Synthesis of 3′-Thioamido-Modified 3′-Deoxythymidine 5′-Triphosphates by Regioselective Thionation and Their Use as Chain Terminators in DNA Sequencing

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Dedicated to Prof. Dr. *F. Seela* on the occasion of his 60th birthday

The thioamide derivatives 3′-deoxy-5′-*O*-(4,4′-dimethoxytrityl)-3′-[(2-methyl-1-thioxopropyl)amino]thymidine (**4a**) and 3′-deoxy-5′-*O*-(4,4′-dimethoxytrityl)-3′-{{6-{[(9*H*-(fluoren-9-ylmethoxy)carbonyl]amino}-1-thioxohexyl}amino}thymidine (**4b**) were synthesized by regioselective thionation of the corresponding amides **3a** and **3b** with 2,4-bis(4-methoxyphenyl)-1,3,2,4-dithiadiphosphetane 2,4-disulfide (*Lawesson*'s reagent). The addition of exact amounts of pyridine to the reaction mixture proved to be essential for an efficient transformation. The thioamides were converted into the corresponding 5′-triphosphates **6a** and **6b**. Compound **6a** was chosen for DNA sequencing experiments, and **6b** was further labelled with fluorescein (→**8**).

Introduction. – Recently, the first complete sequence of a human chromosome has been published [1]. Chromosome 22 is only one step in the huge Human Genome Project, which, itself, is just one of several genome-sequencing projects that are currently being carried out [2]. The *Sanger* procedure, invented in 1977, is still the method of choice to determine the sequence of bases [3][4]. Although the development of automated methods, combined with the dye-primer or dye-terminator chemistry, has greatly accelerated the speed of sequencing, the need for high-throughput and accurate DNA-sequencing techniques is still rapidly growing.

Dye-terminator sequencing is characterized by the enzymatic incorporation of chain-terminating, fluorescent nucleotides. These terminators are attached to fluorescent dyes at their base moiety [5–8]. Apart from this labelling strategy, there are other potential positions within nucleosides that may be used for markers [9]. Different groups have demonstrated that 3′-ether- and 3′-ester-functionalized nucleotides can act as substrates for an enzyme-mediated DNA synthesis [10–12]. We have shown that 3′-amino- and 3′-amido-modified nucleoside 5′-triphosphates are excellent terminators [13][14]. However, the ability of DNA polymerases to hydrolyze ester and amide bonds at the 3′-position limits the use of these 3′-dye terminators for DNA sequencing since the label is cleaved off during the incorporation process [15].

Therefore, we decided to alter the link between dye and sugar moiety to find a linkage that is stable against enzymatic degradation. In this context, we have already reported the synthesis of 3′-thioether- [16], 3′-alkyl- [17], 3′-urea-, and 3′-thiourea-modified [18] 2′,3′-dideoxynucleoside 5′-triphosphates.

Here, we describe an efficient synthesis of 3′-thioamido-modified thymidines, a new class of sugar-modified nucleosides, and the investigation of their substrate acceptance in DNA-sequencing experiments.

Results and Discussion. – The starting material 3′-azido-3′-deoxy-5′-*O*-(4,4′-dimethoxytrityl)thymidine (**1**) was obtained as described before [19][20] and was converted into the amino derivative **2** by hydrogenolytic reduction catalyzed by $PtO_2 \cdot H_2O$ (*Scheme 1*). This procedure allowed the isolation of **2** in high yield without further purification.

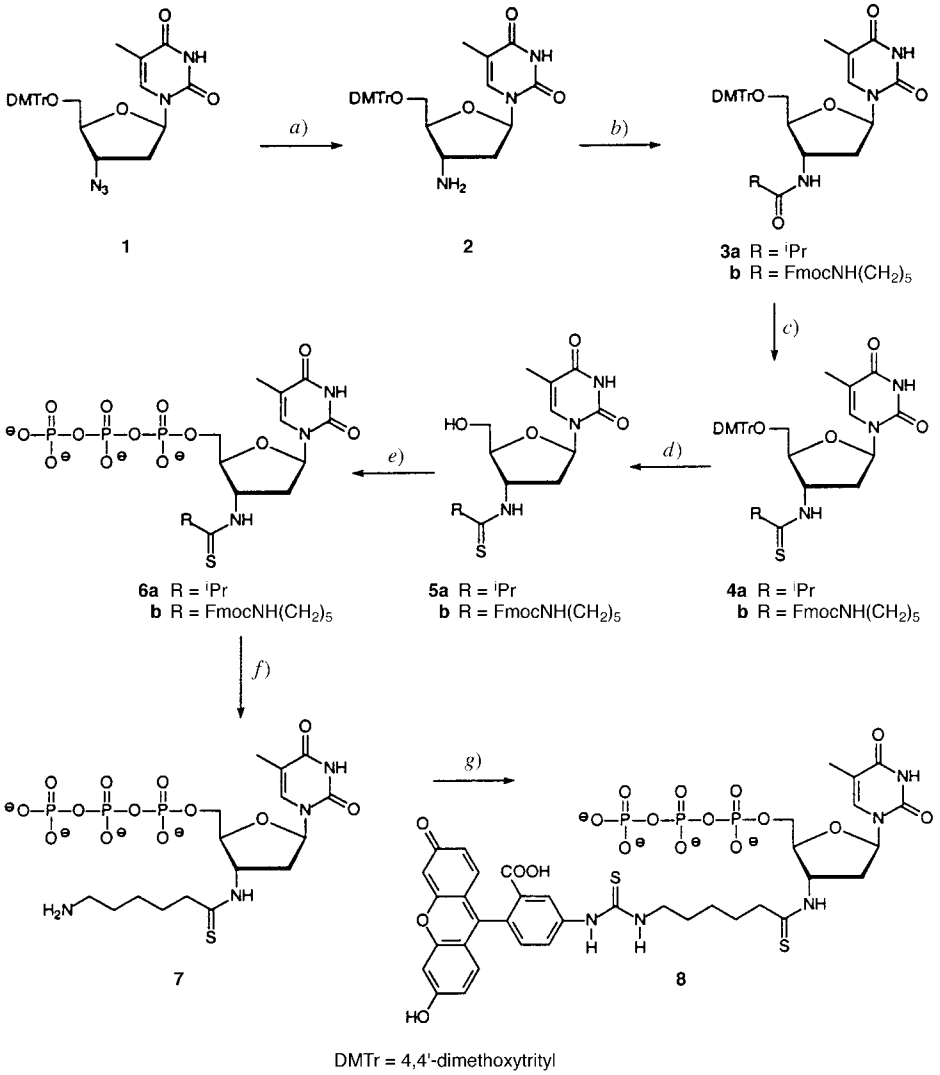
To our knowledge, no synthetic route to thioamido-tethered nucleosides has been published until now. Although a broad spectrum of reactions exists for the synthesis of thioamides [21][22], only the conversion of amides into thioamides seems to be suitable for nucleoside chemistry. Therefore, we synthesized from **2** the readily accessible amide **3a** as a model compound for the investigation of the thionation reaction. In a second synthetic approach, we chose linker **9** to introduce a free amino group for dye labelling at position 3′. This amino function had to be protected to prevent side reactions during the triphosphate synthesis. The protecting group ought to be removable under basic conditions due to the acid lability of triphosphates. Therefore, we chose the Fmoc group (*Scheme 2*), *i.e.*, the linker **10** was introduced *via* an amide coupling at the 3′-position of the sugar moiety, resulting in **3b**.

