57. 3′-Amino-Modified Nucleotides Useful as Potent Chain Terminators for Current DNA Sequencing Methods

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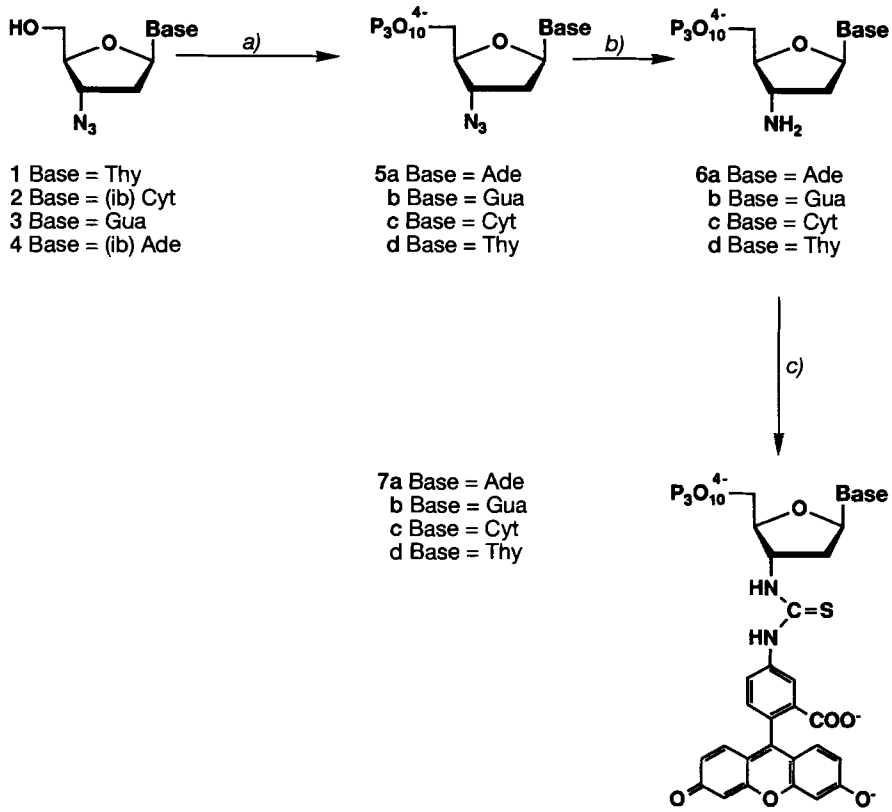
We describe the synthesis of modified nucleoside triphosphates of the four DNA bases containing a 3′-amino group which were prepared from the corresponding 3′-azido derivatives. Introduction of the triphosphate and subsequent reduction of the N_3 to the NH_2 group led directly to the target molecules **6a–d**. Furthermore, 3′-amino-2′,3′-dideoxynucleoside 5′-triphosphates proved to be potent inhibitors of the enzymatic synthesis of DNA catalyzed by the standard sequencing enzymes T7 DNA polymerase, sequenase version 2.0, *Thermus aquaticus* DNA polymerase, and *Thermus thermophilus* DNA polymerase. Both radioactive and fluorescent sequencing methods were applied successfully to the 3′-amino-modified terminators. Investigations in view of using these chain terminators according to *Sanger*'s sequencing method for fluorescence labeling were done.

1. Introduction. – The analysis of human genetic diseases on a molecular basis requires detailed and accurate information about the DNA nucleotide sequence. Radioactive sequencing techniques [1] [2] are being largely replaced by optical methods where sequencing techniques are based on the detection of fluorescent light emitted from fluorophore-labeled DNA fragments during their separation [3–5]. Working fluorophores were previously attached covalently to the 5′-end of oligonucleotide primers [6], to the bases of nucleoside triphosphates [7], or used as fluoresceinamidites [8] during primer synthesis. As an alternative to base-labeling methods of chain terminators, we examined a concept with a 3′-modified sequencing termination system where labeling is enabled by sugar modification. For that we synthesized 3′-amino-3′-deoxythymidine 5′-triphosphate (**6d**), 3′-amino-2′,3′-dideoxycytidine 5′-triphosphate (**6c**), 3′-amino-2′,3′-dideoxyadenosine 5′-triphosphate (**6a**), as well as 3′-amino-2′,3′-dideoxyguanosine 5′-triphosphate (**6b**). In 1984, *Krayevsky* and coworkers showed that amino-modified triphosphates inhibit DNA polymerase I and DNA polymerase α and β [9] [10], but sequencing DNA was not possible without mistakes. Here we describe in detail our results on the synthesis and capability of the 3′-aminonucleotides to serve as very potent and base-specific chain terminators in DNA-sequencing reactions by the use of new sequencing enzymes including fluorescent analysing methods. In addition, we are able to use the 3′-amino group for attaching fluorescent dyes.

2. Results and Discussion. – 2.1. *Synthesis of 3′-Amino-Modified Nucleotides.* The key characteristic of the modified nucleosides is the NH_2 group at the 3′-position of the sugar ring. Introduction of the NH_2 group was achieved by reduction of the azido group [11]. We used different routes to introduce the azido function for each nucleotide. The 3′-azido-3′-deoxythymidine (AZT; **1**) was synthesized as described by *Horwitz et al.* [12], and 3′-azido-2′,3′-dideoxy-N^4-isobutyrylcytidine (**2**) was obtained by inversion of configuration at C(3′) [9]. Base protection with isobutyric anhydride was achieved by the method of *Gait* [13].

In our approach to the purine block, 3′-azido-2′,3′-dideoxyguanosine (**3**) was made through a transglycosylation [14], and 3′-azido-2′,3′-dideoxy-N^6-isobutyryladenosine (**4**) was synthesized by inversion of the configuration at the 3′-position on substitution of the OH with the N_3 group [15]. Base protection with isobutyric anhydride was again achieved by the method of *Gait* [13].

Scheme. *Synthesis of 3′-Modified Nucleoside 5′-Triphosphates*



a) 1. 2-Chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one in dioxane (**5a, c, d**) or $POCl_3$ in $PO(OEt)_3$ (**5b**); 2. tributylammonium pyrophosphate (**5a, c, d**) or tributylammonium pyrophosphate in DMF (**5b**); 3. conc. ammonia (**5a, c**). *b*) PPh_3, pyridine/H_2O. *c*) Fluorescein isothiocyanate, DMF/$NaHCO_3$ buffer.

