

Phosphoramidate Dinucleosides as Hepatitis C Virus Polymerase Inhibitors

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GC dinucleosides exhibiting a phosphoramidate internucleosidic linkage with neutral, amphiphile, positively or negatively charged side chains were synthesized. Their potential inhibitory effect on the hepatitis C virus (HCV) NS5B polymerase was evaluated in vitro and in HCV replicon containing cells. Whereas the amphiphile and the positively charged analogues were found to be inactive, the neutral (**1**) and the negatively charged (**4**) ones inhibited enzyme activity when tested as a diastereoisomeric mixture. The most potent inhibitor proved to be the Sp isomer of the 5'-thiophosphorylated dinucleotide bearing the carboxylic side chain (**8**) (IC₅₀ of 25 μ M in vitro and an EC₅₀ of 9 μ M in HCV subgenomic replicon). Molecular modeling suggests that the phosphoramidate dinucleoside (**8**) is stabilized in the active site by interactions with magnesium ions and lysine and arginine residues of the polymerase.

Introduction

The hepatitis C virus (HCV^a) represents a global health problem; about 3% of the world's population is chronically infected and at risk of developing liver cirrhosis and/or hepatocellular carcinoma. HCV infection is the leading reason for liver transplantation in the West.¹ Current therapy consists of a combination of interferon- α with the nucleoside analogue ribavirin and results in 40–70% of sustained virological responses depending on the genotype.² However, this therapy is associated with significant side effects,³ and there is no approved therapeutic alternative for nonresponders. There is thus an urgent need for new therapeutic molecules active against the HCV infection.

HCV is an enveloped, positive-strand RNA virus; the 9.6 kb genome encodes a single large polyprotein, which is cleaved by cellular and viral proteases into four structural (Core, E1, E2, and p7) and six nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B). The nonstructural proteins assemble into a replication complex that is responsible for the viral RNA genome synthesis (for a review see ref 4). Because they bear essential enzymatic activities for viral replication, the NS3 protease-helicase and the NS5B polymerase are targets of choice for antiviral intervention. Moreover, the NS5B polymerase is the key component of the replicative complex. It is a endoplasmic reticulum membrane-associated RNA-dependent-RNA-

polymerase (RdRp) able to replicate RNA without a primer in a so-called de novo synthesis mechanism.⁵ In vitro, however, the C-terminal truncated recombinant polymerase can use a wide range of hetero- or homopolymeric templates in a primer-dependent or primer-independent fashion.⁶ During de novo RNA synthesis, the RdRp has to synthesize first its RNA primer and then copy the entire RNA template. RNA synthesis can thus be separated into two steps^{7,8} that are structurally and biochemically distinct: the initiation step leading to the RNA primer synthesis and the elongation step of the neosynthesized RNA up to the end of the template.

Intensive drug screening campaigns have led to the discovery of many NS5B inhibitors, some of them being evaluated in clinical settings (reviewed in refs 9, 10). Two main classes of NS5B RdRp inhibitors have been reported: non-nucleoside inhibitors (NNI) and nucleoside inhibitors (NI). NNIs are allosteric, noncompetitive inhibitors that target the alloenzyme, free of RNA substrates (reviewed in ref 11). For most of them, the mode of action requires the binding to the apoenzyme before engagement of the enzyme into an elongative complex. For NIs, the corresponding triphosphates (TPs) are competitors of the natural nucleoside triphosphates and can be used as substrates by the polymerase. After their incorporation into the nascent RNA, they act as nonobligate chain terminators.¹² Alternatively, compounds that target the active site before the enzyme has entered into the elongation steps of RNA synthesis may be potent inhibitors. For example, it has been reported that dinucleotides can be used more efficiently as primer molecules than mononucleotides in in vitro RNA replication assay.⁶ On the basis of these results modified dinucleotides, lacking the 3'-hydroxyl necessary for the polymerization reaction, were synthesized and were shown able to decrease the RNA synthesis measured in vitro, although with modest IC₅₀ values.¹³

We took advantage of this novel approach to synthesize new dinucleotides bearing modifications on the internucleosidic linkage in order to increase both their binding to the active site of the polymerase and their affinity to the RNA template and hence their inhibition properties. Our target compounds were

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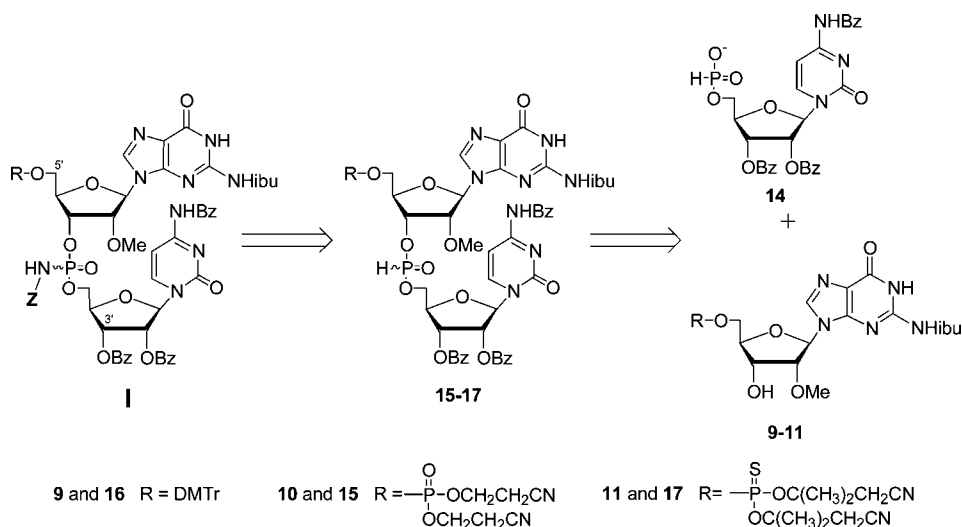
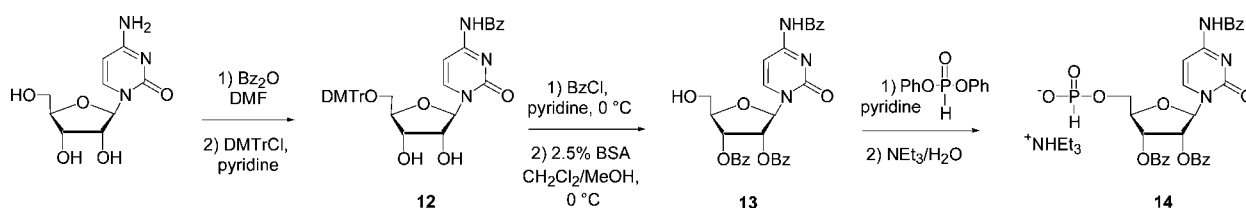
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^a Abbreviations: HCV, hepatitis C virus; NS3, non structural protein 3; NS5B, non structural protein 5B; RdRp, RNA-dependent-RNA-polymerase; NNI, non nucleoside inhibitor; NI, nucleoside inhibitor; MP, monophosphate; TP, triphosphate; GC, guanosin-3'-yl-cytidin-5'-yl dinucleotide; GMP, guanosine monophosphate; GTP, guanosine triphosphate; NTP, nucleoside triphosphate.

Scheme 1. Retrosynthetic Pathway for the Synthesis of Phosphoramidate Dinucleosides **I****Scheme 2.** Synthetic Pathway for the Synthesis of **14**

designed based on several previously published structural data of the NS5B enzyme in its putative RNA synthesis initiation conformation.^{14,15} As it was also previously demonstrated that the 5'-phosphate group of the dinucleotide was required for inhibition,¹³ probably because it allowed hydrogen bonding and electrostatic interaction with residues present in the active site, we decided to study this modification thoroughly. Moreover, such 5'-phosphorylated dinucleotide structures would act as direct substrate to the enzyme, without the need of any intracellular 5'-phosphorylation, as required for NIs. As a modification for the internucleosidic linkage, we chose a phosphoramidate diester linkage. The latter is a chemical modification used either in a prodrug approach for nucleoside analogues^{16–18} or in modified oligonucleotides^{19,20} and allows the introduction of a large variety of side chain functionalities.

Because the 3'-end of the viral RNA negative strand template of HCV is GC^{3'}, the complementary sequence of the dimer is 5'GC. A 2'-*O*-methyl guanosine was chosen instead of natural guanosine to gain higher enzymatic and chemical stability of the final dinucleotide. The phosphoramidate linkage is also more resistant to nucleases,²¹ hence the resulting dinucleotides would have higher stability in biological media. Thus, we first designed and synthesized 5'-phosphorylated dinucleotides made of a 2'-*O*-methyl guanosine and a cytidine, linked together with a phosphoramidate function bearing different side chains.²⁰ To establish a structure–activity relationship (SAR), we used side chains with neutral, cationic, amphiphile, and anionic groups exhibiting different properties. Then, to study the influence of the 5'-environment, phosphoramidate dinucleotide analogues with a 5'-hydroxyl group or bearing a 5'-phosphorothioate monoester instead of the 5'-phosphate monoester were prepared. Then, we evaluated their ability to inhibit the NS5B polymerase activity *in vitro*. A molecular model of the NS5B polymerase and the most potent dinucleotide analogue was constructed to explain the mechanism of inhibition. Finally, the potential

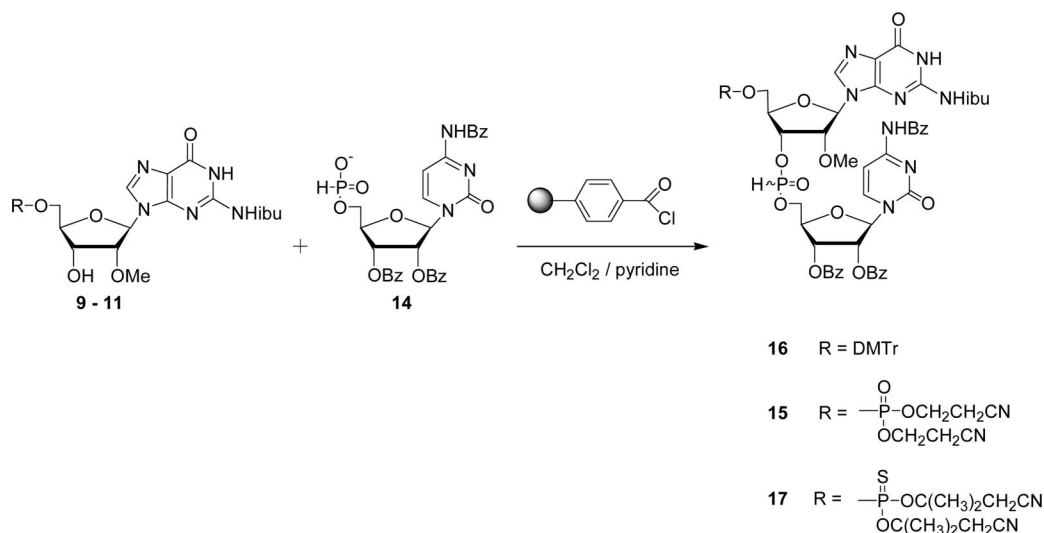
inhibitory effect on HCV replicon replication in cell cultures was evaluated.

Results and Discussion

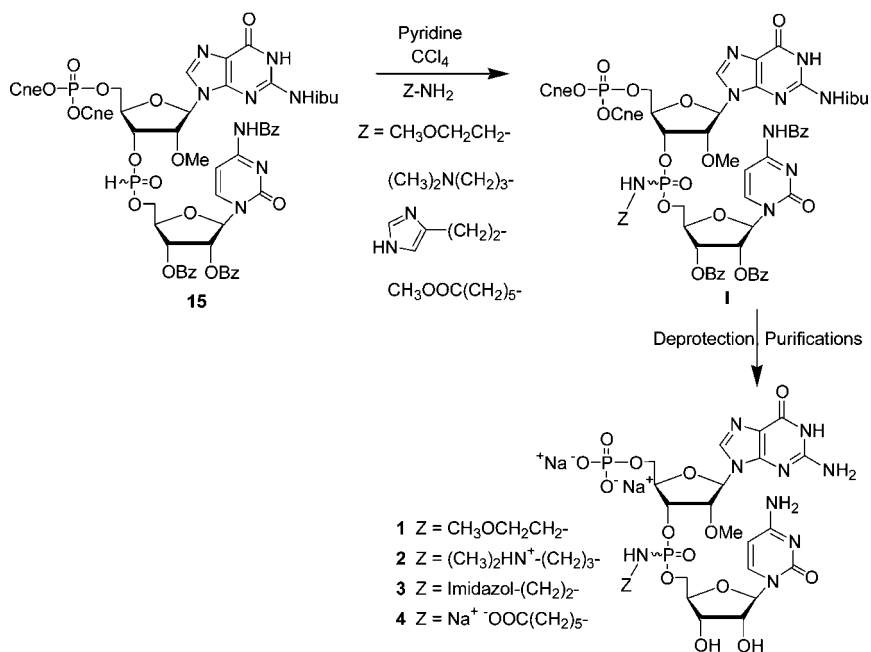
Chemistry. The preparation of these 2'-*O*-Me-GC phosphoramidate dinucleosides proceeded by a strategy using convergent and divergent syntheses. First, a convergent synthesis using 3'-OH-2'-*O*-Me guanosine derivatives (**9–11**) and the 5'-*H*-phosphonate monoester of *N*⁴,2',3'-*O*,*O*-tribenzoyl cytidine (**14**) was applied, affording common intermediates **15–17** with a *H*-phosphonate diester linkage between the 2'-*O*-Me guanosine and the cytidine moieties. Second, the divergent strategy concerned the amidative oxidation,²² allowing, from the common intermediates **15–17**, the preparation of the different dimers **I** with various phosphoramidate side chains (Scheme 1).

