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Recognition and Inhibition of HIV Integrase by Novel Dinucleotides

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Abstract: HIV integrase is involved in the integration of viral DNA into chromosomal DNA, a biological process that occurs by a sequence involving HIV DNA splicing and subsequent integration steps. In the quest for small nucleotide systems with nuclease stability of the internucleotide phosphate bond and critical structural features for recognition and inhibition of HIV-1 integrase, we have discovered novel, nuclease-resistant dinucleotides with defined base sequences that are inhibitors of this key viral enzyme. Synthetic methodologies utilized for the syntheses of the novel dinucleotides include an excellent new phosphorylating agent.

Introduction

The *pol* gene of HIV-1 encodes three viral enzymes, reverse transcriptase (RT), protease, and integrase (IN), that are essential for viral replication. Two of these enzymes, HIV RT and HIV protease, have received considerable attention with respect to the development of inhibitors.[1−4] The third enzyme, HIV IN, which is encoded in the 3′-end of the *pol* gene of the virus, has received much less attention. HIV-1 IN is a relatively small protein (32 000 Da). It is involved in the integration of viral DNA into host cell DNA, a biological process that occurs by a sequence involving DNA cleavage (3′-processing) and coupling (integration) reactions.[5−8] The enzyme apparently recognizes specific sequences (5′-ACTG...CAGT-3′) in the long terminal repeats (LTRs) of viral DNA. In the first step, which involves a pre-integration process and is referred to as 3′-processing (or cleavage step), specific endonuclease activity removes two nucleotides from each end of the double helical viral DNA (synthesized previously by reverse transcription) producing new 3′-hydroxyl termini at the conserved CA dinucleotide. This truncated viral DNA is coupled in the next steps to host cell DNA in the nucleus (integration) which includes the DNA strand transfer reaction in which IN catalyzes covalent bond formation between the processed 3′-ends of retroviral DNA and the 5′-phosphate end of an integrase-cleaved chromosomal DNA. Both the 3′-processing and strand transfer steps involve transesterifications and can be assayed using recombinant HIV-1 IN and a model double-helical DNA (21-mer) corresponding to the U5 region of HIV-1 LTR.[9] The crystal structure (2.5 Å resolution) of HIV-1 IN has been reported.[7,10]

Although studies on the search for clinically useful anti-integrase agents are relatively recent, the availability of the methodology of screening of inhibitors utilizing purified

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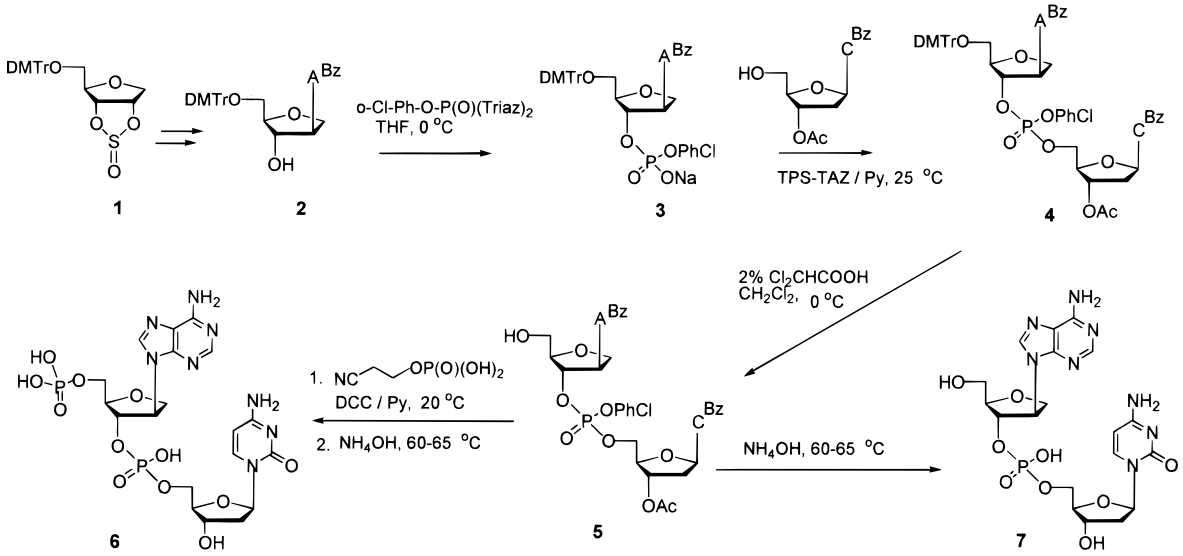
Scheme 1. Summary of the Key Steps in the Synthesis of Dinucleotides **6** and **7**



recombinant integrase has contributed to the identification of some interesting lead compounds which include small and large nucleotides.[11−15] In the quest for a small nucleotide system with resistance to nuclease of the internucleotide phosphate bond and critical structural features for recognition and inhibition of HIV integrase, we designed dinucleotides with features that exploited the ability of integrase to recognize the terminal sequence of the truncated viral DNA during DNA cleavage and prior to strand transfer. The molecular design led to the discovery of conceptually novel, nuclease-resistant, non-natural dinucleotides with defined base sequences that are inhibitors of this key viral enzyme. This paper describes our discoveries in this area involving both chemistry and enzymology.

Results and Discussion

Synthesis. The synthesis of two of the target molecules, **6** and **7**, are summarized in Scheme 1. These and other molecules of this paper were designed with a natural D-deoxynucleoside (3′-OH terminus) bonded through a 5′→3′ internucleotide phosphate linkage to an isomeric L-related deoxynucleoside.[16−18] Dinucleotide **6** and its precursor **7** were synthesized in several steps from protected (*S*,*S*)-isodeoxyadenosine, a structural analogue of natural 2′-deoxyadenosine. This precursor, although previously synthesized by us,[19] was prepared for these studies by a more convenient route using the cyclic sulfite **1**. Synthesis of the dinucleotides utilized the solution-phase phosphotriester approach.[20,21] The solid-phase synthesis using a DNA synthesizer was useful only for sub-milligram quantities of target molecules. The solution phase approach was preferred because this allowed the synthesis of these compounds in sufficient quantities (>50 mg) to allow for complete structural studies as well as reproducible enzyme assays. Thus, isodeoxynucleoside **2** was phosphorylated with the bifunctional phosphorylating agent, 2-chlorophenylphosphoro-bis-triazolide[22,23] to give **3** in 58% yield after purification (Scheme 1).

