71. Sequence-Specific Spin Labeling of Oligothymidylates by Phosphotriester Chemistry

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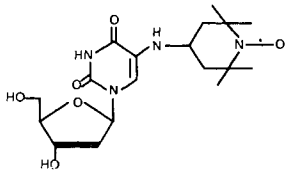
Oligothymidylates (oligo(dT)'s) with the sequence-specifically-incorporated one-atom-tethered C(5)-nitroxide-labeled nucleoside **1** were synthesized by the phosphotriester method. Some modifications of the protocol were required to account for the chemical reactivity of the nitroxide, the stability of which was monitored during the synthesis by electron paramagnetic resonance (EPR) spectroscopy. The EPR specific activity (A_{EPR}) of the FPLC-purified nitroxide-labeled oligomers was determined and found to be in agreement with enzymatically prepared spin-labeled nucleic acids. Annealing the nitroxide-labeled oligo(dT)'s to $(dA)_n$ or oligo(dA) resulted in different EPR-lineshape changes suggesting a strong coupling of the short-tethered nitroxide to global macromolecular motion.

Introduction. – Spin-labeled nucleic acids can be used to study structural and dynamic properties of nucleic acids by electron paramagnetic resonance (EPR) spectroscopy [1–4]. This laboratory has developed a variety of position-4 and -5 nitroxide (spin)-labeled uridine and deoxyuridine derivatives as well as some 5-substituted deoxycytidine analogs as substrates for nucleic-acid-synthesizing enzymes. Either template-independent enzymes, such as polynucleotide phosphorylase [5–7] and terminal deoxynucleotidyltransferase [8], or template-dependent enzymes, such as reverse transcriptase from avian myeloblastosis virus [9] and DNA polymerase I from *E. coli* [10], were used to prepare spin-labeled nucleic acids. Spin-labeled thymidine and deoxycytidine analogs were also incorporated by nick translation with lambda DNA as template [11] [12].

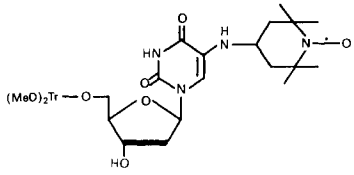
While it is possible to enzymatically incorporate nitroxide radicals sequence-specifically into an oligodeoxynucleotide [13], the choice of sequences for sequence-specific spin labeling is limited due to the nature of the template-dependent reaction, and a successful enzymatic incorporation of spin-labeled substrates depends on the nature of the tether used for attaching the nitroxide to the base. The incorporation of nitroxide-substituted nucleotides by chemical synthesis offers the possibility of preparing labeled nucleic acids of any sequence with any desirable tether. However, one of the difficulties with the nonenzymatic incorporation of nitroxide-labeled nucleoside derivatives is the potential chemical reactivity of the nitroxide radical under the relatively harsh conditions encountered in the chemical synthesis of oligonucleotides by either the phosphotriester or phosphoramidite method. The 'stable' nitroxide radical is a secondary-amine *N*-oxide, which, though quite stable under many conditions, can undergo a variety of chemical reactions [14]. Enzymatically prepared spin-labeled nucleic acids have given a consistent EPR-signal intensity at a given amount of nitroxide incorporation, which has allowed us

to establish EPR-signal standards for spin-labeled nucleic acids. Deoxyuridine, substituted in position 5 with either an acetylene-tethered [15] or a diacetylene-tethered nitroxide [16], was successfully incorporated into oligonucleotides by the phosphoramidite method, although no effort was made by the authors to establish some relationship between EPR-signal intensity and amount of spin-labeled nucleic acid used.

We report here the nonenzymatic incorporation of **1**, a deoxyuridine labeled at the C(5) position with a one-atom-tethered 6-membered-ring nitroxide, into different oligo-(dT)'s by phosphotriester chemistry with some modifications of the protocol to account for the chemical reactivity of the nitroxide radical. The obtained nitroxide-labeled oligomers **4**–**7** were characterized by an A_{EPR} value that is in agreement with data observed previously with enzymatically prepared nucleic acids. The closeness of the nitroxide to the base in **1** made it possible to detect global dynamics of the nucleic acid by EPR, and the shortness of the tether may have been responsible for our previous inability to incorporate **1** enzymatically into nucleic acids [17].