Direct action of H_2S or P_2S_5 on **3** would result in an undesired exchange of the two carbonyl O-atoms at C(2) and C(4) of the base moiety. A more sophisticated thionation reagent is 2,4-bis(4-methoxyphenyl)-1,3,2,4-dithiadiphosphetane 2,4-disulfide (*Lawesson*'s reagent), which converts carbonyl groups into thiocarbonyl groups in high yield [23–25]. Unbranched, alkyl-substituted amides can be thionated by *Lawesson*'s reagent at room temperature within minutes, whereas imides and compounds with unsaturated residues require longer reaction times and/or elevated temperatures. Thus, our aim was to utilize the different reaction conditions for a regioselective conversion at the C(3′) position. *Wörner* showed that 2′,3′,5′-tri-*O*-benzoyl-N^2-benzoylguanosine and -inosine were thionated at the nucleobase C(6)=O function when refluxed with *Lawesson*'s reagent in pyridine for 6 h [26]. Therefore, **3a** was reacted with *Lawesson*'s reagent for 30 min without heating. Humidity had to be strictly eliminated to avoid formation of phosphoric acids and thus removal of the 5′-hydroxy-protecting group. Although no by-products were observed under these reaction conditions, the yield of **4a** was rather poor (22%); 33% of the educt was recovered unchanged.

The synthesis of **4b** under the same conditions resulted in even lower yields, caused mainly by 5′-hydroxy deprotection. Therefore, we tested basic solvents as scavengers for the phosphoric acids. Thionation of **3b** (*Fig. 1*) in pure anh. pyridine instead of anh. THF did not at all indicate any formation of **4b**, even when the amount of *Lawesson*'s reagent was drastically increased. Thus, the reaction of **3b** with *Lawesson*'s reagent was accomplished in mixtures of anh. THF/pyridine. We observed that lowering the amount of pyridine gradually resulted in increased yield. Finally, the addition of 1 equiv. of pyridine and 4 equiv. of *Lawesson*'s reagent in anh. THF as solvent yielded **4b** in 50%; almost 50% of the intact educt was recovered.

Both thioamides **4** were clearly identified by mass spectrometry and NMR spectroscopy. The electrospray-ionization MS (ESI-MS) showed formation of only one

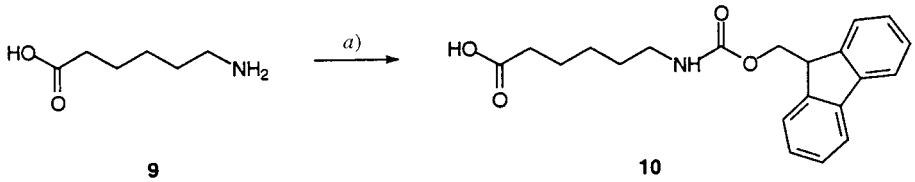
Scheme 1



a) $PtO_2 \cdot H_2O$, H_2, MeOH. *b*) For **3a**: $(^iPrCO)_2O$, pyridine; for **3b**: 6-{[(9*H*-fluoren-9-ylmethoxy)carbonyl]-amino}hexanoic acid (**10**), DMF, dioxane, H_2O, $^iPr_2NEt$, TSTU. *c*) For **4a**: *Lawesson*'s reagent, THF; for **4b**: *Lawesson*'s reagent, THF, pyridine. *d*) 80% aq. AcOH soln. *e*) 1. 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one, dioxane, pyridine, DMF; 2. $(Bu_3NH)_2P_2O_7$, DMF, tributylamine; 3. 1% I_2 in pyridine/H_2O 98 :2; 4. 5% aq. $NaHSO_3$ soln. *f*) piperidine, pyridine, DMF. *g*) FITC, DMF, aq. $NaHCO_3$ soln. (pH 9.3).

