Synthesis of 3′-Sugar- and Base-Modified Nucleotides and Their Application as Potent Chain Terminators in DNA Sequencing

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Dedicated to Prof. *Karl Heinz Drexhage* on the occasion of his 65th birthday

Two 3′-modified and three base-modified ddNTPs were synthesized and tested with several DNA polymerases for incorporation activity. Starting from 3′-azido-3′-deoxythymidine (AZT; **1**), we were able to produce 3′-deoxy-3′-isocyanato-thymidine and 3′-deoxy-3′-isothiocyanatothymidine (**3** and **4**, resp.) in a rapid synthesis based on the solid-support approach (*Scheme 1*). These 3′-functionalities could be used to attach a spacer molecule *via* urea and thiourea groups, respectively. Since the thus-obtained tethered nucleotides **7** and **8** can be used to label with fluorescent dyes (*cf. Scheme 5*), they are convenient building blocks for practical applications in DNA sequencing. Furthermore, we synthesized, *via* **14** (*Scheme 2*), **17** (*Scheme 3*), and **19** (*Scheme 4*), the N^4-modified dideoxycytidine 5′-triphosphate dye derivatives **22**, **23**, and **24**, respectively, with different lengths of linkers between the base residue and the dye (*Scheme 5*). Base-specific termination for the derivatives **22** and **24** was demonstrated (*Fig. 2a* and *2b*).

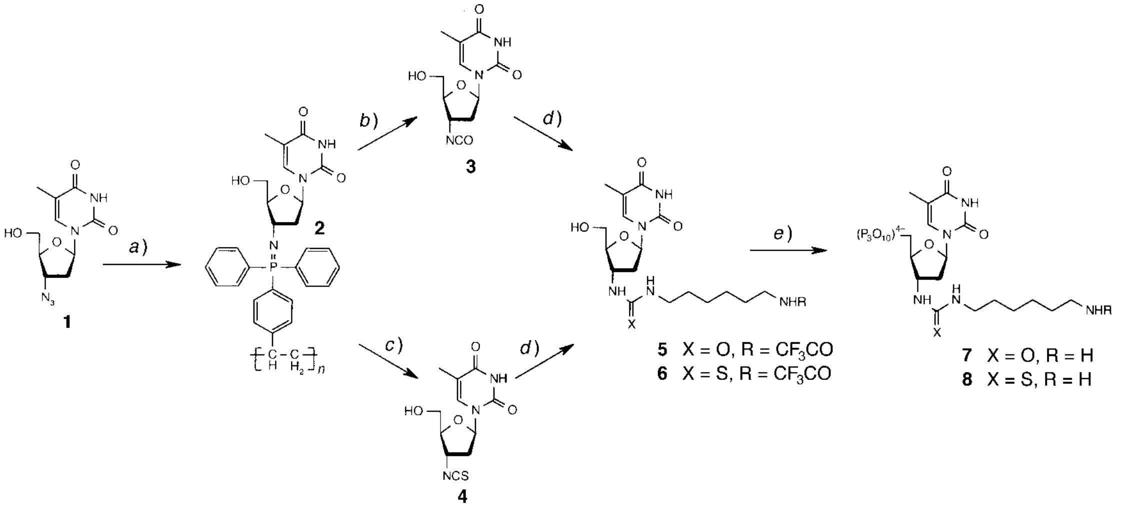
1. Introduction. – The Human Genome Project has greatly contributed to the need for high-throughput DNA sequencing. Radioactive methods have largely been replaced by automated non-isotopic systems using laser-induced fluorescence. Automated DNA sequencing refers to a variation of traditional *Sanger* sequencing [1]. Commercial systems use the dye-primer or dye-terminator approach. Several companies (*Applied Biosystems Inc.* [2][3], *Du Pont de Nemours & Co.* [4], *Pharmacia LKB Biotechnology Inc.* [5], and *Hitachi* [6]) have produced automated DNA sequencers based on one of the two methods mentioned above. Usually, dye-primer sequencing affords four separate extension/termination reactions where each reaction contains four deoxynucleoside triphosphates (dNTPs) and one dideoxynucleoside triphosphate (ddNTP). In the approach of *Applied Biosystems Inc.*, either four separate dye primers corresponding to each ddNTP or four differently labeled dye terminators [7] are used. These dyes are detected and distinguished by analysis of their fluorescence emission spectra. Reading of four dyes in one lane requires fluorophores with different emission maxima resulting from different structures.

Dye-terminator sequencing is characterized by exclusive detection of correctly terminated DNA fragments. There are three different approaches to link a marker to the nucleotide: either at the sugar residue, or at the base moiety, or at the α-phosphate group. To further increase the speed and efficiency of these systems, we intended to use 3′-sugar- as well as base-modified nucleotides linked to a fluorescent chromophore as chain terminators.

2. Results and Discussion. – 2.1. *Synthesis of 3′-Sugar- and Base-Modified Nucleotides.* The 2′-deoxyribonucleoside 5′-triphosphates (dNTPs) modified at their 3′-hydroxy position can act as terminators of enzyme-directed DNA synthesis [8]. The 3′-amino-2′,3′-dideoxynucleoside 5′-triphosphates are well accepted as substrates by commonly used DNA polymerases [9]. However, linking of the dye *via* a 3′-(carbonylamino) bond (carboxamide) is not suitable for most sequencing enzymes because of their ability to hydrolyze these functions [10]. To obviate this property, we intended to create chain terminators that allow dye labeling by modification of the 3′-position of the sugar residue *via* new types of coupling strategies. Thus, we synthesized 3′-modified chain terminators in the thymidine series starting from 3′-azido-3′-deoxythymidine (AZT; **1**) (*Scheme 1*). Usually polymer-bound triphenylphosphine is suitable for the reduction of azido-nucleosides into the corresponding amino-nucleosides [11][12]. In this case, the generated phosphine imide is hydrolyzed by concentrated aqueous ammonia. In a modified *Wittig* reaction, the phosphine imide bond can also be cleaved by other nucleophilic reagents. For the synthesis of the thymidine linker conjugates, AZT (**1**) was immobilized on polymer-bound triphenylphosphine suspended in dioxane. The intermediate **2** was then treated either by passing a stream of dry CO_2 through the suspension or by addition of CS_2 according to the method of *Molina et al.* [13] and *Zehl* and *Cech* [14]. The recovered 3′-deoxy-3′-isocyanato- and 3′-deoxy-3′-isothiocyanatothymidines (**3** and **4**, resp.), respectively, were either isolated without by-products or immediately reacted with a monoprotected hexane-1,6-diamine spacer to yield the urea or thiourea conjugates **5** and **6**, which were transformed to the 5′-triphosphates **7** and **8**, respectively.

Template-dependent DNA polymerases accept as substrates various 2′-deoxynucleoside 5′-triphosphates with modified base residues [15–18]. As an alternative to the above-mentioned sugar-labeling methods, we examined a concept where labeling in the cytidine series is achieved by modification of the exocyclic amino group. There are different approaches to introduce spacers to the NH_2-group at C(4), which start from either uridine or cytidine precursors: treatment of 4-*N*-(arylsulfonyl)cytosine residues [19] or 5′-*O*-(dimethoxytrityl)-4-*O*-(2,6-dimethylphenyl)thymidine [20] with primary amines [21] led to 4-*N*-(aminoalkyl)-substituted cytosine derivatives. Reaction of cytosines with sodium hydrogen sulfite resulted in 5,6-dihydrocytosine-6-sulfonate [22], which could readily be substituted at $NH_2-C(4)$ with a primary amine [23][24]. Treatment of cytidines with 1-(phenoxycarbonyl)-1*H*-tetrazole resulted in conversion of the exocyclic NH_2 group to the corresponding urethane, which could be used to introduce an amino linker under formation of an urea bond [25]. Starting from uracil

Scheme 1. *Synthesis of 3′-Sugar-Modified 3′-Deoxythymidine 5′-Triphosphates* **7** *and* **8**



a) Polymer-bound triphenylphosphine, dioxane. *b*) CO_2, dioxane. *c*) CS_2, dioxane. *d*) N^1-(Trifluoracetyl)-protected hexane-1,6-diamine trifluoroacetate, $(i\text{-}Pr)_2EtN$, pyridine. *e*) 1. 2-Chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one, bis(tributylammonium) pyrophosphate, Bu_3N, I_2, pyridine, dioxane, DMF; 2. Conc. NH_3 soln.

