Synthesis and conformation of phosphate-methylated r(CpU) and r(ApU) : formation of a parallel right-handed duplex for Sp-r(CpU)

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Synthesis and conformation of phosphate-methylated r(CpU) and r(ApU). Formation of a parallel right-handed duplex for S_P r(CpU)

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SUMMARY

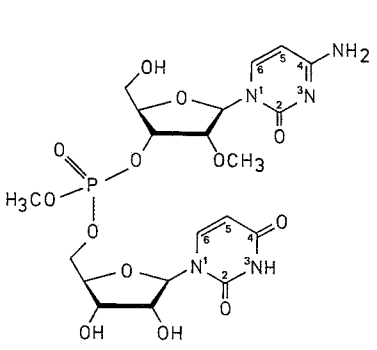
The phosphate-methylated RNA dinucleotides 1 (2'-O-methyl cytidyl 3'→5' uridine O-methyl phosphate) and 2 (2'-O-methyl adenylyl 3'→5' uridine O-methyl phosphate) have been synthesized, and the R_P and S_P diastereoisomers were separated with reversed-phase HPLC. The conformations of the systems have been determined by means of ^{1}H NMR (400 and 600 MHz) and UV hyperchromicity experiments. It was found that a parallel right-handed RNA miniduplex is formed, exclusively for S_P(1). This novel duplex structure is based on the formation of one C-C base pair with two equivalent N_4-H...N_3 hydrogen bonds, and one U-U base pair with two equivalent O_4...H-N_3 hydrogen bonds.

INTRODUCTION

Our interest in phosphate-methylated DNA is based on its significance as site-specific inhibitors of replication and gene expression *in vitro* and *in vivo* (Moody et al., 1989; Smit et al., 1989), as well as on their inherent structural properties, e.g. formation of parallel non-Watson & Crick duplexes, and of left-handed Z-DNA in salt-free aqueous solution (Quaedflieg et al., 1989a). The first observations of a parallel duplex structure for phosphate-methylated DNA were made with phosphate-methylated d(T_n) (n = 2 – 8) (Koole et al., 1986; Koole et al., 1987). Sharp melting transitions were found for these systems (by means of UV hyperchromicity and variable temperature NMR experiments), proving that formation of a parallel duplex occurs irrespectively of the stereochemical configuration of the methylated phosphate groups (R_P, with the

methyl group located inside the helix groove, or S_P, for which the methyl group protrudes from the helix into the solvent). Recently, it was found for phosphate-methylated d(CpC) and d(TpC) that formation of a parallel duplex occurs exclusively in the case of S_P configuration. Molecular modelling studies revealed that the greater propeller twist angle for C-C base pairing (35°) in comparison with T-T base pairing (18°) results in a more narrow helix groove which can therefore not accommodate the methyl group in the case of the R_P configuration (Quaedflieg et al., 1989b).

Stimulated by the results obtained with phosphate-methylated DNA, we have started to study phosphate-methylated RNA systems as well. Synthesis of phosphate-methylated RNA is slightly complicated because of the presence of the 2'-OH group, for which suitable protection is required during synthesis. More seriously, the presence of a free 2'-OH group in combination with a methylated phosphate group results in immediate intramolecular attack of 2'-OH on the phosphotriester function, leading to chain scission or demethylation (Reese et al., 1985). We coped with these problems via the use of a methyl group for *permanent* protection of the 2'-OH function. The selection of a methyl group was largely based on the work of Drake et al. (1974) and of Inoue et al. (1987), who found that a 2'-O-methyl group can be easily accommodated in an RNA double helix, without affecting the duplex stability. Herein, we report the synthesis of the phosphate-methylated RNA dimers 1 (2'-O-methyl cytidyl 3'→5' uridine O-methyl phosphate) and 2 (2'-O-methyl adenylyl 3'→5' uridine O-methyl phosphate) in diastereoisomerically pure form. The structural properties of 1 (R_P, S_P) and 2 (R_P, S_P) in aqueous solution were investigated with high-field NMR (400 and 600 MHz ^1H, 120 MHz ^{31}P), and UV hyperchromicity techniques.