1 = L



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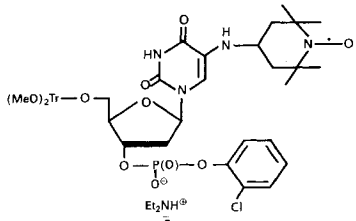

3

dT-dT-dT-dT-L-dT-dT-dT-dT (**4**)
dT-dT-dT-dT-dT-L-dT-dT-dT-dT-dT (**5**)
dT-dT-dT-L-dT-dT-dT-dT-dT-dT (**6**)
dT-dT-dT-dT-dT-dT-dT-L-dT-dT-dT-dT-dT-dT-dT (**7**)

Results and Discussion. – *Fig. 1* shows the motion-restricted EPR spectrum of **4** bound to the glass bead support and indicates that **3**, obtained from nitroxide **1** *via* **2**, was successfully incorporated into the oligomer *via* phosphotriester chemistry. In addition to **4**, the spin-labeled oligonucleotides **5**–**7** were prepared by this approach.

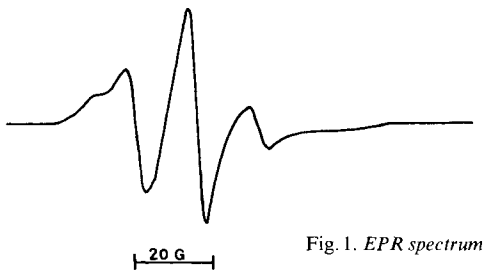

Fig. 1. *EPR spectrum of* **4** *attached to a glass-bead support in dioxane/H_2O 1:1*

For the initial deprotection step, *Method I* described in the *Exper. Part* gave the best yield. The oximate-treatment of *Method I* to deprotect the phosphate groups reduces the risk of phosphodiester bond cleavage [18]. Although dioxane/H_2O is normally used as solvent with oximate, we observed that is was better to substitute it with pyridine due to

partial destruction of the nitroxide label in dioxane/H_2O. Also, the concentration of the final solution was best done to only *ca.* 1 ml before the immediate desalting on a *Bio Gel P-2* column, since minimizing the contact between nitroxide-labeled oligomer and the concentrated oximate solution was found to improve the A_{EPR} value. Deprotection using *Method II* offers the advantage of simplicity, since it allows easy removal of the deblocking reagent, but affords, as reported earlier [18], a smaller yield than *Method I*. About 40 OD_{260} of crude tritylated spin-labeled oligomers were isolated with *Method II* when carrying out the synthesis on a 1-μmol scale.

Although it is possible to perform the final detritylation step with the oligomer still attached to the bead, it has been reported that NH_3 treatment can lead to side reactions without protection of the 5′-OH group [19]. In order to avoid these side products, detritylation was executed after the deblocking of the phosphate groups. No nitroxide destruction was observed in the detritylation reaction, as shown by A_{EPR} value of trityl-on and trityl-off spin-labeled oligomers. Also, the A_{EPR} value was similar for **5** and **6** (*Table*), even though the spin-labeled nucleotide of the former oligomer was exposed to two additional detritylation steps in the oligonucleotide synthesis.

Table. *Average Yield*[a]) *and EPR Properties of Oligo(dT)'s Containing* **1**

	OD_{260}	% **1**/oligomer	h_{-1}/h_0 (±0.01)	A_{EPR}[b]) ($= S/OD$) × 10^{-3} (±20%)
4	3–5	11	0.49	80
5	4–6	9	0.46	80
6	4–6	9	0.49	80
7	5–8	6.7	0.44	65

[a]) Based on a 1-μmol-scale synthesis.

[b]) A_{EPR} is the EPR specific activity expressed as S/OD. S is the digitized absolute h_0 signal intensity measured at room temperature in a flat cell (o.d. 56 × 16 × 2 mm) with a Tm_{110} cavity on a *Bruker-ESP300* spectrometer at X-band (gain 10^5; modulation amplitude 1.6 G; microwave power 30 mW; field width 100 G, resolution 1024 addresses; conversion time 164 ms; time constant 164 ms). Note that the number of nitroxides is directly proportional to the area under the EPR absorption curve; however, it is practical to quantify the EPR signal by its h_0 peak-to-peak height, an approximation that is valid as long as there is no change in the lineshape. OD_{260} is the nucleic-acid concentration in 0.1M NaCl/0.01M $MgCl_2$/0.01M Na-cacodylate (pH 7.0), measured as absorption at 260 nm per ml.

