Synthesis and conformational analysis of phosphate-methylated RNA dinucleotides

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Synthesis and Conformational Analysis of Phosphate-Methylated RNA
Dinucleotides

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Synthesis of RNA dimers having a methyl phosphotriester group as the internucleoside linkage is reported; six pairs of diastereoisomerically pure systems were prepared, i.e., r(CpU) (15), r(ApU) (16), r(CpG) (17), r(ApG) (18), r(CpG) (19), and r(ApG) (20). Compounds 15–20 are stabilized by a 2′-O-methyl group in the 5′-terminal residue. The present systems represent the third class of backbone-modified RNA oligomers, following the 2′-O-methylribonucleotide phosphorothioates and the 2′-O-methylribonucleotide methyl phosphonates. Our synthetic approach comprises the use of 9-fluorenylmethoxycarbonyl (Fmoc) groups for transient protection of the 2′- and 3′-OH groups of the 3′-terminal residues, methanolic KHCO₃ for the simultaneous removal of Fmoc and Lev groups with full preservation of the methyl phosphotriester function, and finally reversed-phase HPLC separation of the Sp and Rp diastereoisomers. The availability of the six dimers in diastereoisomerically pure form enabled us to examine the molecular conformations using high-field NMR and circular dichroism (CD) spectroscopy. These studies led to the following conclusions: (i) NMR J-coupling analysis: the central C₅=C₆ (γ) and C₇=C₈ (β) bonds in 15–20 show less preference for the γ and β rotamers, in comparison with their natural analogues, i.e., base stacking is diminished upon introduction of the phosphate group; (ii) CD analysis: 15–20 show substantially reduced molecular ellipticities when compared to the natural counterparts, which also reveals that base stacking is reduced; (iii) UV and variable-temperature 'H NMR measurements: (Sp)- and (Rp)-19 show self-association, via the formation of a right-handed miniduplex with two C-G base pairs (16-19, Tm = 9.3°C, concn = 36.6 μM; Rp)-19, Tm = 8.7°C, concn = 48.1 μM). The present conformational data on (R)- and (S)-15–20 are in agreement with literature data on other phosphate-triesterified oligonucleotides, e.g., the trimer d(TpGpApTpO) and the tetramer d(TpGpApTpO), while the latter systems also showed little base-base stacking; it was established that they readily form a local duplex with a complementary natural RNA sequence. Hence we anticipate that phosphate-methylated 2′-O-methyl RNA oligomers, longer than the dimer systems described in the present work, will also hybridize easily with complementary natural RNA.

Introduction

Over the past several years there has been a surge of activity in constructing novel types of modified DNA oligomers as antisense inhibitors of gene expression. In most cases, the naturally occurring phosphodiester groups were replaced with, for example, phosphorothioates, phosphorodithioates, phosphoramidates, methyl phosphonates, or alkyl phosphotriesters. The introduction of these modifications generally serves a double purpose: (i) the replacement of the phosphodiester groups renders the oligomer resistant toward breakdown by enzymes (exo- and endonucleases); (ii) transport across cell membranes is usually accelerated, especially for those modifications that result in neutral internucleoside linkages. To achieve inhibition of gene expression at the level of mRNA translation, it is essential that the modified oligomer binds effectively to the RNA target sequence. Very recently, this has prompted several groups to investigate the possible utility of modified oligoribonucleotides for antisense purposes. Due to the inherent instability of natural oligoribonucleotides under physiological conditions, it is absolutely mandatory that the 2′-OH groups are protected. The logical way to accomplish this is methylation, in view of the fact that the methyl group is spatially small and also because it is known that the thermal stability of hybrids of 2′-O-methylribonucleotides with complementary RNA is even greater than of the corresponding RNA–DNA or DNA–RNA duplexes. On the basis of this reasoning, two types of modified 2′-O-methylribonucleotides have been introduced recently. The first type comprises 2′-O-methylribonucleotide phosphorothioates. These systems could be prepared from 2′-O-methylated nucleosides via the H-phosphonate method, exactly analogous to the synthesis of deoxynucleoside phosphorothioates. The second type comprises 2′-O-methylribonucleotide methyl phosphonates, which were prepared recently by Miller et al. These systems were synthesized from the 2′-O-methylribonucleosides by standard methods. The pentamer UpApUpC (all five nucleosides carry a 2′-O-methyl group, p represents a methyl phosphate group, and p represents a phosphodiester group) indeed showed a greater affinity for a complementary strand GAUCA, as compared to the 2′-deoxyribonucleoside methyl phosphonate pentamer d(TpGpApTpC) (p is methyl phosphate, p is phosphodiester). As an extension of our previous work on the synthesis and structural analysis of well-defined short methyl phosphotriester DNA oligomers, we now wish to describe studies of 2′-O-methylribonucleotide methyl phosphotriester. Herein, we describe the synthesis and structural analysis of six pairs of diastereoisomerically pure phosphate-methylated dimers: r(CpU) (15), r(ApU) (16), r(CpG) (17), r(ApG) (18), r(CpG) (19), and r(ApG) (20).

1 Eindhoven University of Technology.
2 DSM Research Laboratories.
3 Received November 27, 1990 (Revised Manuscript Received May 13, 1991)
5 Miller, P. S.; Bhan, P.; Cushman, C.; Lefkowitz, R.; Shiber, M.; Thaden, J. In Proceedings of the 9th International Round Table on Nucleic Acids, Nucleotides & Their Biological Implications, July 30–August 1, Uppsala, 1990, 24.
levulinoyl esters were simultaneously saponified during the synthesis of partially phosphate-methylated DNA fragments. Ribodinucleotides was left completely unaffected and the coupling unit. Furthermore, in lieu of using a experiments, led to considerable demethylation of the methyl phosphotriester function during the Fmoc cleavage. The Sp and Sp diastereoisomers of all six dinucleotides we used the 9-fluorenylmethoxycarbonyl function during the Fmoc cleavage. The Sp and levulinoyl functions and reversed-phase HPLC separation of the Rf and Sf diastereoisomers. In the following, we briefly describe each of the steps.

**Step 1 (Scheme I).** By a slight modification of the procedure described by Yano et al.,

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Figure 1. Structural formulae of the studied phosphate-methylated ribodinucleotides, which are 2'-O-methylated in their upper ribose residue.

(16), r(CpC) (17), r(ApC) (18), r(CpG) (19), and r(ApG) (20). The structural formulae of these systems are presented in Figure 1.

Analogous to our work on phosphate-methylated DNA dinucleotides we used the 9-fluorenemethoxycarbonyl (Fmoc) group for protection of the bases A, C, and G, and we chose the levulinoyl (Lev) group for the protection of both the 2'- and 3'-hydroxyl functions of the 3'-terminal coupling unit. Furthermore, in lieu of using a (1:1) mixture of triethylamine and pyridine (which, in some preliminary experiments, led to considerable demethylation of the methyl phosphotriester function during the Fmoc cleavage), the Fmoc groups were cleaved off by the recently published method described by Kuijpers et al., who used methanolic potassium carbonate in their solid-phase synthesis of partially phosphate-methylated DNA fragments. In this way, the methyl phosphotriester function in our ribodinucleotides was left completely unaffected and the levulinoyl esters were simultaneously saponified during the Fmoc cleavage. The Sp and Rf diastereoisomers of all six ribodinucleotides were separated with reversed-phase HPLC on a milligram scale. This enabled us to perform a detailed conformational analysis by 600-MHz 1H NMR and circular dichroism (CD) spectroscopy.