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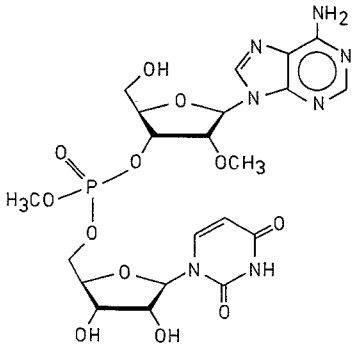

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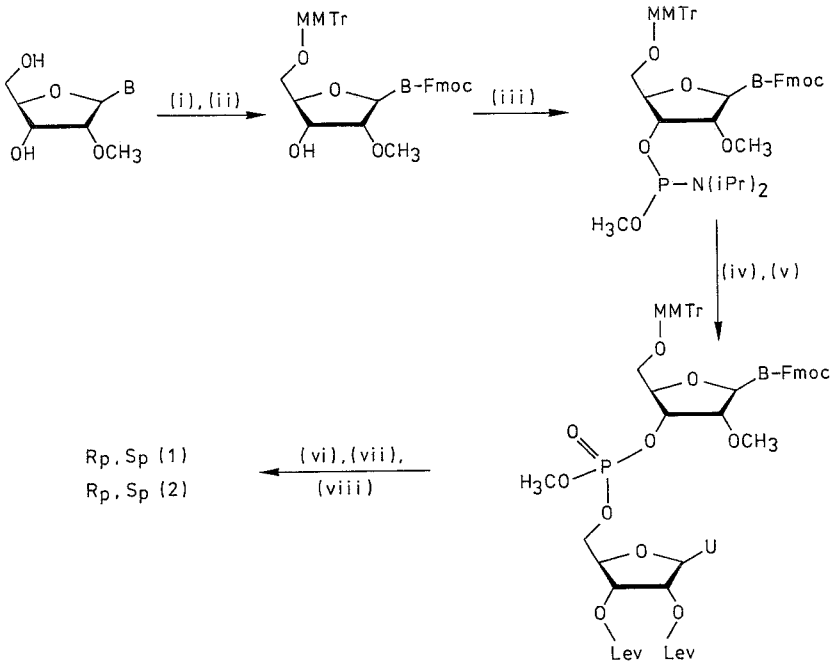
SYNTHESIS

The phosphate-methylated RNA dimers 1 and 2 were synthesized according to Scheme 1. The starting compound 2'-O-methyl adenosine was synthesized according to a literature procedure (Yano et al., 1980). It proved to be possible to prepare 2'-O-methyl cytidine in an analogous way. Based on our previous

work on the synthesis of phosphate-methylated DNA fragments, we selected 9-fluorenyl methoxy carbonyl (Fmoc) for the transient protection of the NH_2-groups of adenine and cytosine. 5'-Tritylation and introduction of the phosphoramidite function on $O_{3'}$ were performed according to standard reaction protocols. For the introduction of uridine as the bottom residue, we selected the levulinyl (Lev) group for the temporary protection of the 2'- and 3'-hydroxyl groups (Hassner et al., 1975). Oxidation of the 3'-5' phosphite triester (two diastereoisomeric forms are present as judged from ^{31}P NMR spectroscopy) was performed by addition of tert. butylhydroperoxide. The Fmoc and levulinyl groups were simultaneously removed by treatment with a mild base. Subsequently, detritylation was performed under acidic conditions. Separation of the R_P and S_P diastereoisomers was performed by reversed-phase HPLC.