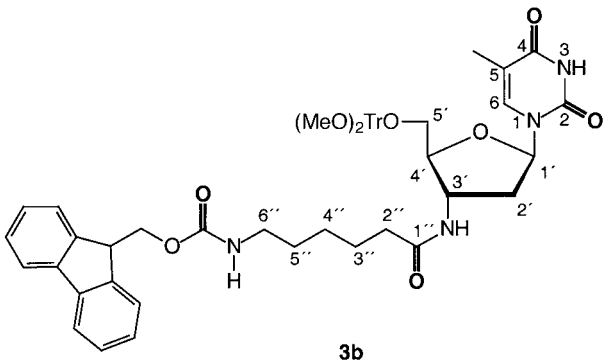
product with an increased weight of 16 Da compared with the educt, representing the exchange of an O-atom against a S-atom. The ^{13}C-NMR spectrum revealed the identity of the carbonyl group involved. The ^{13}C-chemical shifts of C(2) and C(4) of the base moiety remained unchanged, whereas the carbonyl signal at the 3′-terminal moved downfield as expected (*Table*).

Scheme 2



9 **10**

a) *N*-[(9*H*-Fluoren-9-ylmethoxy)carbonyl]succinimide, $NaHCO_3$, H_2O, acetone.



3b

Fig. 1. *5′-O-(4,4′-dimethoxytrityl)-3′-{[6-{[(9*H*-fluoren-9-ylmethoxy)carbonyl]amino}-1-oxohexyl]amino}thymidine* (**3b**)

Table. *Electrospray-Ionization MS* (ESI-MS) *and* ^{13}*C-NMR Carbonyl Signals of Compounds* **3** *and* **4**

	m/z	δ(C) [ppm] C(2)	C(4)	*C*ONH–C(6″)	*C*XNH–C(3′)
3a (X = O)	612.7[a])	150.8	164.1	–	177.4
4a (X = S)	628.3[a])	150.7	164.2	–	213.2
3b (X = O)	896.6[b])	150.8	163.9	156.5	173.2
4b (X = S)	893.6[c])	150.7	164.2	156.4	206.4

[a]) ESI (neg.). [b]) ESI (pos.) and NH_4^+ adduct, M^+ 879.0. [c]) ESI (neg.), M^- 895.1.

The thioamides **4a** and **4b** were deprotected in 80% aqueous AcOH solution at room temperature, and the syntheses of the triphosphates **6a** and **6b** were achieved according to the procedure of *Ludwig* and *Eckstein* [27]. The linker amino function of **6b** was deprotected with 20% piperidine in pyridine and DMF at room temperature (→ **7**) and subsequently coupled with fluorescein isothiocyanate (FITC) in aq. 0.1M $NaHCO_3$ (pH 9.3) and DMF at room temperature for 24 h (→ **8**).

Triphosphate **6a** was substituted for ddTTP as a terminator in DNA-sequencing experiments. No band pattern was detected with sequenase or with thermosequenase. With Taq DNA polymerase, we obtained a band pattern over a range of several hundred bases (*Fig. 2*, *Lanes 7–9*). This pattern did not correlate with the standard ddTTP sequence. It was, however, identical with the band pattern obtained with the 3′-(alkylthio)-3′-deoxythymidine 5′-triphosphate **11** (*Fig. 3*), an alternatively 3′-modified

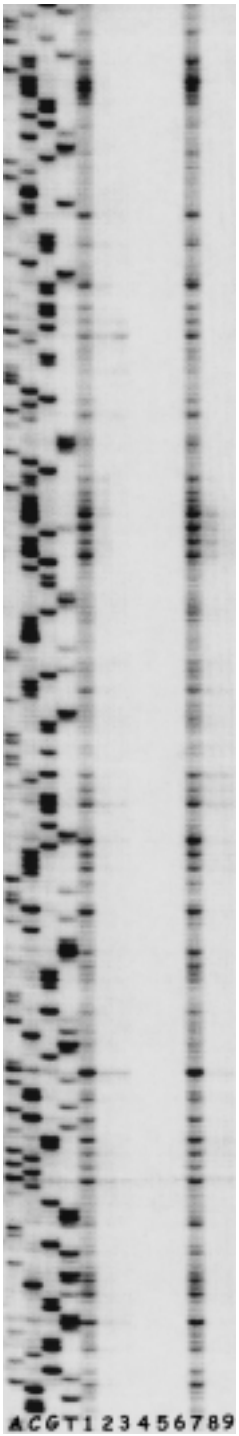
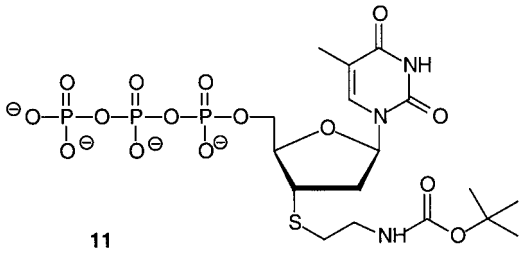


Fig. 2. *DNA Sequencing with IRD-41-labelled primer, M13 mp18 template DNA, Taq DNA Polymerase, and 2′-deoxynucleoside 5′-triphosphates* (dNTP) *as substrates.* Fluorescence detection on a *LI-COR* DNA sequencer. *Lanes A*, *C*, *G*, and *T* terminated with 2′,3′-dideoxyadenosine 5′-triphosphate (ddATP), ddCTP, ddGTP, or ddTTP, respectively; *Lanes 1–3* terminated with **11**, *lanes 7–9* terminated with **6a**. Terminator concentration: *Lanes 1* and *7*, 750 μM; *Lanes 2* and *8*, 3.75 mM; *3* and *9*, 5.25 mM.



11

Fig. 3. *3′-{[2-{[(*tert*-Butoxy)carbonyl]amino}ethyl]thio}-3′-deoxythymidine 5′-triphosphate* (**11**)

nucleotide [16] (see *Fig. 2*, *Lanes 1–3*). Although **6a** and **11** are obviously not specific terminators for the enzymatic DNA synthesis under the chosen conditions, the result cannot be called nonspecific, since two structurally different nucleotides were incorporated at exactly the same positions within the DNA. This excludes a random process. Variation of the terminator concentration only resulted in a different fragment-length distribution. The reason for this could be a conformationally driven selection process.