**Synthesis**

The synthesis of 15-20 in diastereisomerically pure form comprises four essential steps, which can be summarized as follows: (1) Preparation of the 5'-terminal coupling unit by methylation of the 2'-OH group of cytidine and adenosine, followed by the protection of the base amino group with Fmoc and tritylation of the 5'-OH group.

(2) Preparation of the 3'-terminal coupling unit by Fmoc protection of the base amino group of cytidine and guanosine (the imino group of uridine needs no protection) and levulinoylation of the 2'-OH and 3'-OH groups via transient trityl protection of the 5'-OH group. (3) Coupling of the two protected nucleosides via an in situ generated phosphoramidite synthon, followed by oxidation to the phosphotriester. (4) Removal of Fmoc, trityl, and levulinoyl groups and reversed-phase HPLC separation of the Rf and Sp diastereoisomers. In the following, we briefly describe each of the steps.

**Step 1 (Scheme I).** By a slight modification of the procedure described by Yano et al.,

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cytidine was converted into a 3:1 mixture of 2'- and 3'-O-methylcytidine (and some dimethylated side products) by reaction with sodium hydride and methyl iodide. Chromatographic separation and crystallization from absolute ethanol yielded 2'-O-methylcytidine (1a) as a white solid. By the same procedure 2'-O-methyladenosine was obtained as a white solid out of a 5:1 mixture of the 2'-O- and 3'-O-methylated compounds. Fmoc protection of the bases was carried out via transient protection of the 3'- and 5'-hydroxyl groups. In this method the 3',5'-bis(trimethylsilyl) derivatives of 1a and 1b were reacted with 9-fluorenemethoxycarbonyl chloride followed by deprotection of the 3'- and 5'-OH groups. This readily furnished 2a and 2b as white amorphous solids. For the subsequent protection of the 5'-OH group, we used the 4-monomethoxytrityl (MMTr) group, which could be introduced under standard reaction conditions,11 yielding 3a and 3b as white solids.

**Step 2 (Scheme II).** The base amino groups of cytidine and guanosine were protected with Fmoc via transient protection of the 2'-, 3', and 5'-hydroxyl functions,10 yielding 5b and 5c as white amorphous solids. Tritylation of uridine, 5b, and 5c under standard reaction conditions yielded 6a-c as white solids. Subsequent levulinoyl protection of the 2'- and 3'-OH groups was performed with levulinic anhydride in pyridine, yielding 7a-c. Finally, detritylation was accomplished through treatment with aqueous 80% acetic acid (overnight, at room temperature), yielding 8a-c as white solids.

**Step 3 (Scheme III).** For the 3'-5' coupling reactions we have chosen the same synthetic approach as described earlier for the synthesis of phosphate-methylated DNA dinucleotides. This approach has shown13 that bifunctional phosphitylating agents are very effective for the in situ preparation of nucleoside 3'-phosphoramidites. The

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underlying principle is that alkoxybis(dialkylamino)-phosphines are selectively activated by 1H-tetrazole. The 3'-phosphoramidite in situ is then further reacted with the 3'-terminal coupling unit, under activation of an excess of 1H-tetrazole. The advantage of this procedure is that the isolation of the reactive 3'-phosphoramidites is circumvented.

In the present synthesis we reacted the compounds 3a and 3b with a slight excess of methoxybis(diisopropylamino)phosphine in the presence of 1H-tetrazole in dry dichloromethane/acetonitrile mixtures, except in the synthesis of 9, which was performed in dry pyridine. 31P NMR revealed the quantitative formation of the desired phosphoramidites in situ, 4a and 4b, during 20–60 min. As a result of the diastereoisomeric nature, two distinct absorptions were seen in the 31P NMR spectrum around δ 150. The 3'-terminal coupling units 8a–c were added to both 4a and 4b. These reactions required addition of an extra quantity of 1H-tetrazole. In all six cases, 31P NMR spectroscopy showed the complete conversion of 4a and 4b into the desired 3'-5' phosphite triesters (two absorptions around δ 140) within 1–1 1/2 h.

Protection of both 2'- and 3'-OH functions is necessary in order to prohibit the undesired formation of 3'-3' and 3'-2' phosphite triesters. The choice of the levulinoyl group for this purpose was based on the fact that it is compatible with the methyl phosphotriester group (cleavage of levulinoyl and Fmoc with methanolic potassium carbonate, vide infra) and even with the Fmoc group (selective cleavage of levulinoyl with hydrazine, which offers the opportunity of further synthesis into the 3'-direction). In the synthesis of 9, in which dry pyridine was used as the solvent, TLC experiments showed the formation of 9-methylenefluorene and polar side products during the coupling reaction and oxidation. Apparently, the slightly basic medium leads to partial Fmoc cleavage. All phosphite triesters were readily oxidized through reaction with tert-butyl hydroperoxide. This reaction proceeded smoothly without byproducts within 10 min, as was seen in the 31P NMR spectra.

**Scheme III**

* (i) CH3OP[N(iPr)2]2, 1/2 equiv of 1H-tetrazole; (ii) 8a, 8b, or 8c; (iii) t-BuOOH.

Step 4. Our method of deprotection was based on a recent paper by Kuipers et al. in which it is shown that the levulinoyl and Fmoc groups can be simultaneously removed by methanolic potassium carbonate, with complete preservation of the methyl phosphotriester function. The underlying principle of this method is the fact that methanolate, acting as a base, removes the Fmoc groups via a β-elimination reaction and, acting as a hard nucleophile, saponifies the levulinoyl ester. It might also cause

some transesterification on the methyl phosphotriester, leading either to an exchange of the methoxy group or to chain cleavage. The latter was observed only to a very small extent (3% after 6 h).

In the present synthesis we used a 0.05 M solution of potassium carbonate in dry methanol. TLC experiments showed complete cleavage of the levulinoyl groups in 3–10 min and of the Fmoc groups in 2–3 h. Subsequent detritylation was accomplished by overnight stirring in aqueous 80% acetic acid at room temperature. This afforded 15–20 as a mixture of the Sp and Rp diastereoisomers, which were separated on a milligram scale by reversed-phase HPLC. For this the optimal separation conditions (type and concentration of organic modifier, pH) were first developed on an analytical scale. In all cases, acetonitrile was found to be the most suitable organic modifier. Table I lists the optimal parameters (% acetonitrile, pH) of the preparative isocratic reversed-phase separations. Under these conditions, the Sp was eluted prior to the Rp diastereoisomer, except in case of r(CpG).

A detailed description of the optimization procedures will be published elsewhere.16

### Structural Analysis

The second part of our investigation was dedicated to the conformational analysis of the Sp and Rp diastereoisomers of 15–20, using high-resolution 1H NMR at 400 MHz16 or 600 MHz17 as well as circular dichroism (CD) spectroscopy. Spectral assignments were made on the basis of extensive homonuclear decoupling experiments.