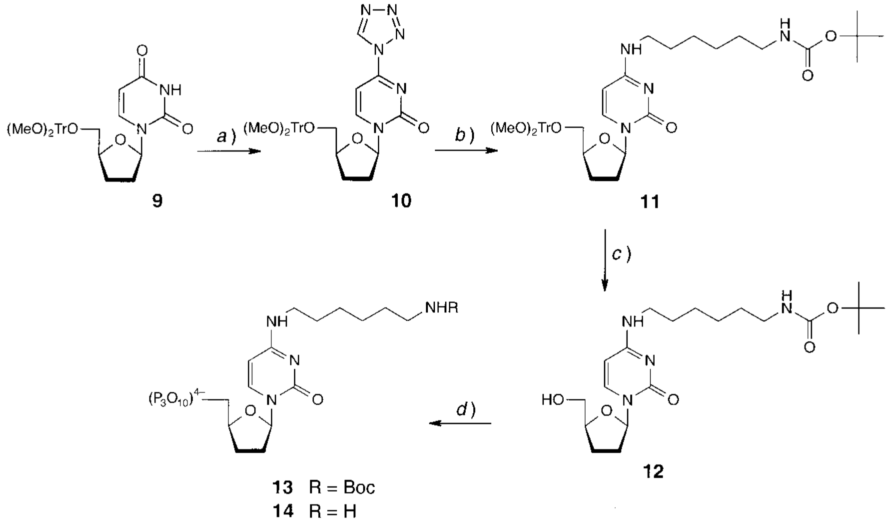
derivatives, the C(4) position could be activated with 1*H*-1,2,4-triazole as well as with phosphoroxychloride [26] or 4-chlorophenyl phosphorodichloridate [27].

For the modification of the exocyclic NH_2 group in the cytidine series, with a substituted hexane spacer, we used a modified *Sung* [28] procedure (*Scheme 2*). Starting from 2′,3′-dideoxyuridine, selective protection of the 5′-OH group was carried out with dimethoxytrityl chloride in pyridine (→**9**). Treatment of compound **9** with 1*H*-1,2,3,4-tetrazole and 2-chlorophenyl phosphorodichloridate gave the 4-*N*-deamino-4-*C*-tetrazoylcytidine derivative **10** which was not isolated but treated immediately with a monoprotected hexane-1,6-diamine to give 4-*N*-{6-{[(*tert*-butoxy)carbonyl]amino}-hexyl}-2′,3′-dideoxy-5′-*O*-(dimethoxytrityl)cytidine (**11**). Subsequently, removal of the dimethoxytrityl group was carried out in 80% AcOH/H_2O for 45 min. Purification of the crude product gave N^4-{6-{[(*tert*-butoxy)carbonyl]amino}hexyl}-2′,3′-dideoxycytidine (**12**) in 35% overall yield, which was transformed to the 5′-triphosphates **13** and **14**.

Introduction of the short spacer arm in the cytidine series was performed by heating 2′,3′-dideoxycytidine and monoprotected propane-1,3-diamine in a hydrogen-sulfite-containing solution in water at 55° for 5 h to give 4-*N*-{3-{[(*tert*-butoxy)carbonyl]amino}propyl}-2′,3′-dideoxycytidine (**15**), which was transformed to the 5′-triphosphates **16** and **17** (*Scheme 3*).

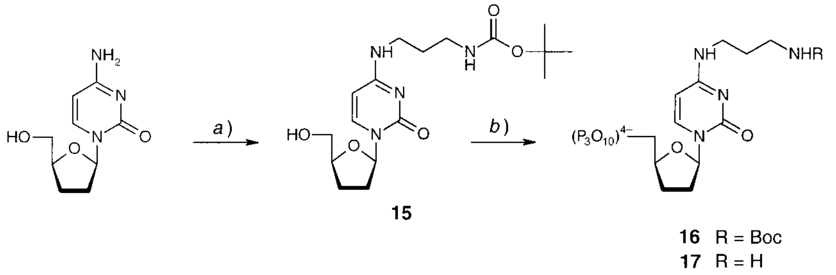
The target nucleosides **5**, and **6** (*Scheme 1*), **12** (*Scheme 2*), and **15** (*Scheme 3*) could be triphosphorylated at the 5′-hydroxy group according to the method of *Ludwig* and *Eckstein* [29]. All triphosphates were purified by ion-exchange chromatography. The purity of the products was established by HPLC and ^1H, ^{13}C-, and ^{31}P-NMR spectroscopy. Removal of the (*tert*-butoxy)carbonyl group from triphosphates **13** and **16** with CF_3COOH in 2 min did not affect the *N*-glycosidic bond in the cytidine

Scheme 2. *Synthesis of* N^4*-(6-Aminohexyl)-2′,3′-dideoxycytidine 5′-Triphosphate* (**14**)



a) 1*H*-1,2,3,4-Tetrazole, 2-chlorophenyl phosphorodichloridate, pyridine. *b*) *N*^{1}-[(*tert*-Butoxy)carbonyl]-protected hexane-1,6-diamine, dioxane. *c*) 80% AcOH/H_2O. *d*) 1. 2-Chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one, bis(tributylammonium) pyrophosphate, Bu_3N, I_2, pyridine, dioxane, DMF; 2. CF_3COOH

Scheme 3. *Synthesis of* N^4*-(6-Aminopropyl)-2′,3′-dideoxycytidine 5′-Triphosphate* (**17**)

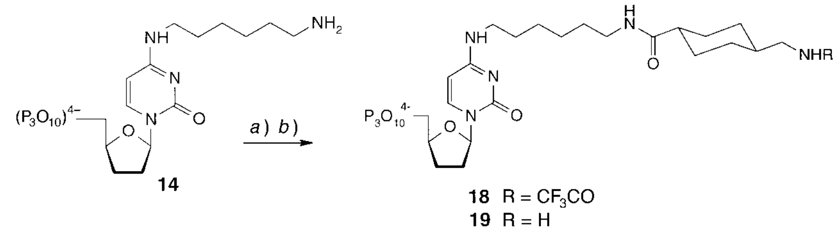


a) NaOH, $NaHSO_3$, *N*^{1}-[(*tert*-butoxy)carbonyl]-protected propane-1,3-diamine, H_2O. *b*) 1. 2-Chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one, bis(tributylammonium) pyrophosphate, Bu_3N, I_2, pyridine, dioxane, DMF; 2. CF_3COOH.

derivatives. The trifluoroacetyl groups of the modified 3′-deoxythymidine 5′-triphosphates obtained from **5** and **6** were removed with concentrated ammonia. Removal of the protecting groups generated a primary amino group at the linker arm, which allowed reaction with various types of compounds, *i.e.*, linkers and dyes with a functional group reacting with a nucleophilic amino group.

There is a strong dependence of the spacer length between dye and nucleotide upon quantum yields and absorption coefficients [30][31]. We, therefore, decided to elongate the spacer arm of the *N*^{4}-modified cytidine derivative **14** with a rigid 4-(aminomethyl)cyclohexylcarbonyl moiety (→ **18** and **19**; *Scheme 4*).