The internucleotide coupling was carried out with triisopropylbenzenesulfonyl tetrazolide (TPS-TAZ)[24] to give the protected dinucleotide **4** in 66% yield. The formation of **4** was confirmed by its NMR and HRMS data. Deprotection with 2% dichloroacetic acid gave **5** (56% yield), and this key intermediate was fully characterized by ^{1}H and ^{31}P NMR and HRMS data. Further deprotection of **5** with NH_4OH gave target molecule **7** (66%), after purification by reversed-phase HPLC (C_{18}, ethanol/water). The product was initially identified by comparison of HPLC ion-exchange retention times with that of natural dinucleotides (Partisil-10 SAX ion-exchange column, phosphate buffer system, retention time = 70 min). The complete structure was established by multinuclear NMR data (^{1}H, ^{13}C, ^{31}P NMR spectra and COSY, HMQC, and HMBC data), quantitative UV spectra, CD spectra, and ESI HRMS data [calculated for **7**: 539.1404 $(M - H)^-$, found: 539.1402]. The quantitative UV spectral data (λ_{max} 263 nm, ϵ 19 400) gave evidence of hypochromicity in these molecules. This and the CD data (Figure 1) suggested the existence of base stacking interactions. For stacking interactions to occur, the carbohydrate moieties must assume an orthogonal relationship, which results in an unusual internucleotide phosphate linkage (Figure 2). The

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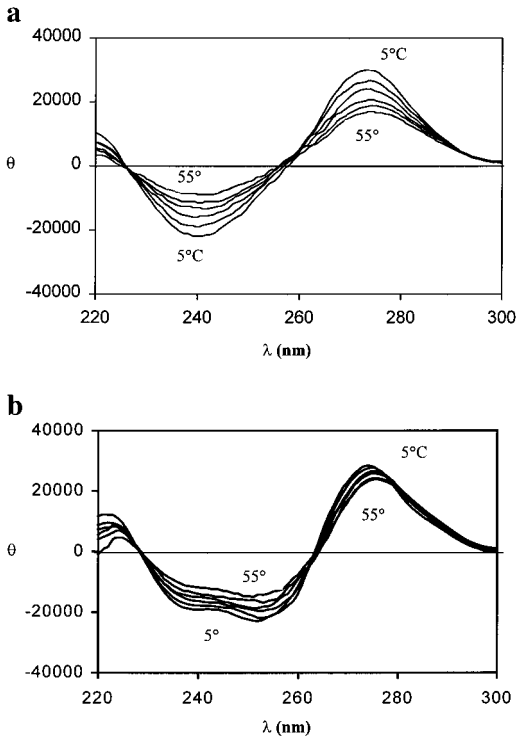

Figure 1. Temperature-dependent CD spectra of dApdC (a) and IsodApdC (b) in 10 mM sodium-cacodylate (pH 7.0) at 0.2 mM final concentration.

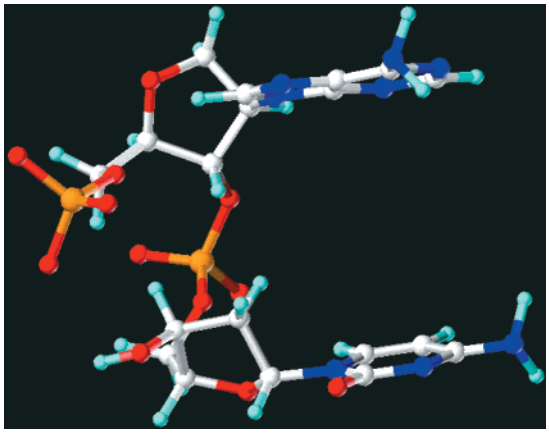

Figure 2. A Minimum-energy conformational representation of the anti-integrase inhibitor, pIsodApdC.

consequences of this with respect to nuclease stability are discussed below.

Target molecule **6** was synthesized by phosphorylation of intermediate **5** with 2-cyanoethylphosphate[25,26] in the presence of DCC followed by deprotection with NH_4OH. This compound was characterized as described above for **7**. The COSY spectrum with assignments is shown in Figure 3.

Three other dinucleotides were synthesized by multistep procedures for these studies. Compound **9** was prepared using methodologies similar to the synthesis of **7**. However, 5′-phosphorylation of **8a** with 2-cyanoethylphosphate as well as other reagents (e.g., phosphoroimidazolides, phosphorotriazolides, phosphoramidites, etc.) was inefficient. We therefore developed a new reagent (**10**), a derivative of 2,2′-sulfonyldiethanol, for this transformation (Scheme 2). Treatment of **8a** with **10** in pyridine in the presence of TPS-TAZ at room temperature for 0.5 h followed by deprotection of the trityl group with dichloroacetic acid (3 min) gave **11** in 70% overall yield (for two steps). The yield of the phosphorylation step was consistently between 80 and 90%. *Reagent **10** is an excellent new phosphorylating agent!* Compound **11** was isolated, purified, and completely characterized. Deprotection with NH_4OH gave target molecule **12**. If the immediate product of the reaction of **8a** and **10** is worked up with 10% NH_4OH instead of aqueous pyridine, the overall phosphorylation approach produces a novel dinucleotide **13**, which bears a methylcytosine base, a result of the ammonolysis of the phosphorylated intermediate involving the 4-position of thymine. That this reaction had indeed occurred, was confirmed unequivocally by the NMR and ESI HRMS data of **13**.

Inhibition Studies with Recombinant HIV Integrase. HIV integrase inhibition assays were conducted with purified recombinant integrase using a 21-mer oligonucleotide substrate as described previously by Pommier and co-workers.[27] The data are summarized in Table 1. Compound **6** was found to have strong inhibitory activity against recombinant wild-type HIV integrase in reproducible assays (IC_{50} 19 μM for 3′-processing and 25 μM for strand transfer). The activity is much greater than for dideoxynucleoside monophosphates[5,6] (e.g., (*S*,*S*)-IsoddAMP, AZT, L-ddCMP, Table 1) and is close to the activity of the corresponding "natural dinucleotide" pdApdC.[28] The significant anti-integrase activity of the unusual dinucleotide **6** and its natural analog suggests base sequence selectivity. This sequence selectivity is consistent with the catalytic mechanism of 3′-processing in which endonuclease activity produces a truncated viral DNA with a terminal CA dinucleotide. Molecular recognition by the integrase of the ultimate and penultimate bases at the 5′-end of the minus strand of noncleaved viral DNA may result in stable complex formation before the strand transfer reaction.[29] Thus, the potent inhibitor activity of **6** may reflect the affinity that HIV integrase has for this dinucleotide sequence. In addition, other data[28] also suggest that two neighboring bases may fulfill a substantial part of the essential interaction requirements when integrase recognizes its viral DNA substrate. Further support for this comes from the inactivity of pCpIsodA, that is, the compound in which the sequence is reversed from compound **6**. Additional support comes from the observation that the AT analogue corresponding to compound **6** (i.e., pIsodApT **12**) exhibits low activity for the cleavage and strand transfer steps. The terminal 5′-phosphate appears to be essential for activity as the precursor of **6**, that is, the dinucleotide that is devoid of the 5′-phosphate group, (i.e., compound **7**) is remarkably less potent. Another interesting observation that arises from these studies is that HIV integrase tolerates modifications in the base moiety, which is in contrast to the nucleotide binding site of HIV reverse transcriptase where there is little tolerance for modified bases. For example, compound **13** which bears a 5-methylcytosine base shows good activity against HIV integrase. The active dinucleotides of Table 1 inhibited all enzymatic activities of integrase and DNA-integrase cross-linking in the same range as indicated in Table 1 when either Mn^{2+} or Mg^{2+} was used as a cofactor (data not shown).