Straightforward conversion of the 3′-azidonucleosides **1**, **2**, and **4** to the corresponding triphosphates **5a**, **c**, **d** (*Scheme*) was performed by the one-pot procedure of *Ludwig* and *Eckstein* [16] in 45–60° yields and removal of the isobutyryl protecting group with aqueous ammonia (**5a**, **c**). Introduction of the triphosphate group to the unprotected guanosine derivative **3** was achieved by a milder procedure. Phosphorylation of **3** with $POCl_3$ in triethyl phosphate gave predominantly nucleoside 5′-phosphorodichloridate. To eliminate the influence of HCl, this phosphorylation was carried out in the presence of the base *N*,*N*,*N*′,*N*′-tetramethylnaphthalene-1,8-diamine as proposed by *Kovacs* and *Ötvös* [17]. The 5′-phosphorodichloridate of **3** was transformed into the nucleotide **5b** by a short treatment with tributylammonium pyrophosphate in DMF followed by neutral hydrolysis and purification by anion-exchange chromatography. All triphosphates showed the characteristic ^{31}P-NMR spectra. The presence of the triphosphate groups was also tested by enzymatic degradation of the nucleotides using alkaline phosphatase followed by HPLC analysis (MeCN/$(Et_3NH)OAc$ buffer).

For reduction to the 3′-aminonucleotides **6a–d**, we applied the *Staudinger* reaction [11] [18] using PPh_3 in pyridine and subsequent hydrolysis with aqueous ammonia. This *Staudinger* reaction is one of the mildest and most selective routes to convert azides to amines. The purification of the products of the previous reaction steps was achieved by anion-exchange chromatography (*Sephadex A-25*, $(Et_3NH)HCO_3$ buffer gradient (0–0.8M)). For labeling the nucleotides with fluorescein, a tenfold excess of fluorescein 5-isothiocyanate (FITC) solution was added to **6a–d** in $NaHCO_3$ buffer (1M, pH 9.6) to give directly the 3′-fluorescein derivatives **7a–d** of the 3′-deoxynucleotide 5′-triphosphates. After purification with *Sephadex G-10*, the yield was *ca.* 70%.

In principle, the 3′-NH_2 group of the nucleotides **6a–d** allows their reaction with various types of compounds, *i.e.* any group reacting with a nucleophilic NH_2 group can be attached. Furthermore, we could never detect an intramolecular cyclization involving the triphosphate and the 3′-NH_2 group. An important aspect is the pH of the solution. Best results for the labeling reaction **6a–d** → **7a–d** were obtained at pH 9.6. The reaction is slower at lower pH due to the protonation of the NH_2 function.

2.2. *Application of 3′-Amino-Modified Nucleotides in Sequencing Reactions Using Conventional Techniques.* In fluorescence-based DNA-sequencing methods, the fluorescent dye is introduced without exception prior to the enzymatic reaction. This procedure leads to problems during the sequencing reaction. Dye-labeled primers sometimes cause problems with the hybridization reaction, and most base-labeled nucleotides are not well accepted by standard enzymes. Our approach to these problems is to use chain terminators with an NH_2 group at the 3′-position of the sugar. The high reactivity of the NH_2 group permits a fluorescent dye to be introduced after the enzymatic reaction. For this purpose, we had to test the ability of amino-modified nucleotides to serve as potent chain terminators for standard sequencing enzymes. Their performance was tested by sequence analysis of the bacteriophage vector M13mp18 using the universal primer and the M13 (lacZ) primer. In *Fig. 1*, the analyzed DNA sequence of M13mp18 vector is shown. Amino terminators **6a–d** were tested with several enzymes: T7 DNA polymerase, sequenase version 2.0, *Thermus aquaticus* (Taq) DNA polymerase and *Thermus thermophilus* (Tth) DNA polymerase. All aminonucleotides **6a–d** were well accepted by the mentioned enzymes and showed perfect termination quality. Here we want to show in detail our studies using T7 DNA polymerase, Taq DNA polymerase, and sequenase version 2.0.

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1 AACGC CAGGG TTTTC CCAGT CACGA CGTTG TAAA ACGACG GCCAG TGCCA AGCTT GCATG 60

61 CCTGC AGGTC GACTC TAGAG GATCC CCGGG TACCGAGCTC GAATTCGTAA TCATGGTCAT 120

121 AGCTGTTTCC TGTGTGAAAT TGTTATCCGC TCACAATTCC ACACAACATA CGAGCCGGAA 180

181 GCATAAAGTG TAAAG CCTGG GGTGCCTAAT GAGTGAGCTA ACTCACATTA ATTGCGTTGC 240

241 GCTCACTGCC CGCTTTCCAG TCGGGAAACC TGTCGTGCCA GCTGCATTAA TGAATCGGCC 300

301 AACGCGCGGG GAGAGGCGGT TTGCGTATTG GGCGC CAGGG TGGTTTTTCT TTTCACCAGT 360

361 GAGACGGGCA ACAGCTGATT GCCCTTCACC GCCTGGCCCT GAGAGAGTTG CAGCAAGCGG 420

421 TCCACGCTGG TTTGCCCCAG CAGGCGAAAA TCCTGTTTGA TGGTGGTTCC GAAATCGGCA 480

481 AAATCCCTTA TAAATCAAAA GAATAGCCCG AGATAGGGTT GAGTGTTGTT CCAGTTTGGA 540

541 ACAAGAGTCC 550
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Fig. 1. *Analyzed sequence of M13mp18 DNA with the hybridization area of the M13 (lacZ) primer* (underlined) *and the universal primer* (twice underlined)