Conclusion. – We demonstrated that 3′-thioamido-modified nucleosides can efficiently be synthesized from 3′-amido-tethered nucleosides by a regioselective thionation strategy applying *Lawesson*'s reagent. Addition of 1 equiv. of pyridine to the reaction mixture was necessary to prevent degradation of the nucleoside and ensure good yields at the same time. Thioamide **6a** was accepted as a substrate by the Taq DNA polymerase. However, specific incorporation did not occur. Rather the band pattern showed a distinct correlation with the 3′-thioether-tethered nucleoside triphosphate **11**.

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Experimental Part

General. Solvents were of anal. grade and were dried and distilled or purchased and stored over molecular sieves. Anal. TLC: Silica gel *60 F_{254}* plates (*Merck*) for TLC. Prep. TLC: covered glass plates (gypsum silica gel *60 PF_{254}* from *Merck*), *Harrison Research 7924T Chromatotron*. FPLC (fast protein liquid chromatography): *Pharmacia* instrument with *LCC-500* controller, *P-500* pumps, and single-path UV monitor *UV-1*. HPLC: *Merck-Hitachi* with *L-4250-UV-VIS* detector and *Shimadzu RF-535* fluorescence monitor. DNA Sequencing: *LI-COR* automated DNA sequencer with M13 mp18 as template, Taq DNA polymerase, 5′-fluorescent-labelled primer, and **6a** or **11** (0.75 mM, 3.75 mM, 5.25 mM) as substitute for ddTTP. UV Spectra and optical density: *Varian Cary 1*; optical density measured at 270 nm (molar absorptivity in H_2O, 8700 l mol^{-1} cm^{-1}). IR Spectra: $\tilde{\nu}$ in cm^{-1}. ^{1}H-NMR Spectra: *Bruker AM250* (250 MHz) or *WH270* (270 MHz); δ in ppm; calibrating signals: $\delta((D_6)DMSO) = 2.50$, $\delta(CDCl_3) = 7.26$. ^{13}C-NMR Spectra: *Bruker AM250* (62.9 MHz) or *WH270* (67.9 MHz); calibrating signals: $\delta((D_6)DMSO)$ 39.5, $\delta(CDCl_3)$ 77.0. ^{31}P-NMR Spectra: *Bruker AMX 400* (162 MHz); calibrating signal: external 85% phosphoric acid. ESI-MS: *Fisons-VG-Platform-II* electrospray-ionization mass spectrometer; in *m*/*z*.

General Purification Procedures. a) FPLC: filtered (0.2-μm *Nalgene* syringe filter) crude product in H_2O was applied to a column filled with *Pharmacia-DEAE-Sephadex-A25* anion-exchange gel, 2.5 × 15 cm, 4°;

$A = 0.05$M $(Et_3NH)HCO_3$ (pH 7.5), $B = 0.5$M $(Et_3NH)HCO_3$ (pH 7.5); flow rate 1 ml/min, $0 \rightarrow 100\%$ *B*, 500 ml).

b) Anion-exchange HPLC: *Synchropak AX 300*, 6 μm (250 × 4.6 mm); $A = 0.05$M KH_2PO_4/50% formamide (pH 5.2), $B = 0.05$M KH_2PO_4/1M $(NH_4)_2SO_4$/50% formamide (pH 5.5); flow rate 1 ml/min, 0–30 min $0 \rightarrow 100\%$ *B*. HPLC retention times t_R [min] refer to anion-exchange HPLC.

c) Reversed-phase HPLC: *LiChrosphere RP-18* 5 μm (3 × 125 mm); $A = 0.1$M $(Et_3NH)OAc$ (pH 5.5), $B = A + 50\%$ MeCN; flow rate 1 ml/min, 0–10 min 5% *B*.

3′-Amino-3′-deoxy-5′-O-*(4,4′-dimethoxytrityl)thymidine* (**2**). The 3′-azido-3′-deoxy-5′-*O*-(4,4′-dimethoxytrityl)thymidine (**1**; 2.5 g, 4.4 mmol) was filled in a heat-dried flask and dissolved in anh. MeOH (30 ml) under Ar. The soln. was degassed repeatedly by alternating evacuation and flushing with Ar. A small amount (two spatula tips) of $PtO_2 \cdot H_2O$ was added to the stirred soln., and H_2 was injected for 1 h. The flask was fitted with a septum and a H_2-filled balloon and stirred overnight at 50°. The mixture was then filtered over *Celite*, the *Celite* washed with MeOH several times, and the combined filtrate evaporated: 2.21 g (93%) of pure **2**. No further purification was necessary. TLC (CH_2Cl_2/MeOH 95:5): R_f 0.18. IR (KBr): 2931, 1694, 1607, 1509, 1445, 1252, 1179, 1033, 829. ^{1}H-NMR ((D_6)DMSO): 7.58 (*d*, H–C(6)); 7.44–7.18 (*m*, 9 arom. H); 6.83 (*d*, 4 arom. H); 6.25 (*dd*, H–C(1′)); 3.78 (*s*, 2 MeO); 3.77–3.68 (*m*, H–C(4′), H–C(3′)); 3.53–3.32 (*m*, 2 H–C(5′)); 2.40–2.15 (*m*, 2 H–C(2′)); 1.52 (*s*, Me–C(5)). ESI-MS (neg.): 542.4 (M^-, $C_{31}H_{32}N_3O_6^-$; calc. 542.6).