The building block **9** is commercially available. The building blocks **10** and **11** were synthesized from *N*²-isobutyryl-2'-*O*-methyl guanosine²³ by a selective 5'-phosphitylation using solid-supported reagents as previously reported.^{24,25}

The *N*⁴,2',3'-*O*,*O*-tribenzoyl-5'-*H*-phosphonate monoester of cytidine (**14**) was prepared according to a five-step procedure with a 63% overall yield (Scheme 2). Cytidine was first selectively benzoylated on the *N*⁴ position by means of benzoic anhydride in DMF.²⁶ After replacement of DMF by pyridine, the crude was directly dimethoxytritylated on the 5'-hydroxyl (90%) by addition of dimethoxytrityl chloride (Scheme 2). After purification, the nucleoside **12** was benzoylated on both 2' and 3' hydroxyls by means of benzoyl chloride, and after workup, the crude was directly treated with benzene sulfonic acid, affording after purification the *N*⁴,2',3'-*O*,*O*-tribenzoyl cytidine **13** (88%). Finally, **13** was treated with diphenylphosphite followed by aqueous hydrolysis,²⁷ affording the expected 5'-*H*-phosphonate monoester of *N*⁴,2',3'-*O*,*O*-tribenzoyl cytidine **14** (80%).

Scheme 3. Coupling of 9–11 and 14 Affording *H*-Phosphonate Diester Dimers 15–17

Scheme 4. Amidative Oxidations of 15 and Deprotection Affording 1, 2, 3, and 4



The coupling of the two building blocks **14** and **9–11** to form *H*-phosphonate diesters **15–17** was carried out using a solid-supported acyl chloride activator²⁸ (Scheme 3). This coupling proceeded in two steps, first, the *H*-phosphonate monoester function of **14** reacted with the solid-supported acyl chloride and, second, the formed mixed anhydride was displaced by the 3'-hydroxyl of **9–11** to yield **15–17** in solution. By this way, the excess of **14** was trapped on the solid support and only dimers **15–17** and pyridinium salt remained in solution. A simple filtration, an aqueous extraction, and a precipitation in diethyl ether afforded the dimers **15–17** with >85% HPLC purity (Figure S1 in Supporting Information). Because this coupling reaction is nonstereoselective, the *H*-phosphonate diesters were all a ~1:1 mixture of *R_p* and *S_p* diastereoisomers on the chiral phosphorus center and their presence was confirmed by ³¹P NMR as two peaks ($\delta_{\text{P-H}}$ between 7.3 and 9.8 ppm).

The crude *H*-phosphonate diesters **15–17** were dissolved in dry pyridine, and a mixture of carbon tetrachloride and amine

was added. The amidative oxidation occurred to form the two diastereoisomers (*S_p* and *R_p*) of the phosphoramidate **I** with some minor formation of phosphodiester linkage corresponding to a side-oxidation due to trace of water. First, the oxidation of **15** was performed in the presence of four different amines: 2-methoxyethylamine, 3-(dimethylamino)-1-propylamine, histamine, or 6-aminocaproic acid methyl ester to afford, after deprotection, the corresponding dimers bearing neutral (**1**), cationic (**2**), amphiphile (**3**), or anionic (**4**) side chains, respectively (Scheme 4). Pure target compounds were obtained after two chromatographic steps (ion exchange and reverse phase). Finally, sodium ion-exchange was performed, yielding the desired compounds as their sodium salt.

To further establish the role of the 5'-terminal function group in a SAR, dimers **16** and **17** were also oxidized in presence of 2-methoxyethylamine and 6-aminocaproic acid methyl ester, affording, after deprotection, phosphoramidate dimers with a neutral or an anionic chain, **5–6** and **7–8**, respectively, bearing

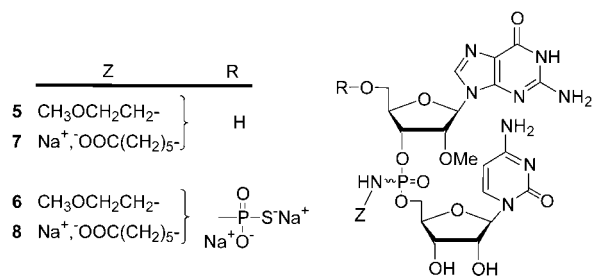


Figure 1. Schematic Structure of Phosphoramidate Dimers 5–8.

a 5'-hydroxyl function for **5** and **7** and a 5'-phosphorothioate monoester function for **6** and **8** (Figure 1).

For each target compound, a mixture of two diastereoisomers exhibiting the Sp and the Rp absolute configurations on the asymmetric phosphorus atom of the phosphoramidate linkage was obtained and this was confirmed by NMR and sometimes visible on the HPLC spectra (as two peaks). The mixture was directly tested for inhibitory evaluation. Henceforth, the more potent compounds tested as isomeric mixture were further separated by HPLC on a preparative reverse phase C₁₈ column, and each diastereoisomer was isolated pure (see Supporting Information for HPLC and NMR examples). For all isolated diastereoisomers, a clear correlation was established between the retention times on RP-HPLC and the ³¹P NMR chemical shift of the phosphoramidate bond, as previously noticed²⁹ (see Table S1 in Supporting Information). This correlation was also observed in the inhibition assays. In analogy to the established behavior of dinucleoside phosphoramidate diastereoisomers,²⁹ the isomer with the shorter retention time on RP-HPLC (named "fast" or F) and with the more upfield ³¹P chemical shift of the P–N bond (named "uf") was assigned as the diastereoisomer with the Rp configuration, while the other (named "slow" or S and "df") was assigned to the one with the Sp configuration. The validity of this assignment is supported by the results of the molecular dynamics (MD) experiments (see below).

In Vitro Determination of IC₅₀. In a previous work using a polyC template, we studied the RNA polymerization catalyzed by NS5B and identified three kinetically distinct steps.⁷ First, the very first phosphodiester bond formation from G₁ to G₂ occurs rapidly, and G₂ products accumulate over time. Second, products from G₂ up to G₆ are formed. Subsequently, a third phase occurs in which G₆ products are elongated in a processive fashion into template-length, runoff products. The formation of G₂, of G₃ to G₆, and of >G₆ products has been referred to as initiation, transition, and elongation of RNA synthesis, respectively.⁷ Bearing GC nucleobases complementary to the 3'-end of a synthetic oligonucleotide template corresponding to the 3'-end of the negative strand of the HCV genome, the phosphoramidate dinucleosides were designed to target the initiation step of RNA synthesis. This initiation phase is defined as the formation of the first phosphodiester bond between two nucleotides, leading to a ribodinucleotide primer.

We measured the production of the pppGC product and the ability of phosphoramidate dinucleosides to inhibit this reaction (Figure 2A). As reported by others for such a template,³⁰ the HCV polymerase is able to initiate RNA synthesis at two sites, giving rise to the desired product pppGC and a minor pppCC byproduct, with no effect on the calculations because the CTP concentration was kept constant throughout all the experiments. When a phosphoramidate dinucleoside is added in the reaction (up to 500 μM), the accumulation of pppGC product is decreased, reflecting the inhibition of RNA synthesis (Figure 2A). The concentration of the pppGC product can be quantified,

and increasing the concentration of the phosphoramidate dinucleosides allowed determining the IC₅₀ values of the different phosphoramidate dinucleosides (Figure 2B–C). The phosphoramidate dinucleosides harboring either positively charged (**2**) or amphiphile, with a more hindered imidazoleethyl (**3**), side chains have no significant effect on pppGC formation. However, when the phosphoramidate linkage is either neutral (**1**) or negatively charged (**4**), a significant and reproducible inhibition was observed, with the best effect measured with the negatively charged phosphoramidate dinucleoside **4** (IC₅₀ = 69 μM). Therefore, only compounds **1** and **4** were selected for further study.

We then studied the influence of the chemical nature of the 5'-moieties on the inhibitory effect of the inhibitors. In the first series, the phosphoramidate dinucleosides were synthesized with a 5'-phosphate monoester. Indeed, it was shown that guanosine monophosphate (GMP) was able to correctly initiate the RNA synthesis with a better efficiency than the natural guanosine triphosphate (GTP),³¹ suggesting a higher affinity of monophosphate compounds for the active site. For SAR studies, the 5'-nonphosphorylated and the 5'-thiophosphate analogues were also synthesized and evaluated for their inhibition *in vitro*. As expected,¹³ the removal of the phosphate monoester group at the 5'-position of **1** or **4** resulted in a loss of activity, with an increase of IC₅₀ to >200 μM and 160 μM for compounds **5** and **7**, respectively (not shown). However, the 5'-thiophosphate substitution increased the inhibition potency of the phosphoramidate dinucleosides (Figure 3) regardless of the nature of the phosphoramidate side chain; for both neutral (**6**) and negatively charged (**8**) phosphoramidate dinucleosides, the IC₅₀ value is decreased up to 2-fold (Figure 3A: 52 μM for **8** and 47 μM for **6**) compared to the 5'-monophosphate phosphoramidate dinucleotides (Figure 3A: 69 μM for **4** and 106 μM for **1**).

For phosphoramidate dinucleosides **1**, **4**, **6**, and **8**, the Sp and Rp diastereoisomeric mixture was separated and distinct isomers were tested. The IC₅₀ values show that the best inhibition potency is always observed for the slowly eluting isomers (i.e., with longer retention time in RP-HPLC) (Figure 3B, noted S for "slow" in the figure). The "slow" dimer is always 2- to 3-fold more potent than the "fast" one (Figure 3B, for example 54 μM for **8F** compared to 25 μM for **8S**). Moreover, this tendency is observed irrespective to the nature of the phosphoramidate linkage. Finally, the separation of the diastereoisomers did not change the SAR trend obtained with the mixtures: series **1** is always the less potent, followed by the negatively charged **4**, the 5'-thiophosphorylated **6** and **8** series being the most potent.

Molecular Dynamics Study of the Polymerase–Phosphoramidate Dinucleoside Complex. Structural studies of the HCV polymerase have indicated that the active site is encircled by a peculiar secondary structure, the flap, on one side, and the fingers on the other.^{14,15} Therefore, the orientation of the phosphoramidate modification may influence the accommodation of the dinucleotide in this extremely closed and constrained active site.

Indeed, only the Sp isomer was straightforwardly docked within the active site of the HCV RdRp (Figure 4). As expected, important hydrogen bond and/or electrostatic interactions between the 5'-phosphate/thiophosphate moiety and three arginine residues (ARG386, ARG394, ARG158) can be observed in our model. In the Sp absolute configuration, the nonbridging oxygen atom of the internucleosidic linkage is situated in a pseudo-equatorial position, directed toward the two magnesium ions present in the polymerization site, thus allowing complexation between them. Moreover, the only way to accommodate smoothly the long phosphoramidate side chain into the HCV

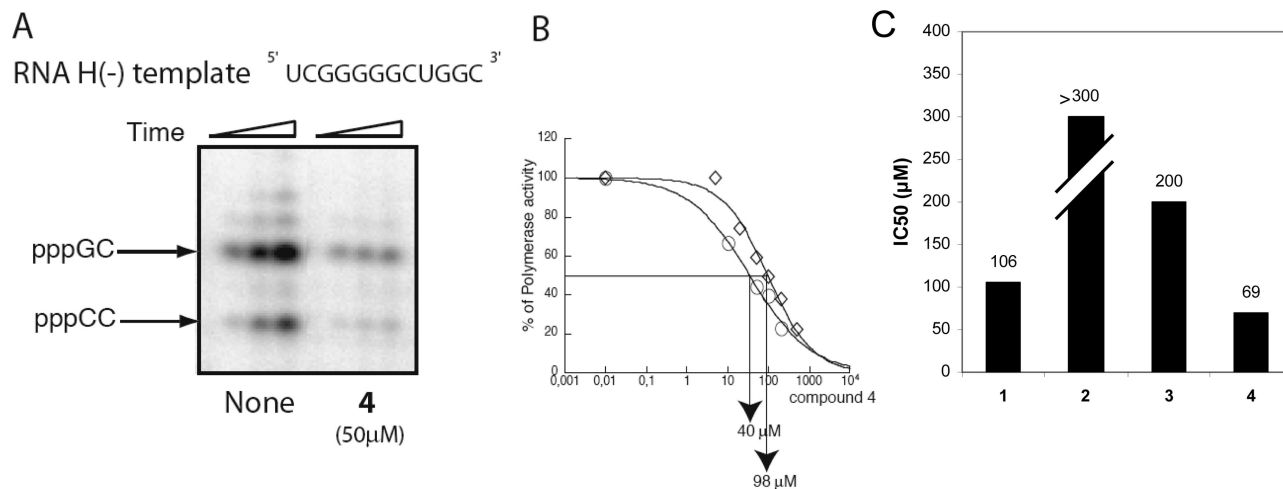


Figure 2. Phosphoramidate dinucleosides decrease the formation of initiation products (pppGC and pppCC) synthesized by wild-type NS5B. (A) Time course of the formation of initiation products by the NS5B polymerase using an RNA template corresponding to the 3' end of the HCV strand (-) genome in the presence of phosphoramidate dinucleoside **4** at the concentration of 50 μM. Initiation products were separated by electrophoresis on denaturated sequencing gel. (B) pppGC products obtained for various concentrations of compound **4** were quantified and values reported as a function of concentration. Curves from 2 independent experiments are shown, and IC₅₀ values calculated from these curves are indicated under the arrow. (C) pppGC products obtained for various concentrations of compounds **1–4** were quantified as described in (B). IC₅₀ values were calculated and are represented as a bar for each phosphoramidate dinucleosides. All data represents average values for at least three separated experiments.