The final purification of the nitroxide-labeled oligomers was achieved by FPLC (=fast protein liquid chromatography) on an anion-exchange *MonoQ* column. Purification by FPLC was by far the most successful approach for isolating spin-labeled oligonucleotides with respect to purity, yield, and A_{EPR}. A poor yield and a low A_{EPR} was observed, when the samples were isolated by silica-gel TLC or reversed-phase HPLC. In addition, the nitroxide-labeled n-mer could not be separated from the unlabeled $(n-1)$-mer by reversed-phase HPLC [20]. Also, purification of a small amount of spin-labeled oligonucleotides by HPLC on a reversed-phase steel column, with MeCN as a component of the solvent system, significantly lowered the A_{EPR} value. The exact nature of this loss is not known, but it seems that MeCN will destroy the nitroxide moiety in the presence of a metal surface. Therefore, all purifications of spin-labeled oligomers were carried out by FPLC on a *MonoQ* column. A typical elution profile for a labeled oligo(dT) such as **5** is shown in *Fig. 2*. In all cases, the desired material (peak I) eluted as the last major peak.

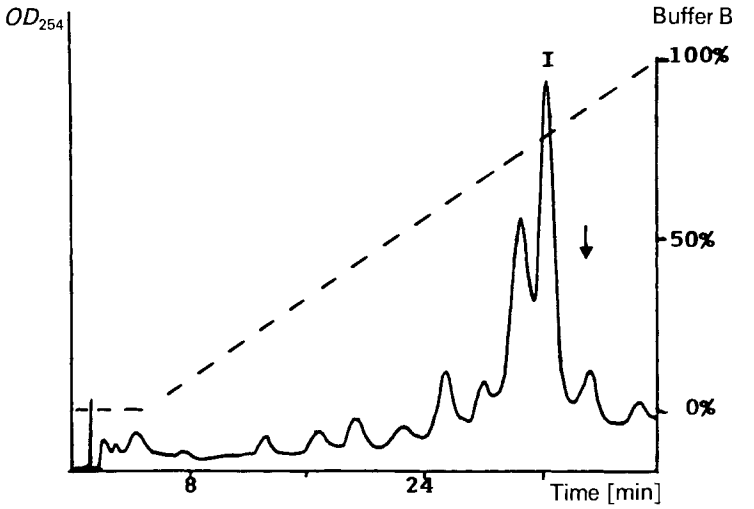

Fig. 2. *FPLC profile of the product mixture of a synthesis of* **5** *on a* MonoQ HR 5/5 *column.* Peak I contains **5** and the arrow marks the $(dT)_{11}$ retention time. Elution buffers: *A*: 0.01N NaOH/0.50N NaCl; *B*: 0.01N NaOH/0.64N NaCl. Flow rate: 1 ml/min.

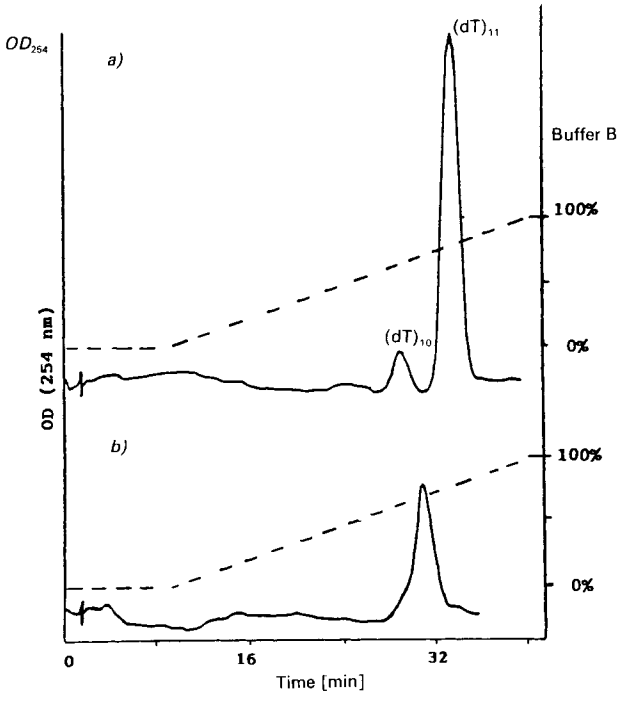

Fig. 3. *Anal. FPLC profiles of* a) $(dT)_{10}$ *and* $(dT)_{11}$, *and* b) **5** *after FPLC purification on a* MonoQ HR 5/5 *column.* Elution buffers and flow rate: see *Fig. 2.*

For reference purposes, the position of the unlabeled analog is marked with an arrow. The peak immediately preceding peak I showed no EPR signal and is believed to consist mainly of the failure sequence without **1**.