3′-Deoxy-5′-O-*(4,4′-dimethoxytrityl)-3′-[(2-methyl-1-oxopropyl)amino]thymidine* (**3a**). A soln. of **2** (400 mg, 0.74 mmol) in anh. pyridine (20 ml) and isobutyryl anhydride (2 ml, 8.5 mmol) was stirred under Ar for 2 d and quenched with MeOH. The soln. was evaporated and the residue purified by prep. TLC (CH_2Cl_2/MeOH 98:2): 0.38 g (84%) of **3a**. TLC (CH_2Cl_2/MeOH 9:1): R_f 0.49. ^{1}H-NMR ($CDCl_3$): 7.65 (*d*, H–C(6)); 7.42–6.79 (*m*, 13 arom. H); 6.43 (*dd*, H–C(1′)); 4.70–4.65 (*m*, H–C(3′)); 4.02 (*d*, H–C(4′)); 3.78 (*s*, 2 MeO); 3.48 (*s*, 2 H–C(5′)); 2.60–2.31 (m, 2 H–C(2′), Me_2CHCO); 1.37 (*d*, Me–C(5)); 1.12 (*d*, Me_2CHCO). ^{13}C-NMR ($CDCl_3$): 177.4 (*C*ONH–C(3′)); 164.1 (C(4)); 158.7 ($(MeO)_2$*Tr*); 150.8 (C(2)); 144.4 ($(MeO)_2$*Tr*); 135.7, 135.4 (C(6), $(MeO)_2$*Tr*); 130.1, 128.2, 127.9, 127.1, 113.3 ($(MeO)_2$*Tr*); 111.5 (C(5)); 87.0, 85.4, 84.6 (C(1′), C(4′), $(MeO)_2$*Tr* (C); 64.1 (C(5′)); 55.2 (MeO); 51.0 (Me_2CHCO); 38.0, 35.1 (C(3′), C(2′)); 19.5, 18.8 (Me_2CHCO); 11.6 (Me–C(5)). ESI-MS (neg.): 612.7 (M^-, $C_{35}H_{38}N_3O_7^-$; calc. 612.7).

3′-Deoxy-5′-O-*(4,4′-dimethoxytrityl)-3′-[(2-methyl-1-thioxopropyl)amino]thymidine* (**4a**). To a soln. of **3a** (200 mg, 0.33 mmol) in anh. THF (10 ml), *Lawesson*'s reagent (66 mg, 0.16 mmol) was added. After stirring at r.t. for 30 min, sat. $NaHCO_3$ soln. (20 ml) was added. The resulting white precipitate was filtered and washed with H_2O. The aq. phase was extracted with CH_2Cl_2 twice, the combined org. layer dried (Na_2SO_4) and evaporated, and the residual solid purified by prep. TLC (CH_2Cl_2/MeOH 98:2): 45 mg (22%) of **4a**. TLC (CH_2Cl_2/MeOH 9:1): R_f 0.54. ^{1}H-NMR ($CDCl_3$): 7.76 (*d*, H–C(6)); 7.46–6.83 (*m*, 13 arom. H); 6.63 (*t*, H–C(1′)); 5.31 (*m*, H–C(3′)); 4.28 (*d*, H–C(4′)); 3.91 (*d*, 1 H–C(5′)); 3.79 (*s*, 2 MeO); 3.42 (*d*, 1 H–C(5′)); 3.05–2.97 (*m*, Me_2CHCS); 2.53–2.48 (*m*, 2 H–C(2′)); 1.42 (*d*, Me–C(5)); 1.26 (*d*, 3 H, Me_2CHCS); 1.23 (*d*, 3 H, Me_2CHCS). ^{13}C-NMR ($CDCl_3$): 213.2 (*C*SNH–C(3′)); 164.2 (C(4)); 158.7 ($(MeO)_2$*Tr*); 150.7 (C(2)); 144.6 ($(MeO)_2$*Tr*); 135.7, 135.5 (C(6), $(MeO)_2$*Tr*); 130.1, 128.2, 128.0, 127.1, 113.3 ($(MeO)_2$*Tr*); 111.7 (C(5)); 87.2, 85.0 (C(1′), C(4′)); 65.5 (C(5′)); 57.0 (Me_2CHCS); 55.3 (MeO); 42.9 (C(2′)); 37.0 (C(3′)); 23.1, 22.6 (Me_2CHCS); 11.7 (*Me*–C(5)). ESI-MS (neg.): 628.3 (M^-, $C_{35}H_{38}N_3O_6S^-$; calc. 628.8).

3′-Deoxy-3′-[(2-methyl-1-thioxopropyl)amino]thymidine (**5a**). A soln. of **4a** (235 mg, 0.373 mmol) in 80% AcOH/H_2O (20 ml) was left stirring for 2 h. About 90% of the solvent was evaporated and the resulting oil co-evaporated (3×) with H_2O to remove residual acid. The residue was purified by prep. TLC (CH_2Cl_2/MeOH 9:1): 113 mg (93%). TLC (CH_2Cl_2/MeOH 8:2): R_f 0.53. ^{1}H-NMR ((D_6)DMSO): 11.28 (*s*, H–N(3)); 10.24 (*d*, CSNH–C(3′)); 7.83 (*d*, H–C(6)); 6.30 (*t*, H–C(1′)); 5.15 (*t*, OH–C(5′)); 4.92–4.87 (*m*, H–C(3′)); 3.95 (*q*, H–C(4′)); 3.69 (*m*, 2 H–C(5′)); 2.89 (*sept.*, Me_2CHCS); 2.39–2.28 (*m*, 1 H–C(2′)); 2.20 (*ddd*, 1 H–C(2′)); 1.78 (*s*, Me–C(5)); 1.12 (*d*, Me_2CHCS). ^{13}C-NMR ((D_6)DMSO): 210.7 (*C*SNH–C(3′)); 163.7 (C(4)); 150.5 (C(2)); 136.0 (C(6)); 109.5 (C(5)); 85.0, 83.9 (C(1′), C(4′)); 62.2 (C(5′)); 55.6 (Me_2CHCS); 41.2 (C(2′)); 36.0 (C(3′)); 22.9, 22.6 (Me_2CHCS); 12.4 (Me–C(5)). ESI-MS (neg.): 326.3 (M^-, $C_{14}H_{20}N_3O_4S^-$; calc. 326.4).

