Newly Synthesized L-Enantiomers of 3'-Fluoro-Modified β-2'-Deoxyribonucleoside 5'-Triphosphates Inhibit Hepatitis B DNA Polymerases But Not the Five Cellular DNA Polymerases α, β, γ, δ, and ϵ Nor HIV-1 Reverse Transcriptase

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Novel β-L-2',3'-dideoxy-3'-fluoro nucleosides were synthesized and further converted to their 5'-triphosphates. Their inhibitory activities against hepatitis B virus (HBV) and duck hepatitis B virus (DHBV) DNA polymerases, human immunodeficiency virus (HIV) reverse transcriptase (RT), and the cellular DNA polymerases α, β, γ, δ, and ϵ were investigated and compared with those of the corresponding 3'-fluoro-modified β-D-analogues. The 5'-triphosphates of 3'-deoxy-3'-fluoro-β-L-thymidine (β-L-FTTP), 2',3'-dideoxy-3'-fluoro-β-L-cytidine (β-L-FdCTP), and 2',3'-dideoxy-3'-fluoro-β-L-5-methylcytidine (β-L-FMetdCTP) emerged as effective inhibitors of HBV/DHBV DNA polymerases (IC_{50} = 0.25−10.4 μM). They were either equally (FTTP) or less (FMetdCTP, FdCTP) effective than their β-D-counterparts. Also the 5'-triphosphate of β-L-thymidine (β-L-TTP) was shown to be a strong inhibitor of these two viral enzymes (IC_{50} = 0.46/1.0 μM). However, all β-L-FdNTPs (also β-L-TTP) were inactive against HIV-RT, a result which contrasts sharply with the high efficiency of the β-D- FdNTPs against this polymerase. Between the cellular DNA polymerases only the β and γ enzymes displayed a critical susceptibility to β-D-FdNTPs which is largely abolished by the β-L-enantiomers. These results recommend β-L-FTdR, β-L-FCdR, and β-L-FMetCdR for further evaluation as selective inhibitors of HBV replication at the cellular level.

Introduction

A series of modified nucleosides with the unnatural L-configuration have been described recently as potent inhibitors of hepatitis B virus (HBV) and human immunodeficiency virus (HIV) replication. These compounds include 2',3'-dideoxy-3'-thia-β-L-cytidine (3TC, Lamivudine),[1] 2',3'-dideoxy-3'-thia-β-L-5-fluorocytidine (L-FTC),[2] 2',3'-dideoxy-β-L-cytidine (L-ddC), 2',3'-dideoxy-β-L-5-fluorocytidine (L-FddC),[3,4] 2'-deoxy-2'-fluoro-5-methyl-β-L-arabinofuranosyluracil (L-FMAU),[5] 2',3'-dideoxy-2',3'-didehydro-β-L-cytidine, and 2',3'-dideoxy-2',3'-didehydro-β-L-5-fluorocytidine.[6] For the most part, they display higher antiviral activity and lower cytotoxicity than their D-counterparts.

The approval of 3TC in combination with AZT for the treatment of HIV infection and its first promising results in the therapy of chronic HBV infection[7] have prompted the synthesis of new nucleosides with the unnatural L-configuration. One of the key factors of this enantioselectivity is the interaction of the 5'-triphosphates of the L-nucleosides with cellular and viral polymerases.

To get direct insights into enantioselectivity at the viral and cellular levels, we decided to prepare the 5'-triphosphates of such 3'-fluoro-modified β-L-pyrimidine nucleosides whose enantiomeric analogues proved to be extremely efficient inhibitors of HIV reverse transcriptase (HIV-RT) (2',3'-dideoxy-3'-fluoro-β-D-thymidine-5'-triphosphate, D-FTTP; 2',3'-dideoxy-3'-fluoro-β-D-uridine-5'-triphosphate, D-FdUTP)[8−10] and HBV DNA polymerase (2',3'-dideoxy-3'-fluoro-β-D-5-methylcytidine-5'-triphosphate, D-FMetdCTP).[11,12] In addition, as D-nucleosides these 3'-fluoro-modified analogues have been shown to be very strong inhibitors of HIV and/or HBV replication at the cellular level.[8,10,13−15] First clinical applications of β-D- FLT as an anti-AIDS drug produced an unexpectedly severe toxicity emphasizing the need for compounds with higher selectivity.[16]

Here we report the synthesis of the 2',3'-dideoxy-3'-fluoro-β-L-analogues of thymidine, uridine, cytidine, and 5-methylcytidine and the inhibitory activity of their 5'-triphosphates (β-L-FTTP, β-L-FdUTP, β-L-FdCTP, and β-L-FMetdCTP) on the HIV reverse transcriptase, HBV and duck hepatitis B virus (DHBV) DNA polymerases, and the cellular DNA polymerases α, β, γ, δ, and ϵ in comparison to the corresponding D-enantiomers.

Chemistry

Methods developed for preparation of the D-nucleosides and their derivatives were modified and used to prepare the new L-analogues for this work, as summarized in Scheme 1. The synthesis of 1-(2,3-dideoxy-

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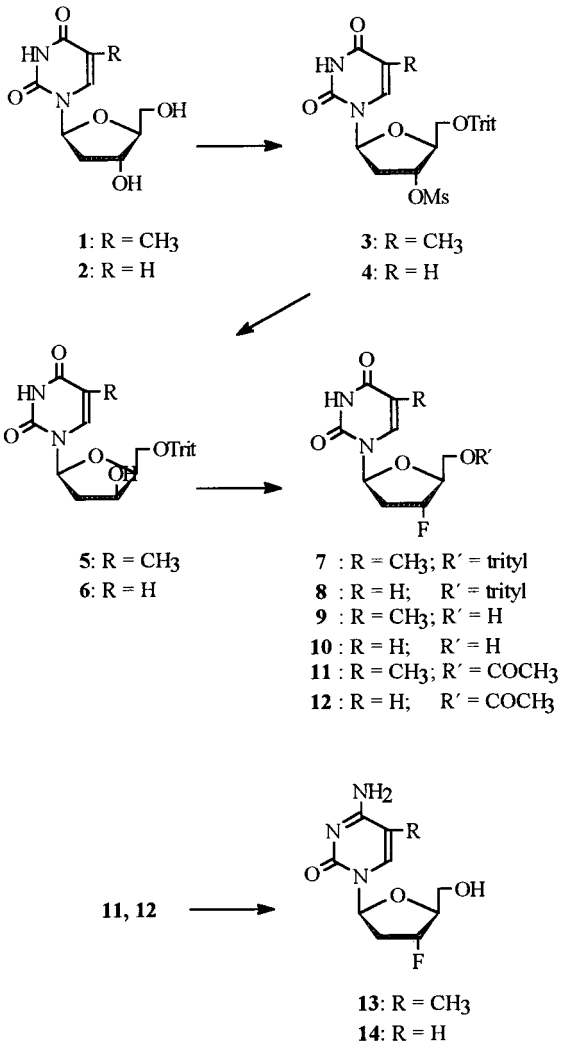
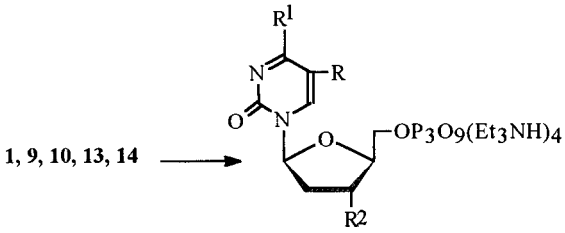
Scheme 1