The retention time of a nitroxide-labeled oligomer relative to the unlabeled oligomer was established by comparing the retention time of unlabeled standards of similar size with that of spin-labeled material. A typical example of such a comparison is shown with *Fig. 3*, displaying FPLC profiles of unlabeled $(dT)_{10}$, and $(dT)_{11}$ and of **5**. It is evident that the nitroxide-labeled 11-mer elutes with an elution time between that of the unlabeled 10- and 11-mers.

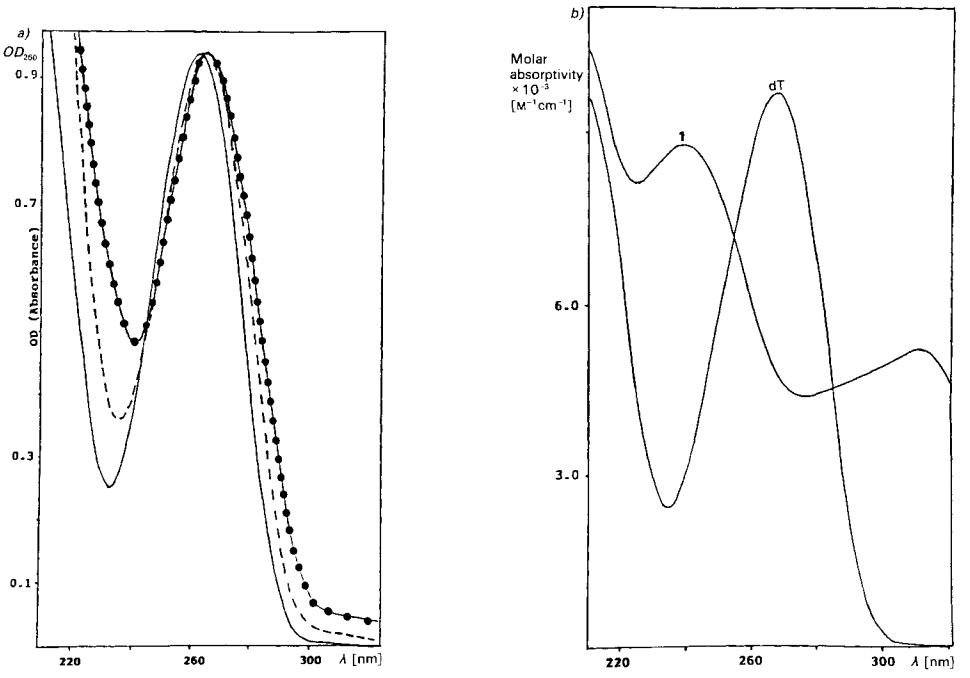

Fig. 4. *UV absorption spectra in H_2O* (pH 6.5): a) $(dT)_{11}$ (——), **5** (●●●), *and* **7** (– – –); b) **1** *and dT*

Fig. 4a shows the UV spectra of FPLC-purified $(dT)_{11}$ and of the spin-labeled oligonucleotides **5** and **7**. The spectra are all normalized to the same absorbance at 260 nm. From the intensity at 238 nm, which corresponds to the UV maximum of **1** (*Fig. 4b*), it is apparent that a proportional relationship exists between this intensity and the relative amount of **1** in the oligomers; namely, the larger the percent labeling, the larger the intensity at 238 nm for a given oligomer. This further corroborates the qualitative relationship between A_{EPR} and the relative amount of **1** present in an oligomer (see *Table*).

In addition to the above-mentioned characterizations, the labeled oligomers were subjected to an enzymatic phosphodiester hydrolysis and subsequent HPLC analysis. *Fig. 5* shows a typical HPLC elution profile of a digest of **5**. If one corrects for the difference in the extinction coefficient of the two bases **1** and dT, their relative amount in the oligomer is supported by their peak areas in *Fig. 5*.