3′-Deoxy-3′-[(2-methyl-1-thioxopropyl)amino]thymidine 5′-Triphosphate (**6a**). Compound **5a** (110 mg, 0.336 mmol) was dried by co-evaporation (3×) with anh. pyridine (0.7 ml)/DMF (2.75 ml). The residue was dried over P_2O_5 under vacuum overnight. The nucleoside was dissolved in anh. pyridine (0.7 ml)/DMF (2.75 ml), a freshly prepared soln. of 1M 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one in anh. dioxane (0.37 ml) was injected under Ar, and the mixture stirred for 20 min. Then a freshly prepared mixture (1.35 ml) of 0.5M $(Bu_3NH)_2P_2O_7$ in dry DMF/anh. Bu_3N 3:1 was instantly added. The mixture was stirred for further 30 min. Oxidation of the P-moiety and ring opening was achieved by adding 6.7 ml of 1% I_2 in pyridine/H_2O 98:2. After 20 min, remaining I_2 was reduced by adding dropwise a 5% aq. $NaHSO_3$ soln. until the brown color changed to

light yellow. After evaporation without heating, H_2O (5 ml) was poured onto the residue, and the soln. containing the dissolved product was separated from the remaining residue by filtering through a 0.2 μm *Nalgene* syringe filter. The soln. was evaporated without heating, the residual solid dissolved in H_2O (5 ml), and this soln. filtered through a 0.2 μm *Nalgene* syringe filter and purified by FPLC (purification procedure *a*), elution concentration 0.26M $(Et_3NH)HCO_3$). The fractions containing product were lyophilized. The yield of **6a** was determined by optical-density measurement (32.9 μmol, 10%). TLC (^{i}PrOH/NH_3/H_2O): R_f 0.08. ^{31}P-NMR (D_2O): −23.3 (*t*, (*β*)); −11.8 (*d*, P(*α*)); −9.6 (*d*, P(*γ*)).

*6-{[(9*H*-Fluoren-9-ylmethoxy)carbonyl]amino}hexanoic Acid* (**10**). *N*-[(9*H*-Fluoren-9-ylmethoxy)carbonyl]succinimide (5 g, 14.82 mmol) and $NaHCO_3$ (1.24 g, 14.82 mmol) were suspended in a soln. of 6-aminohexanoic acid (**9**) (1.95 g, 14.82 mmol) in H_2O/acetone 1:1 (100 ml). The mixture was stirred for 3 h (TLC monitoring). Then the acetone was evaporated, and CH_2Cl_2 was added. The org. phase was washed with 0.1N HCl and H_2O, then dried (Na_2SO_4), and evaporated. Crystallization of the residue from CH_2Cl_2/hexane afforded **10** (5.2 g, 99%). White solid. M.p. 117–120°. TLC (CH_2Cl_2/MeOH 9:1): R_f 0.13. ^{1}H-NMR ($CDCl_3$): 7.75–7.78 (*d*, 2 H, Fmoc); 7.58–7.61 (*d*, 2 H, Fmoc); 7.26–7.43 (*m*, 4 H, Fmoc); 4.80 (br., NH); 4.41–4.43 (*d*, C*H*$_2$OCONH); 4.20–4.24 (*m*, H–C(9) (Fmoc)); 3.07–3.21 (*m*, CH_2(6)); 2.33–2.39 (*t*, CH_2(2)); 1.37–1.66 (*m*, CH_2(3), CH_2(4), CH_2(5)). ^{13}C-NMR ($CDCl_3$): 178.8 (C(1)); 156.34 (CH_2O*C*ONH); 143.8, 141.2, 127.5, 126.9, 124.9, 119.8 (C(Fmoc)); 66.4 (*C*H_2OCONH); 47.1 (C(9) (Fmoc)); 40.6 (C(2)); 33.6 (C(6)); 29.4 (C(5)); 25.9 (C(3)); 24.1 (C(4)). ESI-MS (pos.): 354.1 (M^+, $C_{21}H_{24}NO_4^+$; calc. 354.3).

*3′-Deoxy-5′-*O*-(4,4′-dimethoxytrityl)-3′-{[6-{[(9*H*-fluoren-9-ylmethoxy)carbonyl]amino}-1-oxohexyl]amino}-thymidine* (**3b**). To a soln. of **10** (636 mg, 1.8 mmol) and **2** (1.17 g, 2.15 mmol) in DMF (5 ml), dioxane (15 ml), and H_2O (5 ml), ^{i}Pr$_2$NEt (0.4 ml, 2.4 mmol) and *N*,*N*,*N*′,*N*′-tetramethyl-*O*-succinimidouronium tetrafluoroborate (TSTU; 845 mg, 2.8 mmol) were added. After stirring at r.t. for 30 min (TLC monitoring), H_2O was added. The aq. phase was extracted with CH_2Cl_2 (4×), the combined org. layer dried ($MgSO_4$) and evaporated, and the residue purified by prep. TLC (CH_2Cl_2/MeOH 98:2): 585 mg (37%) of **3b**. TLC (CH_2Cl_2/MeOH 9:1): R_f 0.38. ^{1}H-NMR ($CDCl_3$): 9.90 (br., 1 NH); 7.99 (*s*, H–C(6)); 7.71–7.73 (*d*, 2 H, Fmoc); 6.78–7.61 (*m*, 19 H, $(MeO)_2$*Tr*, Fmoc); 6.36 (*t*, H–C(1′)); 5.08 (br., CH_2OCON*H*); 4.70–4.71 (*m*, H–C(3′)); 4.34–4.36 (*m*, C*H*$_2$OCONH); 4.20–4.22 (*m*, H–C(9) (Fmoc)); 4.00–4.01 (*m*, H–C(4′)); 3.79 (*s*, 2 MeO); 3.42–3.43 (*m*, 2 H–C(5′)); 3.12–3.14 (*m*, CH_2(6″)); 2.15–2.45 (*m*, 2 H–C(2′), CH_2(2″)); 1.25–1.62 (*m*, CH_2(3″), CH_2(4″), CH_2(5″)); 1.37 (*s*, Me–C(5)). ^{13}C-NMR ($CDCl_3$): 173.2 (CONH–C(3′)); 163.9 (C(4)); 158.6 ($(MeO)_2$*Tr*); 156.5 (CH_2O*C*ONH); 150.8 (C(2)); 144.3 ($(MeO)_2$*Tr*); 143.9, 141.2 (C(Fmoc)); 135.6, 135.4 (C(6), $(MeO)_2$*Tr*); 130.1, 128.2, 127.9, 127.0 ($(MeO)_2$*Tr*); 127.6, 126.9, 124.9, 119.9 (Fmoc)); 113.2 ($(MeO)_2$*Tr*); 111.4 (C(5)); 87.0, 85.0, 84.4 (C(1′), C(4′), $(MeO)_2$*Tr*(C)); 66.5 (*C*H_2OCONH); 64.1 (C(5′)); 55.2 (MeO); 47.2 (C(9) (Fmoc)); 40.7 (C(2″)); 38.0, 35.9 (C(3′), C(2′)); 31.4, 29.5, 26.2, 25.0 (C(6″), C(5″), C(4″), C(3″)); 11.6 (*Me*–C(5)). ESI-MS (pos.): 896.6 (M^+, $C_{52}H_{54}N_4O_9 \cdot NH_4^+$; calc. 897.0).