3-fluoro-β-L-ribofuranosyl)thymine was recently described without experimental details, using a fluoro-1-thiopentofuranoside as the starting synthon[17] in Vorbrüggen-type[18] condensation reactions with silylated thymine. Coupling by this method preferentially results in the α-anomer. Because we were interested in the synthesis of β-L-nucleoside 5′-triphosphates, a more general synthetic approach was chosen.

L-5-Methyluridine[19] was deoxygenated using the method developed by Robins et al.[20] to give compound **1**. 2′-Deoxy-β-L-uridine (**2**) was synthesized from L-arabinose according to the methodology of Holy.[21] Protection of the 5′-hydroxyl function with a trityl group, followed by treatment with methanesulfonyl chloride, gave compounds **3** and **4**, respectively. The configuration in the 3′-position was easily inverted by the action of sodium hydroxide in ethanolic solution to yield the 1-(2-deoxy-5-*O*-trityl-β-L-*threo*-pentofuranosyl)nucleosides **5** and **6**. Treatment of compounds **5** and **6**, respectively, with 2 equiv of (diethylamino)sulfur trifluoride (DAST)[22] in dichloroethane at room temperature overnight produced the desired fluoro compounds **7** and **8** without detectable amounts of elimination products in contrast to results obtained in the α-L-series.[23] Detritylation gave the 1-(2,3-dideoxy-3-fluoro-β-L-*erythro*-pentofuranosyl)nucleosides **9** and **10**. Treatment of the acetylated compounds **11** and **12** with

Scheme 2



15 (L-TTP) : R = CH_3; R^1 = OH; R^2 = OH
16 (L-FTTP) : R = CH_3; R^1 = OH; R^2 = F
17 (L-FdUTP) : R = H; R^1 = OH; R^2 = F
18 (L-FdCTP) : R = H; R^1 = NH_2; R^2 = F
19 (L-FMedCTP) : R = CH_3; R^1 = NH_2; R^2 = F

4-chlorophenyl phosphorodichloridate and 1,2,4-triazole in anhydrous pyridine followed by a solution of ammonium hydroxide and dioxane (3:1, v/v) yielded the corresponding cytidine derivatives **13** and **14**.

Nucleoside 5′-triphosphates of the L-nucleosides were synthesized via the monophosphates[24] as described earlier for the corresponding D-counterparts[8,11,12] according to the method of Hoard.[25]

Biochemical Evaluation

Inhibition of Viral Polymerases by the Triphosphates of β-L- and β-D-3′-Fluoro-Modified Pyrimidine Nucleosides and of β-L-Thymidine (β-L-TTP). The 5′-triphosphates of β-D- and β-L-enantiomers of 3′-fluoro-modified thymidine and 2′-deoxycytidine derivatives were evaluated as inhibitors of HIV-RT and the endogenous DNA polymerases of HBV and DHBV. Earlier, the β-D-enantiomers of FTTP, FdUTP, and FMetdCTP were shown to be extremely efficient inhibitors of HIV-RT (FTTP, FdUTP: IC_{50} = 0.05 and 0.07 μM)[8,10] and HBV DNA polymerase (FTTP, FMetdCTP: IC_{50} = 0.15 and 0.03 μM).[11,12] In contrast, β-L-FTTP (**16**) and β-L-FdUTP (**17**) synthesized here (Scheme 2) lacked any inhibitory effects against HIV-RT (see Table 1). Moreover β-L-FdCTP (**18**) and β-L-FMetdCTP (**19**) lost all inhibitory effects possessed by their enantiomeric counterparts (β-D-FdCTP, β-D-FMetdCTP: IC_{50} = 0.5 and 0.6 μM). In our hands also β-L-TTP (**15**) did not interfere with the activity of the HIV-RT in contrast to what has been reported recently.[26] We find, however, that this compound is a strong inhibitor of the HBV and DHBV DNA polymerases (IC_{50} = 0.46 and 1.0 μM).

Further differences in the stereospecific inhibition of the hepatitis B virus DNA polymerases in comparison to HIV-RT were revealed by the evaluation of β-L- and β-D-enantiomers of the 3′-fluoro-modified dTTP and dCTP analogues. β-L- and β-D-FTTP displayed similar inhibitory effects on HBV DNA polymerase (IC_{50} = 0.25 versus 0.15 μM) as well as on DHBV DNA polymerase (IC_{50} = 0.48 versus 0.17 μM). The same was true for the less effective compounds, β-L- and β-D-FdUTP (see Table 1), whereas hepatitis B virus DNA polymerases proved to be more susceptible to the β-D-enantiomers of FdCTP and FMetdCTP than to their L-enantiomers (Table 1).