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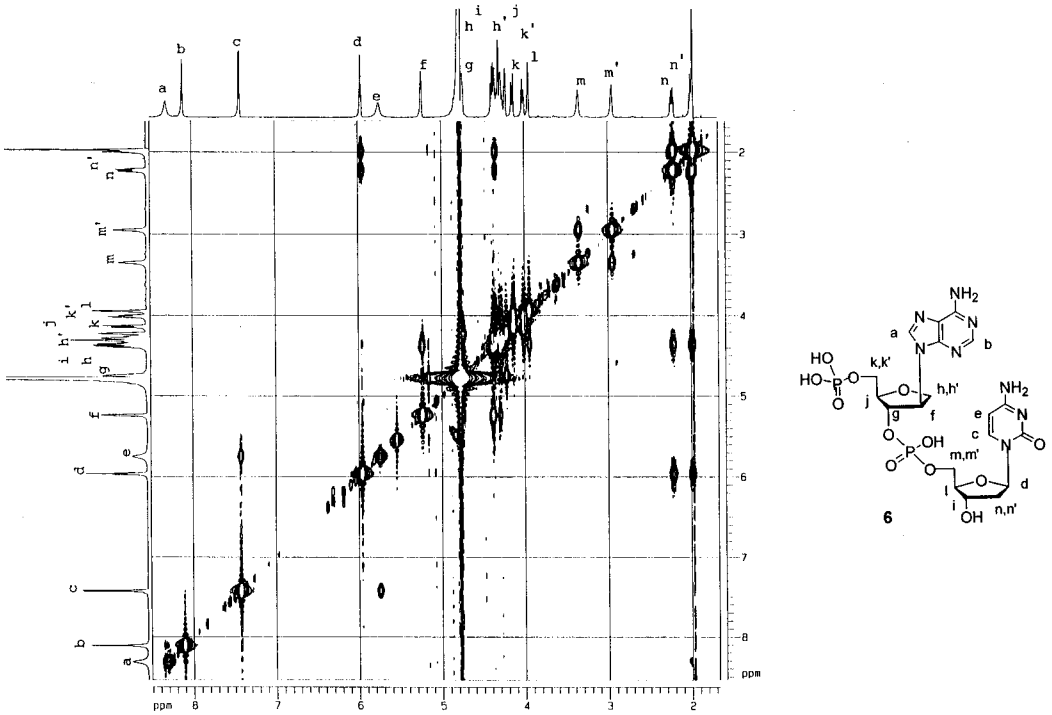
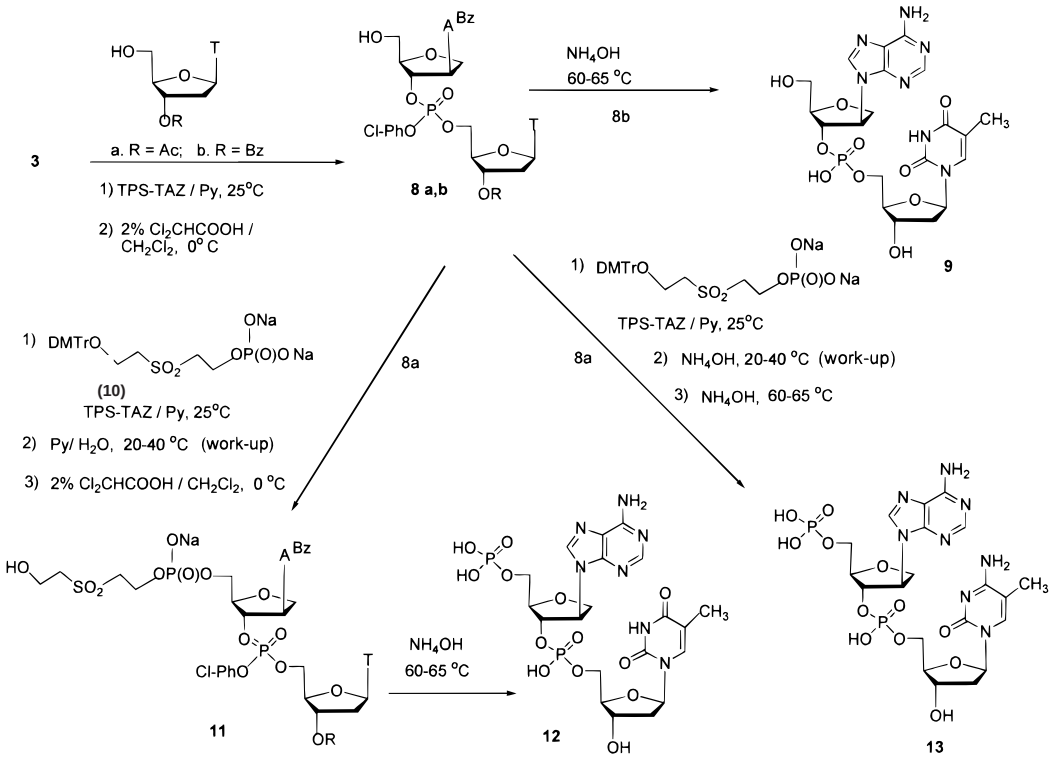


Figure 3. The 600 MHz ^{1}H COSY NMR Spectrum of compound **6** with complete assignments.

Scheme 2. New Phosphorylation Methodology in the Synthesis of Dinucleotides **11** and **13**



This suggests that these compounds bind to the catalytic core of integrase and the inhibition of integrase is metal-independent.

Studies with Exonucleases. Another remarkable aspect of these dinucleotides with isomeric deoxynucleoside components is that the internucleotide bond exhibits resistance to cleavage by mammalian 5′- and 3′-exonucleases [phosphodiesterases (PDE I and II)]. Cleavage reactions were monitored by HPLC as described in the Experimental Section. Analysis of these results revealed that internucleotide phosphate bonds in which one of the components is an isodeoxynucleoside are resistant to degradation by exonucleases. For example, for compound **7**, cleavage of the internucleotide phosphate bond is approximately 33% of that for the natural dinucleotide, dApdC, with PDE I and approximately 20% with PDE II. The results are graphically represented in Figures 4 and 5.