*3′-Deoxy-5′-*O*-(4,4′-dimethoxytrityl)-3′-{[6-{[(9*H*-fluoren-9-ylmethoxy)carbonyl]amino}-1-thioxohexyl}-amino}thymidine* (**4b**). To a soln. of **3b** (1 g, 1.14 mmol) in anh. THF (70 ml) and anh. pyridine (92 μl, 1.14 mmol), *Lawesson*'s reagent (1.84 g, 4.56 ml) was added. After stirring at r.t. for 3 h, sat. $NaHCO_3$ soln. (100 ml) was added. The aq. phase was then extracted with CH_2Cl_2 (2×), the combined org. layer dried ($MgSO_4$) and evaporated, and the residue purified by prep. TLC (CH_2Cl_2/MeOH 98:2): 510 mg (50%) of **4b**. TLC (CH_2Cl_2/MeOH 9:1): R_f 0.44. ^{1}H-NMR ($CDCl_3$): 10.19 (br., 1 NH); 9.22 (br., 1 NH); 6.72–7.67 (*m*, 22 H, H–C(6), $(MeO)_2$*Tr*, Fmoc); 6.49 (*t*, H–C(1′)); 5.42 (*m*, CH_2OCON*H*); 4.83 (*m*, H–C(3′)); 4.27–4.30 (*m*, C*H*$_2$OCONH); 4.21 (*m*, H–C(9) (Fmoc)); 4.10 (*m*, H–C(4′)), 3.70–3.78 (*m*, 1 H–C(5′)); 3.68 (*s*, 2 MeO); 3.31–3.35 (*m*, 1 H–C(5′)); 3.06–3.09 (*m*, CH_2(6″)); 2.61–2.66 (*m*, 2 H–C(2′)); 2.27–2,42 (*m*, CH_2(2″)); 1.27–1.96 (*m*, CH_2(3″), CH_2(4″), CH_2(5″)); 1.35 (*s*, Me–C(5)). ^{13}C-NMR ($CDCl_3$): 206.4 (*C*SNH–C(3)); 164.2 (C(4)); 158.4 ($(MeO)_2$*Tr*); 156.4 (CH_2O*C*ONH); 150.7 (C(2)); 144.6 ($(MeO)_2$*Tr*); 143.9, 141.3 (Fmoc); 135.6, 135.4 (C(6), $(MeO)_2$*Tr*); 130.1, 128.1, 127.9, 127.05 ($(MeO)_2$*Tr*); 127.6, 126.9, 125.0, 119.9 (Fmoc); 113.3 ($(MeO)_2$*Tr*); 111.5 (C(5)); 87.1, 85.8, 85.0 (C(1′), C(4′), $(MeO)_2$*Tr*(C)); 66.5 (*C*H_2OCONH); 65.3 (C(5′)); 55.3 (MeO); 47.22 (C(9) (Fmoc)); 45.4 (C(2′)); 40.7 (C(2″)); 38.6, 37.4 (C(3′), C(6″)); 29.5, 28.9, 25.8 (C(5″), C(3″), C(4″)); 11.7 (*Me*–C(5)). ESI-MS (neg.): 893.6 (M^-, $C_{52}H_{53}N_4O_8S^-$; calc. 894.1).

*3′-Deoxy-3′-{[6-{[(9*H*-fluoren-9-ylmethoxy)carbonyl]amino}-1-thioxohexyl}amino}thymidine* (**5b**). To a soln. of **4b** (140 mg, 0.156 mmol) in CH_2Cl_2 (2 ml), 80% AcOH/H_2O (10 ml) was added. The mixture was stirred at r.t. for 3 h (TLC monitoring) and then evaporated. The resulting oil was co-evaporated with H_2O (3×) and hexane (1×) to remove the residual acid before purifying. The residue was purified by prep. TLC (CH_2Cl_2/MeOH 9:1): 68 mg (74%) of **5b**. TLC (CH_2Cl_2/MeOH 9:1): R_f 0.37. ^{1}H-NMR ($CDCl_3$): 10.25 (*s*, 1 NH); 9.17 (*d*, NH–C(3′)); 7.63–7.69 (*m*, 3 H, H–C(6), Fmoc); 7.47 (*d*, 2 H, Fmoc); 7.15–7.31 (*m*, 4 H, Fmoc); 6.23 (*t*, H–C(1′)); 5.15 (br., OH–C(5′)); 4.92 (*m*, H–C(3′)); 4.26 (*m*, C*H*$_2$OCONH); 4.05–4.11 (*m*, H–C(9) (Fmoc));