Table 1. Effects of β-L-TTP and β-L- and β-D-Enantiomers of 3'-Fluoro-Modified TTP and dCTP Analogues on Viral and Cellular Polymerases

	IC_{50} (μM)[a]							
	viral polymerases			cellular DNA polymerases				
compound	HIV-RT	HBV	DHBV	α	β	γ	δ	ϵ
β-L-TTP (**15**)	>200	0.46 ± 0.08	1.0 ± 0.25	>100	>200	80 ± 15	>200	>100
β-L-FTTP (**16**)	100	0.25 ± 0.06	0.48 ± 0.1	>200	>200	>100	>200	>100
β-D-FTTP	0.05 ± 0.005[b]	0.15 ± 0.02[d]	0.17 ± 0.04	>200[b]	2.20 ± 0.45[b]	1.5 ± 0.26	>200	>100
β-L-FdUTP (**17**)	>200	35 ± 8	>40	>100	>100	>100	>200	>100
β-D-FdUTP	0.07 ± 0.01[c]	25 ± 6[d]	>40	>200[c]	3.0 ± 0.65[c]	3.5 ± 0.9	>100	>100
β-L-FdCTP (**18**)	>100	7.0 ± 1.6	10.4 ± 1.9	>100	>100	>50	>200	>100
β-D-FdCTP	0.5 ± 0.08	0.5 ± 0.1	0.8 ± 0.17	>200	1.0 ± 0.22	2.0 ± 0.34	>100	>100
β-L-FMetdCTP (**19**)	>100	1.9 ± 0.42	2.0 ± 0.45	>200	>200	>50	>200	>100
β-D-FMetdCTP	0.6 ± 0.07	0.03 ± 0.007[e]	0.015 ± 0.005	>200[e]	1.0 ± 0.29[e]	0.5 ± 0.09	100	>100

[a] Concentrations required for a 50% inhibition of the enzymes. Poly(A)·oligo(dT)$_{15}$, poly(dA)·oligo(dT)$_{15}$, or activated DNA and the TTP or dCTP concentrations were used as described in the Experimental Section. For estimation of endogenous HBV/DHBV DNA polymerase activities, the TTP or dCTP concentrations were 0.4 μM. Means of three experiments are given. For DNA polymerase β and γ and the viral polymerases, standard deviations are included. [b] Data from ref 8. [c] Data from ref 10. [d] Data from ref 11. [e] Data from ref 12.

Kinetic studies were performed with **15** and **16**, the most efficient inhibitors of HBV DNA polymerase. The Lineweaver−Burk plots (not shown) indicate a competitive type of inhibition for HBV DNA polymerase by both **15** and **16** with regard to TTP. The K_m value of TTP was estimated to be 0.4 μM, and the K_i values were 0.30 μM for **15** and 0.2 μM for **16**. These results are in contrast to earlier findings demonstrating that HBV DNA polymerase displays some degree of selectivity for the L-enantiomers.[27] Thus L-ddTTP and L-ddCTP derivatives proved to be more active inhibitors of HBV DNA polymerase than their D-counterparts.[28]

Surprisingly, this preference for β-L-enantiomers is abolished by the replacement of the 3'-hydrogen by the bulkier and more electronegative fluoro substituent in these nucleotides. Moreover, FTTP and FdCTP in their L-configurations have even lost all activity as inhibitors of HIV-RT as in the case of ddTTP and ddCTP.[27]

Inhibition of Cellular Polymerases by β-L- and β-D-3'-Fluoro-Modified Pyrimidine Nucleoside Triphosphates. We tested the β-L- and β-D-enantiomers for their inhibition of the cellular DNA polymerases α, β, γ, δ, and ϵ. Table 1 shows that none of the L- or D-enantiomers displayed a significant inhibitory activity against DNA polymerases α, δ or ϵ. In contrast, DNA polymerases β and γ proved to be very susceptible to the fluoro-modified TTP and dCTP analogues but only against their β-D-enantiomers, whereas the corresponding L-enantiomers did not show inhibitory effects against both of these polymerases. These results are in agreement with recent findings demonstrating that the cellular DNA polymerases β and γ are less sensitive against the triphosphates of L-ddC, L-FddC, 3TC, and carbovir compared with their D-counterparts.[29−31] As a consequence, a lower cytotoxicity of 3'-fluoro-modified β-L-analogues might be expected. Experiments are under way to estimate both the antiproliferative activity and the efficiency of the 2',3'-dideoxy-3'-fluoro-β-L-derivatives of thymidine (**9**), 5-methylcytidine (**13**), and cytidine (**14**) to inhibit the HBV replication in Hep G2 2.2.15 cells.

Also β-L-TTP (**15**) did not inhibit any cellular DNA polymerases (Table 1), an observation reported recently to be restricted to the DNA polymerases α, β, δ, and ϵ, whereas a high affinity to the DNA polymerase γ has been reported.[32]

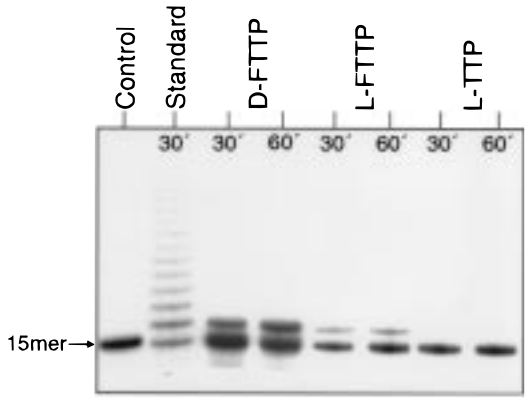


Figure 1. Incorporation of β-D-FTTP and β-L-FTTP (**16**) by HIV-RT into poly(A)·oligo(dT)$_{15}$ in comparison to β-L-TTP (**15**). [5'-^{32}P]-(dT)$_{15}$ (350 nM) was hybridized with poly(A) (3.5 μM) and incubated with 10 μM β-D-FTTP or 50 μM **16** or **15** and 0.1 unit of HIV-RT as described in the Experimental Section for different times. The standard assay was incubated with 10 μM TTP. The products were separated on 20% polyacrylamide−8 M urea gels. The gels were dried and phosphorimaged.

β-L-FTTP (16) and β-D-FTTP as Substrates for HIV-RT. A terminating incorporation of β-D-FTTP into growing oligonucleotide chains by various DNA polymerases was described earlier.[33,34] Here we compared the ability of HIV-RT to incorporate β-L-FTTP (**16**) or β-D-FTTP and β-D-TTP or β-L-TTP (**15**) into poly(A)·oligo(dT)$_{15}$. As can be seen from Figure 1, HIV-RT incorporated significant amounts of **16** but not **15** into poly(A)·oligo(dT)$_{15}$, when given at 50 μM, whereas it used 10 μM β-D-FTTP more efficiently as an alternative substrate. About 30% of the primer was extended by 10 μM β-D-FTTP during 30 min (Figure 1). Such chain-terminating incorporation of L-enantiomers by HIV-RT has been described earlier for the triphosphates of 2',3'-dideoxy-β-L-thymidine, 2',3'-didehydro-2',3'-dideoxy-β-L-thymidine, 3TC, and FTC.[19,35,36] However, these compounds are strong inhibitors of the viral enzyme (K_i values: 0.11, 0.015, 10.6, and 1.6 μM),[7,35,37] whereas the K_i value for **16** was estimated to be 45 μM.