Scheme 4. *Synthesis of an* N^4*-Modified 2′,3′-Dideoxycytidine 5′-Triphosphate with a Rigid Linker Arm*

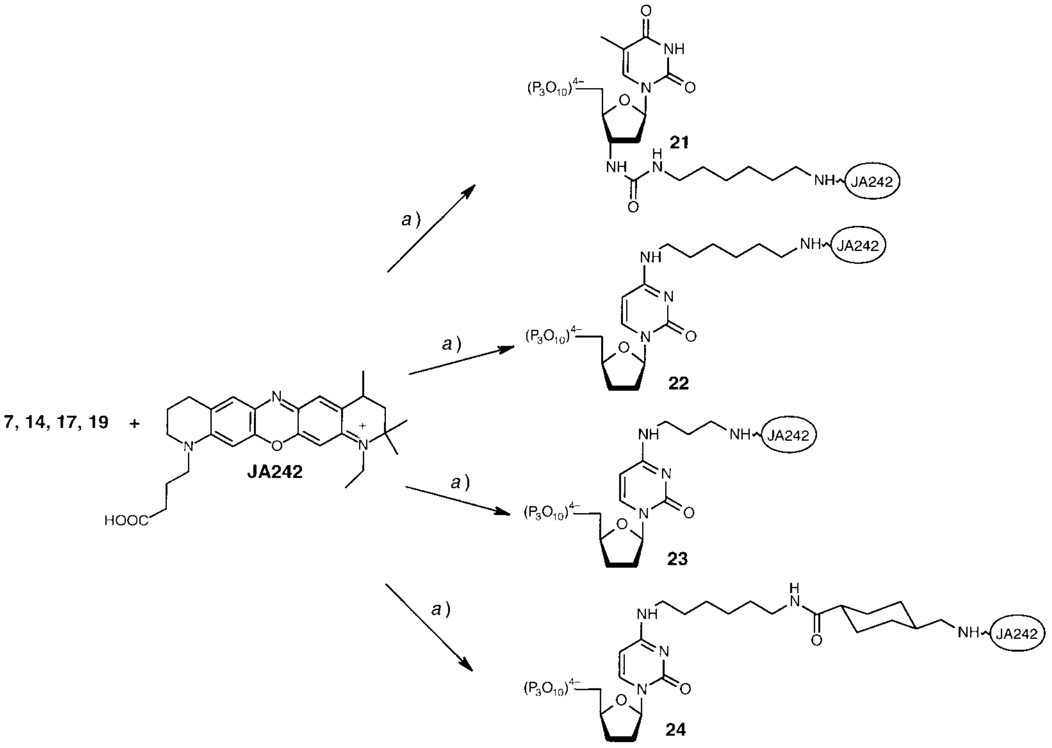


a) 4-{[(Trifluoroacetyl)amino]methyl}cyclohexanecarboxylic acid, TSTU (*N*,*N*,*N*′,*N*′-tetramethyl-*O*-succinimidouronium tetrafluoroborate), (i-Pr)$_2$EtN, dioxane, DMF, H_2O. *b*) Conc. NH_3 soln.

To minimize the electrophoretic-mobility shifts under sequencing gel-electrophoresis conditions, we intended to use newly developed multiplex dyes with high structural similarities. These chromophores are characterized by the same excitation wavelength, but distinguishable fluorescence lifetimes [32]. Detection in the near-IR range simplifies the apparatus requirements and minimizes the background fluorescence. Since the multiplex dye JA242 carries a free carboxy group, coupling is possible *via* a carboxamide linkage. Dye labeling of the modified nucleotides **7**, **14**, **17**, and **19** was performed as shown in *Scheme 5*. Activation was performed according to a procedure described by *Bannwarth* and *Knorr* [33] with *N,N,N′,N′*-tetramethyl-*O*-succinimidouronium tetrafluoroborate (TSTU) – a convenient coupling reagent in the synthesis of peptides. The procedure could be performed easily as a one-pot reaction within a few min. After preactivation of the dye in dioxane/DMF under H_2O-free conditions a solution of the nucleotide in borate buffer (0.1M, pH 8.5) was added. All thus-formed labeled nucleotides **21**–**24** were purified by reversed-phase column chromatography (0.1M (Et_3NH)OAc/MeCN) first and subsequently by anion-exchange chromatography (*Sepharose Q*, 0–1M LiCl-buffer gradient). The labeled nucleotides could be precipitated as lithium salts from acetone/EtOH. Determination of the fluorescence quantum yield of the JA242-labeled nucleoside triphosphates **21**, **22**, and **23** showed values of more than 0.90 with respect to the uncoupled dye (1.00), which indicates that coupling resulted in no significant quenching. However, the quantum yield of the JA242-labeled nucleotide **24** amounted to only 0.71.

2.2. *Application of the Modified Nucleotides in Incorporation Reactions with Terminal Deoxynucleotidyl Transferase.* Terminal deoxynucleotidyl transferase from calf thymus catalyzes a template-independent addition of deoxyribonucleoside triphosphates to the 3′-OH ends of double- or single-stranded DNA. In addition to the natural deoxynucleoside triphosphates, the enzyme also accepts a number of modified dNTPs as substrates. In a simple test, we investigated the incorporation rates of our 3′-sugar- and base-modified dNTPs. The chemically synthesized oligodeoxyribonucleotide (0.4 *OD*) was incubated with a modified nucleoside triphosphate at 37° for 2 h. Since these nucleotide analogues (**21** and **22**) did not possess a 3′-hydroxy group, their incorporation into the elongating DNA strand would stop the labeling reaction. The incorporation assays were analyzed by gel electrophoresis in a denaturing polyacrylamide matrix at 70 V for 3 h. The 3′-modified thymidine triphosphate **21** was

Scheme 5. *Labeling of the 5'-Triphosphates* **7**, **14**, **17**, *and* **19** *with the Dye JA242*



a) TSTU, $(i\text{-}Pr)_2EtN$, DMF, dioxane, H_2O.

not accepted as substrate by terminal deoxynucleotidyl transferase. In contrast, the reaction with the N^4-modified dideoxycytidine triphosphate **22** showed no remaining unlabeled oligonucleotide (*Fig. 1*).

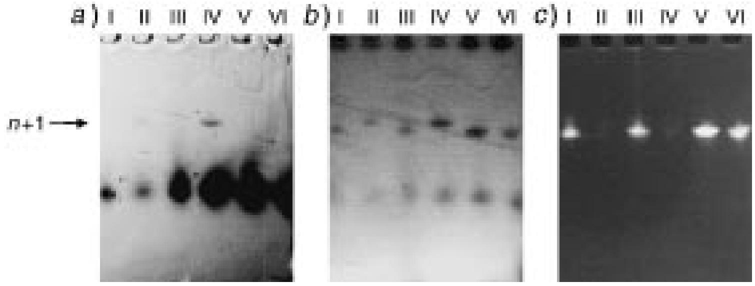


Fig. 1. *Gel electrophoresis of the incorporation reaction of ddTTP* **21** *or ddCTP* **22** *into 5'-d(ACACCCAATTCTGAAAATGGA)-3' with terminal deoxynucleotidyl transferase:* a) *irradiation with white light*; b) *UV irradiation*; c) *reaction of the oligonucleotide bands with ethidium bromide. Lane I*, **21** (0.05 *OD*); *Lane II*, **22** (0.05 *OD*); *Lane III*, **21** (0.05 *OD*); *Lane IV*, **22** (0.2 *OD*); *Lane V*, unlabeled oligodeoxyribonucleotide (0.2 *OD*); *Lane VI:* **21** (0.2 *OD*)

2.3. *Application of the* N^4*-Modified Labeled ddCTPs in Sequencing Reactions.* To apply the *N*^{4}-modified nucleotides in sequencing reactions, we had to test the ability of these nucleotides to serve as potent chain terminators for standard DNA polymerases. Their performance was tested by sequence analysis of the bacteriophage vector M13mp18 with the M13/pUC sequencing primer and two enzymes: *ThermoSequenase* and a new chimeric thermostable DNA polymerase, which had shown improved incorporation of commercially available chromophore-labeled sequencing terminators in previous experiments [34]. Best results were also achieved with *ThermoSequenase* and the new chimeric thermostable DNA polymerase in our cytidine series. Whereas the other enzymes needed 15 to 150 mM final concentrations of the terminator in the sequencing reaction, chimeric DNA polymerase gave best results at 1.5 mM. All experiments were performed under cycle-sequencing conditions including denaturating steps at 95°, demonstrating that the dye-labeled nucleotides have also a good stability at higher temperatures. In two different approaches, the dye-labeled nucleotides **22**–**24** were incubated with either a dye-labeled sequencing primer to test only the acceptance of the terminator or with an unlabeled primer to detect the multiplex dye attached to the terminator. As a reference, a sequencing reaction was carried out with conventional ddNTPs (ddGTP, ddATP, dTTP, and ddCTP). The base-labeled nucleotides **22** and **24** were well accepted by the mentioned enzymes and showed perfect termination quality (*Fig. 2a* and *2b*). Similar results were obtained with the dideoxycytidine derivative **23** labeled with other dyes like Cy5, JA133, and JA169 [32] (data not shown)

First results indicate that the longer the linker the better the acceptance by the tested enzymes. Whereas the readability of the sequence using the labeled ddCTP **23** with the shortest linker was only 600 base pairs, we were able to determine the sequence of up to 800 base pairs using the labeled ddCTP **24** with the longest linker. Regarding both acceptance and quantum yield, best sequencing patterns were shown by the ddCTP derivative **22** with the hexaneamine spacer.

