic acid in one step resulted in a lower yield and a more laborious work-up procedure. Selective reprotection of the amino function was easily done by treating unprotected nucleoside **6** with 9-fluorenylmethoxycarbonyl-*N*-hydroxysuccinimide (Fmoc-*O*-succinimide) and sodium bicarbonate in water and acetone as described by Paquet.[20] Subsequently, 3´-*S*-(2-*N*-(9-fluorenylmethoxycarbonyl)aminoethylthio)-3´-deoxythymidine-5´-triphosphate (**8**) was synthesized. The Fmoc group was only partly cleaved during the work-up procedure of the triphosphate. 5% of the protected product **8** and 17% of the unprotected 3´-*S*-(2-aminoethylthio)-3´-deoxythymidine-5´-triphosphate **9** were isolated. For a clean reaction, piperidine was therefore added previous to the isolation procedure. Alternatively, remaining triphosphate **8** was deblocked with 20% piperidine in pyridine and DMF and finally the nucleotide **9** was coupled with JA242 according to the method of Bannwarth et al.[21] JA242 is a carboxyl functionalized oxazine dye with spectroscopic characteristics similar to the commercially available cyanine dye CY5. The carboxyl group is activated as its *N*-hydroxysuccinimidyl ester and coupled to the free amino group in a mixture of dioxane, DMF and water. The fluorescent nucleoside 5´-triphosphate was purified by analytical anion exchange HPLC by simultaneous UV- and fluorescence detection and a gradient of aqueous 1M LiCl solution as eluent. Fractions containing product were subjected to size exclusion gel filtration on Sephadex G10. The fluorescence spectrum revealed a bathochromic shift (4 nm) in the emission maximum for covalently attached JA242 (667 nm) compared to free dye (663 nm). This corresponds to the results found by Lieberwirth who attached JA242 covalently to amino-modified oligonucleotides.[22]



(I) $HS(CH_2)_2NHBoc$, DBU, DMF, 24 h, 100 °C (80%); (II) 80% aq HOAc, 1h, (91%); (III) (a) van Boom´s reagent (2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one), dioxane, pyridine, DMF, 20 min, (b) $(Bu_3NH)_2P_2O_7$, DMF, tributylamine, 30 min, (c) 1% I_2 in pyridine/H_2O (98:2), 20 min, (d) 5% aq $NaHSO_3$ (13%); (IV) concd TFA, 2 min (79%); (V) Fmoc-*O*-succinimide, H_2O, acetone, $NaHCO_3$, 3 h (73%); (VI) (a) van Boom´s reagent, dioxane, pyridine, DMF, 20 min, (b) $(Bu_3NH)_2P_2O_7$, DMF, tributylamine, 30 min, (c) 1% I_2 in pyridine/H_2O (98:2), 20 min, (d) 5% aq $NaHSO_3$ (22% including subsequent product); (VII) piperidine, pyridine, DMF, 1 h (77%); (VIII) JA242, DMF, dioxane, H_2O, DIPEA, TSTU, 30 min

Compound **5** was chosen for enzymatic incorporation experiments. When **5** was substituted for ddTTP as terminator in a DNA sequencing experiment with Taq-DNA-polymerase, we obtained a band pattern, which could not be correlated with the standard ddTTP pattern. Varying the concentration of **5** did not alter this result. No band pattern was detected when Sequenase or Thermo-Sequenase was employed.

Solvents were of analytical grade and were dried and distilled or purchased and stored over molecular sieve. *t*-Butyl *N*-(2-mercaptoethyl)carbamate was purchased at Fluka Chemie AG, Germany. Silica gel 60 F_{254} plates (Merck) were used for TLC. Preparative silica gel TLC was done with covered glass plates (gypsum silica gel 60 PF_{254} Merck) on a Harrison Research 7924T Chromatotron. NMR solvent signals were used for calibrating as follows: ^{1}H NMR (250, 270 MHz, Bruker AM250, WH270): δ(DMSO-d_6) = 2.50, δ($CDCl_3$) = 7.26; ^{13}C NMR (62.9, 67.9 MHz, Bruker AM250, WH270): δ(DMSO-d_6) = 39.5, δ($CDCl_3$) = 77.0; ^{31}P (162 MHz, Bruker AMX 400), external 85% phosphoric acid. Mass spectra were recorded with a Fisons VG Platform II electrospray ionization mass spectrometer. UV spectra and optical density were measured on a Varian Cary 1. Optical densities were measured at 270 nm (molar absorptivity in water 8700 L mol^{-1} cm^{-1}). Fluorescence spectra were recorded on a Hitachi F-4500. Elemental analysis was performed on Foss Heraeus CHN-O Rapid. FPLC was performed on a Pharmacia instrument with LCC-500 controller, P-500 pumps, and single path UV monitor UV-1. HPLC was performed on a Merck-Hitachi with L-4250 UV-VIS detector and Shimadzu RF-535 fluorescence monitor.