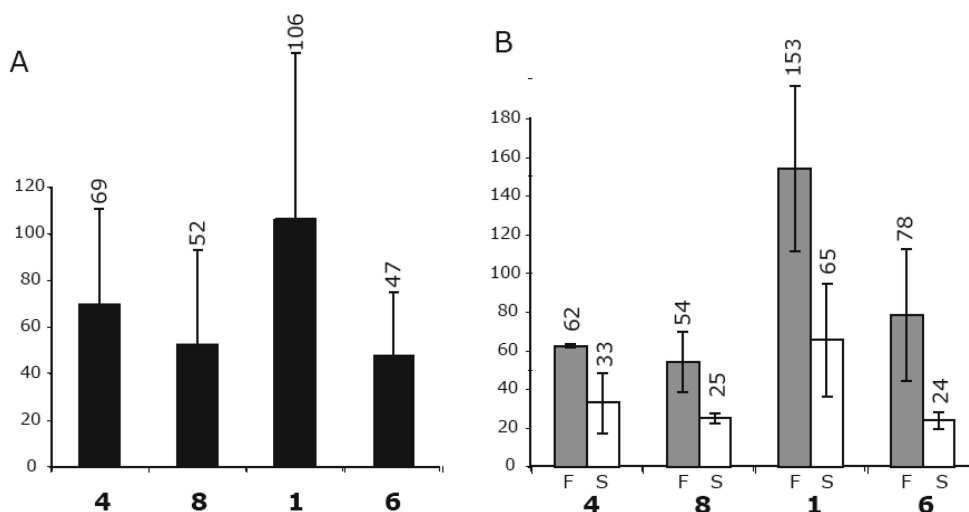


Figure 3. 5'-Thiophosphate modification and separation of the diastereoisomers increase the inhibition potency of phosphoramidate dinucleosides. (A) IC₅₀ values of negatively charged (**4** and **8**) or neutral (**1** and **6**) phosphoramidate dinucleosides. (B) In vitro evaluation of separated diastereoisomers of negatively charged (**4** and **8**) or neutral (**1** and **6**) phosphoramidate dinucleosides. "F" stands for "fast", meaning the fast eluting isomer on RP-HPLC, and "S" stands for "slow", meaning the slowly eluting isomer.

RdRp active site is to direct it toward the nucleotide entry channel, again in the Sp absolute configuration. Thus, the negatively charged side chain interacts with arginine and lysine residues (ARG158, ARG48, LYS155), situated on the incoming pathway for nucleoside triphosphates (NTPs) during polymerization. In a steric clash way, this configuration may block the access of NTPs to the polymerization site, suggesting an explanation to the observed inhibitory effect. In the other orientation, the Rp absolute configuration, the phosphoramidate linkage would be in conflict with Mg²⁺ ions in the active site. Possibly, Rp oriented phosphoramidate linkage could evict Mg²⁺ ions and create direct hydrogen bonds with ASP residues in the active site. Such stabilizing interaction would be probably less efficient than the strong Coulombic attraction originating from sandwiching of Mg²⁺ ions between ASP residues and the oxygen from the phosphoramidate group.

The phosphoramidate dinucleoside mimics the presence of the initiation GC dinucleotide product in the active site of the HCV RdRp. Transition from the initiation to the later phase is initiated by repositioning of the GC product and enables the accommodation of another NTP into the active site. It seems, however, to be disabled by the cooperative action of three potent anchors: the 5'-phosphate/thiophosphate moiety, the carboxylic side chain and the nonbridging oxygen atom from the phosphoramidate linkage both positioned in the Sp absolute configuration. Moreover, the intrusion of some NTPs into the active site seems to be obstructed by the long carboxylate side chains of the phosphoramidate, creating a net of hydrogen bonds with positively charged ARG/LYS residues, which normally paved the way for incoming NTPs into the active site. In that way, ARG/LYS residues interacting with the phosphoramidate side chains are crippled in serving as clips for negatively charged

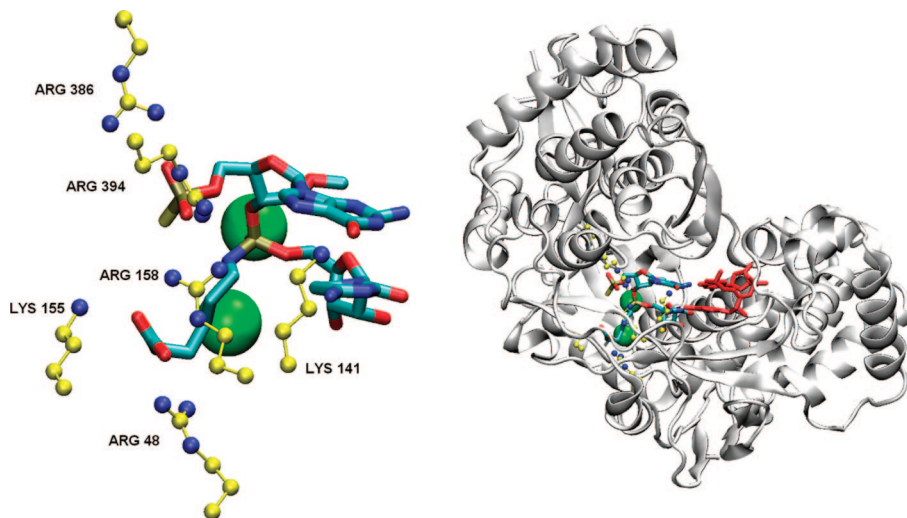


Figure 4. Molecular modeling of **8** in the Sp configuration with its interactions with amino acid residues of the NS5B polymerase in the active site.

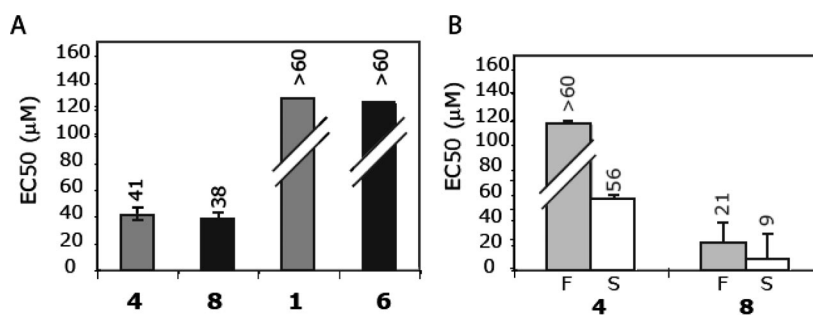


Figure 5. (A) Evaluation of compounds **1**, **4**, **6**, and **8** in Huh-5-2 cells, containing HCV subgenomic replicon, cultured with increasing amount of the corresponding phosphoramidate dinucleoside. (B) Evaluation of the separated diastereoisomers of compounds **4** and **8**. HCV replication was analyzed by quantification of the luciferase activity reporter protein after 72 h, and the signals for each individual concentration of the different compounds were expressed as a percentage of untreated cells and used to calculate the EC₅₀. All data represents average values for at least three independent experiments.

triphosphate moieties of incoming NTPs. Interestingly, it was reported recently that the 5'-phosphoramidate of deoxyadenosine bearing an L-aspartic amino acid could act as a substrate for HIV-1 reverse transcriptase.^{32,33} In this case, aspartic acid mimics the pyrophosphate of NTP, showing that such a carboxylic function could interact with amino acids of the polymerase in the active site.

As an addition to the *in vitro* SAR developed, the model of the polymerase–phosphoramidate dinucleoside complex refined by molecular dynamics simulations settles the better inhibitory activity observed for 5'-phosphorylated/thiophosphorylated, negatively charged phosphoramidates in the Sp absolute configuration (slow eluting diastereoisomers) as the whole of these modifications revealed to be important in our model by crucial interactions with amino acid residues within the binding site.

Activity of Phosphoramidate Dinucleosides on HCV subgenomic Replicon replication. The more potent dinucleotides active against the NS5B polymerase were evaluated in Huh-5-2 cells, a hepatocyte cell line stably carrying the HCV replicon I389luc-ubi-neo/NS3-3'/5.1. This replicon contains a firefly luciferase-ubiquitin-neomycin phosphotransferase fusion protein used as a reporter gene, and its expression is strictly dependent on the level of replicon replication. The expression of the non-structural NS3-5B HCV polyprotein is driven by the EMCV-IRES. The activities of compounds **1**, **4**, and their 5'-thiophosphate analogues **6**, **8** were evaluated in this model (Figure 5). All compounds proved relatively nontoxic (CC₅₀ >60

μM, not shown). In contrast to the biochemical studies, compounds **1** and **6** (diastereomeric mixtures) did not inhibit replicon replication at the highest concentration (60 μM) evaluated. The diastereomeric mixture of anionic phosphoramidate dinucleosides **4** and **8** resulted in inhibition of HCV replicons with EC₅₀ values of ~40 μM (Figure 5A). The “slow” diastereoisomers (**4S** and **8S**) were about 2-fold more active (Figure 5B, 56 and 9 μM, respectively) than the “fast” ones (**4F** and **8F**, >60 and 21 μM, respectively).

The best inhibition was obtained with both isomers of the 5'-thiophosphate negatively charged phosphoramidate dinucleoside (**8S** and **8F**, EC₅₀: 9 and 21 μM, respectively), which appeared to be more potent than the active isomer (**4S**) of the 5'-monophosphate analogue (EC₅₀: 56 μM). This difference may be the result of a better enzymatic stability of the 5'-thiophosphate analogue.³⁴ To our knowledge, no similar inhibitory effect on HCV replication in replicon containing cells was ever reported in the literature using dinucleotides as inhibitors.

Conclusion

In our search for new potent HCV polymerase inhibitors, we have synthesized different phosphoramidate dinucleosides and evaluated their inhibition activity in *in vitro* analysis on purified recombinant NS5B polymerase. Our SAR study revealed that the highest inhibition potency was obtained when the phosphoramidate dinucleosides bear both a negatively charged side chain and a 5'-thiophosphate modification. Moreover, Sp diastereoi-

omers were always found to be more active than Rp diastereoisomers, irrespective to the nature of the phosphoramidate linkage. This was tentatively explained by molecular modeling of the more potent phosphoramidate dinucleoside, **8S**, in the HCV polymerase active site. Finally, this compound proved also more potent to inhibit the HCV replication as measured in Huh-5-2 replicon containing cells. Thus, phosphoramidate dinucleosides bearing a 5-carboxypentyl side chain on the internucleosidic linkage show promising inhibiting activities, on both NS5B polymerase and HCV subgenomic replication, which makes them new potential candidates for anti-HCV therapy.

Experimental Section

HCV 1b Polymerase Plasmid Constructs, Enzyme Preparation, and Reagents. NS5B- Δ 55 gene was tagged with six C-terminal histidines and expressed from the pDest 14 vector (Invitrogen) in *Escherichia coli* BL21(DE3) cells (Novagen). NS5B protein was purified as described previously.⁷ RNA oligonucleotides were obtained from MWG-Biotech. α -³²P-labeled cytosine 5'-triphosphate (3000 Ci/mmol) was purchased from Amersham.