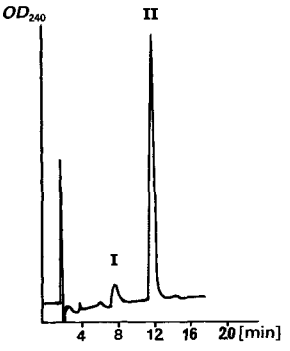

Fig. 5. *HPLC separation of an enzymatic digest of* **5**. Isocratic elution with *0.1* M. (Et_3NH)AcO (pH 6.5) on a 3.9 × 150 mm *Delta-Pak C-18* column (flow rate 0.9 ml/min). Peaks were identified with standards of **1** and dT (**1**, 8 min; dT, 30 min); peak I corresponds to **1** and peak II to dT.

The EPR spectra of **4**–**7** were recorded in the absence and presence of equivalent amounts of the target sequence $(dA)_n$ (*Figs. 6* and *7*). With 9-, 11-, and 15-mers **4**, **5**, and **7**, respectively, containing **1** in the center of the oligomer, the h_{-1}/h_0 ratio shows a size-dependent effect (see *Table*). A qualitative analysis of the EPR lineshapes suggests that the correlation time increases with oligomer size. Also, positioning **1** away from the center toward one of the ends of the oligomer, as in the case of **6**, increases the nitroxide mobility as seen from the h_{-1}/h_0 ratio. Thus, the short-tethered nitroxide present in **1** is strongly coupled to the oligonucleotide and reflects, at least in part, the motion of the oligomer.

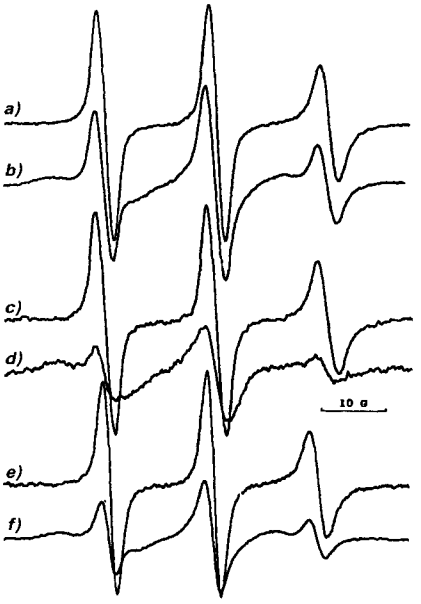

Fig. 6. *EPR spectra of* **4**–**6** a)c)e) *before and* b)d)f) *after annealing with equivalent amounts of* $(dA)_n$. In 100 mM NaCl/10 mM $MgCl_2$/10 mM Na-cacodylate (pH 7) buffer at 20°; *a*)*b*) 19 nmol of **4**; *c*)*d*) 12 nmol of **5**; *e*)*f*) 18 nmol of **6**.

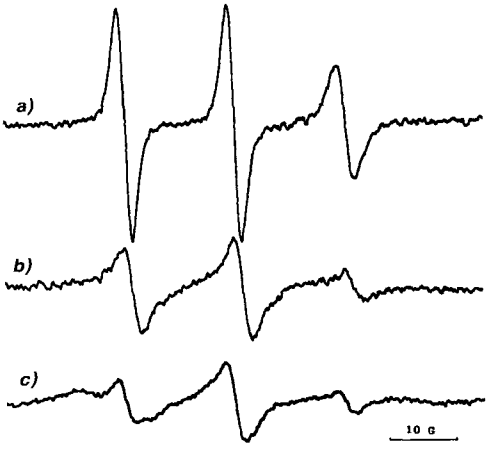

Fig. 7. *EPR spectra of* **7** (22 mmol) a) *before and* b)c) *after annealing with an equivalent amount of* $(dA)_{15}$ *or* $(dA)_n$, *respectively*. In 100 mM NaCl/10 mM $MgCl_2$/10 mM Na-cacodylate (pH 7) buffer at 20°.

The effect of coupling is even more pronounced in the case of the annealed oligomers as seen below.