The resistance to internucleotide phosphate bond cleavage is not associated with chemical alteration of the phosphate bond (e.g., thio modification[30]) or with other structural changes[31,32] (e.g., arabino modification) as is found with nuclease-resistant

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Table 1. Anti-Integrase Inhibition Data for Novel Dinucleotides and Some Selected Compounds

compound	3′-processing (μM)	strand transfer (μM)
pIsodApdC **6**	19	25
IsodApdC **7**	200	200
pIsodApT **12**	150	150
pIsodAp5MeC **13**	60	50
pCpIsodA	>300	>300
(*S*,*S*)-IsoddAMP	>200	>200
AZTMP[12]	>110	140
L-ddCMP[11]	50	45
pdApdC[28]	6	3

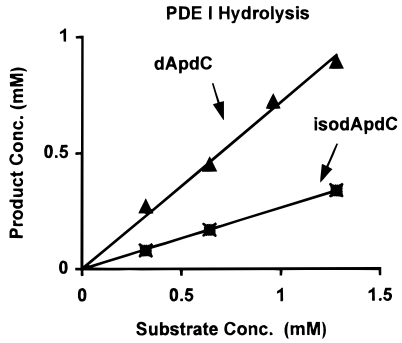

Figure 4. Phosphodiesterase I activity. These assays were carried out using various concentrations of dApdC and isodApdC as substrates. The reactions were analyzed by HPLC using a methanol−water gradient as described in the Experimental Section.

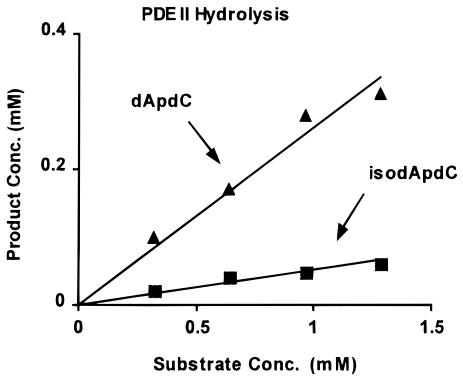

Figure 5. Phosphodiesterase II activity. These assays were carried out using various concentrations of dApdC and isodApdC as substrates. The reactions were analyzed by HPLC using a methanol−water gradient as described in the Experimental Section.

compounds, but with the structural distortion of this phosphate bond as illustrated in Figure 2. It appears that distortion of the internucleotide phosphate bond in compound **7** results in binding to the active site in a less productive mode than the natural counterpart.

In summary, we have synthesized novel, sequence-specific dinucleotides that are inhibitors of HIV integrase. The inhibition data give evidence for sequence selectivity when HIV integrase recognizes its viral DNA substrate, which is consistent with the mechanism of action of integrase. Interestingly, these dinucleotides that possess distorted internucleotide phosphate linkages are more stable with respect to cleavage by both 5′- and 3′-exonucleases. Further studies of conceptually new, nuclease-resistant, sequence-specific dinucleotides as inhibitors of HIV integrase are in progress.

Experimental Section

General. NMR spectra were recorded on a Brüker model AC-300 and AMX-600 spectrometers. Chemical shifts (δ, ppm) are relative to TMS (^{1}H and ^{13}C) or H_3PO_4 (^{31}P). High-resolution FAB and ESI mass spectra were determined on VG ZAB-HF and Micromass, Inc. Autospec high-resolution mass spectrometers. UV spectra were recorded on a Cary 3 UV−visible spectrophotometer. Preparative layer chromatography used plates prepared with E. Merck PF_{254} silica gel. Column chromatography was carried out on columns packed with 240−400 mesh silica gel. HPLC separations were performed on a Waters automated 600E system or a Beckman System Gold HPLC using C_{18} columns or Partisil-10 SAX ion-exchange columns.

Materials. Thymidine, 2′-deoxycytidine hydrochloride, 2,2′-sulfonyldiethanol, TPS-TAZ, 4,4′-dimethoxytrityl chloride, 2-chlorophenyldichlorophosphate, imidazole, 2-cyanoethylphosphate (Ba salt) were purchased from Aldrich or Sigma, and CPG-immobilized T and C from Applied Biosystems Inc. PDE I (bovine intestinal mucosa) and PDE II (bovine spleen) and pAdpC were purchased from Sigma Chemical Co. *N*-Benzoyl-3′-*O*-acetylcytidine[24,33] and 3′-*O*-acetyl- (or benzoyl) thymidine[24,34] were prepared by reported methods.

Synthesis. *N*-Benzoyl-5′-*O*-dimethoxytritylisodeoxyadenosine-3′-(2-chlorophenyl) Sodium Phosphate (3). Compound **2** (1.784 mmol 1.176 mg) was kept overnight under vacuum over P_2O_5 to remove traces of water. 2-Chlorophenyldichlorophosphate (2-ClPhOPOCl$_2$) (2 mmol, 0.32 mL) and 1,2,4-triazole (4.0 mmol, 276 mg)[22,23] were dissolved under nitrogen in anhydrous THF (15−20 mL) and cooled on ice. TEA (4 mmol, 545 mL) was added dropwise, resulting in a copious precipitate. The reaction mixture was stirred for 1−2 h and then filtered into the flask containing **2**. It was kept for 2 h on ice and then quenched with water (2 mL). After 0.5 h, ethyl acetate (200 mL) was added, and the organic layer was washed with saturated aqueous $NaHCO_3$ (10 mL) and brine (10 mL). The solvent was evaporated, and pyridine was removed with toluene. The residue was purified by column chromatography (CH_2Cl_2/10% MeOH) to give **3** (227 mg, 58%) as a white foam (R_f = 0.1, CH_2Cl_2/10% MeOH). ^{1}H NMR ($CDCl_3$): 8.64 (br s, 1H, NHBz), 8.33 (s, 1H, H-8), 7.96 (s, 1H, H-2), 7.95 (s), 7.58−7.12 (m) and 6.75−6.70 (d) (22H, Arom-H), 5.44 (br s, 1H, 2′), 5.25 (br s, 1H, 3′), 4.27 (br s, 2H, 1′), 3.70 (s, 7H, CH_3O, 4′), 3.46 (br s, 2H, 5′). ^{13}C NMR ($CDCl_3$): 167.2 (CO), 153.1 (C-6), 152.2 (C-2), 151.1 (C-4), 142.0 (C-8), 145.5, 138.4, 136.4, 135.8, 135.4, 134.1, 133.1, 130.1, 128.9, 127.9, 127.6 (Arom), 128.2 (C-5), 115.0, 114.8 (DMTr), 86.0 ($Ph_3\underline{C}$), 84.9 (4′), 75.3 (2′), 70.1 (1′), 62.8 (5′), 62.4 (3′), 55.0 (OCH_3). ESI HRMS: $(M + H)^+$ calcd for $C_{44}H_{40}ClN_5NaO_9P$ 848.2243, found 848.2250.