#### Configurational Assignment

Assignment of the configuration at phosphorus was performed according to the method of Summers et al.,18 which we have formerly used in our analysis of diastereoisomerically pure phosphate-methylated DNA dinucleotides. For each pair of ribonucleotides it was found that one diastereoisomer shows a clear NOE contact between Hax of the 3'-phosphorylated residue and the methyl group on phosphorus, while the other diastereoisomer lacks such a contact. The structures with the NOE contact were assigned the Rp configuration. In all six cases the Rp diastereoisomer shows a 31P NMR resonance at higher field than the corresponding Sp diastereoisomer, which is in accordance with our previous results on phosphate-methylated deoxyribonucleotides5 and with literature data on phosphate-ethylated dimers.18

#### NMR Analysis

A closer look at the molecular structure of our phosphate-methylated ribonucleotides reveals that each of these systems has 17 essential degrees of freedom (Figure 2). These are rotation around the Cγ-Oγ bonds β1 and β2, rotation around the Cγ-Cβ bonds γ1 and γ2, a two-state equilibrium between a C2,3'-endo (N) and a C2,3'-exo (Z) pucker, rotation around the C3'-O3 bonds ϑ1 and ϑ2, rotation around the C5'-O5 bonds ϕ1 and ϕ2, rotation around the C3'-N bonds χ1 and χ2 (syn = anti), rotation around the Cγ-Oγ bonds ϵ1 and ϵ2, rotation around P-Oγ (τ1, P-Oγ (τ2), and P-OCH3 (κ), and rotation around the O-CH3 bonds λ1 and λ2. As is well known, only five of these (i.e., γ1, γ2, β1, S1, and S2) can be directly and quantitatively determined with 1H NMR.

The full set of vicinal 1H-1H and 1H-31P coupling constants was derived from the 600-MHz 1H NMR spectrum (measured in D2O at 20 °C). In some cases a routine simulation–iteration algorithm was used in order to extract the precise values for coupling constants. These data were used to determine the conformational properties of the individual ribose rings and backbone bonds γ (Cγ-Cβ) and β (Cγ-Oγ).19

The conformations of the ribose rings (S1 and S2) in 15–20 were analyzed with the help of Figure 3. Essentially, we used the pseudorotation concept of Altana and Sundaralingam20 in which the conformations of the five ring

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torsions are mathematically related to a phase angle of pseudorotation (P) and a puckering amplitude (u_m). The parameter P actually indicates which part of the ring is bent. P lies in the range 0-360°, thus encompassing an entire pseudorotation cycle. From the large number of X-ray crystallographic studies of nucleosides and nucleotides, it is known that P values occur in two distinct and relatively narrow ranges. The first range is centered around P = 18° (C_3-endo ring conformation) and is designated as N (north). The N pucker is characteristic for all RNAs and the A form of DNA. The second range is centered around P = 162° (C_2-endo ring conformation) and is called S (south). This pucker is found, for example, in B DNA. The puckering amplitude identifies the deviation from planarity of the furanose ring. Also from crystallographic studies, it is known that u_m is confined to a narrow range around u_m = 39°. Figure 3 shows the calculated dependence of the proton-proton coupling constants J_{1,y} and J_{4,y} for a fixed value of u_m = 39°. Data points of nucleosides and nucleotides lie roughly on a straight line that connects the calculated points for P = 18° and P = 162°. This confirms that the ribose conformations can be treated as rapid N → S equilibria.

The population density of the C_2-endo puckered form (S) can be extracted from a J_{1,y} vs J_{4,y} plot by taking the ratio of the distance of the data point to the N pucker and the total distance between the N pucker and the S pucker.

The conformation around the y' and y_2 bonds can be best described as a rapid equilibrium over the staggered rotamers γ,+ y_2, and y_. Analogously, the conformation around the y_1 bond is described in terms of a rapid equilibrium over β_1, β_2, and β_3. Table II lists the full details of the calculations. For the standard right-handed A RNA conformation, the γ_+ rotamer has the highest population density. In order to obtain the population distribution over the C_1-C_2 rotamers, we solved the set of equations:

J_{1,y} vs J_{4,y} = x(γ_+), J_{1,y} vs J_{4,y} = x(γ_+), J_{1,y} vs J_{4,y} = x(γ_+), J_{1,y} vs J_{4,y} = x(γ_+),

combined with x(γ_+), x(γ_+), and x(γ_+) = 1. For the coupling constants in the individual γ rotamers, we used the values proposed by Haasnoot et al. (ref 19c), i.e., J_{1,y} (γ_+) = 2.4 Hz, J_{4,y} (γ_+) = 2.6 Hz, J_{1,y} (γ_+) = 10.8 Hz, J_{2,y} (γ_+) = 13.5 Hz, J_{4,y} (γ_+) = 3.9 Hz.
Phosphate-Methylated RNA Dinucleotides

set of vicinal coupling constants and conformational data on both diastereomers of r(CpU), r(ApU), and r(CpC) as well as on their corresponding natural counterparts. As can be seen from Tables II and III, all phosphate-methylated systems have a clear preference for the \( y^+ \) conformation in both the 5'-terminal \( x(y^+) = 0.68-0.79 \) and the 3'-terminal \( x(y^+) = 0.55-0.68 \) residue. This preference, which is almost independent of the configuration at phosphorus, is lower than in the natural counterpart systems, in particular for the 3'-terminal residues \( x(y^+) = 0.72-0.89 \), i.e., there is an increased flexibility around the central \( C_2-C_2 \) bond. The same holds true for the preference for \( b^\ddagger \) around the central \( C_2-C_2 \) bond, which is considerably lower \( x(b^\ddagger) = 0.62-0.68 \) than in the natural analogues \( x(b^\ddagger) = 0.84-0.89 \). This indicates that the \( C_2-C_2 \) and \( C_2-C_2 \) bonding network in our dimers forms a stable conformational unit, which is, however, more flexible than that in the natural analogues.

With respect to the ribose conformations, we must differentiate between the 5'-terminal and the 3'-terminal residues. In the 3'-terminal pC and pU residues there is a moderate bias toward the N form \( x(C_{2,\text{endo}}) = 0.30-0.41 \), while in the 3'-terminal pG residues an approximate 1:1 blend over N and S is found \( x(C_{2,\text{endo}}) = 0.40-0.53 \). In essence, these values do not differ from those of their natural analogues. In the 5'-terminal Cp residues a flexible ribose ring (i.e., no clear preference for N or S, \( x(C_{2,\text{endo}}) = 0.50-0.59 \)) is found in contrast to the clear bias for the N-puckered conformation \( x(C_{2,\text{endo}}) = 0.25-0.28 \) in their natural analogues. Even more remarkably, the 5'-terminal Ap residues possess an unusually strong bias for the S-puckered ribose conformation \( x(C_{2,\text{endo}}) = 0.89-0.92 \) in contrast to their natural counterparts, in which the Ap ribose ring is highly flexible or moderately biased toward the N-puckered form \( x(C_{2,\text{endo}}) = 0.36-0.52 \).

