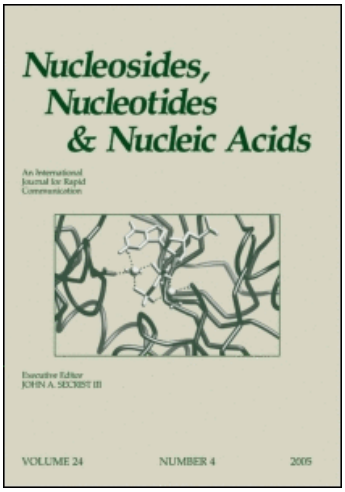

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SYNTHESIS AND ENZYMATIC CHARACTERIZATION OF P_1-THIO-P_2-OXO TRIDEOXYNUCLEOSIDE DIPHOSPHATES HAVING AZT, FdU, OR dT AT THE 3'-POSITION

László Ötvös[a]; Zoltán Bajor[a]; Ferenc Kraicsovits[a]; Gyula Ságí[a]; Zsuzsanna Tegyey[a]
[a] Institute of Chemistry, Hungarian Academy of Sciences Chemical Research Center, Hungary

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SYNTHESIS AND ENZYMATIC CHARACTERIZATION OF P_1-THIO-P_2-OXO TRIDEOXYNUCLEOSIDE DIPHOSPHATES HAVING AZT, FdU, OR dT AT THE 3′-POSITION

László Ötvös,* Zoltán Bajor, Ferenc Kraicsovits, Gyula Sági, and Zsuzsanna Tegyey

Chemical Research Center, Institute of Chemistry, Hungarian Academy of Sciences H-1525 Budapest, P.O. Box 17, Hungary

ABSTRACT

Model compounds for oligonucleotide-prodrugs, P_1-thio-P_2-oxo-trideoxyribonucleoside diphosphates: d[G_sC_oX] and d[T_sA_oX] (X = AZT, FdU or dT) have been prepared, and their hydrolyses by snake venom phosphodiesterase and nuclease S1 are described.

INTRODUCTION

Fundamental differences exist between the mechanism of action of conventional therapeutical agents and antisense oligonucleotides. The majority of currently used drugs modulate the activity of a specific protein by binding directly to the protein. Antisense oligonucleotides reduce the expression of a specific protein by hybridizing to the mRNA or *prem*RNA coding for that protein. In both cases the concentration of a functional protein is reduced in the organism.

3′-Azido-2′,3′-dideoxythymidine (AZT) is the first clinicaly approved drug for the treatment of human immunodeficiency virus (HIV) infections[1]. The effectiveness of AZT and other 2′,3′-dideoxynucleosides of anti-HIV

*Corresponding author.

activity depends on the three-step phosphorylation by cellular enzymes to their 5′-triphosphates. The latter derivatives have the dual ability to act as competitive inhibitors or alternate substrates of HIV reverse transcriptase. Incorporation of a 2′,3′-dideoxynucleoside to viral DNA leads to chain termination[2]. It was found, that a combination of 2′,3′-dideoxyadenosine and S-dC_{14}, a non-complementary phosphorothioate oligomer of antiviral activity, resulted in synergistic inhibition of HIV replication in ATH8 cells system[3]. Similarly, reproducible synergism between AZT and ISIS 2922 (Fomivirsen), a phosphorothioate 21mer, the first oligonucleotide drug against human cytomegalovirus retinitis, was observed for the inhibition of HIV replication in acute infection assays at some concentration combinations[4].

5-Fluorouracil (F-Ura) is used regularly for the treatment of breast cancer, tumors of gastrointestinal tract and other solid tumors[5,6]. F-Ura is converted to 5-fluoro-2′-deoxyuridine 5′-phosphate (FdUMP) in cells, which is the active, cytotoxic form of the drug. FdUMP is a potent inhibitor of thymidylate synthase, an essential enzyme in rapidly proliferating cells[2]. F-Ura may also be fraudulently incorporated into DNA and/or RNA leading to antimetabolite action at the primary genetic level[7,8]. It was demonstrated that the chemosensitivity of human cancer cells to F-Ura could be increased by down-regulating thymidylate synthase expression according to an antisense strategy[9].

The combination of the antiviral/antitumor activity of a nucleoside with the antisense effect of an oligonucleotide might be more effective when the two compounds are connected by a chemical bond of appropriate stability. AZT and 5′-fluoro-2′-deoxyuridine (FdU) were selected to attach by natural phosphodiester bond to the 3′-end of an antisense phosphorothioate[10] oligonucleotide carrier. In this oligonucleotide-prodrug form, FdU and AZT may be protected from premature in vivo catalytic degradations, which could enhance their antitumor/antiviral potential. On the other hand, AZT and FdU will expectedly be liberated, as 5′-phosphates, in cells, because they occupy the 3′-terminal position of the oligomer. FdUMP is the active cytostatic form of F-Ura. AZT 5′-phosphate (AZTMP), as the first member in the phosphorylation chain of AZT, could be effective even in thymidine-kinase deficient cell lines[11]. The outlined approach would be effective only, when AZTMP or FdUMP would be formed in vivo by a significantly higher velocity, than the degradation speed of the antisense phosphorothioate oligonucleotide. This requirement could probably be fulfilled, since natural oligonucleotides are hydrolyzed by nucleases 2–100 times faster, than the phosphorothioate counterparts. A similar approach using antigene oligonucleotides terminated by 3′-AZT has been proposed, recently[12].