Determination of Inhibitory Concentration 50% (IC₅₀). An RNA oligonucleotide corresponding to the 3' end of the negative strand of the HCV genome (RNA H (-) 5'-UCGGGGGCGUGGC-3') was used to analyze the synthesis of the first phosphodiester bond and its inhibition induced by the modified phosphoramidate dinucleotides. The modified dinucleotides concentration, leading to 50% inhibition of NS5B-mediated RNA synthesis, was determined in RdRp buffer (50 mM HEPES pH 8.0, 10 mM KCl, 2 mM MnCl₂, 1 mM MgCl₂, 10 mM DTT, 0.5% Igepal CA630) containing 10 μ M of RNA template, 100 μ M α -³²P-CTP (1 μ Ci), 50 μ M GTP, and various concentration of dinucleotides (0, 5, 10, 20, 50, 100, 200, 500 μ M). Reactions were initiated by the addition of 1 μ M NS5B, incubated at 30 °C for 15 min, and quenched with EDTA/formamide. Products were separated using sequencing gel electrophoresis and quantified using photostimulated plates and a Fuji Imager (Fuji). IC₅₀ was determined using the equation:

$$\% \text{ of active enzyme} = 100 / (1 + (I)^2 / IC_{50}) \quad (1)$$

where *I* is the concentration of the inhibitor. IC₅₀ was determined from curve-fitting using Kaleidagraph (Synergy Software).

Evaluation of Antiviral Activity and Cytostatic Activities of Selected Compounds in HCV Genotype 1b Subgenomic Replicon Carrying Huh-5-2 Cells. Huh-5-2 cells were cultured in RPMI medium (GIBCO) supplemented with 10% fetal calf serum, 2 mM L-glutamine (Life Technologies), 1 \times nonessential amino acids (Life Technologies), 100 IU/mL penicillin and 100 μ g/mL streptomycin, and 250 μ g/mL G418 (Geneticin, Life Technologies). Cells were seeded at a density of 7000 cells per well in a 96-well View Plate (Packard) in medium containing the same components as described above, except for G418. Cells were allowed to adhere and proliferate for 24 h. At that time, culture medium was removed and five serial dilutions (5-fold dilutions starting at 50 μ g/mL or around 60 μ M) of the test compounds were added in culture medium lacking G418. Interferon α -2a (500 IU) was included as a positive control in each experiment for internal validation. Plates were further incubated at 37 °C and 5% CO₂ for 72 h. Replication of the HCV replicon in Huh-5-2 cells resulted in luciferase activity in the cells. Luciferase activity was measured by adding 50 μ L of 1 \times Glo-lysis buffer (Promega) for 15 min followed by 50 μ L of the Steady-Glo luciferase assay reagent (Promega). Luciferase activity was measured with a luminometer and the signal in each individual well is expressed as a percentage of the untreated cultures. The 50% effective concentrations (EC₅₀) were calculated from these data sets. Parallel cultures of Huh-5-2 cells, seeded at a density of 7000 cells/well of classical 96-well cell culture plates (Becton-Dickinson) were treated in a similar fashion except that no Glo-lysis buffer or Steady-Glo luciferase reagent was added. The effect of the compounds on the proliferation of the cells was measured 3 days after addition of the various compounds by means of The CellTiter 96 AQueous

nonradioactive cell proliferation assay (MTS, Promega). In this assay, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was bio-reduced by cells into a formazan that is soluble in tissue. The number of cells correlates directly with the production of the formazan. The MTS stained cultures were quantified in a plate reader.

General Procedures. TLC was performed on Merck silica coated plates 60F₂₅₄ (art. 5554). Compounds were revealed on UV light (254 nm) and after spraying with 10% sulfuric acid ethanol solution and heating. Phosphorus containing compounds were revealed after spraying with the Hanes molybdate reagent³⁵ and heating. Column chromatography was performed on silica gel Merck 60 (art. 9385). Flash chromatography was performed using a Biotage SP1 apparatus and Biotage silica gel columns. Ion exchange purifications were carried using Pharmacia Sephadex DEAE A-25 resin (art. 170), ISCO TRIS Pump and UA-6 detector (254 nm). Triethylammonium bicarbonate (TEAB) 1 M, pH 7.5, was prepared from triethylamine, milli Q water (18.2 M Ω ·cm) and CO₂ gas. Ion exchange reactions were performed using DOWEX 50WX8-200 H⁺ resin (Aldrich), different ion forms were obtained after washing with corresponding 2 M aqueous solutions and then water until neutrality. Polystyrene acyl chloride resin was prepared according to previously published procedure²⁸ and vacuum-dried over P₂O₅. Analytical HPLC were performed using a Nucleosil C₁₈ column, (150 mm \times 4.6 mm, 5 μ m) equipped with a prefilter and a precolumn (Nucleosil, C₁₈, 5 μ m) on a Waters apparatus using a Waters-616 pump and a Waters 966 photodiode array detector. Compounds were eluted using a linear gradient 0–80% of acetonitrile in a 50 mM triethylammonium acetate buffer (pH 7) with a 1 mL/min flow rate. Preparative HPLC purifications were performed with a Waters DeltaPak C₁₈ preparative column (7.8 mm \times 300 mm, 15 μ m) using the analytical HPLC conditions and a 2 mL/min flow rate. All moist-sensitive reactions were carried under anhydrous conditions using dry glassware, anhydrous solvents and Argon atmosphere. Acetonitrile was distilled from CaH₂ and stored over activated 3 Å molecular sieves. Pyridine and triethylamine were distilled from CaH₂. Dichloromethane and carbon tetrachloride were distilled from P₂O₅. All commercially available reagents were used as received. Three Å molecular sieves were dried in a 300 °C oven during 3 h prior to use. FAB-MS and FAB-HRMS spectra were recorded on a JEOL SX102 mass spectrometer using a mixture of glycerol/thioglycerol [50/50, v/v, (G-T)] as a matrix. MALDI/Tof-MS spectra were recorded on a Perspective Biosystems Voyager DE spectrometer using a mixture of 2,4,6-trihydroxyacetophenone/ammonium citrate [10/1, m/m, saturated solution in water/acetonitrile, 1/1, v/v, (THAP/cit) as a matrix. ESI-HRMS spectra were recorded on a Q-ToF Micromass spectrometer. ¹H and ¹³C NMR spectra were recorded at room temperature on a Bruker spectrometer at 200, 400, or 300 MHz (¹H) and 100 or 75 MHz (¹³C). Chemical shifts are given in ppm referenced to the solvent residual peak (CDCl₃ -7.27 and 77.0 ppm; DMSO-*d*₆ -2.49 and 39.5 ppm; D₂O -4.79 ppm + drop of CH₃OH at 49.5 ppm as internal reference³⁶). Coupling constants are given in hertz. Signal splitting patterns are described as singlet (s), doublet (d), triplet (t), quartet (q), broad signal (br), doublet of doublet (dd), doublet of triplet (dt), doublet of quartet (dq), pseudo (p), and multiplet (m). Signal assignments were based on 2D homo-¹H/¹H, NOE or heteronuclear-¹H/¹³C correlations and D₂O exchange. ³¹P NMR spectra were recorded at 101 and 121 MHz on proton-decoupled mode and on proton-coupled mode for *H*-phosphonates. Chemical shifts are given in ppm referenced to external H₃PO₄-80%; coupling constants are given in hertz. UV spectra were recorded on a Varian Cary 300 Bio UV/vis spectrometer.

For convenience, general procedures have been given for the coupling, oxidation, and deprotection reactions. Variations from these procedures and individual purification methods are given in the main text for each compound. Preparative and spectroscopic data for cytidine monomer compounds **12–14** is given as Supporting Information only.

General Procedure for H-Phosphonate Coupling. In a dry flask and under argon, PS acyl chloride resin (6 mol equiv) was

suspended in 10 mL/mmol of anhydrous pyridine/dichloromethane 1:1 (v/v) solution. In another dry flask, a mixture of the appropriate guanosine 3'-hydroxy monomer **9–11** (1 mol equiv) and the cytidine 5'-*H*-phosphonate monomer **14** (1.2 mol equiv) were dissolved in 10 mL/mmol of anhydrous pyridine/dichloromethane solution after drying by azeotropic distillation with anhydrous pyridine. The nucleoside containing solution was then introduced to the resin containing flask, and the mixture was shaken at room temperature for 2 h. The reaction mixture was then diluted with dichloromethane; the resin was filtered off and well rinsed. Filtrates were evaporated to half-volume and then washed twice with water. Organic layers were dried over Na₂SO₄, filtered, and evaporated to dryness. The residue was coevaporated three times with acetonitrile, providing the crude dimer as a white foam (HPLC purity >85%) in a 1:1 diastereomeric mixture. Compounds were used in oxidation steps without further purification.

Oxidation Method A. In a dry flask, appropriate *H*-Phosphonate diester (1 mol equiv) was dried by azeotropic distillation with anhydrous pyridine and dried under vacuum with P₂O₅ for 30 min. It was then dissolved in anhydrous pyridine (5 mL/mmol). To this solution were simultaneously added: anhydrous CCl₄ (25 mol equiv, 2.5 mL/mmol), corresponding amine (10 mol equiv), or 10 M solution of histamine (50 mol equiv) in anhydrous pyridine. Reaction color turned to bright yellow and the mixture was stirred at room temperature for 1 h. The solvents were removed under reduced pressure and the residue was coevaporated twice with acetonitrile. Compounds were directly involved in deprotection steps.

Preparation of 6-Aminocaproic Acid Methyl Ester Hydrochloride.³⁷ First, 400 mg of 6-aminocaproic acid (3 mmol, 1 mol equiv) were suspended in 40 mL of 2,2-dimethoxypropane (10–15 mL/mmol). To the mixture was added 3 mL of 37% HCl and the solution was stirred at room temperature for 18 h. After removal of the solvents under reduced pressure, the residue was precipitated in acetonitrile and recrystallized from acetonitrile/ether. After filtration, the white crystals were dried under vacuum with P₂O₅ at refluxing ethanol for 2 days (490 mg, 90%). ¹H NMR (D₂O, 300 MHz) δ 3.60 (3H, s, CO₂CH₃), 2.90 (2H, t, CH₂-6, *J* = 7.4 Hz), 2.33 (2H, t, CH₂-2, *J* = 7.3 Hz), 1.64 (4H, m, CH₂-3, CH₂-5), 1.32 (2H, q, CH₂-4, *J* = 7.3 Hz). ¹³C NMR (D₂O, 75 MHz) δ 177.3 (CO₂CH₃), 52.1 (CO₂CH₃), 39.3 (CH₂-6), 33.3 (CH₂-2), 26.3 (CH₂-5), 25.0 (CH₂-3), 23.6 (CH₂-4).

Oxidation Method B. In a dry flask, appropriate *H*-phosphonate diester (1 mol equiv) was dried by azeotropic distillation with anhydrous pyridine and dried under vacuum with P₂O₅ for 30 min. It was then dissolved in anhydrous pyridine (2.5 mL/mmol). To this solution were simultaneously added: anhydrous CCl₄ (25 mol equiv, 2.5 mL/mmol), 4 M solution of 6-aminocaproic acid methyl ester hydrochloride (10 mol equiv) in anhydrous pyridine, and anhydrous triethylamine (10 mol equiv). Reaction color turned to bright yellow, and the mixture was stirred at room temperature for 1 h. The solvents were removed under reduced pressure, and the residue was coevaporated twice with acetonitrile. Compounds were directly involved in deprotection steps.

Deprotection Method A. Appropriate compound was dissolved in THF/H₂O 1:1 (v/v) (14 mL/mmol), and 28% aqueous ammonia solution was added (70 mL/mmol). The flask was hermetically closed, and the mixture was stirred 7 h at 50 °C. The solvents were removed under reduced pressure to give the corresponding compound as a brown oil, which was further purified by chromatography.

Deprotection Method B. Appropriate compound was dissolved in methanol (12 mL/mmol), and 80% acetic acid solution (62 mL/mmol) was added; then the mixture was stirred 2 h at room temperature. It was finally diluted with 10 mL of water and washed with dichloromethane (5 times) and ether (3 times). Aqueous layer was evaporated to dryness, and then the residue was purified by chromatography.

HPLC Method for Purification and/or Separation of Diastereoisomers. Waters DeltaPak C₁₈ preparative column was used on a Waters apparatus with a 2 mL/min flow rate (see above). Gradient conditions, run times, and maximal loading of the column are given

for each compound. After purification, the TEAAc buffer was removed after several lyophilizations from water. Collected pure isomers were analyzed by analytical HPLC and retention times for each isomer are given after a coinjection of an equimolar mixture. According to retention times on RP-HPLC, the names "fast eluting" and "slow eluting" were attributed to each isomer.