General Purification Procedures

FPLC: filtrated (0.2 µm Nalgene syringe filter) crude product in H_2O was applied onto a column filled with Pharmacia DEAE Sephadex-A25 anion exchange gel; A = 0.05M TEAB (triethylammonium bicarbonate), pH 7.5, B = 1M TEAB, pH 7.5; flow rate 1 mL/min, 0-500 mL 0-50% B, 500-650 mL 50-100% B.

Anion exchange HPLC: Dionex NucleoPac PA-100 (4 x 250mm), A = H_2O/10% MeCN, B = 1M aq LiCl/10% MeCN, flow rate 1 mL/min, 0-5 min 0% B, 5-20 min 0-25% B, 20-25 min 100% B.

Reverse phase HPLC: LiChrosphere RP-18 5 µm (3 x 125 mm), A = 0.1M TEAA (triethylammonium acetate) pH 5.5, B = A+50% MeCN, flow rate 1 mL/min, 0-10 min 5% B.

HPLC retention times (t_R) refer to anion exchange HPLC (purification procedure 2) and are given in min.

3'-*S*-(2-*N*-(*t*-Butyloxycarbonyl)aminoethylthio)-3'-deoxy-5'-*O*-(4,4'-dimethoxytrityl)thymidine (3)

To a solution of *t*-butyl *N*-(2-mercaptoethyl)carbamate (**1**) (2 mL, 12 mmol) and DBU (1.4 mL, 9.5 mmol) in DMF (10 mL) 5'-*O*-(4,4'-dimethoxytrityl)-2,3'-anhydrothymidine (**2**) (1.00 g, 1.9 mmol) was added. The reaction mixture was heated (bath temperature 100 °C) for 24 h, cooled to r.t., poured into $NaHCO_3$ soln (50 mL) and extracted with EtOAc. The organic layer was washed with H_2O, dried ($MgSO_4$) and evaporated. The residue was purified by preparative silica gel TLC (CH_2Cl_2:MeOH, 98:2) to give **3** (1.07 g, 80%).

^{1}H NMR ($CDCl_3$): δ = 9.19 (s, 1H, NH-3), 7.75 (d, 1H, *J* = 1.2 Hz, H-6), 7.43-6.84 (m, 13H, Ar-H), 6.17 (dd, 1H, *J* = 3.7 Hz, H-1'), 4.80 (t, 1H, *J* = 6.0 Hz, OCO-NH), 3.91 (m, 1H, H-4'), 3.79 (s, 6H, O-CH_3), 3.68-3.54 (m, 2H, H-3', H-5'), 3.32 (dd, 1H, *J* = 2.7 Hz, H-5''), 3.26-3.15 (m, 2H, N-CH_2), 2.54 (t, 2H, *J* = 6.0 Hz, S-CH_2), 2.55-2.37 (m, 2H, H-2', H-2''), 1.46 (d, 3H, *J* = 0.9 Hz, C5-CH_3), 1.42 (s, 9H, *t*-Bu-CH_3).

^{13}C NMR ($CDCl_3$) δ = 163.9 (C4), 158.6 (DMTr), 155.5 (NC(O)O), 150.2 (C2), 144.0 (DMTr), 135.4 (C6), 135.1 (DMTr), 129.9, 128.0, 127.9, 127.1, 113.1 (DMTr), 110.7 (C5), 86.5 (C-DMTr), 85.6, 84.5 (C1', C4'), 79.5 (*C*(CH_3)$_3$), 61.7 (C5'), 55.1 (OCH_3), 40.6 (C3'), 40.3, 39.8 (C2', N-CH_2), 31.8 (S-CH_2), 28.2 (Boc-CH_3), 11.8 (C5-*C*H_3).