***N*-Benzoyl-1′-deoxy-2′-isoadenosine-3′-[(2-chlorophenyl)(*N*-benzoyl-3′-*O*-acetyl-cytidine-5′-yl)] Phosphate (5).** A mixture of **3** (0.770 mmol, 670 mg) and *N*-benzoyl-3′-*O*-acetyl-cytidine (0.770 mmol, 270 mg) was repeatedly evaporated with anhydrous pyridine and then dissolved in pyridine (3 mL) under nitrogen. 2,4,6-Triisopropylbenzensulfonyltetrazolide (TPS-TAZ)[24] (1.54 mmol, 555 mg) was added. After 1 h at room temperature, the reaction mixture was quenched with water (0.5 mL) and then left to stand for 1.5 h and concentrated. The residue was dissolved in ethyl acetate (100 mL) and washed with saturated aqueous $NaHCO_3$ (2 × 20 mL). Solvents were removed by evaporation and coevaporation with toluene, and the residue was purified by column chromatography (CH_2Cl_2/5% MeOH) to give 600 mg (66%) of *N*-benzoyl-5′-*O*-dimethoxytrityl-1-deoxy-2-isoadenosine-3′-[(2-chlorophenyl)(*N*-benzoyl-3′-*O*-acetylcytidine-5′-yl)] phosphate **(4)** as a white foam (R_f = 0.35, CH_2Cl_2/5% MeOH). ^{1}H NMR ($CDCl_3$): 8.67 (s, 1H, A H-8), 8.22 (s, 1H, A H-2), 8.05−8.03 (d, 2H, C H-6, Bz), 7.85−7.83 (d, 1H, C H-5), 7.83−7.80 (m, 3H, Arom), 7.55−7.10 (m, 14H, Arom), 6.78−6.70 (m, 5H, DMTr), 6.20 (q, 1H, C 1′), 5.62−5.50 (m, 1H, A 2′), 5.48−5.38 (m, 1H, C 3′), 5.27−5.22 (d, 1H, A 3′), 4.52−4.35 (m, 2H, A 1′) 4.30 (m. 1H, C 4′), 4.25−4.13 (m, 2H, C H 5′), 3.70 (s, 6H, CH_3O), 3.53−3.35 (m, 2H, A 5′).

The protected dinucleotide **4** (500 mmol, 600 mg) was dissolved in 3 mL of anhydrous methylene chloride and cooled on an ice-bath. Cold

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2% dichloroacetic acid (DCA) in methylene chloride (10−15 mL) was added. The crimson red reaction mixture was kept for 3 min on the ice-bath, poured onto saturated aqueous $NaHCO_3$ (20 mL), and extracted with methylene chloride (2 × 50 mL), and then the solvent was evaporated. The residue was purified by column chromatography (CH_2Cl_2/7% MeOH) to give **5** (225 mg, 56%) as a white foam (R_f = 0.35, CH_2Cl_2/7% MeOH). ^{1}H NMR (D_2O): 8.31 (br s, 1H, A H-8), 8.11 (s, 1H, A H-2), 7.43 (d, J 7.8 Hz, 1 H, C H-6), 5.97 (t, J 6.0 Hz, 1 H, C 1′), 5.75 (br s, 1H, C H-5), 5.24 (q, J 5.4 Hz, 1H, A 2′), 4.76−4.75 (m, 1H, A 3′), 4.40−4.37 (m, 1H, A 1′), 4.36−4.34 (m, 1H, C 3 ′), 4.31−4.28 (m, 1H, A 1′), 4.22 (s, 1H, A 4′), 4.14 (dt, 1H, A 5′a), 4.10 (dt, 1H, A 5′$_b$), 3.95 (s, 1H, C 4′), 3.35 (s, 1H, C 5′$_a$), 2.95 (s, 1H, C 5′$_b$), 2.24−2.20 (m, 1H, C 2′$_a$), 2.01−1.97 (m, 1H, C 2′$_b$). ^{31}P NMR ($CHCl_3$) −11.13 (s), −12.22 (s), ratio 1:1. ESI HRMS: $(M + H)^+$ calcd for $C_{41}H_{39}ClN_8O_{12}P$ 901.2105, found 901.2121.

2′-Deoxycytidylyl(5′→3′)-1′-deoxy-2′-isoadenosine 5′-phosphate (6). Compound **5** (0.16 mmol, 150 mg) and β-cyanoethylphosphate[25,26] (0.8 mmol, 0.6 mL) (prepared from its Ba salt), were evaporated with anhydrous pyridine three times. Dicyclohexylcarbodiimide (DCC) (110 mmol, 230 mg) was added together with 4 mL of pyridine. A copious white precipitate of dicyclohexylurea formed within 10 min. After 48 h, the reaction mixture was filtered, the precipitate was washed with pyridine, and the combined filtrate was evaporated and coevaporated with water to remove pyridine. Cold NH_4OH (29%, d 0.88, 40 mL) was added, and the suspension was heated in a Hastelloy bomb reactor at 60−65 °C for 6 h. The reactor was cooled and opened, and the reaction mixture was filtered. The filtrate volume was reduced by one-fifth to remove excess NH_3. The pH was adjusted to 10 by the addition of 29% NH_4OH (a drop). The reaction mixture was extracted with diethyl ether (2 × 20 mL) and the aqueous layer was evaporated to a small volume and filtered, and the filtrate was treated with LiOH (to saturation). Anhydrous ethanol (10 volumes) was added to the resulting slurry which was centrifuged at 10 000 rpm, 0 °C for 15 min. The supernatant was discarded, and the precipitate was stirred with Dowex 50 H^+ resin (5 mL) and filtered through the same resin (20 mL). The UV-absorbing fractions (60 mL) were concentrated and filtered. The filtrate was purified by reversed-phase HPLC (flow rate 5 mL/min, H_2O) to give **6** (49 mg, 49%) (retention time 35 min, single peak) as a fluffy white material after lyophilization. Ion-exchange HPLC used the following buffers: A 2mM KH_2PO_4, 8 mM KCl, 0.05 M $MgCl_2$, 0.05% acetonitrile, pH 3.0; B: 0.2 M KH_2PO_4, 0.9 M KCl, 0.8 M $MgCl_2$, 5% acetonitrile, pH 3.0; retention time 70 min (cf., ref 17). ^{1}H NMR (D_2O): 8.31 (br s, 1H, A H-8), 8.11 (s, 1H, A H-2), 7.43 (d, J = 7.8 Hz, 1 H, C H-6), 5.97 (t, J = 6.0 Hz, 1 H, C 1′), 5.75 (br s, 1H, C H-5), 5.24 (q, J = 5.4 Hz, 1H, A 2′), 4.76−4.75 (m, 1H, A 3′), 4.40−4.37 (m, 1H, A 1′), 4.36−4.34 (m, 1H, C 3′), 4.31−4.28 (m, 1H, A 1′), 4.22 (s, 1H, A 4′), 4.14 (dt, 1H, A 5′$_a$), 4.10 (dt, 1H, A 5′$_b$), 3.95 (s, 1H, C 4′), 3.35 (s, 1H, C 5′$_a$), 2.95 (s, 1H, C 5′$_b$), 2.24−2.20 (m, 1H, C 2′$_a$), 2.01−1.97 (m, 1H, C 2′$_b$). ^{13}C NMR, D_2O: 166.6 (C C-4), 157.6 (C C-2), 157.6 (A C-6), 154.5 (A C-2), 154.0 (A C-4, not observable), 143.0 (C C-6), 142.8 (A C-8), 120.0 (A C-5) 98.1 (C C-5), 87.5 (A 4′), 87.1 (C 1′), 87.0 (C 4′), 82.3 (A 3′), 73.3 (A 1′), 71.8 (C 3′), 66.8 (C 5′), 66.1 (A 2′), 63.4 (A 5′), 42.0 (C 2′). ^{31}P NMR (D_2O): 8.8 (s, phosphomonoester), −0.9 (s, phospodiester), ratio 1:1. λ_{max}263 (ϵ = 19500). ESI HRMS: $(M - H)^-$ calcd for $C_{19}H_{25}N_8O_{12}P_2$ 619.1067, found 619.1056; $(M - 2H + Na)^-$ calcd for $C_{19}H_{23}N_8NaO_{18}P_2$ 641.0887, found 641.0906.