CONFORMATIONAL ANALYSIS AND DUPLEX FORMATION

Our structural analysis of the R_P and S_P diastereoisomers of 1 and 2 consists of 3 parts, i.e., (i), assignment of the stereochemical configuration at phosphorus; (ii), determination of the backbone and ribose conformation; (iii), UV



Scheme I (i) trimethylchlorosilane, 9-fluorenyl methoxycarbonyl chloride (Fmoc-Cl), H_2O (Koole et al., 1989); (ii) 4-monomethoxytrityl chloride; (iii), bis-(N,N-diisopropylamino)-methoxy phosphine/0.5 eq. 1H-tetrazole (Marugg et al., 1987); (iv) coupling with 2',3'-di-O-levulinyl uridine; (v) oxidation with tert. butylhydroperoxide; (vi), removal of Fmoc and levulinyl (Lev) groups by mild base treatment; (vii), detritylation under acidic conditions; (viii), separation of the R_P and S_P diastereoisomers with reversed-phase HPLC.

hyperchromicity experiments in order to detect a duplex ⇄ coil melting transition.

(i): The stereochemical configuration at phosphorus was assessed according to the method of Summers et al. (1986). For 1 and 2 it was found that only one of the diastereoisomeric forms shows a NOE contact between the methyl group on phosphorus, and $H_{3'}(C)/H_{3'}(A)$, respectively. The diastereoisomer showing the NOE contact was assigned the R_P configuration. In line with our previous data on phosphate-methylated DNA dinucleotides, it was found for 1 and 2 that R_P corresponds with the lower mobility in reversed-phase HPLC and the lower ^{31}P NMR chemical shift value. ($R_P(1)$: $\delta(^{31}P) = 2.04$ ppm; $S_P(1)$: $\delta(^{31}P) =$ $= 2.29$ ppm; $R_P(2)$: $\delta(^{31}P) = 2.15$ ppm; $S_P(2)$: $\delta(^{31}P) = 2.19$ ppm).

(ii): The conformation of the backbone and the ribose rings was deduced from vicinal proton-proton and proton-phosphorus NMR coupling constants. The results show pronounced preferences for β^t ($C_{5'}$-$O_{5'}$ bond) and γ^+ ($C_{4'}$-$C_{5'}$ bond) in all cases. For $R_P(1)$ and $S_P(1)$ both ribose rings predominantly populate a $C_{3'}$-endo (North) conformation, i.e., a standard A-RNA geometry is adopted. In the case of $R_P(2)$ and $S_P(2)$, a $C_{2'}$-endo and $C_{3'}$-endo conformation was encountered for the Ap and pU residues, respectively. Presumably, this difference between 1 and 2 is due to the fact that base-base stacking is stronger for purine 3'-5' pyrimidine than for pyrimidine 3'-5' pyrimidine (Saenger, 1984).

(iii): The possibility of duplex formation in R_P and S_P 1 and 2 was examined with UV hyperchromicity experiments, performed with 0.01 M Tris/HCl buffer solutions (pH 7.5). In principle both diastereoisomeric forms of 1 can show parallel duplex formation via one C-C and one U-U base pair (Quaedflieg et al., 1989b), while both diastereoisomers of 2 are capable of antiparallel duplex formation via two Watson & Crick type A-U base pairs. The UV hyperchromicity curves showed a sigmoidal shape only in the case of $S_P(1)$; the T_m-value was found to be 11.2°C for a concentration of 8.55 μM. The occurrence of a melting transition for $S_P(1)$ and not for $R_P(1)$ was confirmed with variable temperature 400 MHz ^1H NMR experiments. It was found that in $S_P(1)$ the chemical shift vs. temperature profiles of the $H_{1'}(C)$ and $H_6(U)$ protons showed a sigmoidal behaviour, while in $R_P(1)$ continuously increasing and decreasing curves were obtained, when raising the temperature. These results are in good agreement with our previous work on phosphate-methylated DNA dinucleotides for which we found that d(CpC) and d(TpC) show parallel duplex formation exclusively for the S_P diastereoisomer (Quaedflieg et al., 1989b), and that phosphate-methylated d(ApT) did not show duplex formation for both diastereoisomers. The thermodynamic equations as derived by Marky and Breslauer (1987) were used in order to establish the presence of a *duplex* in case of $S_P(1)$, and to determine the van 't Hoff dissociation enthalpy ΔH_d. First, we extracted the ratio $\Delta H_d/(n+1)$ (n = number of strands that associate to form an n-mer complex) from the sigmoidal melting curves. This was done by careful determination of the first derivative of the fraction of single strands vs. temperature plot at the T_m-value (slope = $\Delta H_d/(2+2n)RT_m^2$). First derivatives