Termination efficiency was tested by conventional radioactive techniques and primer fluorescence based method. In *Fig. 2* is demonstrated a section of an autoradiogram produced by manual DNA-sequence analysis using sequenase version 2.0 and 3′-amino-modified chain terminators. As a reference, the sequencing reaction was carried out with conventional $d^2'd^3'NTP$'s (2′,3′-dideoxynucleotide 5′-triphosphates; $d^2'd^3'ATP$ (lane *1*), $d^2'd^3'GTP$ (*2*), $d^2'd^3'CTP$ (*3*), and $d^3'TTP$ (*4*)). In lanes *5–8*, termination was achieved by the use of compounds **6a–d** in sequencing reaction mixtures including Mn^{2+} ($d^2'd^3'ATP(NH_2)^3'$ (lane *5*), $d^2'd^3'GTP(NH_2)^3'$ (*6*), $d^2'd^3'CTP(NH_2)^3'$ (*7*), and $d^2'TTP(NH_2)^3'$ (*8*)). Labeling was achieved using (α-^{35}S)$d^2'ATP$. As described by *Tabor* and *Richardson* [19], incorporation of dideoxynucleotides by T7 DNA polymerase is more efficient, when Mn^{2+} rather than Mg^{2+} is used for catalysis. For this reason, we used Mn^{2+} catalysis in the sequencing reactions. The addition of Mn^{2+} renders especially the 3′-amino-modified nucleotides more potent chain terminators. In all probability, the furanose moiety in the Mn^{2+}-nucleotide complex shows a changed conformation, in which substituents at the 3′-position are put away from the active site of the enzyme. Studies of different concentrations of derivatives **6a–d** in the termination-reaction mixtures revealed that the 3′-aminodideoxynucleotides are accepted by the enzyme like the usual dideoxy analogues when we used 12 μM solutions including Mn^{2+}. *Fig. 3a* shows in detail the concentrations needed for perfect readable sequences, when $d^2'd^3'NTP(NH_2)^3'$'s were used instead of $d^2'd^3'NTP$'s. No significant difference concerning the acceptance of each of the 3′-aminonucleotides by sequenase version 2.0 could be detected. Consequently, we used the same concentrations of chain terminators in each base reaction.

Data for the thermostable Taq DNA polymerase are shown in *Fig. 2b*. In this case, M13mp18 DNA was hybridized with the M13 (lacZ) primer. Each sample was loaded in the order $d^2'd^3'ATP$ (lane *1*), $d^2'd^3'GTP$ (*2*), $d^2'd^3'CTP$ (*3*), $d^3'TTP$ (*4*), $d^2'd^3'ATP(NH_2)^3'$ (*5*), $d^2'd^3'GTP(NH_2)^3'$ (*6*), $d^2'd^3'CTP(NH_2)^3'$ (*7*), and $d^3'TTP(NH_2)^3'$ (*8*). This experiment

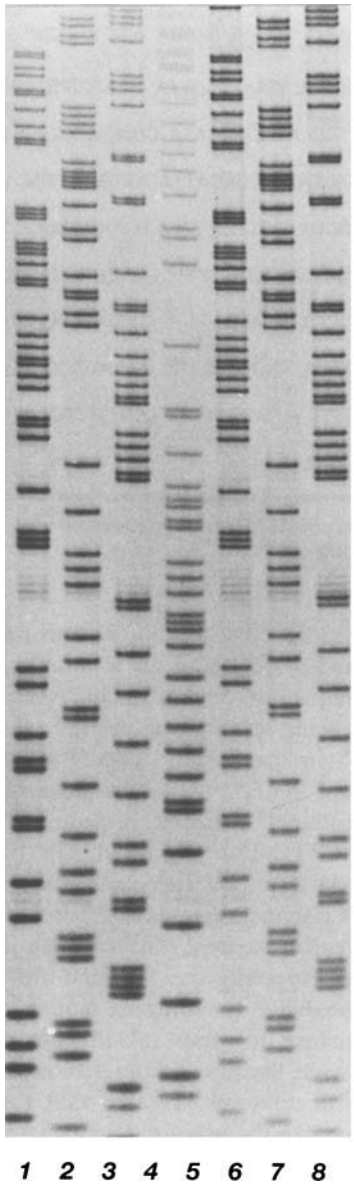

Fig. 2a. *Blow up of a section of an autoradiogram of M13mp18 DNA* (universal primer) *produced by manual DNA-sequence analysis with use of sequenase version 2.0 and 3′-amino-modified chain terminators.* Lanes *1–4*: $d^{2'},d^{3'}$ATP (*1*), $d^{2'},d^{3'}$GTP (*2*), $d^{2'}d^{3'}$CTP (*3*), and $d^{3'}$TTP (*4*) terminated reactions (standard); lanes *5–8*: $d^{2'}d^{3'}$ATP$(NH_2)^{3'}$ (*5*), $d^{2'}d^{3'}(NH_2)^{3'}$ (*6*), $d^{2'}d^{3'}(NH_2)^{3'}$ (*7*), and $d^{2'}d^{3'}$TTP$(NH_2)^{3'}$ (*8*) terminated reactions including manganese.

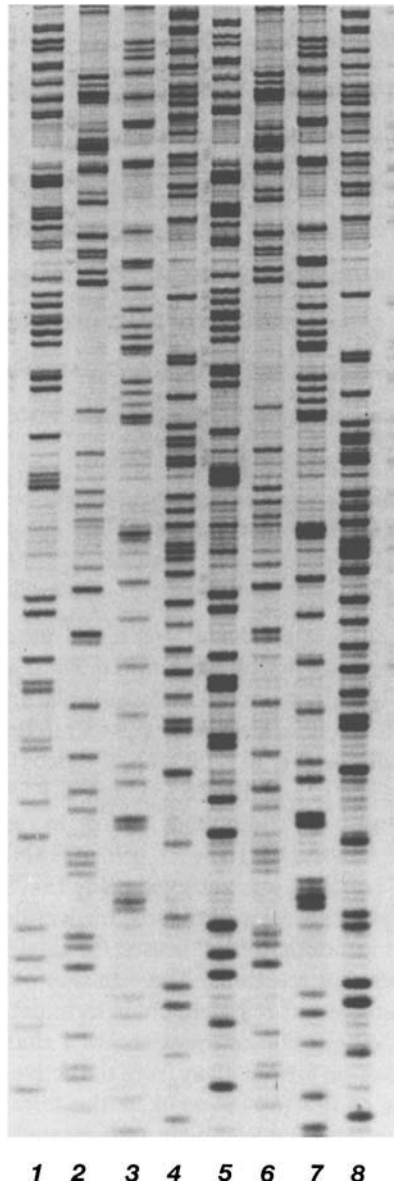

Fig. 2b. *Blow up of a section of an autoradiogram of M13mp18 DNA* (M13 (lacZ) primer) *produced by manual DNA-sequence analysis with use of* Thermus aquaticus *DNA polymerase and 3′-amino-modified chain terminators.* Lanes *1–4*: $d^{2'}d^{3'}$ATP (*1*), $d^{2'}d^{3'}$GTP (*2*), $d^{2'}d^{3'}$CTP (*3*), and $d^{3'}$TTP (*4*) terminated reactions (standard); lanes *5–8*: $d^{2'}d^{3'}$ATP$(NH_2)^{3'}$ (*5*), $d^{2'}d^{3'}$GTP$(NH_2)^{3'}$ (*6*), $d^{2'}d^{3'}$CTP$(NH_2)^{3'}$ (*7*), and $d^{3'}$TTP$(NH_2)^{3'}$ (*8*) terminated reactions.