In order to study the relative enzymatic stability of 3′-terminal AZT and FdU units toward the dominant nuclease activities in vivo, i.e., 3′-exonucleases and endonucleases, two series of P_1-thio-P_2-oxo trideoxyribonucleoside diphosphates, d[T_sA_oX] and d[G_sC_oX] (X = AZT, FdU or dT), as model compounds have been prepared, and their hydrolyses by snake venom

phosphodiesterase (SVDE) and nuclease S1 have been investigated. Both enzymes are frequently used for structural studies in molecular biology and antisense research. Synthesis and enzymatic hydrolyses of these trimer derivatives are described in the present paper.

RESULTS AND DISCUSSION

Synthesis

Two series of chimeric P_1-thio-P_2-oxo trideoxyribonucleoside diphosphates having AZT, FdU or dT at the 3′ terminal position have been synthesized: d[T_sA_oX] and d[G_sC_oX], where X = AZT (**4,6**), FdU (**9,10**) or dT (**5,11**) (Sch. 1 and 2).

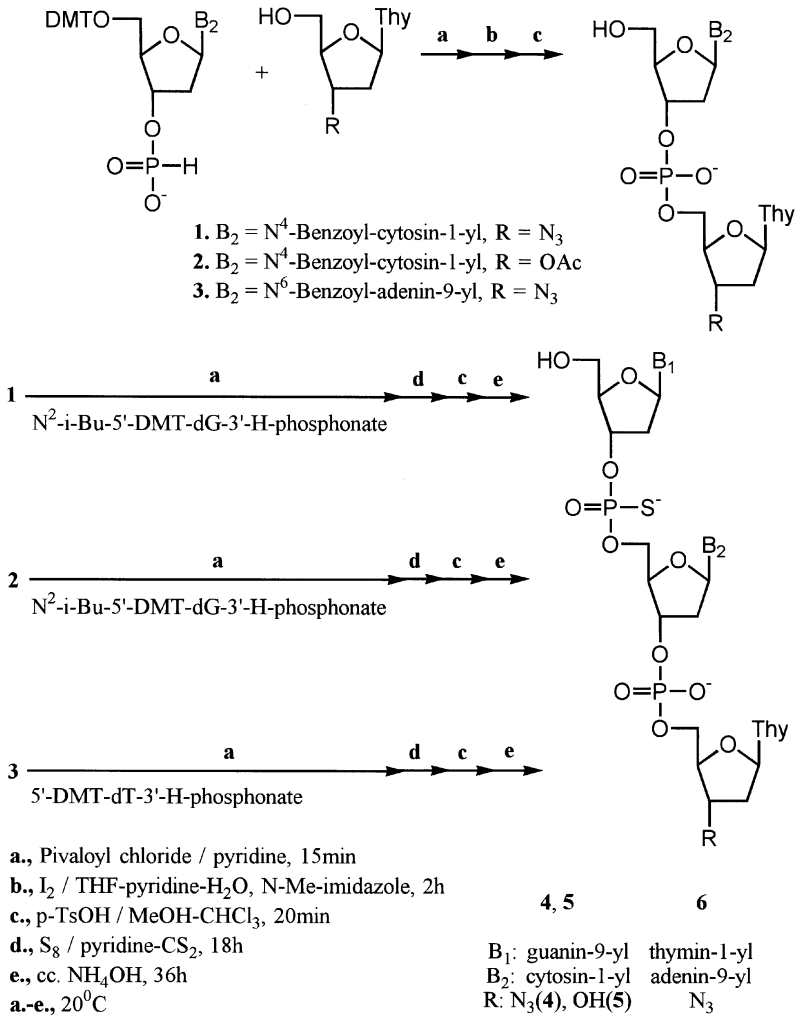
Oligomers terminated by AZT at 3′ position could be synthesized only in the 5′→3′ direction on solid phase.This synthesis route is more laborious and needs less readily accessible starting materials[12]. Therefore, solution phase synthesis has been selected for the preparation of compounds **4,5** and **6** (Sch. 1). Appropriately blocked 2′-deoxycytidine 3′- or 2′-deoxyadenosine 3′-H-phosphonate was coupled with AZT or 3′-O-acetylthymidine (dTAc) to give, after in situ oxidation followed by 5′-deblocking, dimer derivatives **1, 2** or **3**. These dimer derivatives having a free 5′-OH group were isolated by adsorption column chromatography on silica gel and coupled with fully protected 2′-deoxyguanosine 3′- or thymidine 3′-**H**-phosphonate. After thiooxidation by S_8[13] followed by acidic and ammoniacal deprotection, trimers **4, 5** and **6** were isolated by ion-exchange column chromatography on DEAE-Sephadex.

Trimers **9, 10** and **11** were prepared on solid phase according to the phosphoramidite protocol[14] (Sch. 2). Thiooxidation was performed by tetraethylthiuram disulfide (TETD)[15]. 5′-DMT-FdU-LCAA-CPG used for the synthesis of **9** and **10**, was synthesized by derivatization of LCAA-CPG with 5′-O-(4,4′-dimethoxytrityl)-3′-O-succinyl-5-fluoro-2′-deoxyuridine (**8**). Compound **8** was prepared from FdU according to standard procedures. Trimers **9, 10** and **11** were purified by RP-HPLC. Of oligonucleotides containing FdU units, cyclic dimers are known compounds. These were prepared in solution according to the phosphotriester approach[8].