CD Analysis. In order to gather more information about the base-base stacking in the dimers 15-20, we recorded the CD spectra in 0.01 M Tris/HCl buffer solutions (pH 7.5) at 25°C and compared our results with literature data on both the natural counterparts and the analogues that are only 2'-O-methylated in their 5'-terminal residues, measured under exactly the same conditions. The latter systems show a substantially increased molecular ellipticity compared to the natural systems due to extra stacking interactions. Our phosphate-methylated dimers 15-20

(26) The Newman projections of the staggered rotamers around a \( C_2-C_2 \) bond are defined as

\[
\begin{align*}
\beta^+ & : \quad \beta^+ \\
\beta^1 & : \quad \beta^1 \\
\beta^- & : \quad \beta^-
\end{align*}
\]

For the standard A RNA conformation, the \( \beta^1 \) rotamer has the highest population density. The population distribution over the \( C_2-C_2 \) rotamers was solved from the equaions:

\[
J_{\text{P-P}} = \rho \quad \gamma \quad J_{\text{P-P}} + \rho \quad \gamma \quad J_{\text{P-P}} + \rho \quad \gamma \quad J_{\text{P-P}}
\]

along with \( x(\beta') + x(\beta') + x(\beta') = 1 \). We used the \( J_{\text{P-P}} \) values for the \( \beta \) rotamers, as proposed by Lankhorst et al. (ref 19d), i.e., \( J_{\text{P-P}} = J_{\text{P-P}} \quad \gamma \quad J_{\text{P-P}} + \rho \quad \gamma \quad J_{\text{P-P}} = 2.4 \mathrm{Hz} \). (27) Lee, S.-H.; Ezra, F. S.; Kondo, N. S.; Sarma, R. H.; Danyluk, S. S. Biochemistry 1976, 15, 3627.


Figure 4. CD spectra of (Sp)-r(CpU) (--), (Rp)-r(CpU) (-----), natural r(CpU) (--), and the analogue that is only 2'-O-methylated in the upper residue (x-x), recorded at 25°C in 0.01 M Tris/HCl buffer solutions (pH 7.5) (see text).

Figure 5. Left: UV extinction vs temperature profile for (Sp)-r(CpG) at a concentration of 36.6 µM ($T_m$ value 9.3°C). Right: UV extinction vs temperature profile for (Rp)-r(CpG) at a concentration of 48.1 µM ($T_m$ value 8.7°C). All experiments were performed in 0.01 M Tris/HCl buffer solutions (pH = 7.5).

showed CD spectra with a similar form but a lower molecular ellipticity than the natural counterparts, especially for the Ap systems. This indicates that base-base stacking occurs in the same mode but to a lesser extent. This is in perfect agreement with the conclusions previously reached on the basis of our 1H NMR data (vide supra).

The CD spectra of 16-20 along with their analogues are given in the supplementary material. Figure 4 shows the spectra of (Sp)- and (Rp)-15 (r(CpU)) and their analogues as representative examples. Interestingly, in all pairs of diastereoisomers the molecular ellipticity of Sp has a somewhat greater magnitude than that of Rp. This is in contrast to our 1H NMR data in which no significant differences in ribose and backbone ($\gamma$ and $\beta$) conformations were found (vide supra). Apparently, the configuration of the methyl phosphotriester group causes a difference in some of the backbone torsion angles, which cannot be monitored by means of 1H NMR (e.g., the central $\alpha$ and $\xi$ bonds). This is consistent with the results of Weinfeld et al., who found that in dinucleoside ethyl phosphotriesters the $R_P$ exhibits more base unstacking than the $S_P$ form due to a closer proximity of the ethoxy groups to the bases.

Duplex Formation. The possibility of duplex formation in both diastereoisomers of r(ApU) (16) and r(CpG) (19) was first examined with UV hyperchromicity experiments. In principle, all four systems are self-complementary and thus capable of antiparallel duplex formation via two Watson and Crick type A-U or C-G base pairs. The UV hyperchromicity curves showed a sigmoidal shape only in the cases of (Sp)- and (Rp)-r(CpG) (Figure 5).

The $T_m$ values were found to be 9.3°C in case of (Sp)-r(CpG) at a concentration of 36.6 µM and 8.7°C in case of (Rp)-r(CpG) at a concentration of 48.1 µM. This melting transition for both r(CpG) diastereoisomers was further investigated with variable-temperature 400-MHz 1H NMR experiments, in which we particularly focussed

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on the chemical shifts of the anomeric protons (i.e., \(H_1\), of Cp and pG) and the nonexchangeable base protons (i.e., \(H_6\) and \(H_8\) of Cp, H_4 and H_6 of pG). The chemical shifts of these protons are known to be sensitive to changes in base stacking.\(^3\) Therefore, a melting transition gives rise to a \(\delta\) vs temperature profile with a sigmoidal shape for some of these protons. In (S_p)- and (R_p)-r(CpG) this is found to be most clearly visible in the profiles of both anomeric protons (Figure 6). The \(T_m\) values extracted from these curves are 13 °C for (S_p)- and 12 °C for (R_p)-r(CpG). Furthermore, the \(^1\)H NMR spectra in H_2O/D_2O (80:20) at 4 °C showed a single amino resonance at 14.2 ppm, demonstrating two symmetry-related G-C base pairs and thus an antiparallel duplex for both diastereoisomers. The conformational properties of (S_p)- and (R_p)-r(CpG) in the duplex form were determined from the 600-MHz \(^1\)H NMR spectra recorded at 2 °C.\(^3\) The results are shown in Table IV. These data indicate that the miniduplex adopts the standard A RNA geometry, with slightly increased population densities for the \(\gamma^+\), \(\delta^\prime\), and Cp-endo conformers, a reflection of the enhanced intranucleotide stacking in the duplex form as compared to the single stranded conformer.

These results on the behavior of phosphate-methylated r(ApU) and r(CpG) are partly in line with the results of our previous study on the phosphate-methylated DNA congeners d(ApT) which showed no self-association,\(^3\) and d(CpG),\(^6\) which formed a miniduplex with \(T_m\) values of 13 °C for the S_p and 9 °C for the R_p diastereoisomer. These DNA duplexes, however, adopt the left-handed Z geometry. In general, the A \(\rightarrow\) Z transition in natural RNA requires more extreme conditions than the B \(\rightarrow\) Z transition in natural DNA. For instance, Hall et al.\(^3\) showed that the RNA hybrid poly(G-C)-poly(G-C) undergoes a transition to the left-handed Z form in conditions of high ionic strength (3–6 M NaClO4) and high temperatures (over 35 °C). Our studies on phosphate-methylated d(CpG)\(_2\) and r(CpG)\(_2\) show that factors other than phosphate shielding alone determine the transition into a left-handed Z structure.

Figure 6. \(^1\)H NMR chemical shift vs temperature profiles in D_2O of the H_1 protons of (S_p)-r(CpG) (left) and (R_p)-r(CpG) (right). For both diastereoisomers, the upper profile corresponds with the Cp residue and the lower profile with the pG residue.

Table IV. Some Relevant \(^1\)H-\(^1\)H NMR Coupling Constants (in Hz). Measured for (S_p)- and (R_p)-r(CpG) (19) in D_2O at 2 °C, along with the Calculated Time-Averaged Populations of the C_p-endo Puckered Ring Form of the Ribose Rings, the C_{1'-C_4} (\(\gamma\)) Rotamers, and the C_p-\(\alpha\) (\(\beta\)) Rotamers.