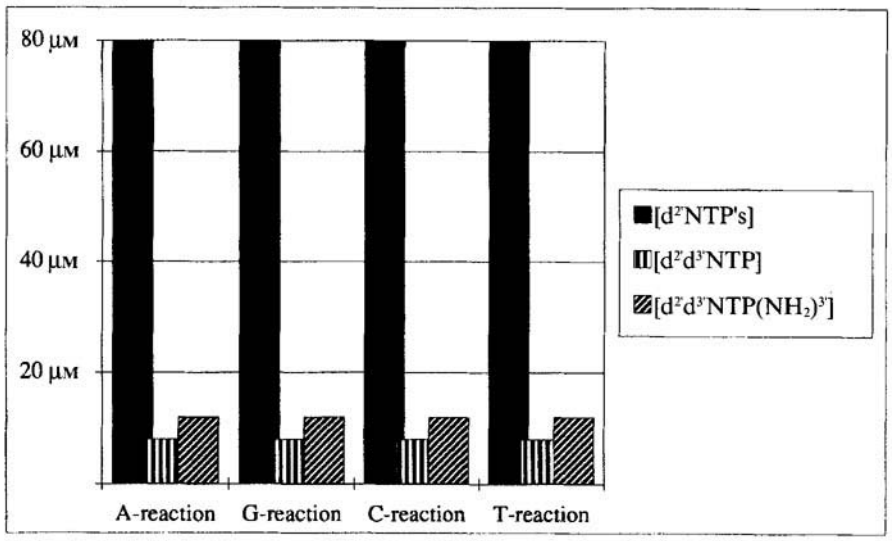

Fig. 3a. *Diagram showing the relation of d²' NTP's/chain terminators* ($d^{2'}d^{3'}$NTP and $d^{2'}d^{3'}NTP(NH_2)^{3'}$) *in sequencing reactions with sequenase version 2.0.* Optimal results with $d^{2'}d^{3'}NTP(NH_2)^{3'}$'s using 12 µM concentrations in termination mixture including manganese; optimal results with $d^{2'}d^{3'}$NTP using 8 µM concentrations in termination mixture. Concentrations of $d^{2'}$NTP's 80 µM in each reaction.

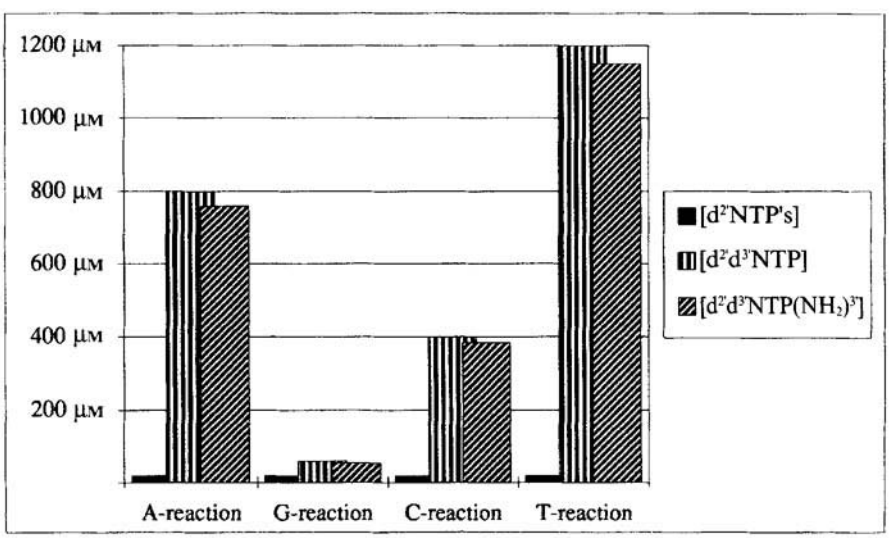

Fig. 3b. *Diagram showing the relation of d²' NTP's/chain terminators* ($d^{2'}d^{3'}$NTP and $d^{2'}d^{3'}(NH_2)^{3'}$) *in sequencing reactions with* Thermus aquaticus *DNA polymerase.* Optimal results with $d^{2'}d^{3'}(NH_2)^{3'}$'s using for A 760 µM, for G 55 µM, for C 385 µM and for T 1150 µM concentrations in termination mixtures. Optimal results with $d^{2'}d^{3'}$NTP using for A 800 µM, for G 60 µM, for C 400 µM, and for T 1200 µM concentrations in termination mixtures. Concentrations of $d^{2'}$NTP's 20 µM in each reaction.

demonstrates that 3′-amino terminators have also good stability at higher temperatures and are well accepted by the enzyme. In this case, we were able to read sequences up to 400 bases. *Fig. 3b* gives in detail the concentrations needed for perfectly readable sequences, if $d^{2'}d^{3'}NTP(NH_2)^{3'}$'s and Taq DNA polymerase were used instead of $d^{2'}d^{3'}NTP$'s. The diagram indicates that $d^{2'}d^{3'}NTP(NH_2)^{3'}$'s are better accepted by the enzyme than dideoxy analogues. Amino triphosphates cannot be used in equal concentrations for each base reaction, because the enzyme distinguishes between the bases as terminators as in the case of $d^{2'}d^{3'}NTP$'s.

A further interesting phenomenon evident from the data in *Figs. 2a* and *2b* is that the migration time for short polymers terminated with $d^{2'}d^{3'}NTP$ and 3′-aminonucleotides is different. Protonated amino groups of 3′-amino strands migrate like $n + 1$ strands because of one compensated negative charge of the phosphate backbone. For sequencing reactions with Taq DNA polymerase, we used standard protocols.