Trimer diastereoisomers were not separated, since antisense oligonucleotides of phosphorothioate type are mixtures of diastereoisomers. Structural verification of trimers **4, 5** and **6** were done by ^1H, ^{31}P-NMR and IR spectroscopies. Trimers **9, 10** and **11** were identified by MS.

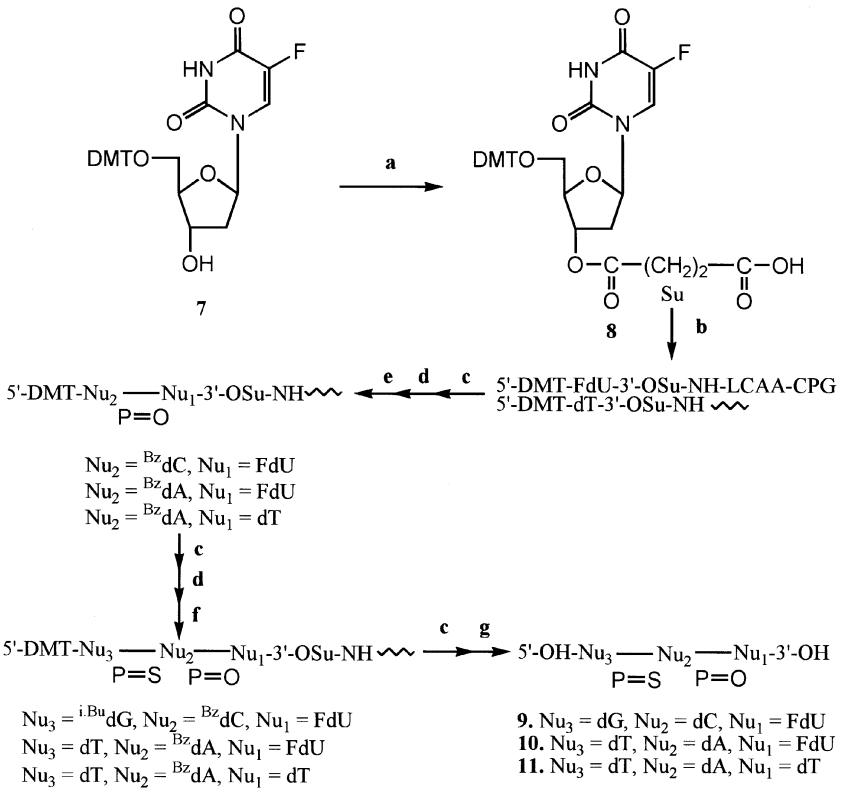
Enzymatic Hydrolysis

As shown in Tables 1 and 2, all the six chimeric trimers are substrates of both SVDE and nuclease S1. Selective cleavage of the phosphodiester bond



Scheme 1. Solution phase synthesis of chimeric P_1-oxo-P_2-thio-trideoxyribonucleoside diphosphates **(4,5,6)**.

was observed under the conditions used for hydrolyses. Thus, a 5′-monophosphate (AZTMP, FdUMP or dTMP) and diastereoisomers of a phosphorothioate dimer (d[GsC] or d[T_sA]) were the products of hydrolysis. Further hydrolysis of R_P-dimers by the R_P-selective SVDE[16] or degradation of the S_P-dimers by the SP-selective nuclease S1[17] could not be detected. It is well known, that oligonucleotides having phosphorothiote backbone are much less efficient substrates, than those with natural phosphodiester bonds, the latter being hydrolyzed by SVDE or nuclease S1 approximately 100 or 14 times faster, respectively[17].



Scheme 2. Solid phase synthesis of chimeric P_1-oxo-P_2-thio-trideoxyribonucleoside diphosphates (**9,10** and **11**).

Data summarized in Table 1 show, that the rate of hydrolysis by SVDE is effected by the nature of the bases/nucleosides on both sides of the scissile bond. Within both series of trimers, compounds having a 3′ dT residue are hydrolyzed by the relatively highest speed. Values of $t_{1/2}$ increased in the order of $\mathbf{5} < \mathbf{9} < \mathbf{4}$ and $\mathbf{11} < \mathbf{10} < \mathbf{6}$, respectively, i.e. dT < FdU < AZT. Rate differences are more pronounced for compounds in which adenine is the base at the 5′ site of the scissile bond (**6, 10, 11**). Trimers of this type are 2–5 times more stable than those with cytosine at the same site. Substitution of 3′ OH by N_3 has a greater effect on the hydrolysis rate than replacement of ring CH_3

Table 1. Hydrolysis of the Phosphodiester Bond in Chimeric P_1-Thio-P_2-oxo Trideoxyribonucleoside Diphosphates by Snake Venom Phosphodiesterase. For Details See Experimental

Compound		$t_{1/2}$(min)
$d[G_sC_oAZT]$	**4**	39
$d[T_sA_oAZT]$	**6**	185
$d[G_sC_oFdU]$	**9**	31
$d[T_sA_oFdU]$	**10**	123
$d[G_sC_oT]$	**5**	17
$d[T_sA_oT]$	**11**	33

Table 2. Hydrolysis of the Phophodiester Bond in Chimeric P_1-Thio-P_2-oxo Trideoxyribonucleoside Diphosphates by Nuclease S1. For Details See Experimental

		$\sim t_{1/2}$(min)	
Compound		**a**	**b**
$d[G_sC_oAZT]$	**4**	15	121
$d[T_sA_oAZT]$	**6**	15	24
$d[G_sC_oFdU]$	**9**	19	159
$d[T_sA_oFdU]$	**10**	10	15
$d[G_sC_oT]$	**5**	21	148
$d[T_sA_oT]$	**11**	9	13

a, faster eluting diastereoisomer.
b, slower eluting diastereoisomer.

by F. Earlier observations are consistent with a requirement by the enzyme for a 5′-phosphorylated nucleoside residue bearing a free or at least non-phosphorylated 3′-OH group[18]. Later it was shown, that a free 3′-OH group is not an essential requirement for the enzyme activity. Similarly to our observations it was shown recently, that oligonucleotides with 3′-terminal AZT group are substrates of SVDE, but quantitative data were not presented[12].