Synthesis of *O*-(*N*⁴,2',3'-*O*,*O*-Tribenzoylcytidin-5'-yl)-*O*-[*N*²-isobutyryl-2'-*O*-methyl-5'-*O*-[bis-(2-cyanoethyl)phosphoryl]guanosin-3'-yl] Hydrogenophosphonate Diester (15**).** Prepared according to general procedure of coupling using monomers **14** (0.35 g, 0.48 mmol, 1.2 equiv) and **10** (0.25 g, 0.4 mmol, 1 equiv), dry PS-acyl chloride resin (1.2 g, 2.5 mmol, 6 equiv), and 10 mL of anhydrous dichloromethane/pyridine 1:1 (v/v). The residue was finally precipitated in ether to give crude **15** as white foam (HPLC purity >85%) (0.30 g, 60%). *t_R*-HPLC: 16.6 min, 10–80% of acetonitrile in 20 min. ¹H NMR (CDCl₃, 200 MHz, peaks of both diastereomers are described) δ 12.24 (2H, s, br, NH-G1), 10.52 (2H, s, br, NHibu), 10.38 (1H, s, NHBz), 9.14, 9.04 (2H, 2s, H-G8), 8.68 (1H, d, H-C6), 7.96–7.87 (12H, m, Hortho), 7.65–7.33 (19H, m, Hpara, Hmeta, H-C5), 7.27 (1H, d, H-P, ¹*J*_{H-P} = 744.6 Hz), 7.19 (1H, d, H-P, ¹*J*_{H-P} = 737.3 Hz), 6.37, 6.26 (2H, 2d, H-C1', *J* = 4.4 Hz), 5.99–5.83 (6H, m, H-G1', H-C2', H-C3'), 5.04, 4.99 (2H, m, H-G3'), 4.65–4.19 (20H, m, H-C4', H-G4', H-C5',5'', H-G5',5'', HαCNE, HβCNE), 3.40, 3.37 (6H, 2s, G2'-O-CH₃), 2.89 (4H, t, HβCNE, *J* = 5.7 Hz), 2.71 (4H, t, Hβ'CNE, *J* = 5.9 Hz), 1.26–1.21 (12H, 2d, CH₃ibu, *J* = 6.9 Hz). ³¹P NMR (CDCl₃, 101 MHz) δ 9.4 (1/4, dq, ¹*J*_{H-P} = 743.8 Hz, ³*J*_{H-P} = 7.5 Hz), 8.4 (1/4, dq, ¹*J*_{H-P} = 746.5 Hz, ³*J*_{H-P} = 8.2 Hz), -2.8 (1/4, m, ³*J*_{H-P} = 7.0 Hz), -3.1 (1/4, m). MALDI⁺ (THAP/cit) *m/z* 1155 (M + H)⁺.

Synthesis of *O*-(*N*⁴,2',3'-*O*,*O*-Tribenzoylcytidin-5'-yl)-*O*-[*N*²-isobutyryl-2'-*O*-methyl-5'-*O*-DMTr]guanosin-3'-yl] Hydrogenophosphonate Diester (16**).** Prepared according to general procedure of coupling from monomer **14** (0.35 g, 0.48 mmol, 1.2 equiv), *N*²-isobutyryl-2'-*O*-methyl-5'-*O*-DMTr guanosine **9** (0.27 g, 0.4 mmol, 1 equiv), dry PS-acyl chloride resin (1.2 g, 2.5 mmol, 6 equiv), and 10 mL of anhydrous dichloromethane/pyridine 1:1 (v/v). Crude **16** was a white foam (HPLC purity >90%) (0.39 g, 77%). *t_R*-HPLC: 14.1 min, 14.3 min, 10–80% of acetonitrile in 10 min. ¹H NMR (CDCl₃, 200 MHz, peaks of both diastereomers are described) δ 12.07 (2H, 2s, br, NH-G1), 9.45 (1H, s, br, NHBz), 8.99 (2H, 2s, H-G8), 8.64 (1H, d, H-C6), 7.95–6.76 (60H, m, Harom), 7.34 (1H, d, H-P, ¹*J*_{H-P} = 740.0 Hz), 7.02 (1H, d, H-P, ¹*J*_{H-P} = 729.8 Hz), 6.42, 6.28 (2H, 2d, H-C1', *J* = 4.2 Hz), 5.91–5.83 (6H, m, H-G1', H-C2', H-C3'), 4.96, 4.82 (2H, m, H-G3'), 4.86–4.38 (12H, m, H-C4', H-G4', H-C5',5'', H-G5',5''), 3.78–3.76 (12H, 4s, O-CH₃DMTr), 3.52, 3.50 (6H, 2s, G2'-O-CH₃), 1.26 (12H, s, CH₃ibu). ³¹P NMR (CDCl₃, 101 MHz) δ 9.4 (1/2, dq, ¹*J*_{H-P} = 740.4 Hz, ³*J*_{H-P} = 7.6 Hz), 7.3 (1/2, dq, ¹*J*_{H-P} = 739.1 Hz, ³*J*_{H-P} = 8.0 Hz). MALDI⁺ (THAP/cit) *m/z* 1272 (M + H)⁺, 969 (M + H-DMTr)⁺.

Synthesis of *O*-(*N*⁴,2',3'-*O*,*O*-Tribenzoylcytidin-5'-yl)-*O*-[*N*²-isobutyryl-2'-*O*-methyl-5'-*O*-[bis-(2-cyano-1,1-dimethylethyl)thionophosphoryl]guanosin-3'-yl] Hydrogenophosphonate Diester (17**).** Prepared using general procedure for coupling from monomers **14** (0.25 g, 0.33 mmol, 1.2 equiv), **11** (0.17 g, 0.28 mmol, 1 equiv), dry PS-acyl chloride resin (0.7 g, 1.7 mmol, 6 equiv), and 7 mL of anhydrous dichloromethane/pyridine 1:1 (v/v). Crude **17** was a white foam (HPLC purity >93%) (0.23 g, 70%). *t_R*-HPLC: 15.9 min, 10–80% of acetonitrile in 15 min. ¹H NMR (CDCl₃, 200 MHz, peaks of both diastereomers are described) δ 12.18–12.16 (2H, 2s, br, NH-G1), 9.59, 9.84 (2H, 2s, br, NHibu), 8.67, 8.65 (2H, 2s, H-G8), 8.15, 8.08 (2H, 2d, H-C6, *J* = 7.6 Hz), 7.97–7.87 (12H, m, Hortho), 7.66–7.34 (19H, m, Hpara, Hmeta, H-C5), 7.35 (1H, d, H-P, ¹*J*_{H-P} = 744.2 Hz), 7.22 (1H, d, H-P, ¹*J*_{H-P} = 736.6 Hz), 6.47, 6.31 (2H, 2d, H-C1', *J* = 4.3 Hz), 5.97–5.83 (6H, m, H-G1', H-C2', H-C3'), 5.61, 5.48 (2H, m, H-G3'), 4.87–4.34 (12H, m, H-C4', H-G4', H-C5',5'', H-G5',5''), 3.46, 3.43 (6H, 2s, G2'-O-CH₃), 3.11–2.71 (8H, m, CH₂CN), 2.68 (2H, m, CHibu), 1.73–1.66 (24H, m, C(CH₃)₂), 1.26–1.21 (12H, 2d, CH₃ibu, *J* = 6.8 Hz). ³¹P NMR (CDCl₃, 101 MHz) δ 48.9, 48.7 (1/2, 2t, ³*J*_{H-P} = 7.3 Hz), 9.8 (1/4, dq, ¹*J*_{H-P} = 744.7 Hz, ³*J*_{H-P} = 7.6 Hz), 8.0

(1/4, dq, $^1J_{H-P} = 737.2$ Hz, $^3J_{H-P} = 8.1$ Hz). MALDI⁺ (THAP/cit) m/z 1227 (M + H)⁺.

Synthesis of *O*-(Cytidin-5'-yl)-*N*-(2-methoxyethyl)-*O*-(2'-*O*-methyl-5'-*O*-phosphorylguanosin-3'-yl) Phosphoramidate, Sodium Salt (1). Prepared according to oxidation method A from *H*-phosphonate diester **15** (0.07 mmol, 1 equiv), 0.5 mL of anhydrous CCl₄, 0.5 mL of anhydrous pyridine, and 2-methoxyethylamine (60 μ L, 0.7 mmol, 10 equiv) and according to deprotection method A using 1 mL of THF/H₂O 1:1 (v/v) and 5 mL of 28% aqueous ammonia. Residue was dissolved in water and washed twice with ether. Aqueous layer was evaporated to dryness and purified on a DEAE-A25 Sephadex column (linear gradient: TEAB 10⁻³ to 0.3 M). High purity final compound was obtained after purification on preparative RP-HPLC (gradient: acetonitrile 5–40% in 20 min; maximal loading of the column: 10 mg of crude product per run). Target compound was obtained as a sodium salt after elution on a DOWEX-Na⁺ column with water. After lyophilization from water, **1** was a white spongy solid (28 mg, 47%) epimeric mixture (ratio ~1:1). *t_R*-HPLC: 8.2 min; 8.5 min, 0–40% acetonitrile in 15 min. UV (H₂O) $\lambda_{max} = 255$ nm ($\epsilon = 15100$). ¹H NMR (D₂O, 300 MHz, peaks of both diastereomers are described) δ 8.19 (2H, s, H-G8), 7.71, 7.64 (2H, 2d, H-C6, *J* = 7.5 Hz), 5.94 (1H, d, H-G1', *J* = 6.4 Hz), 5.90 (4H, pd, H-C1', H-G1', H-C5), 5.73 (1H, d, H-C5, *J* = 7.5 Hz), 5.16 (2H, m, H-G3'), 4.66 (2H, m, H-G2'), 4.52 (2H, m, H-G4'), 4.42–4.18 (10H, m, H-C2', H-C3', H-C4', H-C5',5''), 4.02 (4H, m, H-G5',5''), 3.52 (3H, s, G2'-O-CH₃), 3.49 (4H, pt, CH₂OMe, *J* = 5.1 Hz), 3.47 (3H, s, G2'-O-CH₃), 3.36, 3.33 (6H, 2s, CH₂-OCH₃), 3.18, 3.16 (4H, m, PNH-CH₂, *J* = 5.1 Hz). ¹³C NMR (D₂O, 75 MHz) δ 168.2 (C-C4), 161.1 (C-G6), 157.7 (C-C2), 152.5 (C-G4), 141.6, 141.4 (C-C6), 138.0 (C-G8), 118.1 (C-G5), 96.7, 96.3 (C-C5), 91.1, 90.8 (C-C1'), 85.2, 84.7 (C-G1'), 83.9 (C-G4'), 82.2 (C-C4'), 81.9, 81.8 (C-G2'), 75.1 (C-C3'), 74.8 (C-G3'), 72.8 (CH₂OMe), 66.9, 66.3 (C-C5'), 63.7 (C-G5'), 58.9, 58.6 (G2'-OCH₃, CH₂-OCH₃), 40.8 (PNH-CH₂). ³¹P NMR (D₂O, 121 MHz) δ 11.4 (1/4, P-N), 11.0 (1/4, P-N), 3.3 (1/4, P-O), 3.2 (1/4, P-O). FAB⁺ (GT) m/z 784 (M + H)⁺; 762 (M-Na + H)⁺; FAB⁻ (GT) m/z 760 (M-Na)⁻; 738 (M-2Na + H)⁻. HRMS: FAB⁻ (GT) m/z calcd for (C₂₃H₃₄N₉O₁₅P₂)⁻, 738.1802; obsd, 738.1778.

The two diastereoisomers of the obtained mixture were separated by preparative RP-HPLC using the general conditions described above. Gradient: acetonitrile 7–10% in 30 min; maximal loading of the column: 1 mg of mixture per run.

Fast Eluting Isomer of 1. *t_R*-HPLC: 8.2 min - 0 to 40% acetonitrile in 15 min. ¹H NMR (D₂O, 300 MHz) δ 8.19 (1H, s, H-G8), 7.67 (1H, d, H-C6, *J* = 7.5 Hz), 5.91 (1H, d, H-G1', *J* = 6.9 Hz), 5.89 (1H, d, H-C1', *J* = 2.7 Hz), 5.77 (1H, d, H-C5, *J* = 7.8 Hz), 5.20–5.15 (1H, m, H-G3'), 4.66 (1H, pt, H-G2', *J* = 5.4 Hz), 4.53 (1H, m, H-G4'), 4.42–4.19 (5H, m, H-C2', H-C3', H-C4', H-C5',5''), 4.04 (2H, m, H-G5',5''), 3.55–3.51 (5H, m, G2'-O-CH₃, CH₂OMe), 3.37 (3H, s, CH₂-OCH₃), 3.17 (2H, dt, PNH-CH₂, $^3J_{H-H} = 5.1$ Hz, $^3J_{H-P} = 12.0$ Hz). ³¹P NMR (D₂O, 121 MHz) δ 10.5 (1/2, P-N), 2.0 (1/2, P-O).