<table>
<thead>
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<tr>
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<td>(J_{\gamma})</td>
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</tr>
<tr>
<td>(J_{\delta'})</td>
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</tr>
<tr>
<td>(J_{\delta'})</td>
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<td>4.4</td>
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<tr>
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<td>(x(\delta'))</td>
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Concluding Remarks

Phosphate-methylated RNA dimers such as 15–20 can be conveniently prepared by using 9-fluorenylmethoxycarbonyl (Fmoc) for transient protection of the exocyclic carbonyl (Fmoc) for transient protection of the exocyclic carbonyl groups of the bases A, C, and G, levulinoyl for the NHz groups of the bases A, C, and G, levulinoyl for the NHz groups of the bases A, C, and G, and methanolic K_2CO_3 for the simultaneous removal of both the Fmoc and Lev groups in the last stage of the synthesis. We feel that this methodology could also be used for the preparation of longer oligomers of this type. Our NMR and CD conformational studies show that the contribution of base-stacked states to the conformational equilibria of 15–20 is substantially reduced as a consequence of the introduction of the two methyl groups on O_2 and on the phosphate group. This effect must be primarily due to the methyl on the phosphate group, since it is known that the presence of methyl on merely O_2 enhances base stacking. Most likely, the conformations about the bonds P–O_2 (\(\alpha\)) and/or P–O_2 (\(\beta\)) are perturbed in 15–20. Our CD data indicate that this must be especially the case for the Rp diastereomers, since these systems show base stacking to a lesser extent.

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(33) Koole, L. H.; Quaedflieg, P. J. L. M., unpublished results.
Independent support for our conclusion that phosphorysterified nucleotides are inherently more flexible than their unmodified counterparts can be found in the X-ray crystal structures of two phosphorysterified 5'-mono-nucleotides, i.e., adenosine 5'-([O-diethyl phosphate] and cytidine 5'-([O-dimethyl phosphate]). The phosphorysterified moieties were found to be disordered over two distinct conformations, and the torsion angles α and θ do not fall in the g-g range, which is typical for a regular double-helical conformation.

The poor tendency of (Rp)- and (Sp)-15-20 to adopt a helical stacked conformation is in line with previous work on phosphorysterified DNA oligomers, as described by Jensen and Reed and Miller and co-workers. They reported that triesterification of the backbone phosphate groups does not at all inhibit the formation of Watson and Crick type double-helical structures. The "stiffer" unmodified DNA or RNA apparently forces the inherently more flexible phosphotriester to adopt a helical structure. Modified DNA or RNA apparently forces the inherently more flexible phosphotriester to adopt a helical structure.

An elegant example of this phenomenon was described by Miller et al., who showed that the tetramer d(TPOEtTPOEtCPOEtA) is able to bind specifically to the anticondon region of Escherichia coli 16S rRNA, while the tetramer on its own shows virtually no base stacking. Based on this information, it might be expected that elongation of the phosphate-methylated 2'-O-methyl dimers, as described in the present work, can ultimately lead to a new valuable class of reagents for specific hydrogen bonding to and formation of double helices with their cellular targets.

Experimental Section

Materials and Methods. The 1H NMR spectra were recorded on 400-MHz (compounds 1-14 and (Sp)-15) and 600-MHz (compounds (Rp)-15 and 16-20) NMR spectrometers. Tetramethylsilane was used as the internal standard for samples in organic solvents. For samples in aqueous solution (D2O), the residual HDO peak was set at 4.65 ppm. For the variable-temperature measurements on (Sp)- and (Rp)-r(CpG) in D2O, tetramethylammonium bromide (0.1% w/v) was added, while stirring was continued for 2/4 h at 0 °C and for another 1 h at room temperature. TLC analysis (using a mixture of CH2Cl2 and CH3OH) gave Rf values of 0.45 and 0.5 for the unesterified (R0 = 0.45) and methylated compounds (R0 = 0.52). The purity of all title compounds was determined as >95% by HPLC, 1H NMR, and 31P NMR determinations.

2'-O-Methylcytidine (1a). We used the method described by Yan et al. for the synthesis of 2'-O-methyladenosine, with some modifications. To a cooled (0 °C) solution of cytidine (21.00 g, 66.33 mmol) in 350 mL of DMF was added sodium hydride (1.45 g, 103.75 mmol), and the reaction mixture was stirred for 45 min. Then a slight excess of methyl iodide (12.62 g, 88.92 mmol) was added, and stirring was continued for 24 h at 0 °C and for another 1 h at room temperature. TLC analysis (using a mixture of CH2Cl2 and CH3OH) gave Rf values of 0.45 and 0.5 for the unesterified (R0 = 0.45) and methylated compounds (R0 = 0.51). The purity of all title compounds was determined as >95% by HPLC, 1H NMR, and 31P NMR determinations.

2'-O-Methyl-4',5'-di-O-methylcytidine (2a). We used the method described by Yan et al. for the synthesis of 2'-O-methyladenosine, with some modifications. To a cooled (0 °C) solution of cytidine (1.06 g, 6.64 mmol) in 160 mL of DMF was added chlorotrimethylsilane (1.21 g, 3.77 mmol), and the reaction mixture was stirred for 45 min. Then a slight excess of methyl iodide (2.16 g, 88.92 mmol) was added, and stirring was continued for 24 h at 0 °C and for another 1 h at room temperature. TLC analysis (using a mixture of CH2Cl2 and CH3OH) gave Rf values of 0.45 and 0.5 for the unesterified (R0 = 0.45) and methylated compounds (R0 = 0.51). The purity of all title compounds was determined as >95% by HPLC, 1H NMR, and 31P NMR determinations.

2'-O-Methyl-4',5'-di-O-propyl-2'-deoxyadenosine (3a). We used the method described by Yan et al. for the synthesis of 2'-O-methyladenosine, with some modifications. To a cooled (0 °C) solution of 2'-deoxyadenosine (1.06 g, 6.64 mmol) in 160 mL of DMF was added chlorotrimethylsilane (1.21 g, 3.77 mmol), and the reaction mixture was stirred for 45 min. Then a slight excess of methyl iodide (2.16 g, 88.92 mmol) was added, and stirring was continued for 24 h at 0 °C and for another 1 h at room temperature. TLC analysis (using a mixture of CH2Cl2 and CH3OH) gave Rf values of 0.45 and 0.5 for the unesterified (R0 = 0.45) and methylated compounds (R0 = 0.51). The purity of all title compounds was determined as >95% by HPLC, 1H NMR, and 31P NMR determinations.
Fmoc), 4.91 (1 H, dd, H3), 6.54 (1 H, d, Hit), 7.28 (2 H, m, arom Fmoc), 7.39 (2 H, m, arom Fmoc), 7.43 (1 H, d, H5), 7.58 (2 H, m, arom Fmoc), 7.80 (2 H, d, arom Fmoc), 9.11 (1 H, d, H6) exact mass calcd 563; FAB (M + H)+ = 575.