Sensitivity of thiophosphodiester bond cleavage to the nature of the 5′ base was observed in R_P-dinucleoside phosphorothioates[19]. Our results and those with phosphorothioates call the attention to the possible role of the 5′ base in the hydrolysis by SVDE of the phosphodiester bond of oligonucleotides. Our results do not support the earlier observations according to

which variations of the purines and pyrimidines do not affect the rate of hyrolysis[20].

As shown in Table 2, the phosphodiester bond was cleaved with different rate for the two diastereoisomers of a given trimer, the slower eluting species being more stable. It means that the orientation of the sulfur atom at the neighbouring phosphorus, influences the rate of hydrolysis of the phosphodiester bond. The difference is especially pronounced for the members of d[G_sC_oX] series (**4, 5** and **9**). This rate influencing effect may be ascribed to differences in the stability of the enzyme-substrate complexes derived from the two diastereoisomers. Depending on the chirality at phosphorus either a P-O...Zn^{2+} or a P-S...Zn^{2+} complex may be formed. The greater difference between the hydrolysis rates of d[G_sC_oX] diastereoisomers comparing with d[T_sA_oX] diastereoisomers, may be interpreted on conformational basis.

Asymmetric hydrolysis by nuclease S1 of trimer diastereoisomers rendered possible the configurational assignment at their chiral phosphorus atom. Hydrolysis of the slower eluting trimer diastereoisomers resulted in the formation of the slower eluting phosphorothioate dimer diastereoisomers. A phosphorothioate dimer diastereoisomer with the S_P configuration displays the longer elution time upon C_{18} HPLC[13,19,21–24]. On this basis, the faster eluting trimer diastereoisomers have the R_P configuration, while the slower eluting diastereoisomers have the S_P configuration. For d[T_sA_oX] series, $t_{1/2}$ values increased in the order of **11** < **10** < **6**, similarly as found for both series in the case of SVDE. However, for d[G_sC_oX] compounds, different stability orders were observed for the two diastereoisomers. Comparing the two series, compounds of type d[T_sA_oX] (**6, 10** and **11**) are hydrolyzed by a significantly higher speed, than trimers d[G_sC_oX] (**4, 5** and **9**). The former are notably less stable against nuclease S1 than toward SVDE.

Our results suggest that the liberation of a 5′-mononucleotide from the 3′-terminal of an antisense phosphorothioate oligonucleotide is effected not only by the nature of the 3′-terminal nucleoside, but also by the sequence of the antisense oligo near the 3′ end.

A detailed and systematic investigation of the rate influencing effect of the chirality of neighbouring thiophosphodiester linkage on the hydrolysis of a phosphodiester bond, which is published here on a preliminary level only, is in progress in our Laboratory.

EXPERIMENTAL

Materials. All chemicals were of reagent grade. 5′-O-(4,4′-Dimethoxytrityl)-N-acyl-2′-deoxynucleosides were purchased from Pharma-Waldhof GmbH, AZT and pivaloyl chloride from FLUKA AG, dTAc, FdU, 5′-DMT-dT-CPG and LCAA-CPG from Sigma. Pivaloyl chloride

was freshly distilled before use. Pyridine and THF were distilled from CaH_2 and stored over molecular sieves. 5′-O-(4,4′-dimethoxytrityl)-N-acyl-2′-deoxynucleoside 3′-H-phosphonates were prepared according to the literature[25]. Phosphodiesterase I [from Crotalus adamanteus venom, Type II, Sigma P-6877; 1.0 unit will hydrolyze 1.0 μmole of bis(p-nitrophenyl) phosphate/min, at pH 8.8 and 37°C] and nuclease S1 [Amersham, E2410Y; 1.0 unit converts 1.0 μg of heat denaturated DNA to an acid soluble form at pH 4.6 in 1 min at 37°C] were used for enzymatic hydrolyses[26,27].