2.3. *Application of 3′-Modified Nucleotides in Sequencing Reactions Using Fluorescence Analysis.* Since 3′-aminonucleotides proved to be excellent chain terminators, we tested their performance in automated DNA sequencing. DNA Fragments produced by the enzymatic chain-extension reaction with T7 DNA polymerase were separated by polyacrylamide-gel electrophoresis, detected, and identified when they migrated past the laser detection system of an *A. L. F.* DNA sequencer. Fluorescent signals were provided by addition of a fluorescein-labeled universal primer. In *Fig. 4* is shown the processed output of this experiment. Best results were obtained with 30 μM concentrations of compounds **6a–d** and 150 μM concentrations of $d^{2'}NTP$'s including Mn^{2+} in the sequencing reactions. This output demonstrates the possibility of obtaining a high-resolution DNA sequence with 3′-amino chain terminators and T7 DNA polymerase. The DNA sequence of M13mp18 DNA was analyzed up to 520 bases without detectable errors. No significant difference in the acceptance of each of the 3′-aminonucleotides by T7 DNA polymerase could be detected. In each case, we used equally concentrated amino mixtures.

The performance of compounds **7a–d** was tested by sequence analysis of the bacteriophage vector M13mp18 using the universal primer and T7 DNA polymerase. DNA Fragments produced by the enzymatic chain-extension reaction were identified with an *A. L. F.* DNA sequencer. Fluorescent signals were amplified using a fluorescent universal primer, because reduced fluorescence intensities for nucleotides **7a–d** were measured. In all cases, fluorescence emission was quenched more than 80% in H_2O [20]. Nevertheless, all modified nucleotides were accepted by the enzyme and showed equivalent termination quality compared with $d^{2'}d^{3'}NTP$ terminated reactions (data not shown). To circumvent reduced fluorescence intensities of **7a–d**, experiments with a spacer between the dye molecule and the base were performed in our laboratory [20].

3. Conclusion. – In the present work, we demonstrate that 3′-amino-modified nucleotides are well accepted as substrates for today's sequencing enzymes. For each base, we introduced the azido group at the 3′-position of the corresponding nucleosides, converted the azidonucleosides to the triphosphates and then reduced the azido group by PPh_3 and aqueous ammonia to give amino compounds **6a–d**. These nucleoside triphosphates were then tested as terminators. Here we examined all commonly used sequencing enzymes: Tth, Taq sequenase version 2.0, and T7 DNA polymerase. In all experiments, the aminonucleotides showed perfect termination results and high readability in gels. With

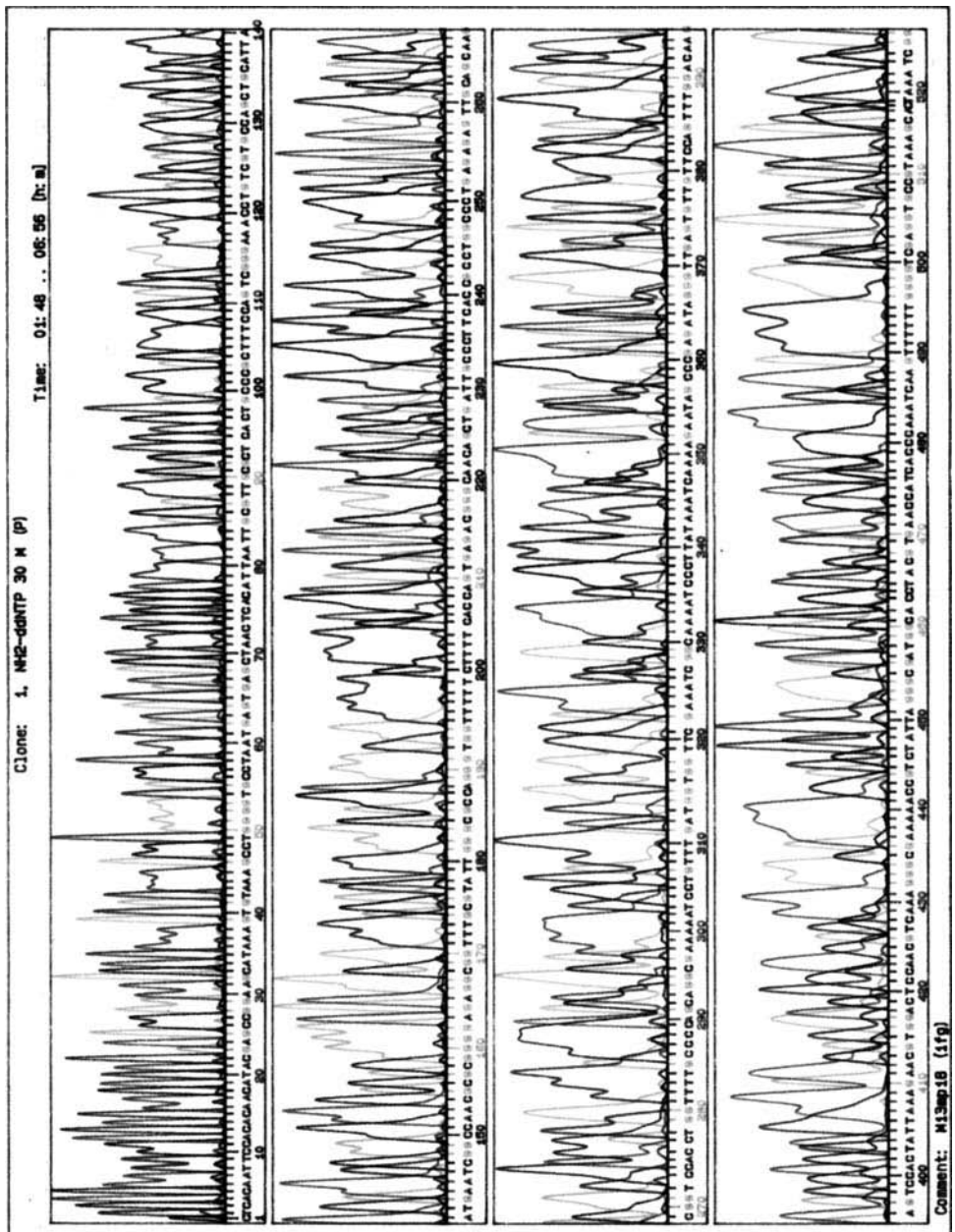

Fig. 4. *Detection of fluorescence emission of an output produced by DNA-sequence analysis with T7 DNA polymerase and 3'-amino-modified chain terminators including manganese from a region of M13mp18* (universal primer) *by the* A.L.F. *DNA sequencer*

an automated DNA sequencer, we were able to read more than 500 bases without error. When amino terminators were used, the presence of Mn^{2+} ions in the sequencing mixtures raised the quality of the sequencing ladders. To show that the 3′-aminopolynucleotides could be labeled, we successfully linked fluorescein 5-isothiocyanate to compounds **6a–d**. Thus, 3′-amino-2′,3′-dideoxynucleotides seem to be ideal chain terminators for current DNA-sequencing technologies. Examination of compounds **7a–d** showed that they were equivalent to d^2d^3NTP as terminators; however, the fluorescence intensities of the labeled nucleotides are highly reduced as a consequence of several fluorescence quenching mechanisms.