5'-O-(4-Monomethoxytrityl)-2'-O-methyl-6-N-(9-fluorenylmethoxycarbonyl)adenosine (5b). A solution of compound 2b (4.05 g, 8.00 mmol) and 4-monomethoxytrityl chloride (2.96 g, 9.80 mmol) in 0.20 mL of pyridine was added to the suspension of the latter (more apolar) compound was accomplished by suspending the crude product decomposed upon heating (approximately 130 °C); 1'H NMR (CDCl3) δ 2.72 (1 H, bs, 3' OH), 3.43 (1 H, dd, H5), 3.54 (1 H, dd, H6), 3.58 (1 H, H, OCH3), 3.79 (3 H, s, OCH3), 4.22 (1 H, m, 2'-OCH3), 7.20 (2 H, d, CH2Fmoc), 7.50 (1 H, d, H2), 4.50 (1 H, dd, H5), 4.61 (2 H, d, CH2Fmoc), 6.18 (1 H, d, H3), 6.81 (2 H, d, arom MMTr), 7.20-7.50 (16 H, m, arom MMTr), 7.63 (2 H, d, arom Fmoc), 7.88 (1 H, s, NH). 

To a solution of uridine (5a, 4.88 g, 20.00 mmol) in 100 mL of pyridine was added 4-monomethoxytrityl chloride (9.26 g, 30.00 mmol), and the reaction mixture was stirred for 18 h in darkness. The mixture was then processed as described for the preparation of 3a. Column separation was performed with a gradient of CH2OH (0 → 2 vol %) in CH2Cl2 as eluent, Rf 0.53 (CHCl3/CH2OH 95:5 v/v), yielding pure 3b (5.76 g, 95%) as a yellowish solid, which decomposed upon heating (approximately 174 °C); 1'H NMR (CDCl3) δ 2.82 (1 H, bs, 3' OH), 3.32 (3 H, s, OCH3), 3.77 (1 H, m, H5), 3.98 (1 H, m, H4), 4.32 (1 H, t, CH Fmoc), 4.39 (1 H, m, H5), 4.60 (1 H, m, H6), 4.66 (2 H, d, CH2 Fmoc), 4.69 (1 H, dd, H5), 5.90 (1 H, d, H6), 6.03 (1 H, d, 5' OH), 7.38 (4 H, m, arom Fmoc), 7.66 (2 H, d, arom Fmoc), 7.79 (2 H, d, arom Fmoc), 8.03 (1 H, s, H5), 8.67 (1 H, NH), 8.78 (1 H, d, H6); exact mass calcd 503; FAB (M + H)+ = 514.

5'-O-(4-Monomethoxytrityl)-2'-O-methyl-6-N-(9-fluorenylmethoxycarbonyl)cytidine (3a). To a suspension of compound 2a (0.80 g, 1.79 mmol) in 24 mL of pyridine was added 4-monomethoxytrityl chloride (0.64 g, 2.07 mmol), and the reaction mixture was stirred for 18 h in darkness. The mixture was then processed as described for the preparation of 3a. Column separation was performed with a gradient of CH2OH (0 → 2 vol %) in CH2Cl2 as eluent, Rf 0.53 (CHCl3/CH2OH 95:5 v/v), yielding pure 3a (5.76 g, 95%) as a yellowish solid, which decomposed upon heating (approximately 115 °C); 1'H NMR (CDCl3) δ 2.82 (1 H, bs, 3' OH), 3.32 (3 H, s, OCH3), 3.77 (1 H, m, H5), 3.98 (1 H, m, H4), 4.32 (1 H, t, CH Fmoc), 4.39 (1 H, m, H5), 4.60 (1 H, m, H6), 4.66 (2 H, d, CH2 Fmoc), 4.69 (1 H, dd, H5), 5.90 (1 H, d, H6), 6.03 (1 H, d, 5' OH), 7.38 (4 H, m, arom Fmoc), 7.66 (2 H, d, arom Fmoc), 7.79 (2 H, d, arom Fmoc), 8.03 (1 H, s, H5), 8.67 (1 H, NH), 8.78 (1 H, d, H6); exact mass calcd 503; FAB (M + H)+ = 504.
solution. After the addition of water (240 mL) and 45 min of stirring, the yellow solution was evaporated to near dryness. Upon addition of water (750 mL) a white precipitate appeared. The mixture was shaken vigorously until no more yellow oil was visible.

After addition of ethyl acetate (360 mL) and shaking, the precipitate formed on the separation layer was isolated by filtration and washed with cold ethyl acetate. After drying in vacuo, the product was recrystallized from ethanol, yielding 20.70 g (488.

'S-0-(4-Monomethoxytrityl)-4-N-(9-fluorenylmethoxycarbonyl)cytidylidine (6b). A mixture of compound 5b (12.35 g, 26.53 mmol) and 4-monomethoxytrityl chloride (10.10 g, 32.71 mmol) in 260 mL of pyridine was stirred for 3 h in darkness and processed as described in the preparation of 6b. Column chromatography of the resulting brown foam, using a mixture of CH2Cl2 and CH3OH (93:7 v/v) as eluent, yielded compound 6b (1.80 g) as a yellow viscous substance. The product was purified by column chromatography, using a gradient of CH3OH (4 -> 6 vol %) in CH2Cl2 as eluent, Rf 0.33 (CHCl3/CH3OH 94:6 v/v). The product was obtained (15.60 g, 80%) of compound 6b as a yellow oil (1.85 g), which consisted mainly of 7c. The product was purified by column chromatography, using a mixture of CH2Cl2 and CH3OH (95:5 v/v) as eluent (Rf 0.22). Compound 8e was obtained as a yellowish solid, yield 0.96 g (63% from 6c), which decomposed upon heating (approximately 110 °C): 'H NMR (CDCl3) δ 2.08 (3 H, CH3), 2.17 (3 H, CH3), 2.5-2.9 (8 H, m, CH2CH2Lev), 3.58 (1 H, m, CH2CH2, CHFmoc), 4.53 (1 H, t, CHFmoc), 4.56 (2 H, d, CH2Fmoc), 5.56 (1 H, dd, CH3), 5.63 (1 H, dd, CH3), 6.12 (1 H, NH), 7.34-7.47 (4 H, m, CHFmoc), 7.81 (2 H, d, aram MMTr), 7.90 (2 H, d, aram MMTr), 8.11 (1 H, s, CH3); exact mass calcd 701; FAB (M+H)+ = 702, (M+Na)+ = 724.

'S-0-(4-Monomethoxytrityl)-2'-0-(9-fluorenylmethoxycarbonyl)cytidylidine (6e). Levulinic anhydride (2.29 g, 10.70 mmol) was added to a solution of compound 6c (2.06 g, 2.67 mmol) in 20 mL of pyridine. After 4 h of stirring the red solution was processed as described in the preparation of 7a. This afforded a yellow oil (1.50 g) which was stirred for 15 h in 50 mL of acetic acid and water (8:2 v/v). After evaporation of all acetic acid (coevaporation with water), the yellow viscous substance was purified by column chromatography, using a mixture of CH2Cl2 and CH3OH (95:5 v/v) as eluent (Rf 0.22). Compound 8c was obtained as a yellowish solid, yield 0.76 g (53% from 6e), which decomposed upon heating (approximately 120 °C): 'H NMR (CDCl3) δ 1.76 (3 H, s, CH3), 2.17 (3 H, s, CH3), 2.5-2.9 (8 H, m, CH2CH2Lev), 3.54 (1 H, m, CH2CH2, CHFmoc), 4.38 (1 H, t, CHFmoc), 4.65 (2 H, d, CH2Fmoc), 5.56 (1 H, dd, CH3), 5.63 (1 H, dd, CH3), 6.12 (1 H, NH), 7.28 (2 H, t, fH4t), 7.39 (2 H, t, fH4t), 7.53 (2 H, d, fH4t), 7.74 (2 H, d, fH4t), 8.10 (1 H, bs, CH3), 8.20 (1 H, d, CH3); exact mass calcd 662; FAB (M+H)+ = 662, (M+Na)+ = 684.