Methods. Solid phase syntheses were carried out on 1.0 μmolar scale using a MilliGen/Biosearch 8700 Synthesizer. TLC was performed on Kieselgel 60 F_{254} chromatoplates. Column chromatography on Kieselgel 60 (0.063–0.2 mm) [Merck] was done in the following solvent systems: **A**, $CHCl_3$/MeOH 9:1; **B**, $CHCl_3$/MeOH 2:1; **C**, $CHCl_3$/MeOH/Et_3N 89:10:1 (v/v). Ion exchange column chromatographic purifications were carried out on DEAE Sephadex A-25 [HCO_3^-] (Pharmacia Fine Chemicals). RP HPLC analyses were performed on a HYPERSIL ODS (250 × 4.0 mm, I.D. 5 μm) column. A guard column (HYPERSIL ODS, 30 × 4.0 mm, I.D. 5 μm) was also used. Compounds were detected at 260 nm on a BISCHOFF LAMBDA 1010 HPLC spectrophotometer. LaChrom L-7100 HPLC pump was used. Gradient conditions: linear gradient of 100% buffer A [100 mM NH_4OAc, pH 7.0/acetonitrile (95:5, v/v)] and 0% buffer B [100 mM NH_4OAc, pH 7.0%acetonitrile (50:50, v/v)] to 85% buffer A/15 % buffer B (Retention times/min/, R_T; FdUMP and dTMP: 2.7; d[G_sC]: 8.7, 10.7; **5**: 12.3, 13.8; **9**: 12.4, 14.3), 80% buffer A/20% buffer B (R_T; FdUMP: 4.5; dTMP: 4.9; d[T_sA]: 12.3, 13.2; **10**: 15.8, 17.7; **11**: 16.4, 18.3) or 65% buffer A/35% buffer B (R_T; AZTMP: 4.6; d[G_sC]: 8.1, 9.3; d[T_sA]: 11.7, 13.2; **4**: 14.5, 15.8; **6**: 17.3, 18.8) over 30 min at a flow rate of 1 mL/min. ^{1}H-(at 400 MHz) and ^{31}P-NMR spectra (at 162 MHz) were recorded on a Varian XL-400 instrument. Chemical shifts are given in ppm relative to Me_4Si or H_3PO_4, as internal standards. Infrared spectra were taken on a Nicolet 205 FT-IR spectrometer in KBr. Mass spectral measurements were done on a PE Sciex API 2000/LC/MS/MS instrument. Ionization: ESI (−), solvent system: MeOH/H_2O.

N^4-Benzoyl-2′-deoxycytidylyl (3′→5′)3′-deoxy-3′-azidothymidine (1). AZT (1.60 g, 6.0 mmol) and N^4-benzoyl-5′-O-(4,4′-dimethoxytrityl)-2′-deoxycytidine 3′-H-phosphonate DBU salt (5.85 g, 6.90 mmol) were dissolved in pyridine (70 mL), and the solution was evaporated to dryness. The residue at evaporation was dissolved in pyridine (70 mL). Pivaloyl chloride (2.21 mL, 18 mmol) was added to the solution at room temperature under stirring. Stirring was continued for 20 min, then CH_2Cl_2 (350 mL) was added. The solution was washed with saturated aqueous

$NaHCO_3$ solution (2×150 mL), dried ($NaSO_4$), filtered and evaporated to give 9.12 g of a white foam. The semisolid foam was dissolved in THF (50 mL). A solution of 0.2 M I_2 in a mixture of THF/pyridine/H_2O/N-methyl-imidazole (89:5:5:1) (33 mL, 1.1 eq.) was added. The solution was stirred at ambient temperature overnight. Excess iodine was reduced by the addition of 0.5 M aqueous $NaHSO_3$ solution (1.2 mL). The reaction mixture was evaporated to a pale yellow syrup. The syrup was dissolved in $CHCl_3$ (60 mL) and MeOH (12 mL). 1.0 M solution of p-TsOH·H_2O in MeOH (10 mL) was added. After 20 min stirring at room temperature the solution was neutralized by Et_3N (1.7 mL) and evaporated to dryness. The amorphous residue at evaporation was taken up in 1.0 M aqueous TEAB solution (50 mL) and extracted with EtOAc (3×25 mL). The aqueous phase was diluted with EtOH (50 mL) and carefully evaporated to a pale yellow syrup. The evaporational residue was dissolved in EtOH (5 mL). The solution was applied onto a silica gel column (130 g) packed in $CHCl_3$. The column was eluted with a linear gradient of systems **A** and **B** (800 mL of each) then with system **B** containing 0.5% Et_3N. Appropriate fractions were combined and evaporated to yield 3.48 g (88%) of **1**, as a white, solid triethylammonium salt. ^{1}H NMR (DMSO-d_6 + $CDCl_3$) δ: 1.26–1.30 (9H, t, Me protons of TEA; 3H, s, 5-CH_3); 2.15–2.77 (4H, m, H2′abs of dC and AZT); 3.02 (6H, q, CH_2 protons of TEA); 3.78 (2H, m, H5′ab of dC); 4.02 (1H, q, H4′ of dC); 4.11 (2H, m, H5′ab of AZT); 4.24 (1H, q, H4′ of AZT); 4.47 (1H, m, H3′ of AZT); 4.95 (1H, m, H3′ of dC); 6.12–6.26 (2H, 2t, H1′s of dC and AZT); 7.38–7.60 (5H, m, benzoyl protons); 7.98 (1H, d, H5 of dC); 8.03 (1H, s, H6 of AZT); 8.40 (1H, d, H6 of dC); 11.13 (1H, bs, NH). ^{31}P-NMR (DMSO-d_6 + $CDCl_3$) δ: −2.68.

N^4-Benzoyl-2′-deoxycytidylyl(3′→5′)3′-O-acetylthymidine (2). The compound was prepared from dTAc (1.12 g, 4.0 mmol) and N^4-benzoyl-5′-O-(4,4′-dimethoxytrityl)-2′-deoxycytidine 3′-H-phosphonate DBU salt (3.90 g, 4.6 mmol) on exactly the same way as described for compound **1**. Yield: 2.46 g (79%) of white, solid trietylammonium salt of **2**. ^{1}H-NMR (DMSO-d_6 + $CDCl_3$) δ: 1.24–1.30 (9H, t, Me protons of TEA; 3H, s, 5-CH_3); 2.05 (3H, s, acetyl CH_3); 2.13 2.80 (4H, m, H2′ab of dC and dT); 2.97 (6H, q, CH_2 protons of TEA); 3.72–3.88 (2H, m, H5′ab of dC); 4.04–4.18 (3H, m, H4′ of dC and H5′ab of dT); 4.24 (1H, q, H4′ of dT); 4.98 (1H, m, H3′ of dT); 5.30 (1H, m, H3′ of dC); 6.12–6.30 (2H, 2t, H1′s of dC and dT); 7.35–7.65 (5H, m, benzoyl protons); 7.94–8.03 (2H, d and s, H5 of dC and H6 of dT); 8.38 (1H, d, H6 of dC), 10.70 (1H, bs, NH). ^{31}P-NMR (DMSO-d_6 + $CDCl_3$) δ: −3.14.