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Experimental Part

1. *General.* T7 DNA Polymerase and Tth DNA polymerase were purchased from *Pharmacia LKB*, sequenase from *United States Biochemical Corporation*, Taq DNA polymerase from *Perkin-Elmer Cetus*, (α-^{35}S)d^2ATP (1200 Ci/mmol) from *Amersham-Buchler*, alkaline phosphatase from *Boehringer Mannheim*, and 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one from *Aldrich*. Dry pyridine, DMF, and dioxan were from *Merck* and used as supplied. Tributylammonium pyrophosphate and fluorescein 5-isothiocyanate (FITC) were purchased from *Sigma*, and *N,N,N′,N′*-tetramethylnaphthalene-1,8-diamine from *Aldrich*. Chromatography: *DEAE-Sephadex*; at 4°. HPLC: *Waters Delta Prep 3000*, *Merck Lichrosphere 100 RP-18*, (5 μm, 250 × 4) endcapped column; MeCN in $(Et_3NH)OAc$ buffer (0.1M, pH 7.0), flow 1 ml/min. TLC: precoated *Merck* TLC aluminium sheets. UV: λ_{max} (ε) in nm. IR: $\tilde{\nu}$ in cm^{-1}. Fluorescence: λ_{max} in nm. ^{1}H-NMR Spectra: *Bruker-AMX-400*, *-AM-300-WB*, or *-WH-270* spectrometers; δ in ppm rel. to Me_4Si and H_3PO_4 as internal reference. Fluorescent DNA-sequencing reactions were analyzed on an *A.L.F.* DNA sequencer (*Pharmacia LKB*).

2. *Azidodeoxyribonucleoside 5′-Triphosphates* **5a**, **c**, **d**: *General Procedure.* A soln. of nucleosides **1**, **2**, or **4** (200 μml) in anh. pyridine was evaporated. The residue was dried (P_2O_5), and during all following steps, a low pressure of Ar was maintained. Into the vessel containing **1**, **2**, or **4**, through a septum, was injected anh. pyridine (200 μl) followed by anh. dioxane (600 μl). Freshly prepared 1M 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one in anh. dioxane (200 μl, 200 μmol) was then injected into the well stirred soln. of the azidonucleoside. After 20 min, a mixture of 0.5M tributylammonium pyrophosphate in anh. DMF (600 μl) and Bu_3N (200 μl) was quickly injected. A soln. of 1% I_2 in pyridine/H_2O (98:2 (*v/v*); 4 ml, 314 μmol) was added after 30 min, and the excess of I_2 destroyed by adding a 5% aq. $NaHSO_3$ soln. In the case of **1**, the soln. was evaporated and the residue dissolved in H_2O. In the case of **2** and **4**, the soln. was evaporated, the residue dissolved in H_2O (10 ml) and stirred for 30 min, conc. NH_3 soln. (20 ml) added, the mixture stirred for 6 h at r.t. and then evaporated and the residue dissolved in H_2O. The aq. triphosphate soln. obtained from **1**, **2**, or **4** was deposited on a *DEAE Sephadex A-25* column (1.8 × 45 cm, HCO_3^-) and eluted with a linear gradient of $(Et_3NH)HCO_3$ (0.05–1M). After evaporation and repeated co-evaporation with EtOH, the corresponding triphosphate was identified and quantified by enzymatic degradation. Yields 45–60%. For enzyme digestion, 4 μl of 20 mM 5′-triphosphate in dest. H_2O were incubated with 5 units (1 μl) of alkaline phosphatase in 100 μl of 1M *Tris*·HCl (pH 7.5) at 37° for 3 h. Then the mixture was analyzed by HPLC: only one product, identical with the starting deprotected nucleoside of the corresponding base.

3′-Azido-2′,3′-dideoxyadenosine 5′-Triphosphate (**5a**): Colorless oil. TLC (dioxane/H_2O/NH_3 6:4:1): R_f 0.17. UV (H_2O): 259.5 (14000). IR (KBr): 2110 (N_3). ^{31}P-NMR (D_2O): −6.1 (*d*, P(γ)); −10.9 (*d*, P(α)); −21.9 (*t*, P(β)).

3′-Azido-2′,3′-dideoxycytosine 5′-Triphosphate (**5c**): Colorless oil. TLC (dioxane/H_2O/NH_3 6:4:1): R_f 0.10. UV (H_2O): 272 (10600). IR (KBr): 2110 (N_3). ^{31}P-NMR (D_2O): −6.08 (*d*, P(γ)); −10.93 (*d*, P(α)); −21.93 (*t*, P(β)).

3′-Azido-3′-deoxythymidine 5′-Triphosphate (**5d**): Colorless oil. TLC (i-PrOH/NH_3/H_2O 7:1:2): R_f 0.05. UV (H_2O): 266.5 (9800). IR (KBr): 2110 (N_3). ^{1}H-NMR (D_2O): 1.91 (*s*, Me); 2.47 (*q*, 2 H–C(2′)); 4.21 (*m*, 2 H–C(5′), H–C(4′)); 4.59 (*m*, H–C(3′)); 6.28 (*t*, H–C(1′)); 7.75 (*s*, H–C(6)). ^{31}P-NMR (D_2O): −10.0 (*d*, P(γ)); −12.8 (*d*, P(α)); −23.7 (*t*, P(β)).