Conclusion. – In the present work, we demonstrated that *N*^{4}-dye-labeled dideoxycytidine 5′-triphosphates were well accepted as substrates by common sequencing enzymes. Among the tested enzymes, *ThermoSequenase* and a newly developed chimeric DNA polymerase showed the best acceptance of base-modified ddCTPs, which is comparable to that of unlabeled ddCTP. For the synthesis of 3′-sugar-modified 3′-deoxythymidine 5′-triphosphates, a rapid method to transform AZT (**1**) into the corresponding 3′-deoxy-3′-isocyanato- or 3′-deoxy-3′-isothiocyanatothymidine **3** and **4**, respectively, by a modified *Wittig* reaction [14] was elaborated. The triphenylphosphine imide **2**, which was obtained from the azido nucleoside **1** using polymer-bound triphenylphosphine, served as the key intermediate. Treatment of phosphine imide **2** of 3′-deoxythymidine with either CO_2 or CS_2 and monoprotected hexane-1,6-diamine led to the corresponding urea and thiourea nucleoside derivatives **5** and **6**, respectively. These tethered nucleosides could be used to label with fluorescent dyes.

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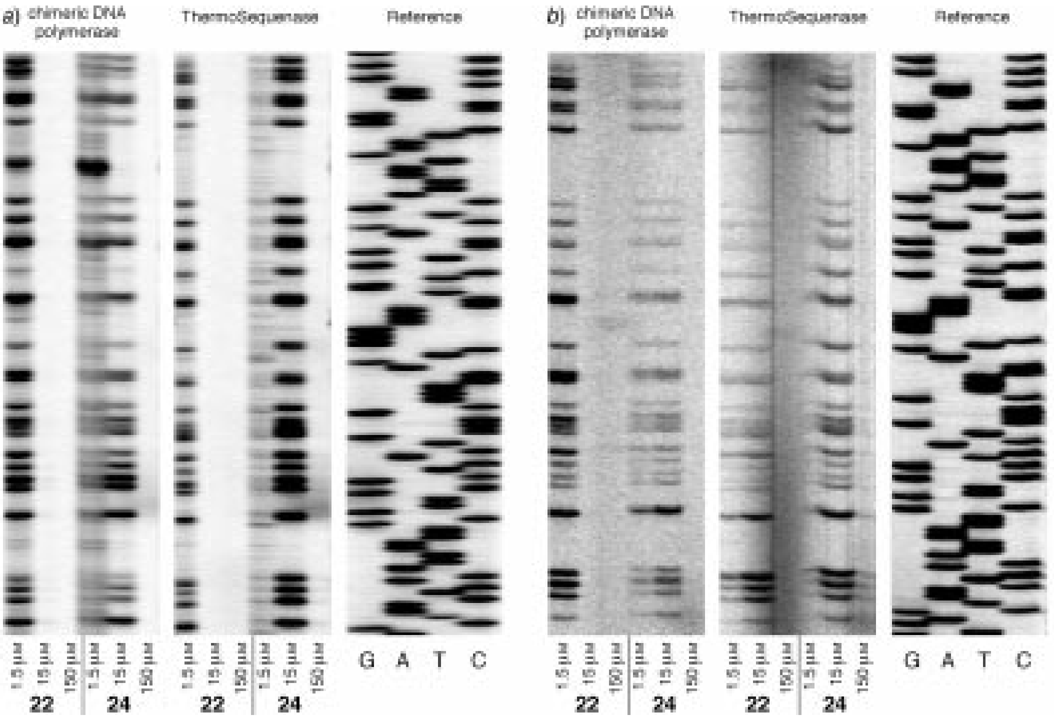

Fig. 2. *Sequencing with* a) *dye-labeled primers and* b) *unlabeled primers.* G, A, T, and C are short forms of ddGTP, ddATP, ddTTP, and ddCTP, resp. For *ThermoSequenase*, the optimal ratio of unlabeled conventional terminators to dNTPs was 1:100. The optimal ratio of N^4-labeled ddCTP to dCTP was in the range of 1:100 to 1:10 (corresponding to 1.5 – 15 μM final N^4-labeled ddCTP concentration), indicating a similar acceptance for N^4-labeled and unlabeled ddCTP (see text).

Experimental Part

General. Terminal deoxynucleotidyl transferase and the newly developed chimeric DNA polymerase were purchased from *Boehringer Mannheim GmbH*, *ThermoSequenase* from *Amersham*, 2′,3′-dideoxyuridine, 2′,3′-dideoxycytidine, and AZT (**1**) from *Boehringer Mannheim GmbH*, and tributylamine, 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one, polymer-bound triphenylphosphine, dry pyridine, DMF, and dioxane from *Fluka*, bis(tributylammonium) pyrophosphate was purchased from *Sigma*, 1*H*-1,2,3,4-tetrazole from *Pharmacia LKB*, and 2-chlorophenyl phosphorodichloridate from *Aldrich*. DNA Synthesis was performed on a *Gene Assembler Plus* (*Pharmacia LKB*), phosphoramidites were purchased from *Pharmacia LKB.* DNA-sequencing reactions were analyzed on a *LiCor 4000 L* sequencer. Column chromatography (CC): DEAE-*Sephadex*, *Sepharose Q* (*Pharmacia LKB*), and DEAE cellulose *DE52* (*Whatman*) at 4°. UV/VIS Spectra: *Spekord UV 160 A* (*Shimadzu*); λ_{max} in nm. Fluorescence spectra: *LS100* (PTI); λ_{max} in nm. NMR Spectra: *Bruker-DPX-300* and *Bruker AMX-600*; δ in ppm rel. to Me_3Si and H_3PO_4 as reference; *J* in Hz. MALDI-TOF-MS: Laser *Tec-Benchtop-II* system (*PerSeptive Biosystems*). Elemental analysis: Elemental analyzer 240 (*Perkin Elmer*).

N-*{6-{[(*tert-*Butoxy)carbonyl]amino}hexyl}-2,2,2-trifluoroacetamide.* *tert*-Butyl (6-aminohexyl)carbamate hydrochloride (1.1 g, 4.35 mmol) was suspended in 0.5M NaOH (50 ml) and stirred at r.t. for 1 h. After extraction with Et_2O (50 ml), the org. phase was evaporated to give *tert*-butyl (6-aminohexyl)carbamate. To a chilled (ice bath) and rapidly stirred soln. of the latter and Et_3N (0.60 ml) in abs. MeOH (9.90 ml), ethyl trifluoracetate (0.65 ml) in MeOH (1.23 ml) was slowly added. The mixture was stirred at r.t. for 40 h. After evaporation, the crude product was purified by CC (silica gel, 0 → 10% MeOH/CHCl$_3$): 1.24 g (91%). White powder. ^{1}H-NMR (CDCl$_3$): 3.28 (*m*, $CF_3CONHCH_2$); 3.05 (*m*, $BocNHCH_2$); 1.27 – 1.58 (*m*, 4 CH_2); 1.37 (*s*, 3 Me).

N-*(6-Aminohexyl)-2,2,2-trifluoroacetamide Trifluoroacetate.* *N*-{6-{[(*tert*-Butoxy)carbonyl]amino}hexyl}-2,2,2-trifluoracetamide (670 mg, 2.20 mmol) was dissolved in CF_3COOH (5 ml) and stirred at r.t. for 20 min. After addition of H_2O (20 ml), the mixture was evaporated. Excess CF_3COOH was removed by repeated co-evaporation with H_2O and EtOH, resp.: 700 mg (98%) of trifluoracetate. White powder. ^{1}H-NMR (CD_3OD): 3.17 (*t*, *J* = 6.78, $CF_3CONHCH_2$); 2.80 (*t*, *J* = 7.54, NH_2CH_2); 1.26 – 1.57 (*m*, 4 CH_2).