2'-N-(9-Fluorenylmethoxycarbonyl)guanosine (5e). Chlorotrimethylamine (28.5 g, 224.56 mmol) was added dropwise to a cooled (0 °C) suspension of guanosine (8.00 g, 28.27 mmol) in 150 mL of pyridine, and the reaction mixture was stirred for 1 h. During this time the reaction mixture was heated to reflux and stirred for 21/2 h. After the addition of water (15 mL) at 0 °C and 30 min of stirring, the clear solution was poured into saturated aqueous NaHCO3 (300 mL) and extracted with three 150-mL portions of ethyl acetate. The combined organic layers were washed with two 100-mL portions of water, dried (MgSO4), and concentrated. The last traces of pyridine were removed by coevaporation with two 100-mL portions of toluene. CH2Cl2 (100 mL) was added to the residue, and the white precipitate was filtered off, washed with CH2Cl2 three times, and dried in vacuo. 'H NMR analysis showed that the solid consisted of 5e (Rf 0.50 in CHCl3/CH3OH 8:2 v/v) and an unidentified side product (5%, Rf 0.40 in CHCl3/CH3OH 8:2 v/v), which were difficult to separate. The semipure 5e (10.14 g, 71%) was used without further purification: 'H NMR (DMSO-d6) δ 3.55 (1 H, m, H2p), 3.65 (1 H, m, H2p), 3.87 (1 H, m, H2p), 4.14 (1 H, m, H2p), 4.45 (1 H, m, H2p), 4.49 (2 H, d, CH2Fmoc), 5.04 (1 H, t, 5' OH), 5.20 (1 H, d, CH), 5.28 (1 H, OH), 5.80 (2 H, d, CH3), 7.34-7.47 (4 H, m, aram MMTr), 7.87 (4 H, m, aram MMTr), 8.3 (1 H, exact mass calcd 505; FAB (M+H)+ = 506, (M+Na)+ = 526.

'S-0-(4-Monomethoxytrityl)-2'-N-(9-fluorenylmethoxycarbonyl)guanosine (6c). A solution of compound 5e (6.00 g, 11.87 mmol) and 4-monomethoxytrityl chloride (4.40 g, 14.25 mmol) in pyridine (70 mL) was stirred for 3 h in darkness and processed as described in the preparation of 6b. Column chromatography of the resulting brown foam, using a mixture of CH2Cl2 and CH3OH (93:7 v/v) as eluent (Rf 0.52), yielded compound 6c as a yellowish solid (6.46 g, 61%), which decomposed upon heating (approximately 110 °C): 'H NMR (DMSO-d6) δ 3.59 (1 H, m, H2p), 3.61 (1 H, m, H2p), 3.7 (1 H, m, H2p), 4.1 (1 H, m, H2p), 4.36 (1 H, m, H2p), 4.49 (2 H, d, CH2Fmoc), 5.04 (1 H, t, 5' OH), 5.20 (1 H, d, CH), 5.27 (1 H, OH), 5.82 (2 H, d, CH3), 7.34-7.47 (4 H, m, aram MMTr), 7.87 (4 H, m, aram MMTr), 8.3 (1 H, exact mass calcd 505; FAB (M+H)+ = 506, (M+Na)+ = 526.
Phosphate-Methylated RNA Dinucleotides

5.89 and 6.00 (1 H, 2 phenomenon) dissolved was 5.0 mL of a 0.0 M solution of K2CO3 in CH3OH (0.50 mmol). The reaction mixture was stirred and the course of the deprotection step was followed with TLC. After the cleavage of both the Lev groups (10 min) and the Fmoc group (2 h), the solution was neutralized (to pH = 6) by addition of Dowex-H+ resin. After filtration over a glass filter the solution was evaporated to yield a white solid. 31P NMR (CDC13) δ 0.31 (peaks overlap). The product was dissolved in 10 mL of 80% acetic acid and stirred for 2 h. The mixture was concentrated in vacuo; vacuum and the last traces of acetic acid were removed by coevaporation (four times) with water. The resulting white residue was then partitioned between water (30 mL) and diethyl ether (15 mL). The aqueous layer was washed with 15 mL of diethyl ether, filtered over a glass filter, and evaporated to afford 134 mg (96%) of a white solid: 31P NMR (CDC13) δ 8.20 (0.16; exact mass calcd 1281; FAB (M+H)+ = 1282).

2'-O-Methyldeoxy-5'-s'-uridine O-(Methyl phosphosphate) (16). Compound (0.900 mmol, 0.232 mmol) dissolved was a 0.0 M solution of K2CO3 in CH3OH (0.50 mmol). Removal of the Lev groups required 10 min of stirring, while the Fmoc group was completely cleaved off after 2 h. Then the solution was neutralized (to pH = 6) by addition of Dowex-H+ resin. After filtration over a glass filter the solution was evaporated to yield a white solid: 31P NMR (CD3OD) δ 0.31 (peaks overlap). The product was dissolved in 10 mL of 80% acetic acid and stirred for 2 h. The mixture was concentrated in vacuo; vacuum and the last traces of acetic acid were removed by coevaporation (four times) with water. 31P NMR (CD3OD): δ 0.31 (peaks overlap). The product was dissolved in 10 mL of 80% acetic acid and stirred for 2 h. The mixture was concentrated in vacuo; vacuum and the last traces of acetic acid were removed by coevaporation (four times) with water. The resulting white residue was then partitioned between water (30 mL) and diethyl ether (15 mL). The aqueous layer was washed with 15 mL of diethyl ether, filtered over a glass filter, and evaporated to afford 134 mg (96%) of a white solid: 31P NMR (CDC13) δ 8.20 (0.16; exact mass calcd 1281; FAB (M+H)+ = 1282).
(1 H, d, H3(Cp)), 7.1-7.8 (28 H, m, arom Fmoc/arom MMTr), 7.90 (1 H, d, H3(Cp)), 8.53 (1 H, d, H3(Cp)).

2'-O-Methylethylidy(3'-5')-cytidine O-(Methyl phosphate) (17). Compound 11 (226 mg, 0.153 mmol) was dissolved in 6.0 mL of a 0.05 M solution of K2CO3 in CH3OH (0.30 mmol). Cleavage of the Lev groups was accomplished after 11/2 h, whereas the removal of both Fmoc groups was accomplished after 21/2 h. The clear solution was neutralized (to pH ~6) by addition of several small portions of Dowex-H+ filtered, over a glass filter, and evaporated to dryness: Rf (CHCl3/CH3OH 8:2 v/v) = 0.10. 31P NMR (CD3OD) δ 0.24 and 0.18. The product was detritylated as described in the synthesis of 16, which afforded 17 as a colorless film (73 mg, 83%): Rf (CHCl3/CH3OH 8:2 v/v) = 0.18. The product was detritylated as described for 16, afforded 17 as a white solid (442 mg, 50%). 31P NMR (CDCl3) δ 11.4 Hz, 3.1 Hz. J y3' = 3.3 Hz, H (pG); 4.13 (1 H, dd, H, (pG)); 4.15 (1 H, dd, H (pG)); 4.7 Hz, Jy2' = 3.1 Hz, Jy2' = 5.9 Hz, H (pC); 5.3 Hz, Jy3' = 6.6 Hz, H (pC); 6.3 Hz, Jy1' = 2.3 Hz, H (pC); 7.0 Hz, Jy3' = 3.3 Hz, Jy3' = 3.3 Hz, H (pC); 3.3 Hz, Jy2' = 11.8 Hz, J (Cp). 