2′-Deoxycytidylyl(5′→3′)-1′-deoxy-2′-isoadenosine (7) (21 mg, 66%) was prepared using **5** (0.06 mmol, 54 mg) and concentrated NH_4-OH (15 mL) in a procedure similar to that described for **6**. Reversed-phase HPLC purification of **7** was done at a flow rate 5 mL/min, using water (solvent A) and ethanol (solvent B) in the following linear binary gradient: 100% A (100 min), 0−60% B (160 min); retention time 150 min, single peak. Ion-exchange HPLC: retention time 22 min (cf. ref 17). ^{1} H NMR, (D_2O): 8.25 (s, 1H, A H-8), 8.10 (s, 1H, A H-2), 7.40−7.39 (d, J = 7.2 Hz, 1H, C H-6), 5.94−5.92 (t, J = 6.3 Hz, 1H, C 1′), 5. 66−5.65 (d, J = 7.2 Hz, 1H, C H-5), 5.26−5.24 (qn, J = 3.6 Hz, 1H, A 2′), 4.84−4.82 (m, 1H, A 3′), 4.39−4.36 (m, 1H, A 1′), 4.29−4.26 (q, J = 5.6 Hz, C 3′), 4.24−4.22 (dd, J_1 = 6.6 Hz, J_2 = 4.2 Hz, A 1′), 4.12−4.09 (m, 1H, A 4′), 4.04−4.02 (m, 2H, A 5′$_a$ and C 5′$_a$) and 3.97−3.924 (m, 2H, A 5′$_b$ and C 5′$_b$), 3.89−3.88 (m, 1H, C 4′), 2.25−2.21 (dt, J_1 = 13.8 Hz, J_2 = 6.0 Hz, 1H, C 2′$_a$) and 1.98−1.93 (dt, J_1 = 13.8 Hz, J_2 = 6.0 Hz, 1H, C 2′$_b$). ^{13}C NMR, (D_2O): 164.2 (C C-4), 157.5 (C C-2), 157.4 (A C-6), 154,6 (A C-2), 154.0 (A C-4), 143.5 (A C-6), 143.4 and 143.0 (A C-8), 120.8 (A C-5), 97.5 (C C-5), 87.7 and 87.5 (A 4′), 87.5 (C 1′), 87.1 and 87.0 (C 4′), 82.0 and 82.00 (A 3′), 73.00 (A 1′), 71.7 (C 3′), 66.7 and 66.6 (C 5′), 64.1 and 64.00 (A 2′), 62.7 (A 5′), 42.2 (C 2′). ^{31}P NMR (D_2O): −0.85 (s). λ_{max} 263 (ϵ = 19400). ESI HRMS: $(M - H)^-$ calcd for $C_{19}H_{24}N_8O_9P$ 539.1404, found 539.1402.

***N*-Benzoyl-1′-deoxy-2′-isoadenosine-3′-[(2-chlorophenyl)(3′-*O*-acetylthymidine-5′-yl)] Phosphate (8a).** The 5′-*O*-dimethoxytrityl derivative of **8a** (423 mg, 50%) was prepared using **3** (770 mmol, 670 mg), 3′-*O*-acetyl-thymidine (770 mmol, 210 mg), and TPS-TAZ (1.54 mmol, 555 mg) in a procedure similar to that described for **4**. Detritylation with methylene chloride/2% DCA furnished **8a** (261 mg, 85% yield) as a mixture of diastereomers. It was purified by column chromatography (CH_2Cl_2/7% MeOH). The slower moving diastereomer of **8a** [125 mg, 40%, R_f = 0.40 (CH_2Cl_2/MeOH)]: ^{1}H NMR ($CDCl_3$): 8.37 (s, 1H A H-2), 8.10 (d, 2H, Bz), 7.57−7.10 (m, 8H, Arom), 7.51 (s, 1H, T H-6), 6.19 (dd, J_1 = 5.8 Hz, J_2 = 2.9 Hz, T H-1′), 5.42−5.39 (m, 2H, A 2′ and T 3′), 5.12−5.10 (m, 1H, A 3′), 4.70−4.64 (m, 1H, A 1′$_a$), 4.59−4.52 (m, 1H A 1′$_b$), 4.41−4.37 (m, 1H A 4′), 4.20−4.11 (m, 2H, A 5′), 4.06−4.01 (m, 1H, T 4′), 3.93−3.83 (m, 2H, T 5′), 2.24 (dd, J_1 = 9 Hz, J_2 = 5.7 Hz, T 2′$_a$), 2.01−1.96 (m, 1H, T 2′$_b$), 2.09 (s, 3H, Ac), 1.64 (s, 1H, T CH_3). ^{31}P NMR ($CDCl_3$): −0.50. The faster migrating diastereomer of **8a** [136 mg, 45% yield, R_f = 0.41 (CH_2Cl_2/MeOH)]: ^{1}H NMR ($CDCl_3$): 10.08 (br s, 1H, NH), 8.78 (s, 1H, A H-8), 8.46 (s, 1H, A H-2), 8.16 (d, 2H Bz), 7.56 (s, 1H, T H-6), 7.54−7.17 (m, 8H, Arom), 5.89 (dd, J_1 = 5.8 Hz, J_2 = 2.9 Hz, T 1′), 5.46−5.44 (m, 1H A 2′), 5.25−5.21 (m, 1H, T 3′), 5.09−5.07 (m, 1H, A 3′), 4.53−4.44 (m, 2H, A 1′), 4.22−4.21 (m, 1H, A 4′), 4.30−4.29 (m, 2H, A 5′), 3.95−3.88 and 3.81−3.76 (m, 3H, T 4′ and 5′), 2.23−2.16 (m, 1H, T H-2′$_a$), 2.02−1.96 (m, 1H, T H-2′$_b$), 2.10 (s, 3H, Ac), 1.73 (s, 3H, T CH_3). ^{31}P NMR ($CDCl_3$): −0.72. ESI HRMS: $(M + H)^+$ calcd for $C_{35}H_{36}ClN_7O_{12}P$ 812.1840, found 812.1845.