Slow Eluting Isomer of 1. *t_R*-HPLC: 8.4 min. 0–40% acetonitrile in 15 min. ¹H NMR (D₂O, 300 MHz) δ 8.22 (1H, s, H-G8), 7.75 (1H, d, H-C6, *J* = 7.5 Hz), 5.98–5.90 (3H, m, H-G1', H-C1', H-C5), 5.21–5.16 (1H, m, H-G3'), 4.68 (1H, pt, H-G2'), 4.55 (1H, m, H-G4'), 4.47–4.27 (5H, m, H-C2', H-C3', H-C4', H-C5',5''), 4.03 (2H, m, H-G5',5''), 3.52 (2H, t, CH₂OMe, *J* = 5.4 Hz), 3.48 (3H, s, G2'-O-CH₃), 3.35 (3H, s, CH₂-OCH₃), 3.17 (2H, dt, PNH-CH₂, $^3J_{H-H} = 5.4$ Hz, $^3J_{H-P} = 11.7$ Hz). ³¹P NMR (D₂O, 121 MHz) δ 10.8 (1/2, P-N), 3.1 (1/2, P-O).

Synthesis of *O*-(Cytidin-5'-yl)-*O*-(2'-*O*-methyl-5'-*O*-phosphorylguanosin-3'-yl)-*N*-[3-(*N,N*-dimethylamino)propyl] Phosphoramidate, Hydrochloride, Sodium Salt (2). Prepared according to oxidation method A from *H*-phosphonate diester **15** (0.07 mmol, 1 equiv), 0.5 mL of anhydrous CCl₄, 0.5 mL of anhydrous pyridine, and 3-(*N,N*-dimethylamino)propylamine (100 μ L, 0.7 mmol, 10 equiv) and according to deprotection method A using 1 mL of THF/H₂O 1:1 (v/v) and 5 mL of 28% aqueous ammonia. Solvents were evaporated to dryness; the residue was dissolved in water and

washed twice with ether. Aqueous layer was evaporated to dryness and purified on a DEAE-A25 Sephadex column (linear gradient: TEAB 10⁻³ to 0.3 M). High purity final compound was obtained after purification on preparative RP-HPLC (gradient: acetonitrile 7–16% in 20 min; maximal loading of the column: 6.4 mg of crude product per run). Residue was then dissolved in 3 mL of 1 M NaCl solution and eluted on a small RP-18 column (gradient: H₂O 100% to 25% of acetonitrile) After lyophilization from water, the suitable salt form of **2** was a white spongy solid (18 mg, 30%) epimeric mixture (ratio ~1:1). *t_R*-HPLC: 7.6 min, 0–40% acetonitrile in 15 min. UV: (H₂O) $\lambda_{max} = 255$ nm ($\epsilon = 14800$). ¹H NMR (D₂O, 400 MHz, peaks of both diastereomers are described) δ 8.20 (2H, s, br, H-G8), 7.58 (2H, d, H-C6, *J* = 7.3 Hz), 5.93 (1H, pd, H-G1', *J* = 5.0 Hz), 5.83–5.66 (5H, m, H-C1', H-G1', H-C5), 5.12, 5.01 (2H, m, H-G3'), 4.56 (2H, m, H-G2'), 4.45–4.06 (12H, 2m, H-G4', H-C2', H-C3', H-C4', H-C5',5''), 3.89 (4H, m, H-G5',5''), 3.48, 3.47 (6H, 2s, G2'-O-CH₃), 3.12–2.81 (8H, m, CH₂-PNH, CH₂-N(CH₃)₂), 2.76 (12H, s, N(CH₃)₂), 1.89 (4H, m, -CH₂-). ¹³C NMR (D₂O, 75 MHz) δ 166.9 (C-C4), 156.5 (C-C2), 148.0 (C-G4), 141.9 (C-C6), 118.1 (C-G5), 96.5 (C-C5), 91.7, 90.6 (C-C1', C-G1'), 81.9 (C-C4'), 81.9, 81.7 (C-C2', C-C4'), 75.1 (C-C3'), 74.6 (C-G3'), 69.8, 69.4 (C-C5', C-G5'), 59.2, 59.1 (G2'-OCH₃), 55.8 (CH₂), 43.4 (N(CH₃)₂), 38.8 (CH₂), 27.4 (CH₂). ³¹P NMR (D₂O, 101 MHz) δ 10.98, 10.87 (1/2, P-N), 3.7 (1/2, P-O). FAB⁺ (GT) m/z 811 (M-Cl)⁺; 789 (M-NaCl + H)⁺; 767 (M-NaCl-Na + 2H)⁺; FAB⁻ (GT) m/z 787 (M-NaCl-H)⁻; 765 (M-2Na-Cl)⁻. HRMS: FAB⁻ (GT) m/z calcd for (C₂₅H₃₉N₁₀O₁₄P₂)⁻ 765.2122; obsd, 765.2129.

Synthesis of *O*-(Cytidin-5'-yl)-*N*-[2-(imidazol-4-yl)ethyl]-*O*-(2'-*O*-methyl-5'-*O*-phosphorylguanosin-3'-yl) Phosphoramidate, Triethylammonium Salt (3). Prepared according to oxidation method A from *H*-phosphonate diester **15** (0.07 mmol, 1 equiv), 0.15 mL of anhydrous CCl₄, 0.15 mL of anhydrous pyridine, and dry histamine base (0.33 g, 3 mmol, 50 equiv) in solution in 0.3 mL of anhydrous pyridine and according to deprotection method A using 1 mL of THF/H₂O 1:1 (v/v) and 5 mL of 28% aqueous ammonia. Solvents were evaporated to dryness; the residue was dissolved in water and washed twice with ether. Aqueous layer was evaporated to dryness and purified on a DEAE-A25 Sephadex column (linear gradient: TEAB 10⁻³ to 0.3 M). High purity final compound (at this stage, the compound was contaminated with 45% of C4-transaminated by histamine, cytosine analogue) was obtained after purification on preparative RP-HPLC (gradient: acetonitrile 6–11% in 30 min; maximal loading of the column: 1 mg of crude product per run). Compound **3** was obtained as a white spongy solid bis-triethylammonium salt (15 mg, 22%) epimeric mixture (ratio ~1:1). Final compound for enzyme assays was obtained as sodium salt after elution on a DOWEX-Na⁺ column with water and lyophilization. *t_R*-HPLC: 7.8 min, 0–40% acetonitrile in 15 min. UV: (H₂O) $\lambda_{max} = 255$ nm ($\epsilon = 12300$). ¹H NMR (D₂O, 300 MHz, peaks of both diastereomers are described) δ 8.45, 8.43 (2H, 2s, H-Im2), 8.04, 8.01 (2H, 2s, H-G8), 7.52, 7.49 (2H, 2d, H-C6, *J* = 7.5 Hz), 7.18 (2H, s, H-Im5), 5.82 (1H, d, H-G1', *J* = 5.6 Hz), 5.75 (3H, m, H-C1', H-G1'), 5.68, 5.57 (2H, 2d, H-C5, *J* = 7.4 Hz), 4.93, 4.84 (2H, 2m, H-G3'), 4.49 (2H, m, H-G2', *J* = 5.2 Hz), 4.38, 4.31 (2H, 2m, H-G4'), 4.21–3.98 (10H, m, H-C2', H-C3', H-C4', H-C5',5''), 3.90 (4H, m, H-G5',5''), 3.41, 3.35 (6H, 2s, G2'-O-CH₃), 3.18 (4H, m, PNHCH₂), 3.09 (4H, q, CH₂CH₃, *J* = 7.1 Hz), 2.82 (4H, t, Im-CH₂, *J* = 6.4 Hz), 1.16 (12H, t, CH₂CH₃, $^3J = 7.3$ Hz). ¹³C NMR (D₂O, 100 MHz) δ 165.8 (C-C4), 158.9 (C-G6), 157.1 (C-C2), 153.9 (C-G4), 140.9, 140.8 (C-C6), 139.0 (C-G8), 133.2 (C-Im4), 133.1 (C-Im2), 116.5, 116.2 (C-Im5), 95.7, 95.5 (C-C5), 90.7 (C-C1'), 84.8, 84.1 (C-G1'), 81.5, 81.4 (C-C2', C-C4'), 74.0 (C-C3'), 73.9 (C-G3'), 68.8, 68.6 (C-C5', C-G5'), 58.2, 58.2 (G2'-O-CH₃), 46.6 (CH₂CH₃), 39.8, 39.7 (PNHCH₂), 26.4 (CH₂-Im), 8.2 (CH₂CH₃). ³¹P NMR (D₂O, 101 MHz) δ 10.4, 10.1 (1/2, P-N), 2.2, 2.1 (1/2, P-O). FAB⁺ (GT) m/z 776 (M-2TEAH + 3H)⁺; FAB⁻ (GT) m/z 774 (M-2TEAH + H)⁻. HRMS: FAB⁺ (GT) m/z calcd for (C₂₅H₃₆N₁₁O₁₄P₂)⁺, 776.1918; obsd, 776.1915.

Synthesis of *O*-(Cytidin-5'-yl)-*O*-(2'-*O*-methyl-5'-*O*-phosphoryl-guanosin-3'-yl)-*N*-(5-carboxypentyl) Phosphoramidate, Sodium Salt (4). Prepared according to oxidation method B from *H*-phosphonate diester **15** (0.18 mmol, 1 equiv), 0.5 mL of anhydrous CCl_4 , 0.4 mL of anhydrous pyridine, dry 6-aminocaproic acid methyl ester hydrochloride (350 mg, 1.8 mmol, 10 equiv) in 0.5 mL of anhydrous pyridine, and anhydrous triethylamine (0.5 mL, 10 equiv). Reaction mixture was treated with 0.3 M NaOH for 2 h at room temperature and then quenched with DOWEX-pyridinium resin. After filtration of the resin, filtrates were evaporated to dryness and treated with saturated ammonia (23 mL) according to deprotection method A. Crude mixture was then purified on a DEAE-A25 Sephadex column (linear gradient: TEAB 10^{-3} to 0.3 M). The two diastereoisomers of the obtained crude mixture (135 mg) were separated by preparative RP-HPLC using the general conditions described above. Gradient: acetonitrile 6–8% in 30 min; maximal loading of the column: 7 mg of crude mixture per run. Each pure isomer was then obtained as sodium salt (white spongy solid) after elution on a DOWEX- Na^+ column with water and lyophilization.

Fast Eluting Isomer of 4. Yield 45 mg, 58%. t_{R} -HPLC: 8.3 min, 0–40% acetonitrile in 15 min. UV: (H_2O) $\lambda_{\text{max}} = 255$ nm ($\epsilon = 14800$). ^1H NMR (D_2O , 300 MHz) δ 8.15 (1H, s, H-G8), 7.69 (1H, d, H-C6, $J = 7.5$ Hz), 5.95 (1H, d, H-G1', $J = 6.3$ Hz), 5.89 (1H, d, H-C1', $J = 2.4$ Hz), 5.83 (1H, d, H-C5, $J = 7.5$ Hz), 5.17–5.12 (1H, m, H-G3'), 4.58 (1H, pt, H-G2', $J = 5.0$ Hz, $J = 5.5$ Hz), 4.54 (1H, m, H-G4'), 4.44–4.25 (5H, m, H-C2', H-C3', H-C4', H-C5', 5''), 4.09 (2H, m, H-G5', 5''), 3.52 (3H, s, $\text{G}2'\text{-OCH}_3$), 3.02–2.93 (2H, dt, PNH-CH_2 , $^3J_{\text{H-H}} = 7.5$ Hz, $^3J_{\text{H-P}} = 11.4$ Hz), 2.19 (2H, t, $\text{CH}_2\text{-COO}$, $J = 7.9$ Hz), 1.60–1.27 (6H, m, $3 \times \text{-CH}_2\text{-}$). ^{13}C NMR (D_2O , 100 MHz) δ 183.8 (COO), 165.9 (C-C4), 158.9 (C-G6), 157.3 (C-C2), 153.9 (C-G2), 151.8 (C-G4), 140.8 (C-C6), 137.3 (C-G8), 116.1 (C-G5) 95.8 (C-C5), 90.4 (C-C1'), 84.2 (C-G1'), 83.3 (pt, C-G4'), 81.7 (d, C-C4', $^3J_{\text{C-P}} = 8.5$ Hz), 81.2 (d, C-G2', $^3J_{\text{C-P}} = 3.7$ Hz), 74.2 (C-C2'), 74.1 (C-G3'), 68.9 (C-C3'), 65.8 (d, C-C5', $^2J_{\text{C-P}} = 4.8$ Hz), 63.1 (d, C-G5', $^2J_{\text{C-P}} = 3.6$ Hz), 58.3 ($\text{G}2'\text{-O-CH}_3$), 40.8 ($\text{CH}_2\text{-PNH}$), 37.5 ($\text{CH}_2\text{-COO}$), 30.8 (d, $\text{CH}_2\text{-CH}_2\text{PNH}$, $^3J_{\text{C-P}} = 5.0$ Hz), 25.9, 25.5 ($2 \times \text{-CH}_2\text{-}$). ^{31}P NMR (D_2O , 101 MHz) δ 10.8 (1/2, P-N), 0.9 (1/2, P-O). FAB^+ (GT) m/z 796 (M-3Na + 4H) $^+$; FAB^- (GT) m/z 794 (M-3Na + 2H) $^-$. HRMS: $\text{ESI}^- m/z$ calcd for ($\text{C}_{26}\text{H}_{38}\text{N}_9\text{O}_{16}\text{P}_2$) $^-$; 794.1912; obsd, 794.2012.