N^6-Benzoyl-2′-deoxyadenylyl(3′→5′)3′-deoxy-3′-azidothymidine (3). The compound was prepared from N^6-benzoyl-5′-O-(4,4′-dimethoxytrityl)-2′-deoxyadenosine 3′-H-phosphonate DBU salt (3.29 g, 4.0 mmol) and AZT (1.07 g, 4.0 mmol) on exactly the same way as

described for compound **1**. Yield: 1.65 g (60.4%) of white solid triethylammonium salt of **3**. ^{1}H-NMR ($CDCl_3$) δ: 1.28–1.32 (9H, t, Me protons of TEA); 1.83 (3H, s, 5-CH_3); 2.35–2.38 (2H, m, H2′ab of AZT); 2.64–2.70 (1H, m, H2′b of dA); 2.93–3.01 (1H, m, H2′a of dA); 3.05 (6H, q, CH_2 protons of TEA); 3.81–3.93 (2H, m, H5′ab of dA); 3.99–4.02 (1H, m, H4′ of AZT); 4.11–4.22 (2H, m, H5′ab of AZT); 4.37 (1H, q, H4′ of dA); 4.44 (1H, m, H3′ of dA); 5.09–5.14 (1H, m, H3′ of AZT); 6.18 (1H, dd, H1′ of AZT); 6.41 (1H, dd, H1′ of dA); 7.44–7.60 (3H, m, benzoyl protons); 7.62 (1H, s, H6 of AZT); 8.06 (2H, d, benzoyl protons); 8.24 (1H, s, H2 of dA); 8.76 (1H, s, H8 of dA); 9.64 (2H, bs, NHs of AZT and dA). ^{31}P-NMR ($CDCl_3$) δ: −0.47.

2′-Deoxyguanylyl(3′→5′)P$_1$-thio-2′-deoxycytidylyl(3′→5′)3′-deoxy-3′-azidothymidine (4). Compound **1** (1.25 g, 1.90 mmol) and N^2-isobutyryl-5′-O-(4,4′-dimethyoxytrityl)-2′-deoxyguanosine 3′-H-phosphonate triethylammonium salt (1.75 g, 2.2 mmol) was dissolved in pyridne (30 mL). The solution was evaporated to dryness. The residue at evaporation was redissolved in pyridine (30 mL). Pivaloyl chloride (0.7 mL, 5.7 mmol) was added, and the reaction mixture was stirred at room temperature for 15 min. After dilution by CH_2Cl_2 (125 mL) the solution was washed with cold saturated aqueous $NaHCO_3$ solution (2 × 50 mL), dried ($NaSO_4$), filtered and evaporated to dryness. The evaporational residue was dissolved in a mixture of pyridine (50 mL) and CS_2 (20 mL). Sulfur powder (0.36 g, 11.2 mmol) was added, and the mixture was stirred at ambient temperature overnight, then evaporated. Evaporation was repeated by toluene (2 × 40 mL). The residue at evaporation was dissolved in $CHCl_3$ (40 mL) and MeOH (10 mL). 1.0 M solution of p-TsOH·H_2O in MeOH (3.8 mL) was added. The solution was stirred at room temperature for 15 min, then neutralized by TEA (0.53 mL, 3.8 mmol) and evaporated to give a yellow, solid residue.The crude product was suspended in conc. aqueous NH_4OH solution (60 mL). The inhomogeneous mixture was stirred in a sealed flask at room temperature overnight, diluted with H_2O (50 mL) and extracted by EtOAc (3 × 40 mL). The aqueous phase was diluted by EtOH (60 mL) and evaporated carefully. The residual syrup was dissolved in 1.0 M aqueous TEAB solution (5.0 mL) and applied onto a column of DEAE-Sephadex (500 mL) equilibrated by 0.1 M aqueous TEAB, pH 7.5. The column was eluted with a linear gradient of aqueous TEAB, pH 7.5 (1400 mL; 0.1→0.8 M). Appropriate fractions were combined and evaporated to dryness. Excess TEAB was removed by coevaporation with MeOH. Yield: 1.63 g (78%). IR (cm^{-1}): 1050 (ν P-O-C); 1225 (ν P=O); 1640, 1680 (ν C=O); 2100 (ν N_3). ^{1}H-NMR (D_2O) δ: 1.17–1.22 (18H, t, Me protons of TEA); 1.88 (3H, s, 5-Me of AZT); 2.15–2.86 (6H, m, H2′ab of dG, dC and AZT); 3.65 (12H, q, CH_2 protons of TEA); 3.85 (2H, m, H5′ab of dG); 4.07–4.26 (4H, m, H5′ab of

dC and AZT); 4.28–4.53 (3H, m, H4′ of dG, dC and AZT); 4.75 (1H, m, H3′ of AZT); 4.82–5.14 (2H, m, H3′ of dG and dC); 5.92 (1H, d, H5 of dC); 6.10–6.35 (3H, 3t, H1′s of dG, dC and AZT); 7.65 (1H, s, H6 of AZT); 7.90 (1H, d, H6 of dC); 7.98 (1H, s, H8 of dG). ^{31}P-NMR (D_2O) δ: −0.32 (O = P-O$^-$); 55.94, 56.59 (O = P-S$^-$).