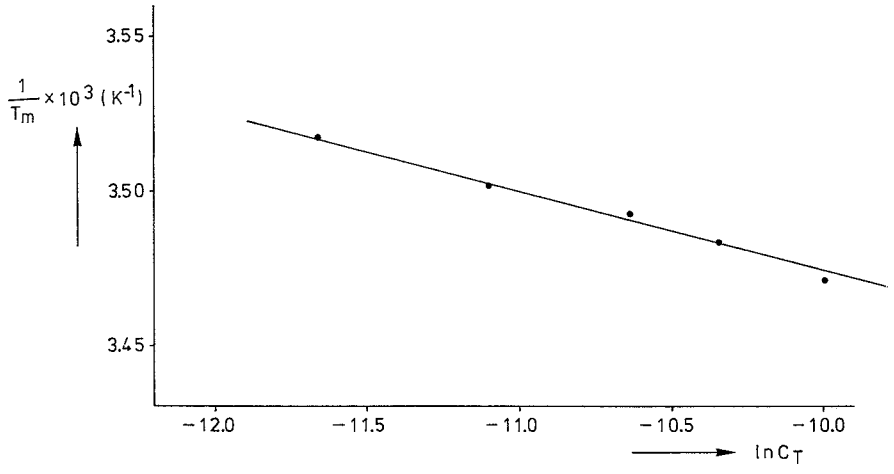


Figure 1. Plot of $1/T_m$ vs. ln C_T as measured for Sp(1). T_m-values were determined by computer fitting of the experimentally determined melting profiles into a sigmoidal curve. C_T-values were obtained from UV extinction measurements.

and T_m-values were obtained by computer fitting of the experimentally determined UV extinction plots into sigmoidal curves. For $S_P(1)$ we found a value of 104 kJ/mol for $\Delta H_d/(n+1)$. Secondly, we measured the T_m-value as a function of the total strand concentration (C_T) of $S_P(1)$. We found that increasing C_T from 8.55 to 45.46 μM results in an increase of T_m from 11.2°C to 15.3°C. These experimental data were used to construct a plot of $1/T_m$ vs. ln C_T (see Fig. 1). According to the Marky and Breslauer model (1987), the slope of this plot equals $-(n-1)R/\Delta H_d$, i.e. a second relationship between ΔH_d and n is obtained in this way. The ratio $(n-1)/\Delta H_d$ was found to be $3.42 \cdot 10^{-3}$ mol/kJ. Solution of n and ΔH_d resulted in $\Delta H_d = 304$ kJ/mol, and $n = 2.04$, i.e. a *duplex* is formed. The stability of the $S_P(1)$ duplex is substantially lower than for the counterpart DNA duplexes of S_P phosphate-methylated d(CpC) ($T_m = 33$°C, $\Delta H_d = 829$ kJ/mol) and phosphate-methylated d(TpC) ($T_m = 26$°C, $\Delta H_d = 793$ kJ/mol). The present data on $S_P(1)$ point to a *parallel* miniduplex structure based on one C-C base pair with two equivalent N_4-H...N_3 hydrogen bonds, and one U-U base pair with two equivalent O_4...H-N_3 hydrogen bonds. This structure shows a two-fold rotational symmetry. The parallel duplex model is confirmed by the high field proton NMR spectrum of $S_P(1)$ at 4°C (well below the T_m-value of 13°C, vide supra) which shows that the two backbone strands reside in magnetically equivalent environments.

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