MS (ESI-): *m/z* = 702.4 (M^- requires 702.8).

Anal: $C_{38}H_{45}N_3O_8S$ (703.86). Calcd for $C_{38}H_{45}N_3O_8S{\cdot}H_2O$: C, 63.23; H, 6.56; N, 5.82. Found: C, 63.55; H, 6.53; N, 5.81.

3'-*S*-(2-*N*-(*t*-Butyloxycarbonyl)aminoethylthio)-3'-deoxythymidine (4)

A solution of **3** (0.90 g, 1.28 mmol) in aq HOAc (80%, 20 mL) was stirred for 1 h at r.t., evaporated, then co-evaporated thrice with H_2O to remove residual acid. The residue was purified by preparative silica gel TLC (CH_2Cl_2:MeOH, 95:5) to give **4** (470 mg, 91%).

^{1}H NMR (DMSO) δ = 11.25 (s, 1H, NH-3), 7.80 (s, 1H, H-6), 6.06 (dd, 1H, *J* = 4.4 Hz, H-1'), 3.76-3.40 (m, 4H, H-3', H-4', H-5', H-5''), 3.12 (m, 2H, N-CH_2), 2.64 (t, 2H, *J* = 6.8 Hz, S-CH_2), 2.47-2.20 (m, 2H, H-2', H-2''), 1.78 (s, 3H, C5-CH_3), 1.38 (s, 9H, *t*-Bu-CH_3).

^{13}C NMR (DMSO) δ = 163.8 (C4), 155.5 (NC(O)O), 150.4 (C2), 136.2 (C6), 109.0 (C5), 85.9 (C1'), 83.6 (C4'), 77.8 (*C*(CH_3)$_3$), 60.4 (C5'), 40.5 (C-3'), 40.4, 40.2 (C2', N-CH_2), (3 peaks covered by DMSO), 30.6 (S-CH_2), 28.2 (Boc-CH_3), 12.3 (C5-*C*H_3).

MS (ESI-): *m/z* = 400.3 (M^- requires 400.5).

Anal: $C_{17}H_{27}N_3O_6S$ (401.49). Calcd: C, 50.86; H 6.78; N 10.47. Found: C, 50.70; H, 6.96; N, 10.21

3'-*S*-(2-*N*-(*t*-Butyloxycarbonyl)aminoethylthio)-3'-deoxythymidine -5'-triphosphate (5) as triethylammonium salt

Nucleoside-5'-triphosphates were synthesized according to Ludwig and Eckstein[18] as follows: 3'-*S*-(2-*N*-(*t*-Butyloxycarbonyl)aminoethylthio)-3'-deoxythymidine (**4**) (100 mg, 0.25 mmol) was dried three times by co-evaporation with anhyd pyridine (0.5 mL) and anhyd DMF (2 mL). The residue was dried over P_2O_5 under vacuum overnight. The nucleoside was dissolved in anhyd pyridine (0.5 mL) and anhyd DMF (2 mL) and a freshly prepared solution of 1M 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one in anhyd dioxane (0.28 mL) was injected under Ar. After stirring for 20 min, a prepared solution of 0.5 M $(Bu_3NH)_2P_2O_7$ in dry DMF was mixed well with tributylamine (3:1) and 1.0 mL of this mixture was instantly added. The reaction mixture was stirred further for 30 min. Oxidation of phosphorus and ring opening was achieved by adding 5 mL of 1% I_2 in pyridine/ H_2O (98:2). After 20 min, the remaining I_2 was reduced by adding a solution of 5% aq $NaHSO_3$ drop by drop until the brown colour changed to a light yellow. Solvents were removed under reduced pressure without heating. H_2O (5 ml) was poured onto the residue and the solution containing the dissolved product was separated from the remaining residue by filtration through a 0.2 µm Nalgene syringe filter. The solution was then purified by FPLC (purification procedure 1), elution concentration = 25% B; analytical amounts of fractions containing triphosphate were further purified by anion exchange HPLC and finally desalted by reverse phase HPLC (purification procedure 2 and 3), t_R = 14.5 min. The yield was determined by optical density measurement (31.1 µmol, 13%), $C_{17}H_{30}N_3O_{15}P_3S$ (641.44).

