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(CpC) (17), r(ApC) (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-fluorenlymethoxycarbonyl (Fmoc) groups for transient protection of the exocyclic NH2 groups of the bases A, C, and G, levulinoyl (Lev) groups for the transient protection of the 2'- and 3'-OH groups of the 3'-terminal residues, methanolic K2CO3 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 C2'-Cp/C3' (γ) and C2'-O2' (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 methyl group on the phosphotriester 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 1H NMR measurements: (Sp)- and (Rp)-19 show self-association, via the formation of a right-handed miniduplex with two C-G base pairs ((Sp)-l9, (Rp)-19, Tm = 9.3 °C, concn = 36.6 μM; (Rp)-19, Tm = 8.7 °C, concn = 48.1 μM). The present conformational data on (Rp)- and (Sp)-15–20 are in agreement with literature data on other phosphate-esterified oligonucleotides, e.g., the trimer d(TpGpApTpC) and the tetramer d(TpGpApTpA). 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.1 In most cases, the naturally occurring phosphodiester groups were replaced with, for example, phosphorothioates, phosphorodithioates, methyl phosphonates, or alkyl phosphotriesters. The introduction of modified oligoribonucleotides for antisense activity is even greater than of the corresponding RNA-RNA or DNA-RNA duplexes.2 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.3 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.4 These systems were synthesized from the 2'-O-methylribonucleosides by standard methods. The pentamer UpApApUpC (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 phosphate 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,5–7 we now wish to describe studies of 2'-O-methylribonucleoside methyl phosphotriesters. Herein, we describe the synthesis and structural analysis of six pairs of diastereoisomerically pure phosphate-methylated dimers: r(CpU) (15), r(ApU)

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levulinoyl esters were simultaneously saponified during the synthesis of partially phosphate-methylated DNA fragments. Ribodinucleotides was left completely unaffected and the published method described by Kuijpers et al. who used a 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 methanolic potassium carbonate in their solid-phase synthesis, followed by oxidation to the phosphotriester. (4) Removal of Fmoc, trityl, and levulinoyl groups and reversed-phase HPLC separation of the Rp 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., 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-fluorenylmethoxycarbonyl 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, 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'- and 3'-hydroxyl functions, 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 shown that bifunctional phosphitylating agents are very effective for the in situ preparation of nucleoside 3'-phosphoramidites. The

* NaH/CH3I; (ii) (CH3)2SiCl; Fmoc-Cl; H2O; (iii) MMTr-Cl.

![Figure 1. Structural formulae of the studied phosphate-methylated ribodinucleotides, which are 2'-O-methylated in their upper ribose residue.](image)

(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-fluorenylmethoxycarbonyl (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 Kuipers 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 experiments was left completely unaffected and the levulinoyl esters were simultaneously saponified during the synthesis of partially phosphate-methylated DNA fragments. The Sp and Rp 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 a transiently generated phosphoramidite synthon, followed by oxidation to the phosphotriester. (4) Removal of Fmoc, trityl, and levulinoyl groups and reversed-phase HPLC separation of the Rp and Sp diastereoisomers. In the following, we briefly describe each of the steps.

**Scheme I**

![Scheme I](image)

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**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 shown that bifunctional phosphitylating agents are very effective for the in situ preparation of nucleoside 3'-phosphoramidites. The


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' phosphate 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' phosphate 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,9 vide infra) and even with the Fmoc group (selective cleavage of levulinoyl with hydrazine,14 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.

Step 4. Our method of deprotection was based on a recent paper by Kuypers et al.8 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

* (i) (CH3)2SiCl; Fmoc-Cl; H2O; (ii) MMTr-Cl; (iii) (CH2COCH2CH2)2O; (iv) CH2COOH/H2O (8:2). As the imino group of uridine needs no protection, steps ii, iii, and iv yielded the corresponding uridine derivatives 6a, 7a, and 8a.

* (i) CH2OP[N(iPr)2]2, 1/2 equiv of 1H-tetrazole; (ii) 8a, 8b, or 8c; (iii) t-BuOOH.
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 deacetylation was accomplished by overnight stirring in aqueous 80% acetic acid at room temperature. This afforded 15–20 as a mixture of the $\text{Sp}$ and $\text{Rp}$ 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 $\text{Sp}$ was eluted prior to the $\text{Rp}$ diastereoisomer, except in case of d(CpG).

A detailed description of the optimization procedures will be published elsewhere.15

### Structural Analysis

The second part of our investigation was dedicated to the conformational analysis of the $\text{Sp}$ and $\text{Rp}$ diastereoisomers of 15–20, using high-resolution $^1$H 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.5 For each pair of ribonucleotides it was found that one diastereoisomer shows a clear NOE contact between $H_\beta$ 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 $\text{Rp}$ configuration. In all six cases the $\text{Rp}$ diastereoisomer shows a $^{31}\text{P}$ NMR resonance at higher field than the corresponding $\text{Sp}$ 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 $\text{C}_\text{p}$-$\text{O}_\text{p}$ bonds $\beta^1$ and $\beta^2$, rotation around the $\text{C}_\text{p}$-$\text{C}_\text{p'}$ bonds $\gamma^1$ and $\gamma^2$, a two-state equilibrium between a $\text{C}_\text{p}$-$\text{endo}$ (N pucker) and $\text{P}$-$\text{endo}$ (S pucker). For 17 ($\text{d}(\text{CpG})$) the data points of both the $\text{Sp}$ (A for $\text{Cp}$-ribose, $\alpha$ for $\text{pC}$-ribose) and the $\text{Rp}$ diastereoisomer (D for $\text{Cp}$-ribose, $\alpha$ for $\text{pC}$-ribose) as well as of their natural counterpart system (O for $\text{Cp}$-ribose, $\bullet$ for $\text{pC}$-ribose) are depicted in the graph. As can be seen the three $\text{pC}$ residues and the $\text{P}$ residue of natural $\text{r}(\text{CpG})$ are clearly biased toward the $\text{N}$ pucker form ($\text{Cp}$-$\text{endo}$ = 0.25–0.30), while the $\text{P}$ residue of (d)($\text{Sp}$)- and (d)($\text{Rp}$)-17 show an approximate 1:1 blend for their ribose rings.

![Figure 2](image)

**Figure 2.** Representation of the 17 essential degrees of conformational freedom in a phosphate-methylated ribodinucleotide, which is 2'-O-methylated in the upper residue.