3'-Azido-2',3'-dideoxyguanosine 5'-Triphosphate (**5b**). To a soln. of azidonucleoside **3** (0.3 mmol) in $PO(EtO)_3$ (1 ml), *N,N,N',N'*-tetramethylnaphthalene-1,8-diamine (0.45 mmol) was added, followed by $POCl_3$ (35 µl, 0.34 mmol). The mixture was stirred at 0° for 3 h. Into the vessel was injected a mixture of 0.5M bis(tributylammonium) pyrophosphate in anh. DMF (3 ml) and Bu_3N (0.3 ml) under vigorous stirring at 4°. After 5 min, an aq. $(Et_3NH)HCO_3$ soln. (40 ml, 0.5M) was added, the solvent evaporated, and the residue purified and characterized as above: **5b** as colorless crystals. TLC (i-PrOH/NH_3/H_2O 7:1:2): R_f 0.04. UV (H_2O): 254 (13700). IR (KBr): 2210 (N_3). ^{31}P-NMR (D_2O): −9.8 (*d*, P(γ)); −12.7 (*d*, P(α)); −23.1 (*t*, P(β)).

3. *3'-Aminodideoxyribonucleoside 5'-Triphosphates* **6a–d**: *General Procedure.* To a stirred soln. of one of the nucleotides **5a–d** (100 µmol) in pyridine (1.25 ml) was added PPh_3 (150 µmol). After all starting material had reacted (5 h), a 5% aq. NH_3 soln. was added and the mixture stirred for additional 2 h at r.t. The soln. was evaporated, the residue dissolved in H_2O, and this soln. poured onto a *DEAE Sephadex A-25* column (1.5 × 30 cm, HCO_3^-). The product was eluted with a linear gradient of (Et_3NH) HCO_3 (0.05–0.8M). After evaporation and elimination of (Et_3NH) HCO_3, the corresponding triphosphate **6a–d** was identified by enzymatic degradation. Yields 70–80%.

3'-Amino-2',3'-dideoxyadenosine 5'-Triphosphate (**6a**): White crystals. TLC (dioxane/H_2O/NH_3 6:4:1): R_f 0.05. UV (H_2O): 259 (13500). ^{1}H-NMR (D_2O): 2.65–3.06 (*m*, 2 H–C(2')); 4.20–5.00 (*m*, H–C(4'), H–C(3'), 2 H–C(5')); 6.28 (*t*, H–C(1')); 8.21 (*s*, H–C(2)); 8.40 (*s*, H–C(8)).

3'-Amino-2',3'-dideoxyguanosine 5'-Triphosphate (**6b**): Transparent oil. TLC (i-PrOH/NH_3/H_2O 7:1:2): R_f 0.01. UV (H_2O): 254 (13900). ^{1}H-NMR (D_2O): 2.68–2.95 (*m*, 2 H–C(2')); 4.15–5.04 (*m*, H–C(4'), H–C(3'), 2 H–C(5')); 6.78 (*t*, H–C(1')); 7.96 (*s*, H–C(8)).

3'-Amino-2',3'-dideoxycytosine 5'-Triphosphate (**6c**): White crystals. TLC (dioxane/H_2O/NH_3 6:4:1): R_f 0.09. UV (H_2O): 272 (11000). ^{1}H-NMR (D_2O): 2.63–2.86 (*m*, 2 H–C(2')); 3.78–4.34 (*m*, H–C(4'), H–C(3'), 2 H–C(5')); 5.78 (*d*, H–C(5)); 5.91 (*t*, H–C(1')); 7.64 (*d*, H–C(6)).

3'-Amino-3'-deoxythymidine 5'-Triphosphate (**6d**): Colorless oil. TLC (i-PrOH/NH_3/H_2O 7:1:2): R_f 0.02. UV (H_2O): 267 (9800). ^{1}H-NMR (D_2O): 1.90 (*s*, Me); 2.65 (*q*, 2 H–C(2')); 4.24–4.46 (*m*, 2 H–C(5'), H–C(4'), H–C(3')); 6.35 (*t*, H–C(1')); 7.71 (*s*, H–C(6)). ^{31}P-NMR (D_2O): −9.0 (*d*, P(γ)); −12.8 (*d*, P(α)); −23.4 (*t*, P(β)).

4. *3'-Fluorescein-Modified Dideoxyribonucleoside 5'-Triphosphates* **7a–d**: *General Procedure.* To one of the nucleotides **6a–d** (1 µmol) in H_2O (2.5 ml) and 1M Na_2CO_3/$NaHCO_3$ buffer (pH 9.6; 2.5 ml), a 10 mg/ml soln. of fluorescein 5-isothiocyanate in *N,N*-dimethylformamide (1 ml) followed by H_2O (4 ml) was added. The soln. was kept in the dark at r.t. for 4 h. After evaporation, the mixture was applied to a column (1 × 20 cm) of *Sephadex G-10* and eluted with H_2O (800 ml). The first fluorescent band was collected and lyophilized. Yields *ca.* 70%.

*3'-{N^3-[3-Carboxylato-4-(3-oxido-6-oxo-6*H*-xanthen-9-yl)phenyl]thioureido}-2',3'-dideoxyadenosine 5'-Triphosphate* (**7a**): Slightly pink powder. TLC (i-PrOH/$NH_3$$H_2O$ 7:1:4): R_f 0.49. UV (0.1M *Tris* · HCl, pH 8): 259, 493. Fluorescence (0.1M *Tris* · HCl, Ph 8): 522.

*3'-{N^3-[3-Carboxylato-4-(3-oxido-6-oxo-6*H*-xanthen-9-yl)phenyl]thioureido}-2',3'-dideoxyguanosine 5'-Triphosphate* (**7b**): Slightly pink powder. TLC (i-PrOH/NH_3/H_2O 7:1:4): R_f 0.54. UV (H_2O): 254.5, 490. Fluorescence (H_2O): 522.5.

*3'-{N^3-[3-Carboxylato-4-(3-oxido-6-oxo-6*H*-xanthen-9-yl)phenyl]thioureido}-2',3'-dideoxycytosine 5'-Triphosphate* (**7c**): Slightly pink powder. TLC (i-PrOH/NH_3/H_2O 7:1:6): R_f 0.52. UV (0.1M *Tris* · HCl, pH 8): 272, 493. Fluorescence (0.1M *Tris* · HCl, pH 8): 523.

*3'-{N^3-[3-Carboxylato-4-(3-oxido-6-oxo-6*H*-xanthen-9-yl)phenyl]thioureido}-3'-deoxythymidine 5'-Triphosphate* (**7d**): Slightly pink powder. TLC (i-PrOH/NH_3/H_2O 7:1:6): R_f 0.62. UV (H_2O): 267, 490. Fluorescence (H_2O): 521. ^{1}H-NMR (D_2O): 1.94 (*d*, Me); 2.31–2.68 (*m*, 2 H–C(2')); 3.78–4.45 (*m*, 2 H–C(5'), H–C(4'), H–C(3')); 5.95–6.05 (*m*, H–C(1')); 7.24–7.70 (*m*, 9 arom. H); 7.78 (*d*, H–C(6)).