^{1}H NMR (D_2O) δ = 7.82 (d, 1H, *J* = 1.2 Hz, H-6), 6.21 (dd, 1H, *J* = 4.7 Hz, H-1'), 4.37-4.22 (m, 2H, H-5', H-5''), 4.15 (m, 1H, H-4'), 3.59-3.53 (m, H-3' partly overlapping with triethylammonium signal), 3.33 (m, 2H, N-CH_2), 2.82 (t, 2H, *J* = 6.4 Hz, S-CH_2), 2.60-2.44 (m, 2H, H-2', H-2''), 1.94 (d, 3H, *J* = 1.0 Hz, C5-CH_3), 1.41 (s, 9H, *t*-Bu-CH_3).

^{13}C NMR (D_2O) δ = 166.6 (C4), 151.4 (C2), 137.3 (C6), 111.3 (C5), 84.9, 84.6 (C1', C4'), 80.9 (*C*(CH_3)$_3$), 62.6 (C5'), 40.9 (C3'), 40.0, 39.1 (C2', N-CH_2), 29.9 (S-CH_2), 28.0 (Boc-CH_3), 11.8 (C5-*C*H_3).

^{31}P NMR (D_2O) δ = -22.2 (t, 1P, β-P), -10.8 (d, 1P, α-P), -9.2 (d, 1P, γ-P).

MS (ESI-): *m/z* = 640.4 (M^- requires 640.4).

3'-*S*-(2-Aminoethylthio)-3'-deoxythymidine (6)

Concd CF_3CO_2H (10 mL) was poured onto solid **4** (1.63 g, 4.06 mmol) and stirred for 2 min. The reaction was quenched by adding Et_2O (100 mL). The obtained white residue was filtered, washed

with Et_2O and dissolved in MeOH (10 ml) for purification by silica gel chromatography (CH_2Cl_2:MeOH, 2:1) to give compound **6** as the trifluoroacetic salt (1.33 g, 79%).

t_R (HPLC) = 9.2 min.

^{1}H NMR (D_2O) δ = 7.48 (s, 1H, H-6), 5.94 (dd, 1H, *J* = 4.4 Hz, H-1´), 3.78-3.22 (m, 4H, H-5´, H-5´´, H-4´, H-3´), 3.03 (t, 2H, *J* = 6.6 Hz, N-CH_2), 2.75 (t, 2H, *J* = 6.6 Hz, S-CH_2), 2.45-2.21 (m, 2H, H-2´, H-2´´), 1.65 (s, 3H, C5-CH_3).

^{13}C NMR (DMSO) δ = 163.8 (C4), 150.4 (C2), 136.2 (C6), 109.0 (C5), 85.7, 83.6 (C1´, C4´), 60.3 (C5´), 40.5 (C3´), 40.4, 40.1 (C2´, N-CH_2), 27.9 (S-CH_2), 12.2 (C5-CH_3).

MS (ESI-): *m/z* = 300.2 (M^- requires 300.4), 414.2 ($M{\cdot}TFA^-$ requires 414.4).

Anal: $C_{12}H_{19}N_3O_4S$ (301.37). Calcd for $C_{12}H_{19}N_3O_4S{\cdot}TFA$: C, 40.48; H, 4.85; N, 10.12. Found: C, 40.10; H, 4.89; N, 9.63.

3´-*S*-(2-*N*-(9-Fluorenylmethoxycarbonyl)aminoethylthio)-3´-deoxythymidine (7)

To a solution of **6** (1.31 g, 4.35 mmol) in H_2O (60 mL) and acetone (60 mL), $NaHCO_3$ (364 mg, 4.36 mmol) and 9-fluorenylmethoxycarbonyl-*N*-hydroxysuccinimide (1.47 g, 4.36 mmol) was added. The reaction mixture was stirred at for 3 h r.t. Further 9-fluorenylmethoxycarbonyl-*N*-hydroxysuccinimide (220 mg, 0.65 mmol) was added since the reaction was incomplete as was evident from TLC. After additional stirring for 1 h, the reaction mixture was adjusted to pH 2 with concd HCl, acetone was removed by evaporation under reduced pressure and the aq residue was extracted twice with CH_2Cl_2 (100 mL). The combined organic layers were dried (Na_2SO_4), evaporated and purified by silica gel chromatography (CH_2Cl_2:MeOH, 95:5) giving compound **7** (1.66g, 73%).