![Figure 3](image)

**Figure 3.** Calculated dependence of $J_{12}'$ and $J_{12}$ on the phase angle of pseudorotation for $\nu_p = 39\text{°}$: The straight line (---) connects the points for $P = 18\text{°}$ (N pucker) and $P = 182\text{°}$ (S pucker). For 17 ($\text{d}(\text{CpG})$) the data points of both the $\text{Sp}$ (A for $\text{Cp}$-ribose, $\alpha$ for $\text{pC}$-ribose) and the $\text{Rp}$ diastereoisomer (D for $\text{Cp}$-ribose, $\alpha$ for $\text{pC}$-ribose) are clearly biased toward the $\text{N}$ pucker form ($\text{Cp}$-$\text{endo}$ = 0.25–0.30), while the $\text{P}$ residue of (d)($\text{Sp}$)- and (d)($\text{Rp}$)-17 show an approximate 1:1 blend for their ribose rings.

### Table I. Optimal Parameters of the Preparative Isocratic Reversed-Phase HPLC Separations

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(16) NMR Facility at the Eindhoven University of Technology, The Netherlands.

(17) Dutch National NMR Facility at the Catholic University of Nijmegen, The Netherlands.


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\(^{21}\) it is known that \(P\) values occur in two distinct and relatively narrow ranges. The first range is centered around \(P = 18°\) (C\(_2\)-endo ring conformation) and is designated as N (north).\(^{22}\) The N pucker is characteristic for all RNAs and the A form of DNA. The second range is centered around \(P = 162°\) (C\(_3\)-endo ring conformation) and is called S (south).\(^{22}\) 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\(^{21}\) around \(u_m = 39°\). Figure 3 shows the calculated dependence of the proton-proton coupling constants \(J_{152}\) and \(J_{381}\) for a fixed value of \(39°\) for \(u_m\).\(^{23}\) 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.\(^{21}\) The population density of the C\(_3\)-endo puckered form (S) can be extracted from a \(J_{152}/J_{381}\) 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 \(\gamma^1\) and \(\gamma^2\) bonds can be best described as a rapid equilibrium over the staggered rotamers \(\gamma^+,\gamma^-\), and \(\gamma^0\).\(^{23,24}\) Analogously, the conformation around the \(\beta^2\) bond is described in terms of a rapid equilibrium over \(\beta^+,\beta^-,\) and \(\beta^0\).\(^{23,26}\) Table II lists the full population distribution over the \(\gamma^\circ\) and \(\beta^\circ\) rotamers.

\[J_{\gamma^\circ \gamma^\circ} = x(\gamma^+)J_{\gamma^\circ \gamma^\circ}^+(\gamma^+) + x(\gamma^-)J_{\gamma^\circ \gamma^\circ}^-(\gamma^-) + x(\gamma^0)J_{\gamma^\circ \gamma^\circ}^0(\gamma^0)\]

combined with \(x(\gamma^+) + x(\gamma^-) + x(\gamma^0) = 1\). For the coupling constants in the individual \(\gamma\) rotamers, we used the values as proposed by Haasnoot et al. (ref 19c), i.e., \(J_{\gamma^\circ \gamma^\circ}^+(\gamma^+) = 2.4\) Hz, \(J_{\gamma^\circ \gamma^\circ}^-(\gamma^-) = 2.6\) Hz, \(J_{\gamma^\circ \gamma^\circ}^0(\gamma^0) = 10.8\) Hz, \(J_{\gamma^\circ \gamma^\circ}^+(\gamma^+) = 1.8\) Hz, \(J_{\gamma^\circ \gamma^\circ}^-(\gamma^-) = 10.5\) Hz, \(J_{\gamma^\circ \gamma^\circ}^0(\gamma^0) = 3.8\) Hz.
set of vicinal coupling constants and conformational data on both diastereoisomers of r(CpU), r(ApU), and r(CpC) as well as on their corresponding natural counterparts.\textsuperscript{27,28} Table III analogously contains all data on r(ApC), r(CpG), and r(ApG). As can be seen from Tables II and III, all phosphate-methylated systems have a clear preference for the $\gamma^+$ conformation in both the 5'-terminal ($x(\gamma^+) = 0.68-0.79$) and the 3'-terminal ($x(\gamma^+) = 0.65-0.68$) 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 ($x(\gamma^+) = 0.72-0.89$), i.e., there is an increased flexibility around the central C$\gamma$-C$\gamma$ bond ($\gamma^+$). The same holds true for the preference for $\beta^\prime$ around the central C$\gamma$-$\beta^\prime$ bond, which is considerably lower ($x(\beta^\prime) = 0.62-0.63$) than in the natural analogues ($x(\beta^\prime) = 0.84-0.89$). This indicates that the C$\gamma$-C$\gamma$ and C$\gamma$-$\beta^\prime$ 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 ($x(C2,-endo) = 0.30-0.41$), while in the 3'-terminal pG residues an approximate 1:1 blend over N and S is found ($x(C2,-endo) = 0.40-0.53$). 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, $x(C2,-endo) = 0.50-0.59$) is found in contrast to the clear bias for the N-puckered conformation ($x(C2,-endo) = 0.25-0.28$) in their natural analogues. Even more remarkably, the 5'-terminal Ap residues possess an unusually strong bias for the S-puckered ribose conformation ($x(C2,-endo) = 0.89-0.92$) in contrast to their natural counterparts, in which the Ap ribose ring is highly flexible or moderately biased toward the N-puckered form ($x(C2,-endo) = 0.36-0.52$).

**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\textsuperscript{29} 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.\textsuperscript{2} Our phosphate-methylated dimers 15-20...
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 (γ and β) 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 α and γ bonds). This is consistent with the results of Weinfeld et al.30 who found that in dinucleoside ethyl phosphotriesters the Rp exhibits more base unstacking than the Sp 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.31 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.32 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


on the chemical shifts of the anomeric protons (i.e., \( H_2 \) of Cp and pG) and the nonexchangeable base protons (i.e., \( H_5 \) and \( H_6 \) of Cp, \( H_5 \) and \( H_6 \) of pG). The chemical shifts of these protons are known to be sensitive to changes in base stacking.\(^{30}\) Therefore, a melting transition gives rise to a \( \delta \) vs temperature profile with a sigmoidal shape for some of these protons. In \((S)\) and \((R)\)-r(CpG) this is found to be most clearly visible in the profiles of both anomeric protons (Figure 6). The \( T_{1\rho} \) values extracted from these curves are 13 °C for \((S)\) and 12 °C for \((R)\)-r(CpG). Furthermore, the \(^1\)H NMR spectra in \( H_2 O/D_2 O \) (80:20) at 4 °C showed one 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 \((S)\)- and \((R)\)-r(CpG) in the duplex form were determined from the 600-MHz \(^1\)H NMR spectra recorded at 2 °C.\(^{17}\) 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 \( \gamma^+ \), \( \beta^+ \), and \( \gamma^- \)-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,\(^ {34}\) and d(CpG),\(^ {8}\) which formed a miniduplex with \( T_{1\rho} \) values of 13 °C for the \( S \) and 9 °C for the \( R \) 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.\(^ {30}\) 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 \( NaClO_4 \)) 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.