In contrast to the incorporation of some modified β-L-dNTPs by HIV-RT, this enzyme and the cellular DNA polymerases α, β and ϵ proved to be unable to utilize unmodified β-L-dNTPs as substrates.[38]

Conclusions

The newly synthesized β-L-enantiomers of 3'-fluoro-modified pyrimidine nucleoside 5'-triphosphates emerged as effective inhibitors of HBV/DHBV DNA polymerases. They proved to be equally or less effective than their β-D-counterparts. However, the β-L-enantiomers were completely inactive against HIV-RT, whereas the corresponding β-D-enantiomers have been described earlier as very efficient inhibitors of both HIV-RT and HBV DNA polymerase.[8−12]

Inhibition of cellular DNA polymerases β and γ by β-D-FdNTPs, which seems to be mainly responsible for their toxicity at the cellular level, is largely abolished by the β-L-FdNTPs. Therefore, a lower antiproliferative activity of 3'-fluoro-modified β-L-nucleosides can be expected than that described earlier for the corresponding β-D-nucleosides. Indeed, preliminary results show that β-L-FTdR displayed much less antiproliferative activity against MT-4 cells (CD_{50} about 500 μM) than β-D-FTdR (CD_{50} about 1 μM[13]). It remains to be seen whether β-L-FTdR, β-L-FMetCdR, and β-L-FCdR share some of the favorable metabolic properties of other β-L-ddC analogues[39] and of L-FMAU,[40] respectively, causing potent activity against HBV replication at the cellular level.

Experimental Section

DNA polymerase α (0.4 unit) from human placenta (provided by Dr. M. Kukhanova, Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow, Russia) was assayed at 37 °C for 15 min in 20 μL containing 50 mM Tris-HCl, pH 7.5, 10 mM KCl, 2 mM $MgCl_2$, 2 mM β-mercaptoethanol, 100 μg/mL BSA, 0.35 μM poly(dA)·oligo$(dT)_{15}$, and 10 μM [^{3}H]TTP (5000 cpm/pmol) for testing of the TTP-derived inhibitors or 1 μg of activated calf thymus DNA, 5 μM [^{3}H]-dCTP (20 000 cpm/pmol), and 50 μM each of the three other dNTPs and the dCTP-derived inhibitors.

DNA polymerase β (0.25 unit) purified from HeLa cells (MoBiTec, Göttingen) was assayed at 37 °C for 15 min in 20 μL containing 50 mM Tris-HCl, pH 8.7, 100 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 0.4 mg/mL BSA, poly(dA)·oligo$(dT)_{15}$, and [^{3}H]TTP or activated DNA and the three dNTPs, [^{3}H]dCTP, and inhibitors as described above.

DNA polymerase γ (0.5 unit; provided by Dr. H. König, Paul Ehrlich Institut, Langen, Germany) was assayed at 37 °C for 20 min in 20 μL containing 50 mM Tris-HCl, pH 8.2, 150 mM KCl, 5 mM Mg-acetate, 1 mM DTT, 100 μg/mL BSA, 1 μg of activated DNA, 50 μM concentrations each of the three dNTPs, and, on the basis of the inhibitor tested (TTP or dCTP analogues), a 5 μM concentration of the fourth one as a labeled triphosphate ([^{3}H]TTP, 5000 cpm/pmol; [^{3}H]dCTP, 20 000 cpm/pmol).

DNA polymerases δ and ε (0.05 unit), each purified from calf thymus according to Weiser et al.,[41] were assayed at 37 °C for 30 min in 25 μL containing 50 mM Bis-Tris, pH 6.5, 6 mM $MgCl_2$, 1 mM DTT, 250 μg BSA/mL, 3.5 μM poly(dA)·oligo$(dT)_{15}$, and 10 μM [^{3}H]TTP (5000 cpm/pmol) or activated DNA, substrates, and the dCTP analogues as described above. Recombinant PCNA (10 μg/mL)[42,43] was added to each DNA polymerase δ assay.

HIV-RT (0.05 unit; Boehringer, Mannheim) was assayed at 37 °C for 60 min in 20 μL containing 50 mM Tris-HCl, pH 8.0, 150 mM KCl, 5 mM $MgCl_2$, 0.5 mM EGTA, 5 mM DTT, 0.3 mM GSH, 0.35 μM poly(A)·oligo$(dT)_{15}$, and 10 μM [^{3}H]TTP (5000 cpm/pmol) for testing of the TTP-derived inhibitors or 1 μg of activated calf thymus DNA, 5 μM [^{3}H]dCTP (10 000 cpm/pmol), and 50 μM each of the three other dNTPs and the dCTP-derived inhibitors.[8]

For estimation of HBV and DHBV DNA polymerase activities, virus particles were pelleted from HBV- or DHBV-positive sera[11] (>100 pg of HBV (DHBV) DNA/mL) and suspended in 1/40 of the original volume in PBS; 20 μL of the virus suspension was lysed in 2.6 μL of 6% 2-mercaptoethanol in 10% Nonidet P-40, and the endogenous DNA polymerase activities were assayed at 37 °C for 60 min in 50 μL containing 50 mM Tris-HCl, pH 8.1, 60 mM KCl, 40 mM $MgCl_2$, 100 μM concentrations of the three dNTPs, and, on the basis of the inhibitor tested (TTP or dCTP analogues), 0.4 μM [^{3}H]TTP or [^{3}H]dCTP (8500 cpm/pmol).[12]

Chemistry. Reactions were monitored by TLC using precoated silica gel 60-F_{254} sheets, and compounds were detected either with UV light or with a 10% sulfuric acid spray, followed by heating on a hot plate. Elemental analyses were performed by the Institut für Angewandte Chemie, Berlin. Melting points were determed with a Boetius microscope hot stage and are uncorrected. Optical rotations were measured on a Perkin-Elmer model 141 polarimeter and are given in units of 10^{-1} deg cm^2 g^{-1}. ^{1}H NMR spectra were recorded on a Varian Unity 500 NMR spectrometer at 300 MHz using DMSO-d_6 as solvent and tetramethylsilane as internal standard; ^{13}C NMR spectra were obtained at 75 MHz on the same spectrometer. All chemical shifts (δ) are reported in parts per million (ppm), and the coupling constants (*J*) are quoted in Hz. Electron impact mass spectra were measured at 70 eV on the GC/MS-Datensystem HP 5985 B. MALDI time-of-flight (TOF) mass spectra of the triphosphates were recorded on a Bruker Reflex (UV laser) instrument using dihydroxybenzoic acid as the matrix. UV spectra were recorded on a Shimadzu UV-161A spectrometer. For column chromatography Merck silica gel 60 (230−400 mesh) was used. Solvents and reagents were purified and dried by standard procedures.