5. *Sequencing Reactions with Nucleotides* **6a–d**. 5.1. *Sequencing with Sequenase Version 2.0.* A mixture of 1 µg of M13mp18 template, 1 µl of universal primer (0.5 pmol), 2 µl of annealing buffer (200 mM *Tris* · HCl pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl), and deionized H_2O to a total volume of 10 µl was heated to 65° and then cooled to 30° within 40 min. To the annealed template and primer were added 1 µl of DTT (= dithiothreitol = *threo*-1,4-dimercaptobutane-2,3-diol; 0.1M), 1 µl of Mn buffer (0.15M sodium isocitrate, 0.1M $MnCl_2$), 2 µl of labeling mix (1.5 µM each d^2'GTP, d^2'CTP, TTP), 1 µl of (α-^{35}S)d^2'ATP (1200 Ci/mmol), and 2 µl of diluted sequenase (5 µl of dilution buffer (10 mM *Tris* · HCl pH 7.5, 5 mM DTT, 0.5 mg/ml BSA (= bovine serum albumine)) + 1 µl of enzyme). The mixture was mixed thoroughly and incubated for 5 min at r.t. Subsequently 2.5 µl of the A, G, C, and T termination mix (A mix: includes 12 µM d^2'd^3'ATP$(NH_2)^{3'}$ (**6a**); G mix: includes 12 µM d^2'd^3'GT$(NH_2)^{3'}$ (**6b**); C mix: includes 12 µM d^2'd^3'CTP$(NH_2)^{3'}$ (**6c**); T mix: includes 12 µm d^3'TTP$(NH_2)^{3'}$ (**6d**); each of the mixes also inlcudes 80 µM d^2'ATP, 80 µM d^2'GTP, 80 µM d^2'CTP, 80 µM TTP, and 50 mM NaCl) was pipetted into 4 test tubes and prewarmed at 37° for at least 1 min. Sequencing reaction was carried out by pipetting 3.5 µl of the labeling mix to each of the vials including

the termination mix and subsequent incubation for 7 min at 37°. Then 4 µl of stop soln. (95% formamide, 20 mM EDTA (ethylenediaminetetraacetic acid), 0.05% bromophenol blue, 0.02% xylene cyanol) were added to each vial, and 3 µl of each mixture were transferred to a separate vial and heated at 80° for 2 min. Immediately afterwards, 2 µl of each mixture was loaded in an appropriate well of a 6% denaturing polyacrylamide sequencing gel.

5.2. *Sequencing with Taq DNA Polymerase.* Into a test tube were pipetted 1 µl of M13 (lacZ) primer (1.3 pmol/µl), 1 µl (α-^{35}S)d$^{2'}$ATP (1200 Ci/mmol), M13mp18 DNA (0.6 pmol), 4 µl of labeling mix (0.57 U/µl Taq DNA polymerase, 0.86 µM d$^{2'}$GTP, 0.86 µM d$^{2'}$TTP, 0.86 µM d$^{2'}$CTP, 143 mM *Tris*·HCl, pH 8.8, 20 mM $MgCl_2$, stabilizers) and distilled H_2O to bring the volume to 20 µl. After mixing and spinning, the mixture was incubated for 5 min at 45°. To each sample, 4 µl of each of the A, G, C, and T termination mixes (A mix: includes 760 µM d$^{2'}$d$^{3'}$ATP(NH$_2$)$^{3'}$ (**6a**); G mix: includes 55 µM d$^{2'}$d$^{3'}$GTP(NH$_2$)$^{3'}$ (**6b**); C mix: includes 385 µM d$^{2'}$d$^{3'}$CTP (**6c**); T mix: includes 1150 µM d$^{3'}$TTP(NH$_2$)$^{3'}$ (**6d**); each of the mixes also includes: 20 µM d$^{2'}$ATP, 20 µM d$^{2'}$GTP, 20 µM d$^{2'}$CTP, and 20 µM TTP) were pipetted into a labeled tube. Into each of the 4 termination mixes as prepared above, 4 µl of the labeling mixture were dispensed, and the tubes were incubated at 72° for 5 min. The tubes were then removed, 4 µl of stop soln. were added to each, the samples were denatured by incubating at 72° for 5 min, and each mixture was loaded into an appropriate well of a 6% denaturing sequencing gel.

5.3. *Sequencing with T7 DNA Polymerase.* The reaction vial for annealing reaction with T7 DNA polymerase contained 1 µg of M13mp18 template, 2 µl of fluorescent universal primer (1 pmol, *Auto Read* Kit, *Pharmacia LKB*), 2 µl of annealing buffer (*Auto Read* Kit), and deionized H_2O to a total volume of 17 µl. The mixture was heated to 60° and then cooled to 30° for 40 min. To the annealed template and primer was added 1 µl of extension buffer (*Auto Read* Kit, containing manganese) and 2 µl of diluted T7 DNA polymerase (5 units/2 µl, *Auto Read* Kit). Meanwhile, 2.5 µl of termination (mix (A mix: includes 30 µM d$^{2'}$d$^{3'}$ATP(NH$_2$)$^{3'}$ (**6a**); G mix: includes 30 µM d$^{2'}$d$^{3'}$GTP(NH$_2$)$^{3'}$ (**6b**) C mix: includes 30 µM d$^{2'}$d$^{3'}$CTP(NH$_2$)$^{3'}$ (**6c**); T mix: includes 30 µM d$^{3'}$TTP(NH$_2$)$^{3'}$ (**6d**); each of the mixes also includes: 1 mM d$^{2'}$ATP, 1 mM d$^{2'}$GTP, 1 mM d$^{2'}$CTP, 1 mM TTP, 50 mM NaCl, and 40 mM *Tris*·HCl pH 7.5) were pipetted into 4 vials and warmed for at least 1 min at 37°. Immediately 4.5 µl of the annealing mix were pipetted into each of the prewarmed termination mixes. After incubation for 7 min at 37°, 4 µl of stop soln. (deionized formamide, blue dextran) was added to each mixture, the mixture then heated for 3 min at 90° and loaded (6 µl) into the appropriate wells of a 5% hydrolink sequencing gel.

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