3′-Deoxy-3′-isothiocyanatothymidine (**4**). A soln. of 3′-azido-3′-deoxythymidine (**1**; 1.54 g, 5.76 mmol) in anh. dioxane (10 ml) was evaporated (3 ×). The residue was dried further over P_4O_{10} *in vacuo* at r.t. for 5 h. In a flask flushed with dried Ar (*via* a desiccator) and fitted with an Ar-filled balloon and a septum, polymer-bound triphenylphosphine (9.5 g, 3 mmol PPh_3/g polymer) was suspended in anh. dioxane (100 ml). The support was allowed to swell by shaking for 15 min. Then **1** in dioxane (50 ml) was added. After loading, 5 equiv. of CS_2 were added by syringe. The suspension was gently shaken for another 6 h and then filtered. The residual solid was washed with dioxane several times and the filtrate evaporated. CC (silica gel, 0 → 5% MeOH/$CHCl_3$) gave 1.22 g (75%) of **4**. White foam. TLC ($CHCl_3$/MeOH 9 : 1): R_f 0.52. M.p. 113°. ^{1}H-NMR ((D_6)DMSO): 7.61 (*s*, H–C(6)); 6.26 (*t*, H–C(1′)); 4.52 (*m*, H–C(3′)); 4.19 (*m*, H–C(4′)); 3.49 – 3.62 (*m*, 2 H–C(5′)); 2.30 – 2.69 (*m*, 2 H–C(2′)); 1.81 (*s*, Me).

3′-Deoxy-3′-{{thioxo{{6-[(trifluoroacetyl)amino]hexyl}amino}methyl}amino}thymidine (**6**). To a soln. of **4** (200 mg, 0.71 mmol) in anh. pyridine (18 ml), (i-Pr)$_2$EtN (110 mg, 146 µl, 1.2 equiv.) was added, followed by *N*-(6-aminohexyl)-2,2,2-trifluoroacetamide trifluoroacetate (280 mg, 1.2 equiv.) in anh. pyridine (18 ml). The soln. was stirred at r.t. overnight. After evaporation, the residue was co-evaporated with toluene to remove traces of pyridine and then purified by CC (silica gel, 0 → 10% MeOH/$CHCl_3$): 230 mg (65%) of **6**. White foam. TLC ($CHCl_3$/MeOH 9 : 1): R_f 0.21. ^{1}H-NMR ((D_6)acetone: 7.93 (*s*, H–C(6)); 6.21 (*t*, H–C(1′)); 4.45 (*m*, H–C(3′)); 3.86 – 3.97 (*m*, H–C(4′), 2 H–C(5′)); 3.32 (*m*, $CF_3CONHCH_2$); 3.49 (*m*, $NHCSNHCH_2$); 2.33 – 2.55 (*m*, 2 H–C(2′)); 1.84 (*s*, Me); 1.35 – 1.61 (*m*, 4 CH_2). ^{13}C-NMR ($CDCl_3$): 164.45 (*s*, C(4)); 158.03 (*s*, C=O); 150.97 (*s*, C(2)); 136.31 (*s*, C(6)); 110.99 (*s*, C(5)); 84.44 (*s*, C(1′)); 61.13 (*s*, C(5′)); 26.48 – 29.20 (4 CH_2); 12.01 (*s*, Me).

3′-Deoxy-3′-{{{{6-[(trifluoroacetyl)amino]hexyl}amino}carbonyl}amino}thymidine (**5**). As described for **4** and **6**, with **1** (1.54 g, 5.76 mmol), dioxane (3 × 10 ml), and polymer-bound triphenylphosphine (9.5 g, 3 mmol PPh_3/g polymer) in anh. dioxane (100 ml). After addition of **1** in dioxane (50 ml), a stream of dry CO_2 was passed through the suspension. The resulting *3′-deoxy-3′-isocyanato-thymidine* (**3**) was not isolated, but immediately treated with *N*-(6-aminohexyl)-2,2,2-trifluoroacetamide trifluoroacetate. CC (silica gel, MeOH/$CHCl_3$) gave 1.5 g (55%) of **5**. White foam. TLC ($CHCl_3$/MeOH 9 : 1): R_f 0.20. M.p. 183 – 185°. ^{1}H-NMR ((D_6)acetone): 6.14 (*t*, H–C(1′)); 4.36 (*m*, H–C(3′)); 3.71 – 3.81 (*m*, H–C(4′), 2 H–C(5′)); 3.32 (*m*, $CF_3CONHCH_2$); 3.14 (*m*, $NHCONHCH_2$); 2.30 – 2.36 (*m*, 2 H–C(2′)); 1.83 (*s*, Me); 1.29 – 1.61 (*m*, CH_2). ^{13}C-NMR ((D_6)acetone): 159.29 (*s*, NHCONH); 136.81 (*s*, C(6)); 110.40 (*s*, C(5)); 87.10 (*s*, C(4′)); 84.53 (*s*, C(1′)); 62.17 (*s*, C(5′)); 50.41 (*s*, C(3′)); 40.19 (*t*, $CF_3CONHCH_2$); 38.60 (*s*, $NHCH_2$); 26.95 – 31.01 (4 CH_2); 12.60 (*s*, Me).

*2′,3′-Dideoxy-5′-*O*-(dimethoxytrityl)uridine* (**9**). To a soln. of 2′,3′-dideoxyuridine (2 g, 9.4 mmol) in anh. pyridine (20 ml), 4-(dimethylamino)pyridine (58 mg), Et_3N (1.85 ml), and dimethoxytrityl chloride (3.83 g) were added. The mixture was left stirring 5 h (TLC monitoring). Then MeOH (4.3 ml) was added and, after 15 min, the mixture was diluted with H_2O (40 ml) and extracted with Et_2O (3 × 120 ml). The combined org. phase was evaporated and the residue purified by CC (silica gel, 0 → 10% MeOH/$CHCl_3$): 4.1 g (85%) of **9**. White foam. TLC ($CHCl_3$/MeOH 9 : 1): R_f 0.71. M.p. 81 – 85°. ^{1}H-NMR ($CDCl_3$): 7.91 (*d*, H–C(6)); 6.02 (*m*, H–C(1′)); 5.28 (*d*, *J*(5,6) = 7.91, H–C(5)); 4.14 (*m*, H–C(4′)); 3.24 – 3.48 (*m*, 2H–C(5′)); 2.34 (*m*, H_a–C(2′)); 1.87 – 2.08 (*m*, H_b–C(2′), H–C(3′)); 3.72 (*s*, 2 MeO); 6.75 – 7.35 (*m*, 13 arom. H). ^{13}C-NMR ($CDCl_3$): 163.59 (C(4)); 158.64 ((MeO)$_2$*Tr*); 150.38 (C(2)); 144.44 ((MeO)$_2$*Tr*); 140.54 (C(6)); 135.50 ((MeO)$_2$*Tr*); 135.33 ((MeO)$_2$*Tr*); 130.12 ((MeO)$_2$*Tr*); 128.14 ((MeO)$_2$*Tr*); 127.96 ((MeO)$_2$*Tr*); 127.07((MeO)$_2$*Tr*); 113.23 ((MeO)$_2$*Tr*); 101.51 (C(5)); 86.34 (C(1′)); 81.15 (C(4′)); 63.64 (C(5′)); 55.25 (MeO); 33.42 (C(2′)); 25.11 (C(3′)). Anal. calc. for $C_{30}H_{30}N_2O_6$: C 70.02, H 5.88, N 5.44; found: C 69.58, H 5.81, N 5.26.