### Concluding Remarks

Phosphate-methylated RNA dimers such as 15–20 can be conveniently prepared by using 9-fluorenylethoxycarbonyl (Fmoc) for transient protection of the exocyclic NH2 groups of the bases A, C, and G, levulinoyl for the \( 2'\)-OH and \( 3'\)-OH groups in the \( 3'\)-terminal residue, and methanolic \( K_2 CO_3 \) 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 one \( O_2 \) enhances base stacking. Most likely, the conformations about the bonds P–O\(_5\) (\( \alpha \)) and/or P–O\(_5\) (\( \beta \)) are perturbed in 15–20. Our CD data indicate that this must be especially the case for the \( R \) diastereoisomers, since these systems show base stacking to a lesser extent.

### Table IV. Some Relevant \(^1\)H–\(^1\)H NMR Coupling Constants (in Hz), Measured for \((S)\)- and \((R)\)-r(CpG) (19) in D\(_2\)O at 2 °C, along with the Calculated Time-Averaged Populations of the \( C_\gamma\)-endo Puckered Ring Form of the Ribose Rings, the \( C_\gamma-C_\gamma \) (\( \gamma \)) Rotamers, and the \( C_\gamma-O_\gamma \) (\( \beta \)) Rotamers

<table>
<thead>
<tr>
<th></th>
<th>( S )</th>
<th>( R )</th>
<th>( S )</th>
<th>( R )</th>
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<tr>
<td>( J_{1\gamma} )</td>
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<td>5.0</td>
<td>4.1</td>
<td>5.2</td>
</tr>
<tr>
<td>( J_{2\gamma} )</td>
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<td>5.1</td>
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<td>4.9</td>
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<tr>
<td>( J_{3\gamma} )</td>
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<td>2.5</td>
<td>3.1</td>
<td>2.5</td>
</tr>
<tr>
<td>( J_{4\gamma} )</td>
<td>3.4</td>
<td>4.4</td>
<td>3.3</td>
<td>4.4</td>
</tr>
<tr>
<td>( J_{5\gamma} )</td>
<td>5.4</td>
<td>5.4</td>
<td></td>
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</tr>
<tr>
<td>( J_{6\gamma} )</td>
<td>4.4</td>
<td>4.4</td>
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<tr>
<td>( z(C_\gamma\text{-endo}) )</td>
<td>0.42</td>
<td>0.53</td>
<td>0.44</td>
<td>0.56</td>
</tr>
<tr>
<td>( x(\gamma^+) )</td>
<td>0.74</td>
<td>0.66</td>
<td>0.72</td>
<td>0.66</td>
</tr>
<tr>
<td>( x(\gamma^-) )</td>
<td>0.22</td>
<td>0.34</td>
<td>0.20</td>
<td>0.34</td>
</tr>
<tr>
<td>( x(\gamma') )</td>
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<td>0.00</td>
<td>0.08</td>
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</tr>
<tr>
<td>( x(\beta^+) )</td>
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<td>0.10</td>
<td>0.10</td>
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</tr>
<tr>
<td>( x(\beta^-) )</td>
<td>0.75</td>
<td>0.75</td>
<td>0.75</td>
<td>0.75</td>
</tr>
<tr>
<td>( x(\beta') )</td>
<td>0.15</td>
<td>0.15</td>
<td>0.15</td>
<td>0.15</td>
</tr>
</tbody>
</table>

(34) Koole, L. H.; Quaedflieg, P. J. L. M., unpublished results.
Independent support for our conclusion that phosphotriesterified nucleotides are inherently more flexible than their unmodified counterparts can be found in the X-ray crystal structures of two phosphotriester 5'-mononucleotides, i.e., adenosine 5'-O(diethyl phosphate) and cytidine 5'-O(dimethyl phosphate). They reported that triesterification of the backbone phosphate groups does not at all inhibit the formation of Watson and Crick type double-helical structures. However, it is important to note that the torsion angles as determined by Jensen and Reed and Miller and co-workers 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 phosphotriesterified DNA oligomers, as described by Jensen and Reed and Miller and co-workers. They reported that triesterification of the backbone phosphate moieties were found to be disordered over two distinct conformations, and the torsion angles do not fall in the g-g range, which is typical for a regular double-helical conformation.

An elegant example of this phenomenon was described by Miller et al., who showed that the tetramer (Rp)TPOEt(TPOEt)CPOEtA is able to bind specifically to the anticondon region of Escherichia coli and yeast tRNA. 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 and formation of double helices with their cellular targets.

### Experimental Section

#### Materials and Methods.

The 'H NMR spectra were recorded on 400-MHz (compounds 1-14 and 18-19) and 600-MHz (compounds (Rp)-15 and 16-20) NMR spectrometers. Tetramethylysilane was used as the internal standard for samples in organic solvents. For samples in aqueous solution (D2O), the residual HDO peak was set at 4.68 ppm. For the variable-temperature measurements on (Sp)- and (Rp)-TPOEt in D2O, tetramethylylammonium bromide (δ = 3.18 ppm) was used as the standard. 31P NMR spectra were recorded at 162 MHz and referenced against 85% H3PO4 as external standard for all column chromatographic separations we used Merck Silica 60, 70-230 mesh ASTM. TLC experiments were performed on Merck silica gel 60 F254 plates. Dimethylformamide (DMF) was distilled from CaH2 under reduced pressure and stored on 4-A molecular sieves. Dichloromethane (CH2Cl2) was distilled from potassium carbonate (K2CO3) and stored on 4-A molecular sieves. Dichloromethane (CH2Cl2) was distilled from potassium carbonate (K2CO3) and stored on 4-A molecular sieves. Methanol (CH3OH) was refluxed on magnesium for 2 h, distilled, and stored on 3-A molecular sieves. 1H-Tetrazole, if used as a solid, was purified by sublimation prior to use. 1H-Tetrazole (0.5 M) in anhydrous acetonitrile (CH3CN) was used as received (Applied Biosystems). tert-Butyl hydroperoxide (t-BuOOH) was received as 75% solution in di-tert-butyl peroxide, Merck-Schuchhardt), as well as 9-fluorenylethoxybenzyl chloride (Janssen) and chlorotrimethylsilane (Janssen). Bis-(N,N-diisopropylaminomethyl)phosphine and leucyl anhydride were prepared as described elsewhere. Reactions were routinely run in an inert atmosphere of dry argon, and, unless otherwise stated, at ambient temperature. Prior to reactions run in pyridine or CH2Cl2 all nucleotide compounds were routinely dried by coevaporation with three portions of the dry solvent.