1-(2-Deoxy-3-*O*-(methylsulfonyl)-5-*O*-(triphenylmethyl)-β-L-ribofuranosyl)thymine (3). A mixture of L-thymidine (**1**) (1.81 g, 7.47 mmol) and triphenylmethyl chloride (2.5 g, 8.97 mmol) in anhydrous pyridine (40 mL) was refluxed for 2.5 h. After the mixture cooled to room temperature, methanesulfonyl chloride (1.8 mL, 22.5 mmol) was added to the ice-cooled solution. The reaction mixture was kept at room temperature overnight. Water (1 mL) was added, and the solution was dropped into 1.2 L of vigorously stirred ice−water. The resulting amorphous precipitate was collected by filtration, washed with water, and dried in vacuo. Compound **2** so obtained (3.9 g, 7 mmol, 93%) was homogeneous by TLC analysis and was used without further purification in the next reaction step. An analytical sample was obtained by preparative TLC. Elution with EtOAc/*n*-hexane (3/1) afforded pure **3** as a foam: $[\alpha]^{20}_D$ +8.74° (*c* = 0.9, $CHCl_3$); ^{1}H NMR (Me_2SO-d_6) δ 1.43 (s, 3H, CH_3) (2H-2' and CH_3SO_2 under Me_2SO), 3.19−3.23 (m, 2H, H-5', H-5''), 4.14 (m, 1H, H-4'), 5.31 (m, 1H, H-3'), 6.15 (dd, 1H, H-1'), 7.14−7.29 (m, trityl), 7.43 (s, 1H, H-6), 11.34 (br s, 1H, NH); ^{13}C NMR (Me_2SO-d_6) δ 11.64 (CH_3), 36.55 (CH_3SO_2), 37.71 (C-2'), 62.98 (C-5'), 80.05 (C-3'), 82.45 (C-4'), 83.55 (C-1'), 86.63 (quaternary trityl), 109.78 (C-5), 127.17−127.91 (aromatic trityl), 135.50 (C-6), 147.66 (CH_3SO_2), 150.22 (C-2), 163.48 (C-4). Anal. ($C_{30}H_{30}N_2O_7S$) C, H, N.

1-(2-Deoxy-5-*O*-(triphenylmethyl)-β-L-xylofuranosyl)-thymine (5). Compound **3** (4.4 g, 7.8 mmol) was dissolved in ethanol (60 mL) and heated at reflux. Sodium hydroxide (0.36 g, 9 mmol) was added, and refluxing was continued for 1.5 h. After cooling to room temperature the reaction mixture was concentrated to 20 mL and poured into water (500 mL). The turbid solution was extracted with chloroform (3 × 75 mL). The extracts were combined and washed successively with a saturated aqueous solution of $NaHCO_3$ and water. The organic phase was dried ($MgSO_4$), filtered, and evaporated. The resulting residue was purified by column chromatography on silica gel with chloroform (1% methanol, 0.1% triethylamine) as eluant to give **5** (2.64 g, 5.45 mmol, 69.7%), which crystallized from acetone: mp 236−239 °C dec; $[\alpha]^{20}_D$ ≃12.51° (*c* = 1, $CHCl_3$); ^{1}H NMR (Me_2SO-d_6) δ 1.59 (s, 3H, CH_3) (2H-2' under Me_2SO), 3.15−3.34 (m, 2H, H-5', H-5''), 4.04 (m, 1H, H-4'), 4.13 (m, 1H, H-3'), 5.16 (s, 1H, 3'-OH), 6.07 (dd, 1H, H-1'), 7.21−7.38 (m, trityl), 7.55 (s, 1H, H-6), 11.23 (br s, 1H, NH); ^{13}C NMR (Me_2SO) δ 12.38 (CH_3), 40.68 (C-2'), 62.94 (C-5'), 68.83 (C-3'), 83.14 (C-4'), 84.04 (C-1'), 85.99 (quaternary trityl),

108.20 (C-5), 126.90−128.19 (aromatic trityl), 136.64 (C-6), 150.39 (C-2), 163.71 (C-4). Anal. ($C_{29}H_{28}N_2O_5$) C, H, N.

1-(2,3-Dideoxy-3-fluoro-5-*O*-(triphenylmethyl)-*β*-L-ribofuranosyl)thymine (7). To a solution of **5** (2.55 g, 5.9 mmol) in dichloromethane (60 mL) was added DAST (1.4 mL), and the reaction mixture was stirred at room temperature for 1 h. The mixture was diluted with chloroform (60 mL) and poured into saturated sodium bicarbonate solution (200 mL). The organic phase was separated, washed with water (20 mL), dried (Na_2SO_4), filtrated, and evaporated in vacuo. The residue was purified by chromatography on a silica gel column using chloroform (1% methanol, 0.1% triethylamine) as eluant. **7** was isolated as a foam (1.45 g, 50.5%): $[\alpha]^{20}_D$ −23.20° (c = 1, $CHCl_3$); ^{1}H NMR (Me_2SO-d_6) δ 1.36 (s, 3H, CH_3), 3.04−3.18 (m, 2H, H-2', H-2''), 3.30−3.62 (m, 2H, H-5', H-5''), 4.17 (dt, 1H, $J_{4'-F}$ = 27.6, H-4'), 5.36 (dd, 1H, $J_{3'-F}$ = 53.7, H-3'), 6.17 (dd, 1H, J = 5.6 and 8.9, H-1'), 7.27−7.35 (m, trityl), 7.44 (s, 1H, H-6), 11.34 (br s, 1H, NH); ^{13}C NMR (Me_2SO-d_6) δ 12.27 (CH_3), 37.36 (C-2'), 63.37 (d, C-5', $J_{F-C5'}$ = 9.8), 82.86 (d, C-4', $J_{F-C4'}$ = 25.7), 86.67 (quaternary trityl), 94.20 (d, C-3', $J_{F-C3'}$ = 174.7), 109.79 (C-5), 127.18−128.13 (aromatic trityl), 135.32 (C-6), 150.27 (C-2), 163.45 (C-4). Anal. ($C_{29}H_{27}N_2O_4F$) C, H, N.