The EPR spectrum in *Fig. 6b* shows **4**, annealed to an equivalent amount of $(dA)_n$ at 20°. Under these temperature and buffer conditions, the T_m of the duplex is close to the temperature of the EPR measurement (details about the T_m values of such duplexes will be published elsewhere). Therefore, not all of **4** is annealed under these conditions. This is apparent from the EPR spectrum, which shows an EPR lineshape reflecting the presence of at least two distinct states of nitroxide motion. A small amount of a slow-motion component is definitely visible in the low-field region of the spectrum. The percentage of the slow-motion component increases with size as shown with the spectra in *Fig. 6d* and *Fig. 7c*. With the spectrum in *Fig. 6f*, the effect of the positioning of **1** within the 11-mer annealed to $(dA)_n$ is explored. It is apparent that moving **1** away from the center decreases the percentage of the slow-motion component in the spectrum. Presently it is not known whether this decrease is caused by a reduced T_m or by placing **1** in an area where end effects are believed to exist due to breathing. The largest percentage of slow-motion component is observed with **7** annealed to $(dA)_n$ (*Fig. 7c*). On the other hand, annealing **7** to $(dA)_{15}$ to form a small double-stranded lattice, gives rise to a EPR spectrum (*Fig. 7b*) which lacks the slow-motion component. This is additional experimental evidence that **1** with its one-atom tether can be used to monitor global motions.

Thus, all EPR spectra shown here support the hypothesis that the nitroxide in **1** is strongly coupled to the base since the EPR lineshape is dominated by the motion of the macromolecule. This observation is similar to the one made by *Spaltenstein et al.* [4] with an acetylene-tethered nitroxide, which allowed the authors to establish a good qualitative relationship between duplex size and EPR lineshape using hydrodynamic theory and isotropically simulated EPR spectra. Whether or not packing a nitroxide ring with a short tether tightly into the major groove of the DNA duplex will cause some structural disruption and impede the base motion on the nanosecond time scale is difficult to evaluate. Preliminary melting studies on systems containing **1** suggest a decrease in the T_m value by 3–6°. While such a perturbation does not interfere with the hybridization assay, provided the hybridization temperature is adjusted accordingly, it may well impede the local base motion.

It is interesting to note that spin-labeled nucleic acids prepared by enzymatic incorporation of spin-labeled substrates require less rigidly tethered nitroxides at position 5 of the pyrimidine base. In addition, such tethered nitroxides cause no detectable perturbation since the same $T_m(OD)$ is determined with labeled and unlabeled RNA [21] and DNA duplexes [11]. A theoretical analysis of the EPR data of these non-perturbed systems with a previously published motional model [7] is straightforward and has led us to conclude that the base motion is on the order of 4 ns in B-DNA and about two times slower in Z-DNA [22].

In summary, **1** can be synthesized economically in large quantities and incorporated sequence-specifically into oligonucleotides. The strong coupling of the nitroxide to the base in **1** and the high A_{EPR} value of oligomers containing **1** are important features to make oligomers labeled with **1** valuable tools for analyzing macromolecular interactions by EPR.

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Experimental Part

1. *General.* Pyridine was first distilled from ninhydrin and then from CaH_2, 1,2-dichloroethane first passed through basic aluminium oxide and then distilled from P_2O_5, and THF distilled from K and benzophenon. Dichloroacetic acid and 1-methyl-1*H*-imidazole were vacuum-distilled under dry N_2; 4,4′-dimethoxytrityl chloride ($(MeO)_2TrCl$), 2-chlorophenyl phosphorodichloridate, and 1,2,4-triazole were purchased from *Aldrich*, 5′-*O*-(dimethoxytrityl)thymidine 3′-[(2-chlorophenyl)triethylammonium phosphate] and 1-(mesitylenesulfonyl)-2-nitro-1,2,4-triazole from *Cruachem*, and 5′-*O*-(dimethoxytrityl)thymidine 3′-*O*-succinyl-controlled pore glass type II, 2′-deoxyuridine, and 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl from *Sigma*. Conc. NH_3 soln. for deblocking and deprotection reactions was stored at 4° after initial opening of the bottle. Prep. and anal. TLC: silica gel from *Analtech*. Reversed phase TLC: *$KC_{18}F$* from *Whatman, Inc.* Column chromatography (CC): silica gel *60* from *Merck* (art. 9385). EPR spectra: *Varian-E-104-Century-Series* spectrometer, interfaced with an *Apple II plus* microcomputer [23], using parameters reported earlier [7], or a *Bruker-ESP300* spectrometer.