Prior to deprotection reactions in methanolic K2CO3, all protected dinucleotides were dried by coevaporation with three portions of dry CH2Cl2. In the fast atom bombardment (FAB) mass spectrometrical experiments, the samples were dissolved in glycerol solution onto a stainless steel probe and bombarded with xenon atoms having 8-keV energy. The separation of the Sp and Rp diastereoisomers of 15-20 was developed on a gradient HPLC system using a built-in diode array detector to spot the phosphate-methylated 2'-O-methylribodinucleotides between traces of highly absorbing Fmoc derivatives or using an absorbance detector set at 270 nm. Preparative chromatography was executed on a high-pressure liquid chromatography consisting of a solvent delivery system equipped with a solvent select valve module for sample introduction, a RSIL C18 10-μm particle size column (250 × 22 mm, Alltech), and an absorbance detector. Fractions were checked for purity on an analytical HPLC system consisting of a Nucleosil 120-3 C18 reversed-phase column (250 × 4 mm Macherey-Nagel) and an absorbance detector that monitored the eluate at 280 nm. Other reversed-phase columns used for development were a 125 × 4 mm Lichrospher C18 5-μm (Merck, Darmstadt) and a 100 × 4.6 mm Microsphere C18 3-μm column (Chrompack). Mobile phases used were acetonitrile (FSA Lab Supplies) or methanol (Lichrosolv, Merck) as organic modifiers and 0.1% (v/v) formic or acetic acid (AnalaR, BDH), 100-200 μL/L triethylamine (zur Synthese, Merck) in Milli-Q water, adjusted to the desired pH with ammonium hydroxide solution (Baker Analyzed Reagent, 25%). The purity of all title compounds was judged to be >95% by HPLC, 1H NMR, and 31P NMR determinations.

2'-O-Methylcytidine (1a). We used the method described by Yano et al. for the synthesis of 2'-O-methyladenosine, with some modifications. To a cooled (0 °C) solution of cytidine (21.00 g, 86.33 mmol) in 350 mL of DMF was added sodium hydride (4.15 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 2.5 h at 0 °C and for another 1 h at room temperature. TLC analysis (using a mixture of CH2Cl2 and CH3OH, 95:5) showed the presence of monomethylated compounds (Rf = 0.84 and 0.52), unmethylated compounds (Rf = 0.37), and unmethylated cytidine (Rf = 0.17). The mixture was filtered and the clear solution was evaporated to dryness. In order to remove the last traces of DMF, the yellow oil was coevaporated (three times) with CH2OH. Then, the residue was precipitated on silica (35 g) by coevaporation with CH2OH (three times). The precipitated powder was suspended in 100 mL of CH2Cl2 and the suspension was applied on a gradient column (200 g) and eluted with a gradient of CH2OH (5 → 33 vol %) in CH2Cl2. The fractions with unmethylated compounds were evaporated to afford a white solid. 31P NMR and 13C NMR experiments showed that this product consisted of an approximately 3:1 mixture of the desired 2'-O-methylcytidine (1a) and 3'-O-methylcytidine. Pure 1a was obtained as a white solid by crystallization from absolute ethanol: yield 5.11 g (23%); mp 252-254 °C, 1H NMR (D2O) δ 5.37 (3 H, s, CH3), 3.65 (1 H, dd, Ha), 3.78 (1 H, dd, Hb), 3.81 (1 H, dd, Hc), 3.94 (1 H, m, Hb), 4.14 (1 H, dd, Hb), 5.82 (1 H, d, Hc), 5.89 (1 H, d, Hb), 7.71 (1 H, d, Hc); exact mass calc’d [M + H]+ = 258. Anal. Calcd: C, 46.69; H, 5.88; N, 16.33. Found: C, 46.51; H, 6.10; N, 16.09.

2'-O-Methyl-4-(9-fluorenylmethoxybenzyl)cytidine (2a). To a cooled (0 °C) suspension of compound 1a (1.06 g, 4.12 mmol) in 60 mL of CH2Cl2 the samples were added chlorotrimethylsilane (1.21 g, 19.88 mmol), and the reaction mixture was stirred for 1 h. After the addition of 9-fluorenylethoxybenzyl chloride (1.21 g, 4.68 mmol) the mixture was stirred for another 2 h. Hydrolysis of the trimethylsilyl groups and excess chlorides was effected by addition of water (20 mL) at 0 °C. After stirring for 18 h, the mixture was evaporated to a yellow oil and coevaporated with toluene. Upon addition of water (50 mL) a white precipitate appeared. The mixture was shaken vigorously until no more yellow oil was visible. After addition of ethyl acetate (25 mL) and vigorous shaking, the precipitate that was formed on the separation layer was isolated by filtration and washed with ethyl acetate. After drying in vacuo, 2a was obtained as a white solid; yield 1.70 g (96%). This product decomposed upon heating (approximately 70 °C): 1H NMR (pyridine-d5) δ 8.77 (3 H, s, 8-CH3) and 8.36 (1 H, d, J = 8.4 Hz, 8'-CH3).
Phosphate-Methylated RNA Dinucleotides

2'-OCH₃, 4.21 (1 H, dd, H₂), 4.28 (1 H, dd, H₃), 4.36 (1 H, t, CH₂CH₂ CFmoc), 4.39 (1 H, dd, H₄), 6.00 (1 H, s, CH₂ CFmoc), 6.54 (1 H, d, H₅), 7.28 (2 H, m, arom Fmoc), 7.39 (2 H, m, arom Fmoc), 7.43 (1 H, d, H₆), 7.68 (2 H, m, arom Fmoc), 7.89 (2 H, d, arom Fmoc), 9.11 (1 H, d, H₇), exact mass calcd 503; FAB (M + H)⁺ = 504.

5'-O-(4-Monomethoxytrityl)-2'-O-methyl-6-N-(9-fluorenlymethoxycarbonyl)adenosine (3b). A solution of compound 2b (4.65 g, 8.60 mmol) and 4-monomethoxytrityl chloride (2.96 g, 9.80 mmol) was added dropwise to a suspension of cytidine (12.0 g, 49.32 mmol) in 240 mL of pyridine, 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 CH₃OH (0 → 2 vol %) in CH₂Cl₂ as eluent, Rf 0.53 (CHCl₃/CH₃OH 95:5 v/v), yielding pure 3b (5.76 g, 95%), as a yellowish solid, which decomposed upon heating (approximately 115 °C); ¹H NMR (CDCl₃) δ 7.27 (1 H, bs, 3' OH), 3.43 (1 H, dd, H₂), 5.34 (1 H, dd, H₃), 3.56 (1 H, s, 2'-OCH₃), 3.79 (3 H, s, CH₃CFmoc), 6.22 (1 H, d, H₄), 7.25 (1 H, d, dd, H₂), 4.50 (1 H, dd, H₃), 4.61 (2 H, d, CH₂ Fmoc), 6.18 (1 H, d, CH₂ Fmoc), 6.81 (2 H, d, arom MMTr), 7.20–7.50 (16 H, m, aron MMTr), 7.63 (2 H, d, arom Fmoc), 8.20 (1 H, s, H₂), 8.34 (1 H, bs, NH), 8.71 (1 H, s, H₃).