1-(2,3-Dideoxy-3-fluoro-*β*-L-ribofuranosyl)thymine (9). A solution of **7** (1.45 g, 2.98 mmol) in aqueous acetic acid (80%, 50 mL) was heated to 90 °C for 1 h. After cooling the solvent was removed under reduced pressure, and the residue was purified chromatographically on silica gel with chloroform (1.5% methanol) as eluant. Evaporation of the appropriate fractions afforded the title compound (360 mg, 49.5%) which crystallized from ethyl acetate: mp 176−177 °C (methanol); UV (H_2O, pH 7) λ_{max} 265 nm (ϵ 7620), λ_{min} 234 nm (ϵ 2070); MS m/z 244 (M^+), 126 (base + H), 119 ($C_5H_5O_2F$, sugar moiety); $[\alpha]^{20}_D$ +1.44° (c = 0.9, methanol); ^{1}H NMR (Me_2SO-d_6) δ 1.85 (s, 3H, CH_3), 2.19−2.26 (m, 2H, H-2', H-2''), 3.54−3.75 (m, 2H, H-5', H-5''), 4.08 (dt, 1H, H-4', $J_{4'-F}$ = 27.9), 5.17 (br s, 1H, 5'-OH), 5.24 (dd, 1H, J = 4.4, $J_{3'-F}$ = 54.2, H-3'), 6.15 (dd, 1H, J = 5.4 and 9.1, H-1'), 7.63 (s, 1H, H-6), 11.35 (br s, 1H, NH); ^{13}C NMR (Me_2SO-d_6) δ 12.16 (CH_3), 36.78 (C-2', J = 20.1), 60.75 (C-5', J = 11.1), 83.58 (C-1'), 84.67 (C-4', J = 22.4), 94.75 (C-3', $J_{F-C3'}$ = 173.8), 109.66 (C-5), 135.64 (C-6), 150.37 (C-2), 163.54 (C-4). Anal. ($C_{10}H_{13}N_2O_4F$) C, H, N.

1-(5-*O*-Acetyl-2,3-dideoxy-3-fluoro-*β*-L-ribofuranosyl)thymine (11). A mixture of **9** (240 mg, 0.98 mmol) and acetic anhydride (1.5 mL, mmol) in pyridine (6 mL) was kept at room temperature overnight. After removal of the solvent under reduced pressure, the residue was purified by silica gel column chromatography with chloroform (1% methanol) as eluant to give **11** as a white foam (262 mg, 93%): $[\alpha]^{20}_D$ −8.74° (c = 0.9, $CHCl_3$); ^{1}H NMR (Me_2SO-d_6) δ 1.73 (s, 3H, CH_3), 2.02 (s, 3H, CH_3, acetyl), 2.33−2.43 (m, 2H, H-2', H-2''), 4.13−4.19 (m, 2H, H-5', H-5''), 4.26 (dt, 1H, H-4', $J_{4'-F}$ = 26.8), 5.28 (dd, 1H, H-3', $J_{3'-F}$ = 54.7), 6.24 (dd, 1H, H-1', $J_{1'-2'}$ = 8.3, $J_{1'-2''}$ = 6.8), 7.42 (d, 1H, H6), 11.32 (br s, 1H, H−N3); ^{13}C NMR (Me_2SO-d_6) δ 11.70 (CH_3), 20.14 (CH_3, acetyl), 35.70 (C-2', $J_{F-2'}$ = 20.5), 62.77 (C-5'), 81.09 (C-4', $J_{F-4'}$ = 24.1), 83.77 (C-1'), 93.01 (C-3', $J_{F-3'}$ = 175.5), 109.46 (C-5), 135.24 (C-6), 149.98 (C-2), 163.15 (C-4), 169.63 (C═O, acetyl). Anal. ($C_{12}H_{15}N_2O_5F$) C, H, N.

1-(2,3-Dideoxy-3-fluoro-*β*-L-ribofuranosyl)-5-methylcytosine (13). A mixture of 1-(5-*O*-acetyl-2,3-dideoxy-3-fluoro-*β*-L-ribofuranosyl)thymine (286 mg, 1 mmol), 1,2,4-triazole (136 mg, 2 mmol), and 4-chlorophenyl dichlorophosphate (0.244 mL, 1.53 mmol) in pyridine (6 mL) was kept for 3 days at room temperature. Ammonium hydroxide (40 mL) was added, and the solution was allowed to stand overnight. The solvent was removed in vacuo. The resulting residue was dissolved in water (50 mL) and applied on a column of DOWEX W ×8 (H^+-form). Elution with water (800 mL) and with ammonia (5%, 300 mL) gave **13** as crude product. This material was purified by chromatography (silica gel, eluant: chloroform/15% methanol) to give **13** which was isolated as the hydrochloride from methanol/HCl (314 mg, 41%): mp 139−142 °C (2-propanol); MS m/z 243 (M^+ − HCl); UV (H_2O, pH 7) λ_{max} 278 nm (ϵ 8350), λ_{min} 253 nm (ϵ 4320); UV (H_2O, pH 1) λ_{max} 286 nm (ϵ 11 360), λ_{min} 244 nm (ϵ 800); $[\alpha]_D$ −31.91° (c = 1.0, MeOH); ^{1}H NMR (Me_2SO-d_6) δ 1.85 (s, 3H, CH_3), 2.11−2.17 (m, 1H, H-2'), 2.20−2.25 (m, 1H, 2-H''), 3.59−3.52 (m, 2H, H-5', H-5''), 4.13 (m, 1H, $J_{4'-F}$ = 27.4, H-4'), 5.18 (d, 1H, OH-5'), 5.22 (m, 1H, $J_{3'-F}$ = 53.7, H-3'), 6.13 (dd, 1H, $J_{1'-2'}$ = 5.4, $J_{1'-2''}$ = 5.9, H-1'), 7.79 (s, 1H, H-6), 8.09 (br s, 1H, NH_2), 8.23 (br s, 1H, NH_2); ^{13}C NMR (Me_2SO-d_6) δ 30.59 (CH_3), 42.88 (C-2', $J_{F-2'}$ = 20.3), 65.86 (C-5'), 90.17 (C-1'), 90.50 (C-4'), 99.97 (C-3', $J_{F-3'}$ = 174.2), 107.30 (C-5), 144.81 (C-6), 156.17 (C-2), 167.46 (C-4). Anal. ($C_{10}H_{14}N_3O_3F$·HCl) C, H, N.