***N*-Benzoyl-1-deoxy-2-isoadenosine-3′-[(2-chlorophenyl)(3′-*O*-benzoylthymidine-5′-yl)] Phosphate (8b).** The 5′-*O*-dimethoxytrityl derivative of **8b** (118 mg, 100%) was prepared using **3** (0.12 mmol, 109 mg), 3′-*O*-benzoylthymidine (0.1 mmol, 35 mg), and TPS-TAZ (0.3 mmol, 100 mg) in a procedure similar to that described for **4**. Detritylation (CH_2Cl_2/2% DCA,15 mL) and purification by column chromatography (CH_2Cl_2/5%MeOH) gave **8b** (30 mg, 35%) (mixture of diastereomers, ratio 1:1) as a white foam, R_f = 0.35 (CH_2Cl_2/5%MeOH). ^{1}H NMR ($CDCl_3$): 11.06 (br s, 1H, NHBz), 10.17 (br s, 1H, T NH), 8.76 and 8.74 (s and s, 1H, A H-8), 8.39 and 8.37 (s and s, 1H, A H-2), 8.04−7.99 (m, 4H, Bz), 7.62 and 7.59 (s and s, 1H, T-6), 7.69−7.14 (m, 10H, Arom), 6.54−6.37 (m, 1H, T 1′), 5.88 (br s, 1H, 5′-OH), 5.58 (br s, 2H, A 2′ and T 3′), 4.66−4.60 and 3.99−3.91 (9H, unresolved sugar protons signal), 2.66−2.36 (m, 2H, T 2′), 1.84 and 1.82 (d and d, 3H, CH_3). ^{31}P NMR ($CDCl_3$): −8.95 (s), −9.93 (s). ESI HRMS: $(M + H)^+$ calcd for $C_{40}H_{38}ClN_7O_{12}P$ 874.1996, found 874.2021.

Thymidylyl(5′→3′)-1′-deoxy-2′-isoadenosine-5′-phosphate (12) was prepared from **8a** in four steps. Sulfonyldiethanol (20 mmol, 3.08 g) was evaporated with anhydrous pyridine several times and dissolved in 10 mL of pyridine under nitrogen. 4,4′-Dimethoxytrityl chloride (10 mmol, 3.39 g) was added, and the reaction was left overnight at room temperature. The reaction mixture was partitioned between cold methylene chloride (300 mL) and saturated aqueous $NaHCO_3$ (100 mL), and the organic phase was concentrated. The residue was purified by column chromatography (CH_2Cl_2/3% MeOH) to give 2-*O*-(4,4′-dimethoxytrityl) sulfonyldiethanol (4.0 g, 87%) as a colorless oil. ^{1}H NMR ($CDCl_3$): 7.43−7.24 (9H, Arom), 6.35−6.30 (m, 4H, Arom), 4.12 (m, 2H, $DMTrOCH_2$), 3.81 (s, 6H, CH_3O), 3.68 (t, J = 6 Hz, 2H, CH_2OH), 3.40 (t, J = 6 Hz, 2H, $DMTrOCH_2CH_2$), 3.10−3.05 (m, 2H, CH_2CH_2OH). ESI HRMS: $(M + Na)^+$ calcd for $C_{25}H_{28}O_6NaS$ 479.1497, found 479.1510.

The compound from the previous step (5 mmol, 2.28 g) was evaporated with anhydrous pyridine several times and dissolved in 5 mL of pyridine. 1,2,4-Triazole (40 mmol, 276 mg) and TEA (40 mmol, 545 mL) were dissolved in 100 mL of anhydrous THF under nitrogen

and cooled. $POCl_3$ (10 mmol, 0.092 mL) was added dropwise at −5 °C. After stirring for 10 min, the reaction mixture was filtered under nitrogen into the solution prepared above. After 1 h at 0 °C, the reaction mixture was partitioned between cold ethyl acetate (300 mL) and $NaHCO_3$ (saturated, 100 mL), washed with brine (1 × 100 mL), and concentrated. The residue was purified by column chromatography (CH_2Cl_2/12%MeOH) to give 2-*O*-(4,4′-dimethoxytrityl) sulfonyldiethanol disodium phosphate (**10**) as a colorless glassy material (3.0 g, 100%). ^{1}H NMR ($CDCl_3$): 7.45−7.25 (m, 9H, Arom), 6.35−6.30 (m, 4H, Arom), 4.20−4.10 (m, 2H, $DMTrOCH_2$), 3.65−3.45 (m, 2H, CH_2-OP), 3.20−3.15 (m, 2H, $DMTrOCH_2C\underline{H}_2$), 3.10−3.05 (m, 2H, $C\underline{H}_2$-CH_2OP). ^{31}P ($CDCl_3$): −4.90 (br s).

The compound from the previous step (0.5 mmol, 300 mg) (**10**) and **8a** (0.1 mmol, 80 mg) were evaporated several times with anhydrous pyridine and then dissolved in 4 mL of pyridine under nitrogen. TPS-TAZ (1 mmol, 340 mg) was added and the reaction mixture was kept for 0.5 h at room temperature. Workup of the reaction mixture and the following steps were done as described for **5** to give **11** as a colorless glassy material (0.85 mg, 70% from **8a**). Compound **11** (0.7 mmol, 85 mg) was fully deprotected as described for **5**, and worked up as described for **6**. Reversed-phase HPLC purification (flow rate 5 mL/min, H_2O) gave **12** (retention time 30 min, major peak) as a white fluffy material after lyophilization (21 mg, 48%). ^{1}H NMR (D_2O): 8.34 (s, 1H, A H-8), 8.10 (s, 1H, A H-2), 7.24 (s, 1H, T H-6), 6.01 (t, J = 6.1 Hz, 1H, T 1′), 5.27 (qn, J = 3.6 Hz, A 2′), 4.98 (qn, J = 3.6 Hz, A 3′), 4.41−4.32, 4.25−4.09 and 4.02−3.95 (m,m,m, 9H, unresolved sugar signals), 2.21−2.14 (m, 1H, T 2′$_a$), 1.97−1.89 (m, 1H, T 2′$_b$), 1.67 (s, 1H, CH_3-T). ^{31}P NMR (D_2O): −0.19 (br s, phosphomonoester), −1.94 (s, phosphodiester), ratio 1:1. λ_{max} 263 (ϵ = 18000). ESI HRMS: $(M - H)^-$ calcd for $C_{20}H_{25}N_7O_{10}P$ 635.1051, found 635.1078. Minor peak (retention time 35 min) gave **13** (1.5 mg, 3%, see data below).