5'-O-(4-Monomethoxytrityl)uridine (6a). 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 4 h in darkness. The mixture was then concentrated in vacuo, and the solid was recrystallized from absolute ethanol. After a suspension procedure as described in the preparation of 1a, the product was impregnated on silica (40 g) and eluted with a gradient of CH₃OH (5 → 15 vol %) in CH₂Cl₂. Evaporation of the fractions with monomethoxylacetones compounds (Rf = 0.29 in CHCl₃/CH₃OH 85:15 v/v) yielded a white solid.

1H NMR analysis revealed that this product consisted of a 5:1 mixture of the desired 2'-O-methyladenosine (1b) and 3'-O-methyladenosine. Pure 1b was obtained as a white solid by crystallization from absolute ethanol: yield 7.46 g (27%); mp 202–203 °C; ¹H NMR (D₂O) δ 3.20 (3 H, s, CH₃), 3.68 (1 H, dd, H₂), 3.78 (1 H, dd, H₃), 4.14 (1 H, m, H₄), 4.33 (1 H, dd, H₅), 4.46 (1 H, dd, H₆), 5.93 (1 H, dd, H₇), 7.98 (1 H, s, H₈), 8.12 (1 H, s, H₉); exact mass calcld 516; FAB (M + H)⁺ = 517.

2'-O-Methyladenosine (1b). A solution of adenosine (26.70 g, 100.00 mmol) in 450 mL of DMF was reacted with sodium hydride (4.80 g, 120.00 mmol) and methyl iodide (15.60 g, 110.00 mmol) for 4 h at 55 °C. After the workup procedure as described in the preparation of 1a, the product was purified on silica (40 g) and eluted with a gradient of CH₃OH (5 → 15 vol %) in CH₂Cl₂. Evaporation of the fractions with monomethoxylacetones compounds (Rf = 0.29 in CHCl₃/CH₃OH 85:15 v/v) yielded a white solid.

1H NMR analysis revealed that this product consisted of a 5:1 mixture of the desired 2'-O-methyladenosine (1b) and 3'-O-methyladenosine. Pure 1b was obtained as a white solid by crystallization from absolute ethanol: yield 7.46 g (27%); mp 202–203 °C; ¹H NMR (D₂O) δ 3.20 (3 H, s, CH₃), 3.68 (1 H, dd, H₂), 3.78 (1 H, dd, H₃), 4.14 (1 H, m, H₄), 4.33 (1 H, dd, H₅), 4.46 (1 H, dd, H₆), 5.93 (1 H, dd, H₇), 7.98 (1 H, s, H₈), 8.12 (1 H, s, H₉); exact mass calcld 516; FAB (M + H)⁺ = 517.
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 (500 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 (90%) of pure 5b, which decomposed upon heating (approximately 112 °C): 1H NMR (DMSO-d6) δ 3.60 (1 H, dd, H3), 3.74 (1 H, dd, H4), 3.90 (1 H, m, H5), 3.98 (2 H, m, CH2 Fmoc), 4.29 (1 H, t, CH Fmoc), 4.36 (3 H, m, HS3, 3.77 (3 H, m, a")), 4.50 (1 H, m, Hr), 5.20 (1 H, bs, OH), 5.62 (1 H, m, H3), 6.04 (1 H, d, H1J, 7.23 (2 H, t, arom Fmoc), 7.39 (2 H, t, arom Fmoc), 7.74 (2 H, d, arom Fmoc), 8.10 (1 H, bs, NH); exact mass calcd 777; FAB (M + H)+ = 778.

2'-3'-Di-O-diethylamineyl-4'-N-(9-fluorenylmethoxycarbonyl)-cytidine. A mixture of compound 6b (12.35 mmol) and 4-monomethoxytrityl chloride (10.10 g, 28.27 mmol) was added to a solution of compound 4a (2.08 g, 2.67 mmol) in 9 mL of pyridine. After 4 h of stirring the red solution was processed as described in the preparation of 7a. This afforded a yellowish solid, yield 0.96 g (53% from 6b), which decomposed upon heating (approximately 120 °C): 1H NMR (CDCl3) δ 5.34 (1 H, dd, H3), 3.49 (1 H, dd, H4), 3.77 (3 H, s, CH3), 4.37 (3 H, s, CH Fmoc), 4.04 (1 H, m, H3), 4.22 (1 H, m, H4), 4.34 (1 H, t, CH Fmoc), 4.49 (2 H, d, CH2 Fmoc), 4.54 (1 H, m, H3), 5.20 (1 H, bs, OH), 5.62 (1 H, m, H3), 6.04 (2 H, d, arom MTT), 7.2-7.5 (16 H, m, arom MTT), 7.81 (2 H, d, arom Fmoc), 7.92 (2 H, d, arom Fmoc). 8.10 (1 H, bs, NH); exact mass calcd 777; FAB (M + H)+ = 778.}

5'-O-(4-Monomethoxytrityl)-2'-N-(9-fluorenylmethoxycarbonyl)guanosine (6c). A solution of compound 5c (6.00 g, 11.87 mmol) and 4-monomethoxytrityl chloride (4.40 g, 14.25 mmol) in pyridine (70 mL) was stirred for 36 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 (95:5 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): 1H NMR (DMSO-d6) δ 3.18 (1 H, m, H3), 3.26 (1 H, m, H4), 3.72 (3 H, s, OCH2 MMT), 4.04 (1 H, m, H3), 4.22 (1 H, m, H4), 4.34 (1 H, t, CH Fmoc), 4.49 (2 H, d, CH2 Fmoc), 4.54 (1 H, m, H3), 5.20 (1 H, bs, OH), 5.62 (1 H, m, H3), 6.04 (2 H, d, arom MTT), 7.2-7.5 (16 H, m, arom MTT), 7.81 (2 H, d, arom Fmoc), 7.92 (2 H, d, arom Fmoc). 8.10 (1 H, bs, NH); exact mass calcd 777; FAB (M + H)+ = 778.