1-(2-Deoxy-3-*O*-(methylsulfonyl)-5-*O*-(triphenylmethyl)-*β*-L-ribofuranosyl)uracil (4). 2'-Deoxy-*β*-L-uridine (11.41 g, 50 mmol) was dissolved in pyridine (250 mL), and triphenylmethyl chloride (16.7 g, 60 mmol) was added. The mixture was heated to 100 °C for 1 h. The solution was chilled to 0 °C followed by the addition of methanesulfonyl chloride (12 mL, 60 mmol). After workup similar to that for **2**, precipitation in water yielded **4** as a solid (21.4 g, 39 mmol, 78%): ^{1}H NMR (Me_2SO-d_6) δ (2H-2', 3H (mesyl group) under Me_2SO), 3.32−3.35 (m, 2H, H-5', H-5''), 4.17 (t, 1H, H-4'), 5.30 (d, 1H, H-3'), 5.46 (d, 1H, H-5, J_{5-6} = 8.1), 6.15 (dd, 1H, H-1'), 7.32−7.38 (m, 15H, trityl), 7.64 (d, 1H, H-6, J_{6-5} = 8.1), 11.41 (s, 1H, NH); ^{13}C NMR (Me_2SO) δ 36.49 (mesyl), 37.62 (C-2'), 62.70 (C-5'), 79.54 (C-3'), 82.42 (C-4'), 84.16 (C-1'), 86.56 (quaternary trityl), 101.74 (C-5), 127.89−128.17 (aromatic trityl), 140.42 (C-6), 150.15 (C-2), 163.38 (C-4). Anal. ($C_{29}H_{28}N_2O_7S$) C, H, N.

1-(2-Deoxy-5-*O*-(triphenylmethyl)-*β*-L-xylofuranosyl)uracil (6). A mixture of **4** (28.0 g, 51 mmol), sodium hydroxide (3.4 g, 85 mmol), and ethanol (400 mL) was refluxed for 2 h. After workup similar to that for **3**, purification by silica gel column chromatography (chloroform−methanol−triethylamine, 98:2:0.1) gave **6** (11.2 g, 23.8 mmol, 46.6%) which was crystallized from methanol: mp 224−226 °C; $[\alpha]^{20}_D$ −6.37° (c = 1, $CHCl_3$); ^{1}H NMR (Me_2SO-d_6) δ (2H-2' under Me_2SO), 3.20−3.34 (m, 1H, H-5'), 3.37−3.40 (m, 1H, H-5''), 4.08−4.12 (m, 1H, H-4'), 4.18 (m, 1H, H-3'), 5.27 (d, 1H, OH-3'), 5.55 (d, 1H, H-5, J_{5-6} = 8.1), 6.12 (dd, 1H, H-1'), 7.36−7.44 (m, 15H, trityl), 7.73 (d, 1H, H-6, J_{6-5} = 8.1), 11.28 (s, 1H, NH); ^{13}C NMR (Me_2SO) δ 40.79 (C-2'), 60.79 (C-5'), 68.73 (C-3'), 83.40 (C-4'), 84.28 (C-1'), 85.98 (quaternary trityl), 100.88 (C-5), 126.92−128.19 (aromatic trityl), 140.95 (C-6), 150.41 (C-2), 163.14 (C-4). Anal. ($C_{28}H_{26}N_2O_5$) C, H, N.

1-(2,3-Dideoxy-3-fluoro-5-*O*-(triphenylmethyl)-*β*-L-ribofuranosyl)uracil (8). Compound **6** (10.3 g, 21.8 mmol) was suspended in dichloromethane (250 mL), and DAST (3.3 mL, 25 mmol) was added. Treatment according to the procedure for **7** gave **8** as a solid material (4.9 g, 10.4 mmol, 47.7%): $[\alpha]^{20}_D$ −28.51° (c = 0.9, $CHCl_3$); ^{1}H NMR (Me_2SO-d_6) δ 2.33−2.38 (m, 1H, H-2'), 2.41−2.48 (m, 1H, H-2''), 3.20−3.24 (m, 1H, H-5'), 3.32−3.38 (m, 1H, H-5''), 4.26 (dt, 1H, H-4', $J_{F-4'}$ = 26.2), 5.35 (dd, 1H, H-3', $J_{F-3'}$ = 54.3), 5.43 (d, 1H, H-5, J_{5-6} = 8.2), 6.16 (m, 1H, H-1'), 7.35−7.39 (m, 15 H, trityl), 7.59 (d, 1H, H-6, J_{6-5} = 8.2), 11.38 (br s, 1H, NH); ^{13}C NMR (Me_2SO) δ 36.91 (C-2', $J_{F-2'}$ = 20.9), 63.16 (C-5', $J_{F-5'}$ = 9.8), 82.98 (C-4', $J_{F-4'}$ = 25.2), 84.26 (C-1'), 86.60 (quaternary trityl), 93.37 (C-3', $J_{F-3'}$ = 175.1), 127.94−128.14 (aromatic trityl), 140.14 (C-6), 150.18 (C-2), 162.78 (C-4). Anal. ($C_{28}H_{25}N_2O_7F$) C, H, N.

1-(2,3-Dideoxy-3-fluoro-*β*-L-ribofuranosyl)uracil (10). Compound **8** (4.9 g, 10.4 mmol) was treated according to the procedure for **7** to give **10** (2.1 g, 9.1 mmol, 87.7%) as a white solid which crystallized from methanol: mp 187−188 °C; $[\alpha]^{20}_D$ −11.27° (c = 1, H_2O); UV (H_2O, pH 7) λ_{max} 261 nm (ϵ 10 100), λ_{min} 230 nm (ϵ 2400); ^{1}H NMR (Me_2SO-d_6) δ 2.18−2.23 (m, 1H, H-2'), 2.32−2.39 (m, 1H, H-2''), 3.61 (m, 2H, H-5', H-5''), 4.16 (dt, 1H, H-4', $J_{4'-F}$ = 27.6), 5.20 (d, 1H, OH-5'), 5.28 (dd, 1H, H-3', $J_{3'-F}$ = 54.3), 5.67 (d, 1H, H-5, J_{5-6} = 8.1), 6.19 (m, 1H, H-1'), 7.85 (d, 1H, H-6, J_{6-5} = 8.1), 11.36 (br s, 1H, NH); ^{13}C NMR (Me_2SO) δ 37.14 (C-2', $J_{F-2'}$ = 20.1), 60.73 (C-5', $J_{F-5'}$ = 11.3), 83.99 (C-1'), 84.88 (C-4', $J_{F-4'}$ = 22.9), 94.79 (C-3', $J_{F-3'}$ = 173.7), 102.04 (C-5), 140.12 (C-6), 150.33 (C-2), 162.91 (C-4). Anal. ($C_9H_{11}N_2O_4F$) C, H, N.