Slow Eluting Isomer of 4. Yield 26 mg, 34%. t_{R} -HPLC: 8.5 min, 0–40% acetonitrile in 15 min. UV: (H_2O) $\lambda_{\text{max}} = 255$ nm ($\epsilon = 14500$). ^1H NMR (D_2O , 300 MHz) δ 8.21 (1H, s, H-G8), 7.75 (1H, d, H-C6, $J = 7.5$ Hz), 5.98–5.93 (3H, m, H-G1', H-C1', H-C5), 5.19–5.15 (1H, m, H-G3'), 4.61 (1H, pt, H-G2'), 4.55 (1H, m, H-G4'), 4.46–4.25 (5H, m, H-C2', H-C3', H-C4', H-C5', 5''), 4.04 (2H, m, H-G5', 5''), 3.48 (3H, s, $\text{G}2'\text{-OCH}_3$), 3.01–2.93 (2H, dt, PNH-CH_2 , $^3J_{\text{H-H}} = 7.2$ Hz, $^3J_{\text{H-P}} = 11.4$ Hz), 2.15 (2H, t, $\text{CH}_2\text{-COO}$, $J = 7.5$ Hz), 1.58–1.25 (6H, m, $3 \times \text{-CH}_2\text{-}$). ^{13}C NMR (D_2O , 100 MHz) δ 183.8 (COO), 166.0 (C-C4), 159.0 (C-G6), 157.5 (C-C2), 153.9 (C-G2), 151.8 (C-G4), 140.9 (C-C6), 137.6 (C-G8), 116.1 (C-G5), 96.2 (C-C5), 89.9 (C-C1'), 84.4 (C-G1'), 83.7 (pt, C-G4'), 81.8 (d, C-C4', $^3J_{\text{C-P}} = 8.1$ Hz), 81.2 (d, C-G2', $^3J_{\text{C-P}} = 4.6$ Hz), 74.5 (C-G3'), 74.1 (C-C2'), 69.0 (C-C3'), 65.4 (d, C-C5', $^2J_{\text{C-P}} = 5.5$ Hz), 63.0 (d, C-G5', $^2J_{\text{C-P}} = 1.5$ Hz), 58.3 ($\text{G}2'\text{-O-CH}_3$), 40.9 ($\text{CH}_2\text{-PNH}$), 37.5 ($\text{CH}_2\text{-COO}$), 30.9 (d, $\text{CH}_2\text{-CH}_2\text{PNH}$, $^3J_{\text{C-P}} = 5.0$ Hz), 25.9, 25.5 ($2 \times \text{-CH}_2\text{-}$). ^{31}P NMR (D_2O , 101 MHz) δ 11.3 (1/2, P-N), 2.6 (1/2, P-O). FAB^+ (GT) m/z 796 (M-3Na + 4H) $^+$; FAB^- (GT) m/z 794 (M-3Na + 2H) $^-$. HRMS: $\text{ESI}^- m/z$ calcd for ($\text{C}_{26}\text{H}_{38}\text{N}_9\text{O}_{16}\text{P}_2$) $^-$; 794.1912; obsd, 794.2003.

Synthesis of *O*-(Cytidin-5'-yl)-*N*-(2-methoxyethyl)-*O*-(2'-*O*-methyl-guanosin-3'-yl) Phosphoramidate (5). Prepared according to oxidation method A from *H*-phosphonate diester **16** (0.08 mmol, 1 equiv), 0.2 mL of anhydrous CCl_4 , 0.5 mL of anhydrous pyridine, and 2-methoxyethylamine (70 μL , 0.8 mmol, 10 equiv) and according to deprotection method A using 1.5 mL of THF/MeOH, 1:1 (v/v) and 5 mL of 28% aqueous ammonia, then according to deprotection method B using 5 mL of 80% acetic acid solution. The residue was purified by preparative RP-HPLC using the general

conditions described above. Gradient: acetonitrile 10–16% in 30 min; maximal loading of the column: 6.5 mg of crude product per run. After lyophilization from water, pure **5** was a white spongy solid (32 mg, 60%) epimeric mixture (ratio 1:1). t_{R} -HPLC: 9.5 min, 0–40% acetonitrile in 15 min. UV: (H_2O) $\lambda_{\text{max}} = 255$ nm ($\epsilon = 16600$). ^1H NMR (D_2O , 300 MHz, peaks of both diastereoisomers are described) δ 7.92, 7.90 (2H, 2s, H-G8), 7.59, 7.52 (2H, 2d, H-C6, $J = 7.5$ Hz), 5.83–5.75 (5H, m, H-C1', H-G1', H-C5), 5.63 (1H, d, H-C5, $J = 7.4$ Hz), 4.98 (2H, m, H-G3'), 4.50 (2H, m, H-G2'), 4.34–4.06 (12H, m, H-G4', H-C2', H-C3', H-C4', H-C5', 5''), 3.79–3.69 (4H, m, H-G5', 5''), 3.43–3.36 (10H, m, $\text{G}2'\text{-O-CH}_3$, CH_2OMe), 3.27, 3.25 (6H, 2s, $\text{CH}_2\text{-OCH}_3$), 3.05 (4H, m, PNH-CH_2). ^{13}C NMR (D_2O , 75 MHz) δ 165.9 (C-C4), 158.9 (C-G6), 157.4, 157.2 (C-C2), 153.9 (C-G2), 151.4 (C-G4), 141.3, 140.8 (C-C6), 137.6, 137.2 (C-G8), 116.4 (C-G5), 96.0, 95.5 (C-C5), 90.7, 90.6 (C-C1'), 85.3, 84.8 (C-G1'), 84.3 (C-G4'), 83.9, 83.8 (C-C4'), 81.5, 81.4 (C-G2'), 80.9 (C-C2'), 74.0, 73.7 (C-C3'), 73.4 (C-G3'), 72.1 (CH_2OMe), 69.0, 68.9 (C-C5'), 60.7, 60.5 (C-G5'), 58.3–57.9 ($\text{G}2'\text{-O-CH}_3$, $\text{CH}_2\text{-O-CH}_3$), 40.1 (PNH-CH_2). ^{31}P NMR (D_2O , 121 MHz) δ 11.0 (1/2), 10.6 (1/2). FAB^+ (GT) m/z 782 (M + Na) $^+$; 760 (M + H) $^+$; FAB^- (GT) m/z 658 (M-H) $^-$. HRMS: FAB^+ (GT) m/z calcd for ($\text{C}_{23}\text{H}_{35}\text{N}_9\text{O}_{12}\text{P}$) $^+$; 660.2143; obsd, 660.2120.

Synthesis of *O*-(Cytidin-5'-yl)-*O*-(2'-*O*-methyl-guanosin-3'-yl)-*N*-(5-carboxypentyl) Phosphoramidate, Sodium Salt (7). Prepared according to oxidation method B from *H*-phosphonate diester **16** (0.08 mmol, 1 equiv), 0.2 mL of anhydrous CCl_4 , 0.2 mL of anhydrous pyridine, dry 6-aminocaproic acid methyl ester hydrochloride (150 mg, 0.8 mmol, 10 equiv) in 0.3 mL of anhydrous pyridine, and anhydrous triethylamine (0.1 mL, 10 equiv). Reaction mixture was treated with 0.3 M NaOH for 2 h at room temperature and then quenched with DOWEX-pyridinium resin. After filtration of the resin, filtrates were evaporated to dryness and treated with saturated ammonia (5 mL) according to deprotection method A, then according to deprotection method B using 5 mL of 80% acetic acid solution. The residue was purified by preparative RP-HPLC using the general conditions described above. Gradient: acetonitrile 10–16% in 30 min; maximal loading of the column: 3 mg of crude product per run. After lyophilization from water, pure **7** was a white spongy solid (15 mg, 25%) epimeric mixture (ratio 1.5:1). t_{R} -HPLC: 8.8 min, 8.9 min, 0–40% acetonitrile in 15 min. UV: (H_2O) $\lambda_{\text{max}} = 254$ nm ($\epsilon = 22000$). ^1H NMR (D_2O , 300 MHz, peaks of both diastereoisomers are described) δ 7.93, 7.92 (2H, 2s, H-G8), 7.61, 7.57 (2H, 2d, H-C6, $J = 7.5$ Hz), 5.85–5.77 (5H, m, H-C1', H-G1', H-C5), 5.70 (1H, d, H-C5, $J = 7.2$ Hz), 4.98 (2H, m, H-G3'), 4.50 (2H, m, H-G2'), 4.34–4.03 (12H, m, H-G4', H-C2', H-C3', H-C4', H-C5', 5''), 3.76–3.69 (4H, m, H-G5', 5''), 3.42, 3.37 (6H, 2s, $\text{G}2'\text{-O-CH}_3$), 2.87 (4H, m, PNH-CH_2), 2.07, 2.05 (4H, 2t, $\text{CH}_2\text{-COO}$, $J = 7.2$ Hz), 1.45–1.20 (12H, m, $3 \times \text{-CH}_2\text{-}$). ^{31}P NMR (D_2O , 121 MHz) δ 11.4 (1/2), 10.9 (1/2). FAB^+ (GT) m/z 738 (M + H) $^+$; 716 (M-Na + 2H) $^+$; FAB^- (GT) m/z 736 (M-H) $^-$; 714 (M-Na) $^-$. HRMS: FAB^+ (GT) m/z calcd for ($\text{C}_{26}\text{H}_{39}\text{N}_9\text{O}_{13}\text{P}$) $^+$; 716.2405; obsd, 716.2419.

Synthesis of *O*-(Cytidin-5'-yl)-*N*-(2-methoxyethyl)-*O*-(2'-*O*-methyl-5'-*O*-thiophosphoryl-guanosin-3'-yl) Phosphoramidate, Sodium Salt (6). Prepared according to oxidation method A from *H*-phosphonate diester **17** (0.06 mmol, 1 equiv), 0.15 mL of anhydrous CCl_4 , 0.4 mL of anhydrous pyridine, and 2-methoxyethylamine (55 μL , 0.6 mmol, 10 equiv) and according to deprotection method A using 2 mL of THF/ H_2O 1:1 (v/v) and 5 mL of 28% aqueous ammonia. At this stage, 50% of the second dimethylcyanoethyl protection was still present, so solvents were removed under reduced pressure and 6 mL of ammonia solution were added and the mixture was stirred at 50 $^\circ\text{C}$ for 8 more hours. Solvents were evaporated to dryness; the residue was dissolved in water and washed with ether. High purity final compound was obtained after purification on preparative RP-HPLC using the general conditions described above. Gradient: acetonitrile 6–11% in 30 min; maximal loading of the column: 4.7 mg of crude product per run. Final compound was obtained as a sodium salt after elution on a DOWEX- Na^+ column with water. After lyophilization from water, pure **6** was a white

spongy solid (28 mg, 58%) epimeric mixture (ratio 1:1). t_R -HPLC: 8.7 min, 8.5 min, 0–40% acetonitrile in 15 min. UV: (H₂O) λ_{\max} = 255 nm (ϵ = 17600). ¹H NMR (D₂O, 300 MHz, peaks of both diastereoisomers are described) δ 7.92, 7.90 (2H, 2s, H-G8), 7.59, 7.52 (2H, 2d, H-C6, J = 7.5 Hz), 5.83–5.75 (5H, m, H-C1', H-G1', H-C5), 5.63 (1H, d, H-C5, J = 7.4 Hz), 4.98 (2H, m, H-G3'), 4.50 (2H, m, H-G2'), 4.34–4.06 (12H, m, H-G4', H-C2', H-C3', H-C4', H-C5', 5''), 3.79–3.69 (4H, m, H-G5', 5''), 3.43–3.36 (10H, m, G2'-O-CH₃, CH₂OMe), 3.27, 3.25 (6H, 2s, CH₂-OCH₃), 3.12–3.04 (4H, m, PNH-CH₂). ¹³C NMR (D₂O, 75 MHz) δ 165.7 (C-C4), 158.9 (C-G6), 157.2 (C-C2), 153.9 (C-G2), 140.9 (C-C6), 138.1 (C-G8), 96.0, 95.8 (C-C5), 90.3, 90.1 (C-C1'), 84.7 (C-G1'), 84.1 (C-G4'), 83.4 (C-C4'), 81.6 (C-G2'), 74.1 (C-C3'), 72.2 (CH₂OMe), 68.9, 68.8 (C-C5', C-G5'), 58.3–57.9 (G2'-O-CH₃, CH₂-O-CH₃), 40.1 (PNH-CH₂). ³¹P NMR (D₂O, 121 MHz) δ 44.6 (1/2), 10.7, 10.4 (1/2). FAB⁺ (GT) m/z 822 (M + Na)⁺; 800 (M + H)⁺; 778 (M-Na + 2H)⁺; FAB⁻ (GT) m/z 798 (M-H)⁻; 776 (M-Na)⁻; 754 (M-2Na + H)⁻. HRMS: FAB⁻ (GT) m/z calcd for (C₂₃H₃₄N₉O₁₄P₂S)⁻, 754.1421; obsd, 754.1377.