^{1}H NMR ($CDCl_3$): δ = 9.28 (s, 1H, NH-3), 7.77-7.26 (m, 9H, H-6, arom. fluorenyl), 6.03 (dd, 1H, *J* = 3.0 Hz, H-1´), 4.42 (d, 2H, *J* = 6.8 Hz, CH_2 fluorenyl), 4.20 (t, 1H, *J* = 6.8 Hz, CH fluorenyl), 3.97 (m, 1H, H-5´), 3.80 (m, 2H, H-5´´, H-4´), 3.59-3.27 (m, 3H, H-3´, N-CH_2), 2.71 (t, 2H, *J* = 6.3 Hz, S-CH_2), 2.59-2.50 (m, 1H, H-2´), 2.43-2.31 (m, 1H, H-2´´), 2.07 (s, 3H, C5-CH_3).

^{13}C NMR ($CDCl_3$): δ = 164.1 (C4), 156.8 (NC(O)O), 150.4 (C2), 143.5, 141.3 (Fmoc), 136.7 (C6), 127.7, 127.0, 124.9, 120.0 (Fmoc), 110.4 (C5), 86.5, 85.6 (C1´, C4´), 66.9 (Fmoc), 60.7 (C5´), 47.1 (Fmoc), 40.8 (C3´), 40.2, 39.0 (C2´, N-CH_2), 31.9 (S-CH_2), 12.5 (C5-CH_3).

MS (ESI+): *m/z* = 524.2 (M^+ requires 524.6).

Anal: $C_{27}H_{29}N_3O_6S$ (523.61). Calcd for $C_{27}H_{29}N_3O_6S{\cdot}H_2O$: C, 59.87; H, 5.77; N, 7.76. Found: C, 60.25; H, 5.67; N, 7.91

3´-*S*-(2-*N*-(9-Fluorenylmethoxycarbonyl)aminoethylthio)-3´-deoxythymidine-5´-triphosphate (8) as triethylammonium salt

Compound **8** was synthesized and purified following the same procedure as described for **5** with 3´-*S*-(2-*N*-(9-fluorenylmethoxycarbonyl)aminoethylthio)-3´-deoxythymidine (**7**) (0.19 mmol, 100 mg), anhyd pyridine (0.5 mL), anhyd DMF (2 mL), 1 M 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one in anhyd dioxane (0.21 mL), 0.5 M $(Bu_3NH)_2P_2O_7$ in anhyd DMF/tributylamine (3:1) (0.76 mL), 1% I_2 in pyridine/ H_2O (98:2) (3.8 mL). Elution concentration = 55% B; t_R = 17.5 min. The yield was determined by optical density measurement. Compound **8** (9.5 µmol, 5%), $C_{27}H_{32}N_3O_{15}P_3S$ (763.56) and **9** (32.3 µmol, 17%).

^{1}H NMR (D_2O) δ: = 7.89-7.40 (m, 9H, H-6, arom. fluorenyl), 6.07 (d, 1H, *J* = 4.7, H-1´), 4.52 (m, 2H, H-5´, H5´´), 4.31-4.16 (m, 3H, H-4´, CH_2 fluorenyl), 4.04 (s, 1H, CH fluorenyl), 3.47-3.37 (m, 1H, H-3´), 3.11-3.01 (m, 4H, N-CH_2, S-CH_2), 2.40-2.36 (m, 2H, H-2´, H-2´´), 1.75 (s, 3H, C5-CH_3).

^{13}C NMR (D_2O): δ = 166.0 (C4), 158.1 (NC(O)O), 150.9 (C2), 143.7, 141.9 (Fmoc), 136.9 (C6), 128.1, 127.4, 124.9, 120.1 (Fmoc), 111.3 (C5), 84.9, 84.4 (C1´, C4´), 66.3 (Fmoc), 65.0 (C5´), 46.9 (Fmoc), 40.7 (C3´), 40.2, 39.8 (C2´, N-CH_2), 27.8 (S-CH_2), 12.8 (C5-CH_3).