5-Methyl-2′-deoxycytidinylyl (5′→3′)-1′-deoxy-2′-isoadenosine-5′-phosphate (13). This compound was prepared starting with the same procedure as for **12**, that is, with **8a** (0.1 mmol), the phosphorylating reagent (0.5 mmol), and TPS-TAZ (1 mmol). However, the workup after the first step was different and involved treatment with cold 15% NH_4OH (5 mL) followed by 2 h at room temperature. The reaction mixture was concentrated and partitioned between ethyl acetate (100 mL) and brine (30 mL). The organic phase was separated and concentrated, and the residue was purified by column chromatography (CH_2Cl_2/12%MeOH/1%TEA) to give a colorless glassy material (110 mg, 85%) that was deprotected as described for **6**. Reversed-phase HPLC purification (5 mL/min, H_2O) gave **13** (retention time 35 min) as a white fluffy material after lyophilization (25 mg, 46%). ^{1}H NMR (D_2O): 8.29 (s, 1H, A H-8), 8.03 (s, 1H, A H-2), 7.18 (d, J = 1.0 Hz, 1H, T H-6), 5.94 (t, J = 6.3 Hz, 1H, T H-1′), 5.19 (qn, J = 2.4 Hz, A 2′), 4.91−4.87 (m, 1H, A 3′), 4.33−4.24, 4.19−4.03 and 3.97−3.88 (m,m,m, 9H, unresolved sugar signals), 2.15−2.07 (m, 1H, T 2′$_a$), 1.93−1.84 (m, H, T 2′$_b$), 1.59 (s, 3H, T CH_3). ^{31}P NMR (D_2O): 9.17 (br s, phosphomonoester, −1.49 (s,) phospodiester). λ_{max} 263 (ϵ = 18500). ESI HRMS: $(M - H)^-$ calcd for $C_{20}H_{27}N_8O_{12}P_2$: 633.1161, found 633,1221; $(M - 2H)^{2-}$, calcd for $C_{20}H_{26}N_8O_{12}P_2$: 316.5030; found 316.5015.

Thymidylyl(5′→3′)-1′-deoxy-2′-isoadenosine (9). Compound **9** (15 mg, 75%) was prepared using **8b** (0.035 mmol, 30 mg) and 29% NH_4OH (10 mL) and purified by reversed-phase HPLC as described for **6** (retention time 120 min, single peak). ^{1}H NMR (D_2O): 8.30 (s, 1H, A H-8), 8.13 (s, 1H, A H-2), 7.21 (s, 1H, T H-6), 5.98 (t, J = 7.5 Hz, 1H, T 1′), 5.30−5.26 (m, 1H, A 2′), 4.25−4.19 and 4.10−3.92 (m,m,m, 10H, unresolved sugar proton signals), 2.21−2.15 (m, 1H, T 2′$_a$), 2.08−1.99 (m, 1H, T 2′$_b$), 1.67 (s, 3H, T CH_3). ^{31}P (D_2O): 1.93 (s). λ_{max} 263 (ϵ = 17990). ESI HRMS: $(M - H)^-$calcd for $C_{20}H_{26}N_7O_{13}P_2$: 554.0995; found 554.0980.

Hydrolytic Cleavage Catalyzed by Phosphodiesterases (PDE I and II). Phosphodiesterase I Assays. The reaction mixtures were prepared using 20 μL (0.32 mM), 40 μL (0.64 mM), 60 μL (0.96 mM), and 80 μL (1.28 mM) of dApdC and isodApdC (and other modified dinucleotides) substrate concentrations in 250 μL of 50 mM Tris-Cl buffer (pH 7.6) containing 10 mM $MgCl_2$. Reactions were initiated by the addition of 40 μL of enzyme (5 units/mL) to each reaction mixture at 37 °C for 1 h. The reactions were stopped by addition of 250 μL of 1 M phosphate buffer pH 4.0 containing 5 mM EDTA. The reactions were analyzed by HPLC using a C_{18} column (3.9 × 300 mm) with a gradient of the following: 0−20 min, 100% water; 20−40 min, 90:10 water:methanol; 40−60 min, 80:20 water:methanol, and 60−70 min, 70:30 water:methanol. The flow rate was 0.6 mL/min. The retention times for both PDE assays were as follows: 2′-deoxyadenosine and 2′-isodeoxyadenosine, 52 min; 2′-deoxycytidine, 28 min; 2′-deoxycytidine 5′-monophosphate, 9 min; 2′-deoxyadenosine 3′-monophosphate, 2′-isodeoxy-adenosine 3′-monophosphate, 26 min; dApdC and IsodApdC, 46 min.

Phophodiesterase II Assays. The assay mixtures were prepared using 20 μL (0.32 mM), 40 μL (0.64 mM), 60 μL (0.96 mM), and 80 μL (1.28 mM) of dApdC and isodApdC and other substrate concentrations in the 250 μL of 0.1 M acetate buffer (pH 6.0) containing 10 mM $MgCl_2$. The reactions were initiated by the addition of 5 μL of enzyme (5 units/mL) to the reaction mixture at 37 °C for 1 h. The reactions were stopped by addition of 250 μL of 0.5 M Tris-Cl buffer (pH 10.0) containing 5 mM EDTA and analyzed by HPLC as described above.

Anti-Integrase Studies. Anti-integrase studies were carried out as described previously by Pommier and co-workers.[27]

In brief, IN was preincubated at a final concentration of 200 nM with the inhibitor in reaction buffer (50 mM NaCl, 1 mM HEPES, [pH 7.5], 50 μM EDTA, 50 μM dithiothreitol, 10% glycerol [wt/vol], 7.5 mM $MnCl_2$, 0.1 mg of bovine serum albumin per ml, 10 mM 2-mercaptoethanol, 10% DMSO, and 25 mM MOPS [morpholinepropanesulfonic acid] [pH 7.2]) at 30 °C for 30 min. Then, the 5′-end ^{32}P-labeled linear oligonucleotide substrate (20 nM) was added, and incubation was continued for 1 h. Reactions were quenched by the addition of an equal volume (16 μL) of loading dye (98% deionized formamide, 10 mM EDTA, 0.025% xylene cyanol, 0.025% bromophenol blue). An aliquot (5 μL) was electrophoresed on a denaturing 20% polyacrylamide gel (0.09 M Tris-borate [pH 8.3], 2 mM EDTA, 20% acrylamide, 8 M urea). Gels were dried, exposed in a PhosphorImager cassette, and analyzed with a Molecular Dynamics PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Percent inhibition was calculated using the following equation: inhibition = $100[1 - (D - C)/(N - C)]$, where C, N, and D are the fractions of 21-mer substrate converted to 19-mer (3′-processing product) or strand transfer products for DNA alone, DNA plus IN, and IN plus drug, respectively. The 50% inhibitory concentrations (IC_{50}) were determined by plotting the log of drug concentration versus percent inhibition and identifying the concentration which produced an inhibition of 50%.

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