The two diastereoisomers of the obtained mixture were separated by preparative RP-HPLC using the general conditions described above. Gradient: acetonitrile 8–11% in 30 min; maximal loading of the column: 1 mg of mixture per run. Up to 15% of an unknown byproduct was observed after the separation of the diastereoisomers on the preparative RP-HPLC. Unfortunately, it could not be removed from the target compound.

Fast Eluting Isomer of 6. t_R -HPLC: 8.1 min, 0–40% acetonitrile in 15 min. ¹H NMR (D₂O, 300 MHz) δ 8.30 (1H, s, H-G8), 7.71 (1H, d, H-C6, J = 7.5 Hz), 5.92–5.90 (2H, m, H-G1', H-C1'), 5.82 (1H, d, H-C5, J = 7.8 Hz), 5.30–5.26 (1H, m, H-G3'), 4.74 (1H, m, H-G2'), 4.56 (1H, m, H-G4'), 4.41–4.16 (5H, m, H-C2', H-C3', H-C4', H-C5', 5''), 4.08–4.05 (2H, dd, H-G5', 5''), 3.55–3.50 (5H, m, G2'-O-CH₃, CH₂OMe), 3.37 (3H, s, CH₂-OCH₃), 3.21–3.14 (2H, dt, PNH-CH₂, ³ J_{H-H} = 5.4 Hz, ³ J_{H-P} = 12.0 Hz). ³¹P NMR (D₂O, 121 MHz) δ 43.7 (1/2, P-S), 10.4 (1/2, P-N).

Slow Eluting Isomer of 6. t_R -HPLC: 8.3 min, 0–40% acetonitrile in 15 min. ¹H NMR (D₂O, 300 MHz) δ 8.30 (1H, s, H-G8), 7.76 (1H, d, H-C6, J = 7.8 Hz), 5.98–5.91 (3H, m, H-G1', H-C1', H-C5), 5.27 (1H, m, H-G3'), 4.73 (1H, pt, H-G2'), 4.58 (1H, m, H-G4'), 4.44–4.24 (5H, m, H-C2', H-C3', H-C4', H-C5', 5''), 4.11–4.08 (2H, m, H-G5', 5''), 3.53 (2H, t, CH₂OMe, J = 5.4 Hz), 3.48 (3H, s, G2'-O-CH₃), 3.35 (3H, s, CH₂-OCH₃), 3.20–3.14 (2H, dt, PNH-CH₂, ³ J_{H-H} = 5.4 Hz, ³ J_{H-P} = 12.0 Hz). ³¹P NMR (D₂O, 121 MHz) δ 43.7 (1/2, P-S), 10.7 (1/2, P-N).

Synthesis of *O*-(Cytidin-5'-yl)-*O*-(2'-*O*-methyl-5'-*O*-thiophosphorylguanosin-3'-yl)-*N*-(5-carboxypentyl) Phosphoramidate, Sodium Salt (8). Prepared according to oxidation method B from *H*-phosphonate diester **17** (0.04 mmol, 1 equiv), 0.1 mL of anhydrous CCl₄, 0.1 mL of anhydrous pyridine, dry 6-aminocaproic acid methyl ester hydrochloride (100 mg, 0.4 mmol, 10 equiv) in 0.2 mL of anhydrous pyridine, and anhydrous triethylamine (70 μ L, 10 equiv). Reaction mixture was first treated with 70 μ L of DBU in 0.25 mL of *N,O*-bis-(trimethylsilyl)-acetamide and 1.2 mL of dry pyridine for 2 h, then with 0.3 M NaOH (5 mL) for 2 h at room temperature, and then quenched with DOWEX-pyridinium resin. After filtration of the resin, filtrates were evaporated to dryness and treated with saturated ammonia (5 mL) according to deprotection method A. Crude mixture was then purified on a DEAE-A25 Sephadex column (linear gradient: TEAB 10⁻³ to 0.3 M). High purity final compound was obtained after purification on preparative RP-HPLC (gradient: acetonitrile 6–12% in 30 min; maximal loading of the column: 5 mg of crude product per run). Final compound was obtained as a sodium salt after elution on a DOWEX-Na⁺ column with water. After lyophilization from water, **8** was a white spongy solid (13 mg, 37%) epimeric mixture (ratio ~1.8:1). t_R -HPLC: 7.9 min, 8.1 min, 0–40% acetonitrile in 15 min. UV: (H₂O) λ_{\max} = 255 nm (ϵ = 15500). ¹H NMR (D₂O, 300 MHz, peaks of both diastereoisomers are described) δ 8.19, 8.18 (2H, 2s, H-G8), 7.67, 7.63 (2H, 2d, H-C6, J = 7.5 Hz), 5.88–5.77 (6H, m, H-C1', H-G1', H-C5), 5.14 (2H, m, H-G3'), 4.47 (2H, m,

H-G2'), 4.31–4.17 (12H, m, H-G4', H-C2', H-C3', H-C4', H-C5', 5''), 4.01 (4H, m, H-G5', 5''), 3.40, 3.39 (6H, 2s, G2'-O-CH₃), 2.92–2.83 (4H, m, PNH-CH₂), 2.12, 2.09 (4H, 2t, CH₂-COO, J = 7.2 Hz), 1.51–1.41 (8H, m, -CH₂-), 1.28–1.16 (4H, m, -CH₂-). ¹³C NMR (D₂O, 75 MHz) δ 182.7 (COO), 165.4 (C-C4), 158.9 (C-G6), 156.7 (C-C2), 153.9 (C-G2), 151.8 (C-G4), 141.1 (C-C6), 137.6 (C-G8), 116.4 (C-G5), 95.8 (C-C5), 90.3 (C-C1'), 84.4, 84.3 (C-G1'), 83.3 (C-C4', C-G4'), 81.7 (C-G2'), 81.2 (C-C2'), 75.1 (C-C3', C-G3'), 74.1 (CH₂COO), 68.9 (C-C5'), 65.7, 63.5 (C-G5'), 58.3 (G2'-O-CH₃), 40.8 (PNH-CH₂), 36.6 (-CH₂-), 30.7, 25.7, 25.1 (3 \times -CH₂-). ³¹P NMR (D₂O, 121 MHz) δ 45.9 (1/2), 11.2, 10.7 (1/2). FAB⁺ (GT) m/z 900 (M + Na)⁺; 878 (M + H)⁺; 856 (M-Na + 2H)⁺; 834 (M-2Na + 3H)⁺; FAB⁻ (GT) m/z 754 (M-Na)⁻; 832 (M-2Na + H)⁻; 810 (M-3Na + 2H)⁻. HRMS: FAB⁻ (GT) m/z calcd for (C₂₆H₃₈N₉O₁₅P₂S)⁻, 810.1683; obsd, 810.1676.

The two diastereoisomers of the obtained mixture were separated by preparative RP-HPLC using the general conditions described above. Gradient: acetonitrile 6–11% in 30 min; maximal loading of the column: 1 mg of mixture per run. Up to 5% of an unknown decomposition byproduct was observed after the separation of the diastereoisomers on preparative RP-HPLC. Unfortunately, this byproduct could not be removed from the target compound.

Fast Eluting Isomer of 8. t_R -HPLC: 7.6 min, 0–40% acetonitrile in 15 min. ¹H NMR (D₂O, 300 MHz) δ 8.30 (1H, s, H-G8), 7.72 (1H, d, H-C6, J = 7.8 Hz), 5.94 (1H, d, H-G1', J = 7.2 Hz), 5.91 (1H, d, H-C1', J = 2.7 Hz), 5.87 (1H, d, H-C5, J = 7.8 Hz), 5.27–5.23 (1H, m, H-G3'), 4.72 (1H, m, H-G2'), 4.56 (1H, m, H-G4'), 4.42–4.20 (5H, m, H-C2', H-C3', H-C4', H-C5', 5''), 4.09 (2H, dd, H-G5', 5''), 3.49 (3H, s, G2'-O-CH₃), 3.00–2.92 (2H, dt, PNH-CH₂, ³ J_{H-H} = 6.9 Hz, ³ J_{H-P} = 11.7 Hz), 2.18 (2H, t, CH₂-COO, J = 7.5 Hz), 1.57–1.26 (6H, m, 3 \times -CH₂-). ³¹P NMR (D₂O, 121 MHz) δ 44.6 (1/2, P-S), 10.7 (1/2, P-N).

Slow Eluting Isomer of 8. t_R -HPLC: 8.1 min, 0–40% acetonitrile in 15 min. ¹H NMR (D₂O, 300 MHz) δ 8.30 (1H, s, H-G8), 7.76 (1H, d, H-C6, J = 7.5 Hz), 5.98–5.92 (3H, m, H-G1', H-C1', H-C5), 5.25 (1H, m, H-G3'), 4.72 (1H, m, H-G2'), 4.57 (1H, m, H-G4'), 4.46–4.25 (5H, m, H-C2', H-C3', H-C4', H-C5', 5''), 4.08 (2H, dd, H-G5', 5''), 3.48 (3H, s, G2'-O-CH₃), 3.01–2.93 (2H, dt, PNH-CH₂, ³ J_{H-H} = 6.9 Hz, ³ J_{H-P} = 12.0 Hz), 2.15 (2H, t, CH₂-COO, J = 7.2 Hz), 1.57–1.27 (6H, m, 3 \times -CH₂-). ³¹P NMR (D₂O, 121 MHz) δ 43.9 (1/2, P-S), 11.2 (1/2, P-N).

Molecular Dynamics. The model of the HCV RdRp polymerase–phosphoramidate dinucleoside complex is based on the analogy with the crystal structure of the bacteriophage initiation complex.³⁸ Bacteriophage and HCV RdRp^{14,39} were superimposed using the structural alignment method (embedded in the VMD and Chimera software packages^{40,41}) and nucleic acids from the bacteriophage initiation complex were incorporated into the active site of HCV RdRp as proposed in reference.⁴²

Several necessary modifications (anchoring of the tethers to the internucleotide linkage as well as addition of the 2'-O-CH₃ group to rG) were made. This required necessary completion and modification of all_nuc94.in (molecular topology) and parm94.dat (force field) files. Several bond (NS_P), angle and dihedral angle (CT-NS-P-OS, X-CT-NS-P) terms were taken from ref 43 in which the AMBER parameters for a similar internucleotide linkage modification were developed. Remaining charges, as well as bond, angle, and dihedral terms for the side chains, were taken from the AMBER database⁴⁴ and did not require modification (similarity of tethers with the GLU side chain was exploited). Such approximation was judged sufficient for the geometrical factors analyzed here.

Simulated system was surrounded by ~16769 TIP3P water molecules,⁴⁵ which extended to a distance of approximately 10 Å (in each direction) from the HCV RdRp atoms. This gives a periodic box size of ~98 Å, ~77 Å, ~97 Å. New *.inperd (initial coordinates) and *.prmtop (molecular topology, force field etc.) files for the whole simulated system including modified residues were created by use of the TLEAP module (AMBER software package⁴⁴).

Fully solvated trajectories (lasting for 4 ns) were computed with the aid of the NAMD software package.⁴⁶ Conventional computational procedures were used: periodic boundary conditions, cut off distances of 10 Å for the nonbonded interactions, and the particle-mesh-Ewald method for the summation of the Coulombic interactions,⁴⁷ MD time step = 0.001 ps. Initially, for 5 ps, the system was heated up to 310 K using a Langevin temperature equilibration scheme while restraining the position of the solute. The MD was then continued for 4 ns at constant T and constant P with all restraints removed.

Figures were produced with the aid of the VMD software package.⁴¹

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Supporting Information Available: Preparative and spectroscopic data on compounds **12–14**; correlation table between HPLC retention time, δ ³¹P NMR values and suggested absolute configuration for separated isomers of compounds **1**, **4**, **6**, and **8**. Purity data from HPLC analysis and ¹H NMR and ³¹P NMR spectra of all new target compounds. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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