Table 2. MALDI TOF Mass Spectral Analyses of the 3′-Fluoro-Modified β-L-Nucleoside Triphosphates

compound	calcd for $(M - H)^-$	found $(M - H)^-$
16 (L-FTTP)	$C_{10}H_{15}N_2O_{13}FP_3$: 483.16	482.9
17 (L-FdUTP)	$C_9H_{13}N_2O_{13}FP_3$: 469.13	469.0
18 (L-FdCTP)	$C_9H_{14}N_3O_{12}FP_3$: 468.14	468.4
19 (L-FMedCTP)	$C_{10}H_{16}N_3O_{12}FP_3$: 482.18	482.2

1-(5-*O*-Acetyl-2,3-dideoxy-3-fluoro-β-L-ribofuranosyl)-uracil (12). Compound **10** (1 g, 4.34 mmol) was treated with acetic anhydride (8 mmol) in pyridine (10 mL) for 10 h at room temperature, and the solvent was evaporated to dryness under reduced pressure. The residue was purified by silica gel column chromatography (chloroform−methanol, 4:1) to give **12** (1.02 g, 3.75 mmol, 87.2%) which crystallized from methanol: mp 114−115 °C; $[\alpha]^{20}_D$ −7.08° (c = 1, $CHCl_3$); ^{1}H NMR (Me_2SO-d_6) δ 2.05 (s, 3H, CH_3), 2.34−2.38 (m, 1H, H-2′), 2.43−2.49 (m, 1H, H-2″), 4.19−4.22 (m, 2H, H-5′, H-5″), 4.36 (dt, 1H, H-4′, $J_{4'-F}$ = 27.6), 5.33 (dd, 1H, H-3′, $J_{3'-F}$ = 53.9), 5.70 (d, H-5, J_{5-6} = 8.1), 6.18 (m, 1H, H-1′), 7.65 (d, 1H, H-6, J_{6-5} = 8.1), 11.40 (br s, 1H, NH); ^{13}C NMR (Me_2SO) δ 20.56 (CH_3), 36.41 (C2′, $J_{F-2'}$ = 20.4), 63.23 (C-5′, $J_{F-5'}$ = 10.7), 81.75 (C-4′, $J_{F-4'}$ = 25.9), 84.69 (C-1′), 94.0 (C-3′, $J_{F-3'}$ = 175.5), 102.22 (CO, acetyl), 140.26 (C-5), 150.36 (C-6), 162.96 (C-2), 170.08 (C-4). Anal. ($C_{11}H_{13}N_2O_5F$) C, H, N.

1-(2,3-Dideoxy-3-fluoro-β-L-ribofuranosyl)cytosine (14). A solution of compound **12** (2.6 g, 9.85 mmol), 1,2,4-triazole (1.38 g, 20 mmol), and 4-chlorophenyl dichlorophosphate (2.44 mL, 15 mmol) in pyridine (60 mL) was kept for 3 days under an argon atmosphere at room temperature and worked up according to the procedure for **13**. Compound **14** was crystallized from methanol yielding 1.38 g (6.02 mmol, 61%): mp 217−219 °C; UV (H_2O, pH 7) λ_{max} 270 nm (ε 10 860), λ_{min} 249 nm (ε 1700); UV (H_2O, pH 1) λ_{max} 280 nm (ε 12 700), λ_{min} 250 nm (ε 5650); $[\alpha]^{20}_D$ −35.34° (c = 1.0, H_2O); ^{1}H NMR (Me_2SO-d_6) δ 2.09−2.20 (m, 1H, H-2′), 2.39−247 (m, 1H, H-2″), 3.52−3.57 (m, 1H, H-5′), 3.60−3.64 (m, 1H, H-5″), 4.14 (dt, 1H, H-4′, $J_{F-4'}$ = 27), 5.13 (t, 1H, 5′-OH), 5.27 (dd, 1H, H-3′, $J_{F-3'}$ = 53.7), 5.43 (d, 1H, H-5, J_{5-6} = 7.3), 6.21 (m, 1H, H-1′), 7.20 (br d, 2H, NH_2), 7.77 (d, 1H, H-6, J_{6-5} = 7.3); ^{13}C NMR (Me_2SO) δ 37.71 (C-2′, $J_{F-2'}$ = 20.0), 60.88 (C-5′, $J_{F-5'}$ = 9.1), 84.73 (C-4′, $J_{F-4'}$ = 23.0), 84.96 (C-1′), 94.25 (C-5), 95.0 (C-3′, $J_{F-3'}$ = 173.4), 140.22 (C-6), 154.89 (C-2), 165.48 (C-2). Anal. ($C_9H_{12}N_3O_3F$) C, H, N.

5′-Triphosphates. The 5′-triphosphates were isolated by anion-exchange chromatography on a column (2 × 60 cm) of Fractogel TSK DEAE-650 (M) (Merck) using a linear gradient (0.05−0.3 M) of triethylammonium bicarbonate buffer (pH 7−8) as eluant. The triphosphates were eluted at buffer concentrations of 0.2−0.26 M. The fractions containing the triphosphate were pooled and concentrated under diminished pressure. Residual triethylammonium bicarbonate was removed by repeated coevaporation with deionized water. Purity of the triphosphate solutions was controlled by anion-exchange high-performance liquid chromatography on a WAX column (DuPont, Wilmington) using the buffer system: (A) K_2HPO_4/KH_2PO_4 (pH 7.0, 20 mM) and (B) K_2HPO_4/KH_2PO_4 (pH 7.0, 1 M); linear gradient, 0−50% B in 35 min; flow rate, 1.5 mL/min. The retention times for L-FTTP (**16**), L-FdUTP (**17**), L-FdCTP (**18**), and L-FMedCTP (**19**) were in the range between 25.0 and 25.60 min. Reverse-phase HPLC was conducted using a Kontron liquid chromatograph. Analyses were performed on a Spherisorb 50DS2 column using the following buffer system: (A) aqueous triethylammonium acetate (0.1 M, pH 7) and (B) acetonitrile/water (95/5, v/v); linear gradient, 100−90% buffer A in 30 min; flow rate, 1 mL/min. The retention times for compounds **16**, **17**, **18**, and **19** were 15.01, 12.75, 11.08, and 22.91 min, respectively. The triphosphates used for biochemical evaluation were separated from possible contaminations by this HPLC system. The new L-dNTPs were characterized by MALDI TOF mass spectrometry (Table 2).

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