Semi-pure 5c (10.14 g, 71%) was used without further purification: 1H NMR (DMSO-d6) δ 3.35 (1 H, m, H3), 3.65 (1 H, m, H4), 3.91 (1 H, m, H5), 4.14 (1 H, m, H6), 4.35 (1 H, t, CH Fmoc), 4.47 (1 H, m, H3), 4.49 (2 H, d, CH2 Fmoc), 5.04 (1 H, t, 5' OH), 5.20 (1 H, d, OH), 5.50 (1 H, t, OH), 5.86 (1 H, d, H3), 7.34-7.47 (4 H, m, arom Fmoc), 7.87 (4 H, m, arom Fmoc), 8.37 (4 H, m, CH2 Fmoc), 8.57 (1 H, m, CH3 Fmoc), 8.67 (1 H, m, CH3 Fmoc).
Phosphate-Methylated RNA Dinucleotides

H$_2$PO$_4$(H$_2$O) 5.38 (1 H, 2 x d, H$_2$(pU)), 5.69 (1 H, 2 x d, H$_2$(pU)), 5.89 and 6.00 (1 H, 2 x d, H$_2$(pU)), 6.03 (1 H, s, H$_2$(pC)), 5.85 (2 H, d, amom MTR), 6.86 (1 H, d, H$_2$(pC)), 7.2-7.5 (16 H, amom Fmoc/amom MTR), 7.37 (2 H, d, amom Fmoc), 7.49 (1 H, d, H$_2$(pC)), 7.78 (2 H, d, amom Fmoc), 8.50 (1 H, d, H$_2$(pC)); exact mass calcd, 1268; FAB (M + H$^+$) $\ast$ 1289.

2'-O-Methyladenosyl-(3'-5')-uridine O-(Methyl phospho) (16).

Compound (19) ($\sim$600 mg, 0.22 mmol) was dissolved in a 0.05 M solution of K$_2$CO$_3$ in CH$_2$OH (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: R$_f$(CHCl$_3$/CH$_3$OH 8.2 v/v) = 0.16; $^{31}$P NMR (CD$_2$OD) $\delta$ 0.31 (peaks overlapped). The product was dissolved in 10 mL of 80% acetic acid and stirred for 1 h. The reaction mixture was evaporated to dryness, coevaporated (four times) with water and partitioned between water and CHCl$_3$. The aqueous layer and the precipitate on the separation layer were collected.

The reaction mixture was stirred for 40 min. Formation of the phosphoramidite coupling synthon in situ 4b was evident from the $^{31}$P NMR data (two doublets), $\delta$ 149.3 and 149.2. Then a solution of compound 8b (0.93 g, 1.40 mmol) in 3 mL of CH$_2$Cl$_2$ was added. After 3 h of stirring, the mixture was evaporated to yield a white solid: R$_f$(CHCl$_3$/CH$_3$OH 82 v/v) = 0.26 and -0.10; $^1$H NMR (CDCl$_3$) $\delta$ 2.06 (6 H, 4 Me$_2$(pU)), 2.18 (1 H, d, H$_2$(pC)), 2.5-2.8 (8 H, m, 2 x CH$_2$(CH$_2$)$_2$Lev), 3.45 (3 H, 2 x s, CH$_3$(pN)), 3.79 (2 H, 2 x d, POCH$_3$), 4.12 (1 H, t, CH Fmoc), 4.61 (2 H, d, CH$_2$(Fmoc), 4.92 and 5.20 (2 H, 2 x s, H$_2$(Ap)), 5.20 (1 H, m, H$_2$(Ap), 5.30 (1 H, 2 x d, H$_2$(pU)), 5.40 and 5.47 (1 H, 2 x d, H$_2$(pU)), 5.75 (1 H, 2 x d, H$_2$(pU)), 6.11 (1 H, d, H$_2$(Ap)), 6.12 (1 H, 2 x d, H$_2$(pU)), 6.32 (2 H, d, amom MTR), 7.2-7.5 (16 H, amom Fmoc/amom MTR), 7.52 (1 H, d, H$_2$(pP)), 7.70 (2 H, d, amom Fmoc), 7.73 (2 H, d, amom Fmoc), 8.17 (1 H, 2 x s, H$_2$(Ap)), 8.48 (1 H, bs, NH), 8.62 (1 H, 2 x s, H$_2$(Ap)), 9.06 (1 H, bs, NH); exact mass calcd 1281; FAB (M + H$^+$) $\ast$ 1292.

5'-O-(4-Monomethoxymethyl)-2'-O-methyl-4-d N-(9-fluorenylmethoxycarbonyl)adenylyl-(3'-5')-2',3'-dio-levulinoyluridine O-(Methyl phospho) (11).

To a solution of compound 3a (1.00 g, 1.33 mmol) in 5 mL of CH$_2$Cl$_2$ were added 1H-tetrazole (0.65 mL of a 0.56 M solution in CH$_3$CN, 0.33 mmol) and a solution of bis(N,N-diisopropylamino) methoxyphosphine (0.19 g, 0.72 mmol) in 0.5 mL of CH$_2$Cl$_2$, and the reaction mixture was stirred for 30 min. Formation of the phosphoramidite coupling synthon in situ 4b was evident from the $^{31}$P NMR data (two diastereoisomers (CD$_2$I): $\delta$ 152.0 and 151.1). Then a solution of compound 8a (0.30 g, 0.66 mmol) in 2 mL of CH$_2$Cl$_2$ and 1H-tetrazole (0.65 mL of a 0.56 M solution in CH$_3$CN, 0.33 mmol) were transferred into the reaction vessel, and the mixture was stirred for 30 min. Stirring the reaction mixture was transferred to the corresponding phosphite triester (two diastereoisomers (CD$_2$I): $\delta$ 142.0 and 141.4), which were readily oxidized through the addition of tBuOOH (0.5 mL) and 5 min of stirring. The mixture was evaporated to dryness and the residue was coevaporated with toluene (twice) and CHCl$_3$ (three times). The crude product (0.80 mL of a 0.50 M solution in CH$_3$CN) (1.3 mmol) was used for rechromatography, using a mixture of MeOH and CH$_2$OH (98:2 v/v) as eluent (R$_f$ = 0.36). This afforded 0.44 g (53%) of pure 10 as a white solid, which decomposed upon heating (approximately 90 °C). $^{31}$P NMR (CD$_2$I) $\delta$ 0.26 and -0.10; $^1$H NMR (CD$_2$I) $\delta$ 2.18 (6 H, 4 x s, 2 x CH$_2$(CH$_2$)Lev), 2.5-2.8 (8 H, m, 2 x CH$_2$(CH$_2$)Lev), 3.45 (3 H, 2 x s, CH$_3$(pN)), 3.79 (2 H, 2 x d, POCH$_3$), 4.12 (1 H, t, CH Fmoc), 4.61 (2 H, d, CH$_2$(Fmoc), 4.92 and 5.20 (2 H, 2 x s, H$_2$(Ap)), 5.20 (1 H, m, H$_2$(Ap), 5.30 (1 H, 2 x d, H$_2$(pU)), 5.40 and 5.47 (1 H, 2 x d, H$_2$(pU)), 5.75 (1 H, 2 x d, H$_2$(pU)), 6.11 (1 H, d, H$_2$(Ap)), 6.12 (1 H, 2 x d, H$_2$(pU)), 6.32 (2 H, d, amom MTR), 7.2-7.5 (16 H, amom Fmoc/amom MTR), 7.52 (1 H, d, H$_2$(pP)), 7.70 (2 H, d, amom Fmoc), 7.73 (2 H, d, amom Fmoc), 8.17 (1 H, 2 x s, H$_2$(Ap)), 8.48 (1 H, bs, NH), 8.62 (1 H, 2 x s, H$_2$(Ap)), 9.06 (1 H, bs, NH); exact mass calcd 1281; FAB (M + H$^+$) $\ast$ 1292.