*4-*N*-{6-{[(*tert*-Butoxy)carbonyl]amino}hexyl}-2′,3′-dideoxy-5′-*O*-(dimethoxytrityl)cytidine* (**11**). A soln. of **9** (611 mg, 1.19 mmol) in pyridine was evaporated. After addition of 1*H*-1,2,3,4-tetrazole (582 mg, 8.31 mmol, 7 equiv.; sublimated and dried over P_4O_{10}), the mixture was dissolved in pyridine and evaporated again. The rapidly stirred soln. of the residue in pyridine (3.9 ml) was treated at 0° by slow addition with 2-chlorophenyl phosphorodichloridate (469 mg, 1.91 mmol) in anh. pyridine (770 µl). The mixture was left stirring overnight (TLC monitoring). After cooling in an ice bath, MeOH (2 ml) was added and the mixture evaporated and dried *in vacuo*. The residue was dissolved in $CHCl_3$ and the soln. washed carefully with sat. $NaHCO_3$ soln. and brine and evaporated. The obtained tetrazolyl derivative **10** was dissolved in dioxane (2 ml) and treated with *tert*-butyl (6-aminohexyl)carbamate (2.8 mmol) at r.t. overnight. After completion of the reaction, the soln. was evaporated and the residue dried *in vacuo*. Purification by CC (silica gel, 0 → 10% MeOH/$CHCl_3$), gave 320 mg (38%) of **11**. White foam. TLC ($CHCl_3$/MeOH 9 : 1): R_f 0.76. UV/VIS (MeOH): 275. ^{1}H-NMR ($CDCl_3$): 7.93 (*d*, *J*(5,6) = 7.53, H−C(6)); 7.34 – 6.76 (*m*, 13 H, $(MeO)_2Tr$); 6.18 (*t*, H−C(1′)); 5.57 (*d*, H−C(5)); 4.97 (br. *t*, OH−C(5′)); 4.18 (*m*, H−C(4′)); 3.73 (*s*, 2 MeO); 3.51 – 3.26 (*m*, 2 H−C(5′), 2 $NHCH_2$); 2.40 (*m*, H_a − C(2′)); 2.14 – 1.93 (*m*, H_b−C(2′), H−C(3′)).

*4-*N*-{6-{[(*tert*-Butoxy)carbonyl]amino}hexyl}-2′,3′-dideoxycytidine* (**12**). A soln. of **11** (200 mg, 267 mmol) in 80% AcOH/H_2O (10 ml) was stirred at r.t. for 45 min. Then H_2O (20 ml) was added, the mixture evaporated, and the residue coevaporated with H_2O several times. Purification of the crude product by CC (silica gel, 0 → 10% MeOH/$CHCl_3$) gave 93 mg (85%) of **12**. White foam. TLC ($CHCl_3$/MeOH 9 : 1): R_f 0.40. UV/VIS (MeOH): 273. ^{1}H-NMR ($CDCl_3$): 8.12, 7.66 (2*d*, H−C(6)); 5.97 (*m*, H−C(1′)); 5.66 (*m*, H−C(5)); 4.12 (*m*, H−C(4′)); 3.93 (*m*, H_a−C(5′)); 3.66 (*m*, H_b−C(5′)); 3.35, 3.11 (2*t* each, CH_2N−C(4)); 2.99 – 3.07 (*m*, $BocNHCH_2$); 2.27 – 2.42 (*m*, H_a−C(2′)); 2.05 (*m*, H_b−C(2′)); 1.86 (*m*, 2 H−C(3′)); 1.45 – 1.57 (*m*, 4 CH_2, Me_3C). ^{13}C-NMR ($CDCl_3$): 176.28 (C(4)); 163.78 (C(2)); 143.39, 139.89 (both C(6)); 95.12, 89.85 (both C(5)); 87.70 (C(1′)); 82.35 (C(4′)); 63.54 (C(5′)); 40.30 (CH_2N−C(4)); 32.75 (C(2′)); 29.87 (CH_2); 29.02 (CH_2); 28.42 (Me_3C); 26.20 (CH_2); 25.01 (C(3′)). Anal. calc. for $C_{20}H_{34}N_4O_5$: C 58.52, H 8.35, N 13.65; found: C 58.10, H 8.31, N 12.35.

Dideoxyribonucleoside 5′-Triphosphates: General Procedure. Each nucleoside (100 μmol) was dissolved in anh. pyridine (2 ml) and evaporated (3 ×). The residue was dried further over P_4O_{10} *in vacuo* at r.t. for 5 h. In a flask flushed with dried Ar (*via* a desiccator) and fitted with an Ar-filled balloon, anhydrous pyridine (100 μl) was added by syringe through the septum, followed by anh. dioxane (300 μl). To the well-stirred soln. of the nucleoside, freshly prepared 1M 2-chloro-4*H*-1,2,3-dioxaphosphorin-4-one in anh. dioxane (110 μl, 110 μmol) was added. A white precipitate was formed during the reaction. After 15 min, 0.5M bis(tributylammonium) pyrophosphate in anh. DMF (300 μl) and Bu_3N (100 μl) were injected quickly. The white precipitate dissolved immediately. After 20 min, the cyclic intermediate was oxidized by adding freshly prepared 1% I_2 soln. in pyridine/H_2O 98 : 2 (2 ml). After 15 min, the excess I_2 was reduced by 5% $NaHSO_3$ soln. in H_2O (1 ml).

If not stated otherwise, the reaction soln. was evaporated and the residue dissolved in H_2O (2 ml). The resulting soln. of triphosphates with a CF_3CO protecting group at the linker arm was treated with conc. NH_3 soln. (20 ml) for 6 h (see **14** for the removal of the Boc group). The final product was purified by CC (DEAE-*Sephadex A-25*, 2.5 × 25 cm, 4°, linear gradient of 0 → 1M LiCl (3 ml/min) over 8.5 h; 30-ml fractions). The product fractions were lyophilized. On addition of acetone/EtOH 3 : 1 (10 ml) to a soln. of the residue in H_2O (1 ml), a white precipitate of the nucleoside triphosphate was formed.

3′-{{[(6-Aminohexyl)amino]carbonyl}amino}-3′-deoxythymidine 5′-(Tetralithium Triphosphate) (**7** · 4 Li^+): Yield 60%. White powder. ^{1}H-NMR (D_2O): 6.14 (*t*, H−C(1′)); 3.99 – 4.20 (*m*, H−C(4′), 2 H−C(5′)); 2.18 – 2.38 (*m*, 2 H−C(2′)); 1.80 (*s*, Me−C(5)); 7.57 (*s*, H−C(6)); 2.98 (*m*, NH_2CH_2); 2.85 (*t*, *J* = 7.53, $NHCH_2$); 1.18 – 1.60 (*m*, 4 CH_2); ^{31}P-NMR (D_2O): − 7.60 (*d*, P(γ)); − 9.89 (*d*, P(α)); − 20.98 (*t*, P(β)).

3′-{{[(6-Aminohexyl)amino]thioxomethyl}amino}-3′-deoxythymidine 5′-(Tetralithium Triphosphate) (**8** · 4 Li^+): Yield 50%. White powder. ^{1}H-NMR (D_2O): 6.14 (*t*, H−C(1′)); 3.99 – 4.28 (*m*, H−C(4′), 2 H−C(5′)); 2.42 – 2.61 (*m*, H_a−C(2′)); 2.18 – 2.32 (*m*, H_b−C(2′)); 2.85 (*t*, $NHCH_2$); 1.25 – 1.57 (*m*, 4 CH_2); 7.55 (*s*, H−C(6)). ^{31}P-NMR (D_2O): − 10.31 (*d*, P(γ)); − 10.87 (*d*, P(α)); − 22.78 (*t*, P(β)).

*4-*N*-{6-{[(*tert*-Butoxy)carbonyl]amino}hexyl}-2′,3′-dideoxycytidine 5′-(Tetralithium Triphosphate)* (**13** · 4 Li^+): Yield 52%. White powder. UV/VIS (H_2O): 272. ^{1}H-NMR (D_2O): 5.95 (*m*, H−C(1′)); 4.24 (*m*, H−C(4′)); 4.11 – 4.17 (*m*, H_a−C(5′)); 3.94 – 4.02 (*m*, H_b−C(5′)); 3.18 (*t*, $BocNHCH_2$); 2.90 (*t*, CH_2N−C(4)); 2.23 – 2.35 (*m*, H_a−C(2′)); 1.74 – 2.00 (*m*, H_b−C(2′), H−C(3′)); 1.13 – 1.46 (*m*, 2 CH_2, Me_3C); 7.70 (*d*, *J*(5,6) = 7.54, H−C(6)); 5.86 (*d*, H−C(5)). ^{31}P-NMR (D_2O): − 4.14 (*d*, P(γ); − 9.74 (*d*, P(α)); − 19.44 (*t*, P(β)).