3.97 (*m*, H–C(4′)); 3.84 (*m*, 2 H–C(5′)); 3.02–3.04 (*m*, CH_2(6″)); 2.55–2.58 (*m*, CH_2(2″)); 2.18–2.38 (*m*, 2 H–C(2′)); 1.78 (*s*, Me–C(5)); 1.15–1.68 (*m*, CH_2(3″), CH_2(4″), CH_2(5″)). ^{13}C-NMR ($CDCl_3$): 206.4 (*C*SNH–C(3′)); 164.3 (C(4)); 156.6 (CH_2OCONH); 150.8 (C(2)); 143.7, 141.1 (Fmoc); 136.0 (C(6)); 127.6, 126.9, 124.8, 119.9 (Fmoc); 111.0 (C(5)); 85.6, 84.7 (C(1′), C(4′)); 66.6 (*C*H_2OCONH); 62.4 (C(5′)); 47.0 (C(9) (Fmoc)); 45.6 (C(2′)); 40.6 (C(2″)); 36.9 (C(3′)); 29.0, 25.6 (C(5″), C(3″), C(4″)); 12.5 (*Me*–C(5)). ESI-MS (neg.): 591.5 (M^-, $C_{31}H_{35}N_4O_6S^-$; calc. 591.6).

*3′-Deoxy-3′-{{6-{[(9*H*-fluoren-9-ylmethoxy)carbonyl]amino}-1-thioxohexyl}amino}thymidine 5′-Triphosphate* (**6b**). As described for **6a**, from **5b** (150 mg, 0.253 mmol), after co-evaporation with anh. pyridine (0.5 ml) and dissolution in anh. pyridine (0.5 ml)/DMF (2.55 ml), with 1M 2-chloro-4*H*-1,2,3-benzodioxaphosphorin-4-one in anh. dioxane (0.3 ml), 0.5M $(Bu_3NH)_2P_2O_5$ in dry DMF/Bu_3N (3:1 (1.07 ml), 5.4 ml of 1% I_2 in pyridine/H_2O 98:2), and FPLC (elution concentration 0.28M (Et_3NH) HCO_3): **6b** (48 μmol, 19%). TLC (^{i}PrOH/NH_3/H_2O): R_f 0.15. ^{31}P-NMR (D_2O): –21.8 (*t*, P(*β*)); –10.8 (*d*, P(*α*)); –5.7 (*d*, P(*γ*)). ESI-MS (neg.): 831.5 (M^-, $C_{31}H_{38}N_4O_{15}P_3S^-$; calc. 831.7).

3′-[(6-Amino-1-thioxohexyl)amino]-3′-deoxythymidine 5′-Triphosphate (**7**). To a mixture of pyridine (20 ml), DMF (12 ml), and piperidine (8 ml), **6b** (48 μmol) was added. This soln. was stirred at r.t. for 1 h and then evaporated without heating. The residual solid was dissolved in H_2O (4 ml), filtered through a 0.2-μm *Nalgene* syringe filter, and purified by FPLC (purification procedure *a*); elution concentration 0.14M (Et_3NH)HCO_3). The fractions containing product were lyophilized. The yield of **7** was determined by optical-density measurement (39 μmol, 81%). TLC (^{i}PrOH/NH_3/H_2O): R_f 0.13.

^{1}H-NMR (D_2O): 7.86 (*s*, H–C(6)); 6.26 (*t*, H–C(1′)); 4.95–5.00 (*m*, H–C(3′)); 4.46 (*m*, H–C(4′)); 4.18–4.28 (*m*, 2 H–C(5′)); 2.97–3.15 (*m*, CH_2(6″)); 2.66–2.74 (*m*, CH_2(2″), 1 H–C(2′)); 2.40–2.47 (*m*, 1 H–C(2′)); 1.91 (*s*, Me–C(5)); 1.33–1.84 (m, CH_2(3″), CH_2(4″), CH_2(5″)). ^{13}C-NMR (D_2O): 207.0 (*C*SNH–C(3′)); 166.5 (C(4)); 151.4 (C(2)); 137.6 (C(6)); 111.6 (C(5)); 85.3, 81.4 (C(1′), C(4′)); 66.06 (C(5′)); 42.54 (C(2′)); 39.1 (C(2″)); 35.5 (C(3′)); 28.1, 26.4, 24.3 (C(3″), C(4″), C(5″)); 11.5 (Me–C(5)). ^{31}P-NMR (D_2O): –22.0 (*t*, P(*β*)); –10.6 (*d*, P(*α*)); –8.2 (*d*, P(*γ*)). HPLC (purification procedures *b* and *c*): t_R 4.8. ESI-MS (neg.): 609.2 (M^-, $C_{11}H_{33}N_4O_{13}P_3S^-$; calc. 609.4).

Reaction of **7** *with Fluorescein Isothiocyanate: 3′-{[6-{{{[3-Carboxy-4-(6-hydroxy-3-oxo-3*H*-xanthen-9-yl)phenyl]amino}thioxomethyl}amino}-1-thioxohexyl]amino}-3′-deoxythymidine 5′-Triphosphate* (**8**). To a soln. of **7** (10 μmol) in aq. $NaHCO_3$ soln. (6 ml, pH 9.3), a soln. of fluorescein isothiocyanate (12 mg) in DMF (6 ml) was added. The mixture was stirred for 24 h at r.t. The purification was done by HPLC (purification procedures *b* and *c*): **8**: HPLC: t_R (**8**) 19.5; t_R (fluorescein isothiocyanate) 12.5. ESI-MS (neg.): 998.3 (M^-, $C_{37}H_{39}N_5O_{18}P_3S_2^-$; calc. 998.8).

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