^{31}P NMR (D_2O): δ = -22.5 (t, 1P, β-P), -10.8 (d, 1P, α-P), -10.1 (d, 1P, γ-P).

MS (ESI-): *m/z* = 762.3 (M^- requires 762.5).

3´-*S*-(2-Aminoethylthio)-3´-deoxythymidine-5´-triphosphate (9) as triethylammonium salt

A solution of **8** (16 mg, 13.7 µmol) in pyridine (5 mL), DMF (3 mL) and piperidine (2 mL) was stirred for 1 h at r.t., evaporated, redissolved in H_2O (5 mL) and filtered through a 0.2 µm Nalgene syringe filter. The solution was then purified by FPLC (purification procedure 1), elution concentration = 20% B; analytical amounts of fractions containing triphosphate were further purified by anion exchange HPLC and finally desalted by reverse phase HPLC (purification procedure 2 and 3); t_R = 12.1 min. The yield was determined by optical density measurement (10.6 µmol, 77%), $C_{12}H_{22}N_3O_{13}P_3S$ (541.3).

^{1}H NMR (D_2O): δ = 7.81 (d, 1H, *J* = 1.1 Hz, H-6), 6.21 (dd, 1H, *J* = 4.4 Hz, H-1´), 4.32 (m, 2H, H-5´, H-5´´), 4.19 (m, 1H, H-4´), 3.71 (m, 1H, H-3´), 3.27 (m, 2H, N-CH_2), 3.02 (t, 2H, *J* = 6.8 Hz, S-CH_2), 2.65 (m, 1H, H-2´), 2.46 (m, 1H, H-2´´), 1.94 (d, 3H, *J* = 1.1 Hz, C5-CH_3).

^{13}C NMR (D_2O): δ = 166.5 (C4), 151.4 (C2), 137.3 (C6), 111.1 (C5), 84.7, 84.6 (C1´, C4´), 64.1 (C5´), 39.5 (C3´), 38.6, 38.3 (C2´, N-CH_2), 28.1 (S-CH_2), 11.5 (C5-CH_3).

^{31}P NMR (D_2O): δ = -21.9 (t, 1P, β-P), -10.8 (d, 1P, α-P), -7.8 (d, 1P, γ-P).

MS (ESI-): *m/z* = 540.2 (M^- requires 540.3).

Labeling of 3´-*S*-(2-Aminoethylthio)-3´-deoxythymidine-5´-triphosphate (9) with JA242, (10), isolated as lithium salt

Compound **9** (4.24 µmol) was dissolved in DMF (15 µl), dioxane (35 µl) and H_2O (15 µl). DIPEA (0.8 µl, 4.75 µmol), TSTU (1.3 mg, 4.3 µmol) and JA242 (1.56 mg, 3.5 µmol) were added and the solution was stirred under exclusion of light for 30 min at r.t., and further over night at 4 °C. The solvent was removed (Speed Vac) and the residue was dissolved in H_2O (50 µl), vortexed well and centrifuged. The supernatant was separated and purified by anion exchange HPLC (purification procedure 2), t_R = 13.0 min. The product was desalted by size exclusion gel filtration on Sephadex G10 eluting with 0.05M TEAB. UV-absorbing fractions were collected, $C_{39}H_{54}N_6O_{15}P_3S$ (971.88).

UV (H_2O): $\lambda_{abs\text{-}max1}$ = 664nm, $\lambda_{abs\text{-}max2}$ = 263nm.

Fluorescence (H_2O): $\lambda_{em\text{-}max}$ = 677nm.

MS (ESI+): *m/z* = 970.8 (M^+), $[C_{39}H_{50}N_6O_{15}P_3S]H_4$ requires 971.9;
977.2 (M^+), $[C_{39}H_{50}N_6O_{15}P_3S]H_3Li$ requires 977.8;
983.3 (M^+), $[C_{39}H_{50}N_6O_{15}P_3S]H_2Li_2$ requires 983.8;
989.3 (M^+), $C_{39}H_{50}N_6O_{15}P_3S]HLi_3$ requires 989.7;
995.2 (M^+), $[C_{39}H_{50}N_6O_{15}P_3S]$ Li_4 requires 995.6.

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