2′-Deoxyguanylyl(3′→5′)P_1-thio-2′-deoxycytidylyl(3′→5′)thymidine (5). Starting from **2** (0.57 g, 0.73 mmol) and N^2-isobutyryl-5′-O-(4,4′-dimethoxytrityl)-2′-deoxyguanosine 3′-H-phosphonate trietylammonium salt (0.67 g, 0.83 mmol) the synthesis was performed on exactly the same manner as described for compound **4**. Yield: 0.57 g (74%) of white, solid trietylammoniumsalt of **5**. IR (cm^{-1}): 1050 (ν P-O-C); 1220 (ν P = O); 1640, 1680 (ν C = O). ^{1}H-NMR (D_2O) δ: 1.25–1.35 (18H, t, Me protons of TEA); 1.88 (3H, s, 5-CH_3 of dT); 2.25–2.88 (6H, m, H2′ab of dG, dC, and dT); 3.24 (12 h, q, CH_2 protons of TEA); 3.84 (2H, m, H5′ab of dG); 4.04–4.27 (5H, m, H5′ab of dC and dT); 4.30–4.62 (3H, m, H4′ of dG, dC and dT); 4.75 (1H, m, H3′ of dT); 4.82–5.15 (2H, m, H3′ of dG and dC); 5.95 (1H, d, H5 of dC); 6.17–6.38 (3H, 3t, H1′s of dG, dC and dT); 7.68 (1H, s, H6 of dT); 7.92 (1H, d, H6 of dC); 8.05 (1H, s, H8 of dG). ^{31}P-NMR (D_2O) δ: −0.22 (O = P-O$^-$); 55.96, 56.55 (O=P-S$^-$).

Thymidylyl(3′→5′)P_1-thio-2′-deoxyadenylyl(3′→5′)3′-deoxy-3′-azidothymidine (6). Starting from **3** (1.15 g, 1.67 mmol) and 5′-O-(4,4′-dimethoxytrityl)-thymidine 3′-H-phosphonate DBU salt (1.30 g, 1.75 mmol) compound **6** was prepared analogously as compound **4**. Yield: 0.29 g (64.4 %). ^{1}H-NMR (DMSO-d_6) δ: 1.15 (18H, t, Me protons of TEA); 1.80 and 1.83 (6H, 2s, 5-CH_3 of AZT and dT); 2.10–2.95 (6H, overlapping m-s, H2′ab of dT, dA and AZT); 3.01 (12H, q, CH_2 protons of TEA); 3.50–3.65 (2H, m, H5′ab of dT); 3.90–4.05 (4H, m, H5′ab-s of dA and AZT); 4.0–4.3 (2H, m, H4′ of dT and dA); 4.55(1H, m, H4′ of AZT); 4.90–5.10 (3H, overlapping m-s, H3′ of dT, dA and AZT); 6.16, 6.22 and 6.39 (3H, 3m, H1′s of dT, dA and AZT); 7.25 (2H, s, NH_2 of dA), 7.78 (2H, s, H6 of dT and AZT); 8.18 (1H, s, H2 of dA); 8.42 and 8.52 (1H, 2s, H8 of dA); 11.30 (2H, s, NHs of dT and AZT). ^{31}P-NMR (DMSO-d_6) δ: −2.24 (O=P-O$^-$); 54.70, 55.05 (O=P-S$^-$).

5′-O-(4,4′-Dimethoxytrityl)-5-fluoro-2′-deoxyuridine (7). FdU (7.38 g, 30.0 mmol) was dried by coevaporation with pyridine (2 × 70 mL). The dried material was dissolved in pyridine (80 mL). Et_3N (7.05 mL, 51.0 mmol), then 4,4′-dimethoxytrityl chloride (11.18 g, 33.0 mmol) were added and the coloured reaction mixture was stirred with the exclusion of atmospheric moisture at room temperature for 18 h. The reaction was followed by TLC (system **A**, $\mathbf{R}_F$: 0.11 [FdU], 0.47 [7]). More 4,4′-dimethoxytrityl choride (1.52 g, 4.5 mmol) was added, and the mixture was

stirred for an additional 2 h. The reaction mixture was diluted by MeOH (15 mL), and after 15 min stirring, it was evaporated to dryness. The evaporational residue was dissolved in $CHCl_3$ (250 mL). The solution was washed with saturated aqueous $NaHCO_3$ solution (2×80 mL), H_2O (80 mL), dried (Na_2SO_4) and evaporated. The residue at evaporation was coevaporated with toluene (2×50 mL). The yellow syrup obtained was taken up in a mixture of $CHCl_3/Et_3N$ 98:2 (12 mL). The solution was applied onto a silica gel column (320 g). The column was eluted by a linear gradient of $CHCl_3/Et_3N$ 98:2 $\rightarrow$ $CHCl_3/Et_3N$/MeOH 88:2:10 (1400 mL). Appropriate fractions were combined and evaporated to yield 14.31 g (87%) of **7**, as a white, solid foam. ^{1}H-NMR ($CDCl_3$) δ: 2.20–2.50 (2H, m, H2′ab); 3.25–3.55 (2H, m, H5′ab); 3.80 (6H, s, CH_3O protons); 4.05 (1H, q, H4′); 4.55(1H, m, H3′); 6.00 (1H, bs, OH); 6.32 (1H, t, H1′); 6.80–7.50 (13H, m, aromatic protons); 7.78 (1H, d, H6).