\(4\cdot 20.0 \text{ mL of a 0.05 M solution of } K_2CO_3 \text{ in } CH_3OH (0.50 \text{ mmol}) \) and stirred. Cleavage of both Lev groups appeared to be complete within 3 min, whereas the removal of both Fmoc groups was accomplished after 24 h. The clear solution was neutralized (to \(pH \approx 6\)) by addition of several small portions of Dowex-H\(^+\), filtered over a glass filter, and evaporated to dryness: \(R_f \text{ (CHCl}_3/CH_3OH 8.2 v/v) = 0.10; \) \(31\text{P NMR (CD}_2OD) \delta 0.24 \) and 0.18. The product was dried as described in the synthesis of \(16\), which afforded \(17 \) as a colorless film (73 mg, 83%); \(R_f \text{ (CHCl}_3/CH_3OH 8.2 v/v) = 0.18\). The product was purified by vacuum sublimation (300 mm-Hg, 97°C) for 3 h.

Cleavage of both Lev groups appeared to be complete within 24 h, whereas the removal of both Fmoc groups was accomplished after 24 h. The clear solution was neutralized (to \(pH \approx 6\)) by addition of several small portions of Dowex-H\(^+\), filtered over a glass filter, and evaporated to dryness: \(R_f \text{ (CHCl}_3/CH_3OH 8.2 v/v) = 0.10; \) \(31\text{P NMR (CD}_2OD) \delta 0.24 \) and 0.18. The product was dried as described in the synthesis of \(16\), which afforded \(17 \) as a colorless film (73 mg, 83%); \(R_f \text{ (CHCl}_3/CH_3OH 8.2 v/v) = 0.18\). The product was purified by vacuum sublimation (300 mm-Hg, 97°C) for 3 h. This afforded \(0.94 \text{ g (61\%)} \) of pure \(17\) by eluent. This afforded \(0.94 \text{ g (61\%)} \) of pure \(17\) by eluent.

\(\text{Phosphate) (17).}\)

\(5\) \(-\text{(4-Monomethoxytrityl)\text{-}2\text{-} \text{-methyl-6\text{-}N\text{-} (9\text{-fluorenylethoxycarbonyl)adenylyl\text{(3\text{-}5\text{-})\text{-} 2\text{-}2\text{-}di\text{-} \text{-oxylucinoyl\text{-}4\text{-}N\text{-} (9\text{-fluorenylethoxycarbonyl)cystidine O\text{-} (Methyl phosphate) (12).} \) 1H-Tetrazole (0.90 mL of a 0.50 M solution in CH_2CN, 0.45 mmol) and a solution of bis(N,N-diisopropylamino)methoxyphosphine (0.33 g, 1.26 mmol) in 1.5 mL of CH_2Cl_2 were added to a solution of compound 3b (0.79 g, 1.02 mmol) in 4 mL of CH_2Cl_2 and the reaction mixture was stirred for 40 min. Formation of the phosphoramidite coupling synthon in 3b was evident from the \(31\text{P NMR spectrum of the crude product.}\)

A 1H NMR spectrum of the crude product 3b (0.73 g, 0.10 mmol) in 6 mL of CH_2Cl_2 and 1H-tetrazole (6.00 mL of a 0.50 M solution in CH_2CN, 3.00 mmol) were added to the reaction mixture, and stirring was continued for 1/2 h. \(31\text{P NMR showed complete conversion to the phosphite triester (CDCl}_3 \delta 142.0 \) and 141.6. One peak (3-fold lower intensity, (CDCl}_3 \delta 142.0) of a side product was visible as well. Then tBuOOH (1.0 mL) was added, and the reaction mixture was stirred for another 25 min. \(31\text{P NMR data showed that all phosphite triesters had been converted into phosphite triesters (CDCl}_3 \delta 0.2 \) and 0.02; side product, (CDCl}_3 \delta 0.5). The mixture was concentrated to near dryness and coevaporated with toluene (three times) and CHCl_3 (twice). The resulting yellow foam was purified by column chromatography, using a mixture of ethyl acetate and CH_2OH (96:4 v/v) as eluent. This afforded 0.94 g (61%) of pure 12 as a white solid, \(R_f \text{ (ethyl acetate/CH}_2OH 96:4 v/v) = 0.08.} \)

A 1H NMR spectrum of the crude product 3b (0.73 g, 0.10 mmol) in 6 mL of CH_2Cl_2 and 1H-tetrazole (6.00 mL of a 0.50 M solution in CH_2CN, 3.00 mmol) were added to the reaction mixture, and stirring was continued for 1/2 h. \(31\text{P NMR showed complete conversion to the phosphite triester (CDCl}_3 \delta 142.0 \) and 141.6. One peak (3-fold lower intensity, (CDCl}_3 \delta 142.0) of a side product was visible as well. Then tBuOOH (1.0 mL) was added, and the reaction mixture was stirred for another 25 min. \(31\text{P NMR data showed that all phosphite triesters had been converted into phosphite triesters (CDCl}_3 \delta 0.2 \) and 0.02; side product, (CDCl}_3 \delta 0.5). The mixture was concentrated to near dryness and coevaporated with toluene (three times) and CHCl_3 (twice). The resulting yellow foam was purified by column chromatography, using a mixture of ethyl acetate and CH_2OH (96:4 v/v) as eluent. This afforded 0.94 g (61%) of pure 12 as a white solid, \(R_f \text{ (ethyl acetate/CH}_2OH 96:4 v/v) = 0.08.} \)

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Phosphate-Methylated RNA Dinucleotides