*4-*N*-(6-Aminohexyl)-2′,3′-dideoxycytidine 5′-(Tetralithium Triphosphate) Trifluoroacetate* (**14** · 4 Li^+ · CF_3COOH): For the removal of the Boc group, **13** · 4 Li^+ in CF_3COOH (1 ml) was stirred at r.t. for 2 min. On addition of Et_2O (10 ml) **14** · 4 Li^+ · CF_3COOH precipitated. The precipitate was filtered and washed with Et_2O several times. Purification of the crude product was achieved by ion-exchange CC (*Sephadex* 0 → 1M LiCl gradient): 95% of **14** · 4 Li^+ · CF_3COOH. White powder. UV/VIS (H_2O): 282. ^{1}H-NMR (600 MHz, D_2O): 8.28 (*d*, *J* = 8.12, 0.4 H, H−C(6)); 8.04 (*d*, *J* = 8.04, 0.6 H, H−C(6)); 6.34 (*d*, 0.4 H, H−C(5)); 6.14 (*d*, 0.6 H, H−C(5)); 5.96 (*m*, H−C(1′)); 4.33 (*m*, H−C(4′)); 4.20 – 4.26 (*m*, H_a−C(5′)); 3.99 – 4.05 (*m*, H−C(5′)); 3.31 – 3.37 (2*t*, CH_2N−C(4)); 2.87 – 2.89 (*dd*, CH_2NH_2); 2.34 – 2.41 (*m*, H_a−C(2′)); 2.08 – 2.14 (*m*, H_a−C(2′)); 1.97 – 2.02 (*m*, H_a−C(3′)); 1.88 – 1.95 (*m*, H_a−C(3′)); 1.54 – 1.62 (*m*, 2 CH_2); 1.30 – 1.32 (*m*, 2 CH_2). ^{13}C-NMR (300 MHz, D_2O): 157.25 (C(4)); 148.89 (C(2)); 142.53 (C(6)); 98.31, 95.72 (both C(5)); 87.81 (C(1′)); 81.58

(C(4′)); 66.45 (C(5′)); 43.25, 42.74 (both *C*H_2N–C(4)); 39.69 (CH_2NH_2); 27.19 (CH_2); 26.86 (CH_2); 25.65 (C(2′)); 25.45 (CH_2); 25.32 (CH_2); 24.42, 24.14 (both C(3′)). ^{31}P-NMR (300 MHz, D_2O): – 9.36 (P(α), P(γ)); – 21.47 (P(β)).

*2′,3′-Dideoxy-4-*N*-{6-{{{4-{[(trifluoroacetyl)amino]methyl}cyclohexyl}carbonyl}amino}hexyl}cytidine 5′-(Tetralithium Triphosphate)* (**18** · 4 Li$^+$). To a soln. of 4-{[(trifluoroacetyl)amino]methyl}cyclohexanecarboxylic acid (33 mg, 130 μmol) in DMF/dioxane 1 : 1 (1720 μl), (i-Pr)$_2$EtN (27 μl, 154 μmol) and TSTU (47 mg, 154 μmol) were added. After 10 min, complete conversion to the corresponding *N*-hydroxysuccinimide-derived ester had occurred (TLC), and 1M **14** · 4 Li$^+$ · CF_3COOH in H_2O (430 μl) was added. After 10 h, the mixture was diluted with H_2O and lyophilized. Purification of the crude product was performed by ion-exchange CC (*Sepharose Q*), linear gradient of 0 → 1M LiCl over 4.5 h. A clear soln. of the residue in H_2O (1 ml) was added to acetone/EtOH 3 : 1 (10 ml) yielding a white precipitate: 65% of **18** · 4 Li$^+$. White powder. UV/VIS (H_2O): 272. ^{1}H-NMR (D_2O): 7.72 (*d*, H–C(6)); 5.99 (*m*, H–C(1′)); 5.90 (*d*, H–C(5)); 4.22 – 4.32 (*m*, H–C(4′)); 4.12 – 4.21 (*m*, H_a–C(5′)); 3.95 – 4.07 (*m*, H_b–C(5′)); 3.32 (*m*, H–C(1)(Chx)); 3.21 (*m*, CH_2N–C(4)); 2.99 – 3.11 (*m*, 2 C*H*$_2$NHCO); 2.30 (*m*, H_a–C(2′), H–C(4)(Chx)); 1.87 – 2.07 (*m*, H_b–C(2), 2 H–C(3′)); 1.68 (*m*, 2 CH_2); 1.47 (*m*, 2 CH_2); 1.38 (*t*, CH_2); 1.15 – 1.30 (*m*, 3 CH_2). ^{31}P-NMR (D_2O): – 3.9 (P(γ)); – 9.3 (P(α)); – 19.1 (P(β)). ^{19}F-NMR (D_2O): – 76 (CF_3).

*4-*N*-{6-{{[4-(Aminomethyl)cyclohexyl]carbonyl}amino}hexyl}cytidine 5′-(Tetralithium Triphosphate)* (**19** · 4 Li$^+$). A soln. of **18** · 4 Li$^+$ in conc. NH_3 soln. (2 ml) was stirred at r.t. for 6 h. After evaporation, the crude product was purified by ion-exchange CC (*Sepharose Q*, 0 → 1M LiCl gradient): 85% of **19** · 4 Li$^+$. White powder. ^{1}H-NMR (D_2O): 7.79, 7.72 (2*d*, H–C(6)); 5.95 (*m*, H–C(1′)); 5.86 (*d*, H–C(5)); 3.72 – 4.30 (*m*, H–C(4′), 2 H–C(5′)); 3.28 (*m*, H–C(1)(Chx)); 3.19 (*m*, CH_2N–C(4)); 3.02 (*t*, C*H*$_2$NHCO); 2.20 – 2.40 (*m*, H_a–C(2′), H–C(4)(Chx), C*H*$_2$NH$_2$); 1.87 – 2.10 (*m*, H_b–C(2′), H–C(3′)); 1.62 – 1.75 (*m*, 2 CH_2); 1.30 – 1.50 (*m*, 2 CH_2); 1.21 (*m*, 4 CH_2). ^{31}P-NMR (D_2O): – 3.8 (*d*, P(γ)); – 9.3 (*d*, P(α)); – 19.0 (*t*, P(β)).

Coupling with JA242-S Dye: General Procedure. The multiplex dye JA 242-S (67 mg, 150 μmol) (= 1-(3-carboxypropyl)-11-ethyl-3,4,8,9,10,11-hexahydro-8,10,10-trimethyl-2*H*-dipyrido[3,2-*b*: 2′,3′-*i*]phenoxazinium inner salt) was dissolved in DMF/dioxane 1 : 1 (2 ml). To this soln., (i-Pr)$_2$EtN (77 μl, 450 μmol) and TSTU (57 mg, 190 μmol) were added. After 10 min, complete conversion into the corresponding *N*-hydroxysuccinimide-derived ester had occurred (TLC), and the nucleoside 5′-triphosphates **7**, **14**, **17**, or **19** (50 μmol) was added. After 3 h the mixture was diluted with H_2O and lyophilized. The residue was purified by reversed-phase CC (*C18* silica gel; 0.1M (Et_3NH)OAc/MeCN) followed by ion-exchange CC (*Sepharose Q*, linear gradient of 0 → 1M LiCl over 4.5 h). The clear soln. of the residue in H_2O was added to acetone/EtOH 3 : 1, yielding the blue precipitate of the dye-labeled nucleoside triphosphate.