31P NMR showed the complete conversion of compound 17 into the phosphate triester (31P NMR (CDCl3) δ -0.43; 1H NMR (CD3OD) δ 3.43 (3 H, 2'-OCH3), 3.70 (1 H, d, H3(Cp)), 3.81 (1 H, d, H3(Cp)), 4.19 (1 H, m, H2(Cp)); 4.30 (1 H, d, H3(Cp)), 4.51 (1 H, d, H3(Cp)); 4.95 (1 H, m, H2(Cp)), 5.70 (1 H, d, H3(Cp)); 5.73 (1 H, d, H3(Cp)); 7.59 and 7.72 (2 H, d, H2(Cp)/H2(Cp)); Cp residue Jy4' = 5.1 Hz, Jy4' = 5.1 Hz, H (pC); 5.3 Hz, Jy4' = 4.6 Hz, H (pC); 7.0 Hz, Jy4' = 3.1 Hz, Jy, = 3.7 Hz, Jy, = 12.9 Hz; pC residue Jy, = 3.3 Hz, Jy, = 6.6 Hz, Jy, = 2.3 Hz; Jy, = 5.0 Hz, Jy, = 5.0 Hz, H (pC); 3.3 Hz, Jy, = 11.8 Hz, J (Cp). 

The product was detritylated by further passage through a glass filter the solution was evaporated to afford 0.94 g (61%) of pure compound 18 (228 mg, 0.153 mmol) was dissolved in 0.5 mL of CH2Cl2, and the reaction mixture was stirred for another 25 min. After addition of tBuOOH (2.0 mL) and 10 min of stirring, 31P NMR showed complete conversion into the phosphate triester (31P NMR (CDCl3) δ -0.43; 1H NMR (CD3OD) δ 3.36 (3 H, s, H2(Cp)), 3.73 (1 H, dd, H3(Cp)), 3.80 (3 H, d, POCH3, Jy4' = 11.4 Hz), 4.12 (1 H, dd, H2(Cp)), 4.14 (1 H, dd, H2(Cp)), 4.20 (1 H, d, H3(Cp)), 4.35 (1 H, d, H3(Cp)), 4.40 (1 H, m, H3(Cp)), 4.44 (1 H, d, H2(Cp)), 4.59 (1 H, dd, H2(Cp)), 5.14 (1 H, d, H3(Cp)), 5.74 (1 H, d, H3(Cp)), 5.80 (1 H, d, H3(Cp)), 5.93 (1 H, d, H3(Cp)), 6.08 (1 H, s, H2(Ap)), 6.23 (1 H, s, H2(Ap)); Ap residue Jy2' = 4.7 Hz, Jy2' = 4.7 Hz, H (pG); Jy, = 2.3 Hz, H (pG); Jy, = 3.3 Hz, H (pG); Jy, = 3.3 Hz; H (pG); Jy, = 6.6 Hz, Jy, = 2.3 Hz; Jy, = 5.0 Hz, Jy, = 5.0 Hz, H (pC); 3.3 Hz, Jy, = 11.8 Hz, J (pC). 

2'-O-Methylethylidy(3'-5')-guanosine O-(Methyl phosphate) (19). To a solution of compound 3a (0.44 g, 0.59 mmol) in 3.0 mL of CH2Cl2 were added 1H-tetrazole (0.59 mL of a 0.50 M solution in CH2CN, 0.45 mmol) and a solution of bis(N,N-diisopropylamino)methoxyphosphine (13). 31P NMR (CD3OD) δ 0.16. The product was detritylated by further passage through a glass filter the solution was evaporated to afford a white solid (240 mg): 31P NMR (CD3OD) δ 0.29 and 0.27; Rf (CHCl3/CH3OH 8:2 v/v/ = 0.18. The product was detritylated as described for 16, afforded 19 as a white solid (503 mg, 96%). The product was detritylated as described for 16, afforded 19 as a white solid (503 mg, 96%): Rf (CHCl3/CH3OH 8:2 v/v/ = 0.18. The product was detritylated as described for 16, afforded 19 as a white solid (503 mg, 96%): Rf (CHCl3/CH3OH 8:2 v/v/ = 0.18. The product was detritylated as described for 16, afforded 19 as a white solid (503 mg, 96%).
Phosphate-Methylated RNA Dinucleotides

\[ \text{Fmoc/2 × CH Fmoc, 4.88 (1 H, dd, H_{x}(Ap)), 5.02 (1 H, m, H_{y}(Ap)), 5.19 (1 H, m, H_{p}(Ap)), 5.69 (1 H, 2 x dd, H_{p}(pG)), 5.9-6.2 (2 H, 4 x d, H_{y}(Ap)/H_{y}(pG)), 6.70 (2 H, 2 x d, arom MMTT), 7.1-7.8 (28 H, m, arom Fmoc/amom MMTTr), 8.0-8.1 (2 H, 2 x s, H_{x}(Ap)/H_{x}(pG)).} \]

\[ \text{5'-O-(4-Monome thy oxytrityl)-3'-O-methyl-6-N-(2'-O-fluorenylm ethoxy carbonyl) adenylyl-(3'-5')-2'-O-(2'-O-fluorenylim ethoxymethyl) C6(14). 1H-Tetrazole (0.60 mL of a 0.50 M solution in CH_3CN, 0.30 mmol) and a solution of bis(N,N-disopropylamino)methyloxiphosphine (0.17 g, 0.65 mmol) in 0.5 mL of CH_2Cl_2 were added to a solution of compound 3b (0.44 g, 0.57 mmol) in 4.0 mL of CH_2Cl_2 and the reaction mixture was stirred for 1.5 h. Formation of the phosphoramidite coupling silyl 4b was evident from the 31P NMR spectrum (CDCl_3, δ 152.0 and 151.1). Then a solution of compound 8c (0.41 g, 0.58 mmol) in 5.0 mL of CH_2Cl_2 and 1H-tetrazole (2.50 mL of a 0.65 M solution in CH_3CN, 1.25 mmol) were added to the reaction mixture and stirring was continued for 40 min.} \]

\[ \text{31P NMR analysis showed the complete conversion of 4b into the corresponding phosphor triester (CDCl_3, δ 142.0 and 141.2), which was readily oxidized through the addition of TBA(0H) (2.0 mL) and 0.5 mL of stirring. The mixture was evaporated to dryness and coevaporated with toluene (four times) and CH_2Cl_2 (three times). The crude product was purified by column chromatography using a gradient of CH_2OH (4 → 6 vol %) in CH_2Cl_2 as eluent, Rf = 0.31 (CH_2Cl_2/CH_3OH 96:4 v/v). Pure 14 (0.62 g, 70%) was obtained as a white solid, which decomposed upon heating (approximately 115 °C).} \]

\[ \text{Supplementary Material Available:} \]

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