Glass filter, and evaporated to dryness: $R_f$ (CHCl$_3$/CH$_3$OH 3:2 v/v) = 0.21. $^3$P NMR (CDCl$_3$) $\delta$ 13.0 and 0.03. Detritylation as described for 16 yielded 19 as a white solid (60 mg, 74%). $R_f$ = 0.09 (CHCl$_3$/CH$_3$OH 1:1 v/v). ($S_p$)-19: $^3$P NMR (D$_2$O) $\delta$ -0.28; $^1$H NMR (D$_2$O) $\delta$ 3.38 (3 H, s, 2'-OCH$_3$), 3.64 (1 H, dd, H$_2$(Ap)), 3.71 (3 H, d, POCH$_3$, $J$ = 1.14 Hz), 3.72 (1 H, dd, H$_2$(Cp)), 4.04 (1 H, t, H$_2$(Cp)), 4.13 (1 H, m, H$_2$(Cp)), 4.28 (1 H, m, H$_2$(pG)), 4.34 (1 H, dddd, H$_2$(pG)), 4.38 (1 H, dddd, H$_2$(pG)), 4.42 (1 H, t, H$_2$(pG)), 5.80 (1 H, d, H$_2$(Cp)), 5.84 (1 H, d, H$_2$(Cp)), 7.65 (1 H, d, H$_2$(Cp)), 7.84 (1 H, s, H$_2$(pG)); the H$_2$(pG) and H$_2$(Cp) signals reside under the HDO peak; Cp residue $J_{CH} = 4.5$ Hz, $J_{CH^2} = 5.1$ Hz, $J_{CH^3} = 3.0$ Hz, $J_{CH^4} = 3.7$ Hz, $J_{CH^5} = -12.9$ Hz; pG residue $J_{CH} = 4.9$ Hz, $J_{CH^2} = 5.0$ Hz, $J_{CH^3} = 5.0$ Hz, $J_{CH^4} = -11.5$ Hz. ($R_p$)-19: $^3$P NMR (D$_2$O) $\delta$ -0.46; $^1$H NMR (D$_2$O) $\delta$ 3.73 (3 H, s, 2'-OCH$_3$), 3.57 (1 H, dd, H$_2$(Cp)), 3.60 (1 H, dd, H$_2$(Cp)), 3.74 (3 H, d, POCH$_3$, $J = 11.4$ Hz), 3.99 (1 H, m, H$_2$(Cp)), 4.06 (1 H, t, H$_2$(Cp)), 4.25 (1 H, m, H$_2$(pG)), 4.34 (2 H, m, H$_2$(pG)), 4.52 (1 H, t, H$_2$(pG)), 5.82 (1 H, d, H$_2$(pG)), 5.84 (1 H, d, H$_2$(Cp)), 6.75 (1 H, d, H$_2$(Cp)), 7.85 (1 H, s, H$_2$(pG)); the H$_2$(pG) and H$_2$(Cp) signals reside under the HDO peak; Cp residue $J_{CH} = 4.5$ Hz, $J_{CH^2} = 5.3$ Hz, $J_{CH^3} = 4.0$ Hzug; $J_{CH^4} = 3.3$ Hz, $J_{CH^5} = 3.6$ Hz, $J_{CH^5} = -12.8$ Hz; pG residue $J_{CH} = 5.3$ Hz, $J_{CH^2} = 5.5$ Hz, $J_{CH^3} = 5.4$ Hz, $J_{CH^4} = 2.8$ Hz, $J_{CH^5} = 5.4$ Hz, $J_{CH^5} = 5.4$ Hz.

5'-O-(4-Mono- methoxytrityl)-2'-O-methyl-6-N-(9-fluorenethylmethoxy carbonyl)adenyl-3'-(5'-)2'-N-(9-fluorenylmethoxy carbonyl)guanosine O-(Methyl phosphor) (14). 1H-Tetrazole (0.60 mL of a 0.50 M solution in CH$_3$CN, 0.30 mmol) and a solution of bis(N,N- disopropylamino)methoxyphosphine (0.17 g, 0.55 mmol) in 0.5 mL of CH$_2$Cl$_2$ were added to a solution of compound 3b (0.44 g, 0.57 mmol) in 4.0 mL of CH$_2$Cl$_2$ and the reaction mixture was stirred for 11/2 h. Formation of the phosphoramidite coupling slym 4b was evident from the $^3$P NMR spectrum (CDCl$_3$ $\delta$ 152.0 and 151.1). Then a solution of compound 8c (0.41 g, 0.58 mmol) in 5.0 mL of CH$_2$Cl$_2$ and 1H-tetrazole (2.50 mL of a 0.60 M solution in CH$_3$CN, 1.25 mmol) were added to the reaction mixture and stirring was continued for 40 min. $^3$P NMR analysis showed the complete conversion of 4b into the corresponding phosphite triester (CDCl$_3$ $\delta$ 142.0 and 141.2), which was readily oxidized through the addition of tBuOOH (2.0 mL) and 11/2 h of stirring. The mixture was evaporated to dryness and coevaporated with toluene (four times) and CH$_2$Cl$_2$ (three times). The crude product was purified by column chromatography using a gradient of CH$_3$OH (4 → 6 vol %) in CH$_2$Cl$_2$ as eluent, $R_f$ = 0.31 (CH$_2$Cl$_2$/CH$_3$OH 96:4 v/v). Pure 4 (0.62 g, 70%) was obtained as a white solid, which decomposed upon heating (approximately 115 °C). $^1$H NMR (CDCl$_3$) $\delta$ -0.8 and -1.4; $^1$H NMR (CDCl$_3$) $\delta$ 2.14 (6 H, 4 × s, 2 × CH$_2$(Lev), 2.4–2.7 (2 H, m, H$_2$(Ap)/H$_2$(pG), 2.7–2.9 (8 H, m, 2 × CH$_2$(Lev)), 3.36 (3 H, 2 × s, 2'-OCH$_3$), 3.49 (2 H, m, H$_2$(Ap)/H$_2$(pG)), 3.69 (3 H, 2 × s, CH$_3$O MMTT), 3.82 and 3.88 (3 H, 2 × d, POCH$_3$, $J$ = 11.4 Hz), 4.00 (1 H, m, H$_4$(Ap)), 4.3–4.7 (9 H, m, H$_2$(pG)/H$_2$(pG)/H$_2$(pG)/2 × CH$_2$(Lev), 5.00 (1 H, m, H$_4$(Ap)), 5.3–5.7 (5 H, m, H$_2$(pG)/H$_2$(pG)/H$_2$(pG)/2 × CH$_2$(Lev), 5.68 (1 H, 2 × d, H$_2$(Ap)), 5.68 (1 H, 2 × d, H$_2$(Ap)), 5.9–6.2 (2 H, 2 × d, H$_2$(Ap)/H$_2$(pG)), 6.70 (2 H, 2 × d, arom MMTT), 7.1–7.8 (28 H, m, arom Fmoc/aron MMTT), 8.0–8.1 (2 H, 2 × s, H$_2$(Ap)/H$_2$(Ap)), 8.54 (1 H, 2 × s, H$_2$(pG)).

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Supplementary Material Available: $^1$H NMR spectra of all title compounds, CD spectra of 16-20, and HPLC diagrams of 15-20 (61 pages). Ordering information is given on any current masthead page.