*3′-Deoxy-3′-{{{{6-{[4-(11-ethyl-3,4,8,9,10,11-hexahydro-8,10,10-trimethyl-2*H*-dipyrido[3,2-*b*:2′,3′-*i*]phenoxazin-1-ium-1-yl)-1-oxobutyl]amino}hexyl}amino}carbonyl}amino}thymidine 5′-(Tetralithium Triphosphate)* (**21** · 4 Li$^+$): Yield 18%. Blue powder. UV/VIS (H_2O): 668. Fluorescence (H_2O): 683. ^{1}H-NMR (MeOH): 7.60 (*s*, H–C(6)); 7.49 (*s*, 1 arom. H); 7.39 (*s*, 1 arom. H); 6.96 (*s*, 1 arom. H); 6.78 (*s*, 1 arom. H); 6.12 (*t*, H–C(1′)); 4.07 – 4.12 (*m*, 2 H–C(5′)); H–C(4′), 2 H–C(3′)); 3.58 – 3.65 (*m*, 2 CH_2); 3.09 (*t*, NHC*H*$_2$); 2.99 (*t*, NHC*H*$_2$, *m*, CH); 2.86 (*t*, CH_2); 2.23 – 2.33 (*m*, 2 H–C(2′), CH_2); 1.92 – 2.01 (*m*, 2 CH_2); 1.80 (*s*, Me–C(5)(Thy)) 1.19 – 1.48 (*m*, 6 CH_2, 4 Me). ^{13}C-NMR (MeOH): 175.08 (NHC(O)CH_2); 166.08 (C(4)); 161.04 (HNC(O)NH); 156.32, 155.85 (arom. C); 152.58 (C(2)); 150.49, 149.82 (arom. C); 138.49 (C(6)); 135.99 (arom. C); 135.24 (arom. C); 131.99 (arom. CH); 130.96 (arom. C); 129.45 (arom. CH); 112.28 (C(5)); 97.96, 96.98 (arom. CH); 86.23 (C(1′)); 84.30 (C(4′)); 67.40 (C(5′)); 60.65 (quart. C); 53.87 (alicycl. CH_2); 52.56 (C–(3′)); 52.28 (CH_2); 45.81 (CH_2); 42.78 (CH_2); 40.89 ($NHCH_2$); 40.66 ($NHCH_2$); 39.52 (C(2′)); 33.55 (CH_2); 31.24 (CH_2); 30.40 (CH_2); 29.65 (CH); 28.74 (CH_2); 28.43 (Me); 27.75 (CH_2); 27.59 (CH_2); 26.42 (Me); 23.51 (CH_2); 22.15 (CH_2); 19.68 (Me); 14.70 (Me); 12.90 (*Me*–C(5)(Thy)). ^{31}P-NMR (MeOH): – 8.14 (*d*, P(γ)); – 9.62 (*d*, P(α)); – 21.04 (*t*, P(β)). MALDI-TOF-MS: 1054.74 ($[M + H]^+$).

*2′,3′-Dideoxy-4-*N*-{6-{[4-(11-ethyl-3,4,8,9,10,11-hexahydro-8,10,10-trimethyl-2*H*-dipyrido[3,2-*b*:2′,3′-*i*]phenoxazin-1-ium-1-yl)-1-oxobutyl]amino}hexyl}cytidine 5′-(Trilithium Triphosphate)* (**22** · 3 Li$^+$): Yield 15%. Blue powder. TLC (i-PrOH/25% NH_3 soln./H_2O 7 : 1 : 2): R_f 0.02. UV/VIS (H_2O): 668. Fluorescence (H_2O): 683. ^{1}H-NMR (CD_3OD): 7.93 (*d*, H–C(6)); 7.49 (*s*, 1 arom. H); 7.38 (*s*, 1 arom. H); 6.94 (*s*, 1 arom. H); 6.76 (*s*, 1 arom. H); 6.02 (*m*, H–C(1′)); 5.90 (*d*, H–C(5)); 4.03 – 4.30 (*m*, 2 H–C(5′), H–C(4′)); 3.62 (*m*, 2 CH_2); 3.09 – 3.16 (*m*, 2 NHC*H*$_2$); 2.85 – 2.97 (*m*, CH_2, CH); 2.27 – 2.32 (*m*, H_a–C(2′), CH_2); 1.84 – 2.02 (*m*, H_b–C(2′), 2 H–C(3′), 2 CH_2); 1.20 – 1.49 (*m*, 24 H, CH_2, Me). ^{13}C-NMR (CD_3OD): 174.30 (NH*C*(O)CH_2); 159.88 (C(4)); 156.36, 155.89 (arom. C); 147.96 (C(2)); 142.89, 141.63 (both C(6)); 132.20 (arom. CH); 130.96 (arom. C); 129.68 (arom. CH); 97.86, 96.91 (arom. CH); 96.12 (C(5)); 88.50 (C(1′)); 60.69 (quart. C); 53.84 (alicycl. CH_2); 52.26 (CH_2); 45.87 (CH_2); 42.74 (CH_2); 40.63 ($NHCH_2$); (C(2′)); 33.49 (CH_2); 30.54 (CH_2); 29.61

(CH); 28.74 (CH$_2$); 28.45 (Me); 27.78 (CH$_2$); 27.62 (CH$_2$); 26.38 (Me); 23.48 (CH$_2$); 22.15 (CH$_2$); 19.68 (Me); 14.65 (Me). ^{31}P-NMR (D$_2$O): -8.22 (*d*, P(γ)); -9.34 (*d*, P(α)); -20.36 (*t*, P(β)). MALDI-TOF-MS: 982.96 ($[M+H]^+$).

*2′,3′-Dideoxy-4-*N*-{6-{{{4-{{[4-(11-ethyl-3,4,8,9,10,11-hexahydro-8,10,10-trimethyl-2*H*-dipyrido[3,2-*b*:2′,3′-*i*]phenoxazin-1-ium-1-yl)-1-oxobutyl]amino}methyl}cyclohexyl}carbonyl}amino}hexyl}cytidine 5′-(Trilithium Triphosphate)* (**24**$\cdot$3 Li$^+$): Yield 15%. Blue powder. TLC (i-PrOH/25% NH$_3$ soln./H$_2$O 7:1:2): R_f 0.02. UV/VIS (H$_2$O): 668. Fluorescence (H$_2$O): 683. ^{31}P-NMR (D$_2$O): -3.6 (*d*, P(γ)); -9.4 (*d*, P(α)); -18.7 (*t*, P(β)). MALDI-TOF-MS: 1121.93 ($[M+H]^+$).

3′-End-Labeling Reaction with Terminal Deoxynucleotidyl Transferase. Oligonucleotide 5′-d(ACACCCAATTCTGAAAATGGA)-3′ (0.4 *OD*, 1.535 nmol, 11.4 μg) was dissolved in H$_2$O (166 μl). At 37°, 50 μl 5 × reaction buffer (= 1 × final concentration), 25 μl of 2.5 mM CoCl$_2$, 4 μl of 10 mM dye-labeled nucleoside triphosphate soln. (**21** or **22**, resp.), and 125 units of terminal deoxynucleotidyl transferase (5 μl) were added. After 2 h, no unlabeled oligodeoxyribonucleotide remained.

Sequencing Reactions with Nucleotides **22**, **23**, *or* **24**. *Sequencing with* ThermoSequenase *and Chimeric DNA Polymerase.* The optimal nucleotide ratio for sequencing reactions with *ThermoSequenase* and chimeric DNA polymerase was 1:100 (1.5 μM ddNTP:150 μM dNTP each). The unlabeled ddNTPs in the termination mixes were replaced by 1.5 μM, 15 μM, or 150 μM **22**, **23**, or **24**. A template/primer/enzyme mix containing 0.2 μg of M13mp18 DNA (100 fmol), 2 μl of unlabeled or labeled M13/pUC sequencing primer, 2 μl 10 × reaction buffer, 2 μl of *ThermoSequenase* or chimeric DNA polymerase (4 U/μl), and deionized H$_2$O to a total volume of 17.5 μl was prepared. Of each termination mix, a sample (4 μl) was dispensed into a test tube, and the template/primer/enzyme mixture (3.5 μl) was added. Cycle sequencing was performed in 30 cycles: at 95° for 30 s (denaturating), at 55° for 30 s (annealing), at 72° for 60 s (elongation). To remove the excessive dye terminators in the nucleotide **22** and **24** reaction, 92 μl of H$_2$O, 10 μl of 3M NaOAc, and 250 μl of abs. EtOH (r.t.) were added to each tube. The samples were centrifuged at 15000 rpm for 15 min, washed with 300 μl of fresh 70% EtOH/H$_2$O, and the pellets of dye-labeled DNA were dried. Each pellet was diluted in 6 μl of stop soln. and denaturated by heating for 2 min at 97°, and 2 μl of each mixture was loaded in an appropriate well of a 6% denaturating polyacrylamide sequencing gel. Sequencing was performed using a modified automated *LiCor-4000-L* sequencer.

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