5′-O-(4,4′-Dimethoxytrityl)-3′-O-succinyl-5-fluoro-2′-deoxyuridine (8). Compound **7** (257.0 mg, 0.47 mmol) was dissolved in CH_2Cl_2 (10 mL). Et_3N (0.13 mL, 0.94 mmol), DMAP (57.3 mg, 0.47 mmol) and succinic anhydride 94.0 mg, 0.94 mmol) were added to the solution. The mixture was stirred with the exclusion of atmospheric moisture at ambient temperature. After 5 h stirring, additional quantities of DMAP (30.5 mg, 0.24 mmol) and succinic anhydride (50.0 mg, 0.50 mmol) were added. The reaction mixture was stirred overnight, then diluted with CH_2Cl_2 (30 mL). The solution was washed with 10% aqueous NaH_2PO_4 solution (2×15 mL), H_2O (1×15 mL), dried (Na_2SO_4) and evaporated to give a yellow foam(0.37 mg). The crude product was purified by dry column flash chromatography (30 g Kieselgel system **C**). Appropriate fractions were pooled and evaporated. Traces of Et_3N were removed by coevaporation with $CHCl_3$ (3×30 mL). Yield: 337 mg (95.7%) of **8**, as a white, solid triethylammonium salt. ^{1}H-NMR ($CDCl_3$) δ: 1.30 (9H, t, Me protons of Et_3N); 2.22–2.45 (2H, m, H2′ab); 2.50–2.70 (4H, m, succinyl CH_2 protons); 3.08 (6H, q, CH_2 protons of Et_3N); 3.28–3.60 (2H, m, H5′ab); 3.80 (6H, s, CH_3O protons); 4.20 (1H, m, H4′), 5.50 (1 H, m, H3′); 6.25 (1H, t, H1′); 6.78–7.50 (13H, m, aromatic protons); 7.82 (1H, d, H6); 8.95 (1H, bs, NH).

Binding of 8 to LCAA-CPG. LCAA-CPG (1.0 g) was suspended in CH_2Cl_2 (20 mL) containing **8** (0.21 g, 0.28 mmol) and N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) (0.50 g, 2.0 mmol). The suspension was slowly rotated at room temperature with the exclusion of atmospheric moisture for 2 days. The mixture was filtered with suction. The solid was washed with CH_2Cl_2 (2×10 mL) and suspended in a mixture of CAP A and CAP B solutions (10 mL of each). After 3 h rotation the solid was filtered off, washed with THF (3×15 mL) and dried in a vacuum desiccator over P_2O_5.

The capacity of loaded LCAA-CPG was found to be 42 μmoles/g (DMT assay).

Mass Spectral Characterization of Compounds Synthesized on Solid Phase

2′-Deoxyguanylyl(3′→5′)P_1-thio-2′-deoxycytidylyl(3′→5′)5-fluoro-2′-deoxy uridine (9): $C_{28}H_{35}FN_{10}O_{16}P_2S$, 880; $[M\text{-}H]^-$ 879.
Thymidylyl(3′→5′)P_1-thio-2′-deoxyadenylyl(3′→5′)5-fluoro-2′-deoxyuridine (10): $C_{29}H_{36}FN_9O_{16}P_2S$, 879; $[M\text{-}H]^-$ 878.
Thymidylyl(3′→5′)P_1-thio-2′-deoxyadenylyl(3′→5′)thymidine(11): $C_{30}H_{39}N_9O_{16}P_2S$, 875; $[M\text{-}H]^-$ 874.

Enzymatic Hydrolysis

Enzymatic reactions were run at 37°C, and were monitored by RP-HPLC. Chimeric trimers (2.0 A_{260} units of **6**, **10** or **11**; 1.7 A_{260} units of **4**, **5** or **9**) were incubated with SVDE (0.004 unit for **6**, **10** and **11**; 0.001 unit for **4**, **5** and **9**) in 50 mM Tris-HCl, 5 mM $MgCl_2$, pH 8.0, in a total volume of 0.5 mL. Aliquots (50 μL) were withdrawn at intervals (0, 5, 10, 15, 30, 60, 120, 240 and 480 min), added to 100 mM EDTA (10 μL) and analyzed by RP-HPLC. Chimeric trimers (1.3 A_{260} units of **6**, **10** or **11**; 1.0 A_{260} unit of **4**, **5** or **9**) were incubated with nuclease S1 (200 units for **6**, **10** and **11**; 10 units for **4**, **5** and **9**) in 30 mM NaOAc, 0.28 M NaCl, 1.0 mM $ZnSO_4$, pH 4.6, in a total volume of 0.2 mL. Aliquots (20 μL) were removed at intervals, as above, added to 0.1 EDTA (5 μL) and analyzed by RP-HPLC. Values of $t_{1/2}$ are equal to 50% cleavage of the phosphodiester bond. Since the degradations of the diastereoisomers of a given trimer by nuclease S1 proceed with different rates, $t_{1/2}$ values are only approximate numbers in these cases. Hydrolysis products were identified by HPLC comparison with authentic specimens.

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