2. *1-(2′-Deoxy-β-*D*-ribofuranosyl)-5-{[2,2,6,6-tetramethyl-1-(oxyl)piperidin-4-yl]amino}pyrimidine-2,4-(1*H*,3*H*)-dione* (**1**) was prepared by some refinement of a procedure published earlier [24]. The 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl (1.6 g, 9.35 mmol) was added to 2′-deoxyuridine (960 mg, 3.93 mmol) in H_2O (1 ml). The pH of the suspension was adjusted to pH 5 with 1N H_2SO_4 before heating it in a *Thermolyne Modular Dri-Bath* at 100°. The pH was periodically checked and kept at 5 for 10 h before readjusting the mixture with 6N NaOH to pH 8. After lyophilization, the dry residue was dissolved in a minimum amount of MeOH and purified by CC (silica gel, step gradient of MeOH and $CHCl_3$; TLC monitoring (silica gel *60 GF*, 15% MeOH/$CHCl_3$)). After evaporation of the fractions containing **1**, the dry residue was dissolved in 3 ml of 16% MeOH/$CHCl_3$ containing 0.1% (*v*/*v*) of Et_3N (solvent *A*) and submitted again to CC (silica gel): 400 mg (26%) of **1**. Amorphous orange powder. M.p. 126–129°. The UV, NMR, and MS of **1** were reported previously [13].

3. *1-[2′-Deoxy-5′-*O*-(4,4′-dimethoxytrityl)-β-*D*-ribofuranosyl]-5-{[2,2,6,6-tetramethyl-1-(oxyl)piperidin-4-yl]amino}pyrimidine-2,4(1*H*,3*H*)-dione* (**2**). A soln. of **1** (280 mg, 0.7 mmol) and 4,4′-dimethoxytrityl chloride (180 mg, 1.18 equiv.) in pyridine (8 ml) was stirred at r.t. for 6 h. The reaction was terminated by addition of 2 ml of MeOH. After evaporation, the residue was taken up in $CHCl_3$, washed with sat. $NaHCO_3$ and H_2O, and purified by CC (silica gel *60*, 5% MeOH/$CHCl_3$ containing 0.1% of Et_3N). This yielded, after precipitation from hexane with 0.1% of Et_3N, 350 mg (71%) of **2**. Anal. TLC (silica gel, MeOH/$CHCl_3$ 1:9 (*v*/*v*) with 0.1% of pyridine): R_f 0.65.

4. *1-[2′-Deoxy-5′-*O*-(4,4′-dimethoxytrityl)-β-*D*-ribofuranosyl]-5-{[2,2,6,6-tetramethyl-1-(oxyl)piperidin-4-yl]amino}pyrimidine-2,4(1*H*,3*H*)-dione 3′-[(2-Chlorophenyl) Triethylammonium Phosphate]* (**3**). Overnight, **2** (350 mg, 0.50 mmol) and 1,2,4-triazole (129 mg, 1.86 mmol) were dried separately over P_2O_5 and KOH. Then freshly distilled THF (8 ml), distilled Et_3N (245 µl, 1.75 mmol), and 2-chlorophenyl phosphorodichloridate (126 µl, 0.77 mmol) were added to the 1,2,4-triazole. This mixture was periodically shaken for 30 min and then centrifuged. The supernatant was transferred to the vessel containing **2**. After 1.5 h stirring at r.t., the reaction was terminated with 1 ml of cold 90% pyridine/H_2O (*v*/*v*) and the mixture evaporated. The residue was extracted with $CHCl_3$, the $CHCl_3$ layer washed with 0.1M $(Et_3NH)HCO_3$ buffer (pH 8) and H_2O, and the product purified by CC (silica gel, 200 ml of 4% EtOH in $CHCl_3$ with 0.1% of Et_3N, then 300 ml of 7% EtOH in $CHCl_3$ with 0.1% of Et_3N and 300 ml of 20% EtOH in CH_2Cl_2 with 0.1% of Et_3N). After evaporation of the fractions containing **3**, the residue was dissolved in a minimum amount of CH_2Cl_2 and precipitated by dropwise addition of this soln. to pentane (15 ml) with 0.1% of Et_3N under stirring. This procedure afforded 150 mg (30%) of **3**. TLC (silica gel, *60 GF*, 10% EtOH in $CHCl_3$ and 0.1% of pyridine): one spot R_f 0.06. Reversed phase TLC (*$KC_{18}F$*, 20% (*v*/*v*) 0.04M $(NH_4)HCO_3$ in acetone): ^{1}H-NMR (reduced **3**, (D_6)DMSO): 11.4 (br., NH(3)); 9.4 (br., OH–N(1) of piperidinyl); 7.63–6.60 (*m*, 13H of $(MeO)_2Tr$, H–C(6)); 6.24 (*t*, H–C(1′)); 4.83–3.95 (*m*, H–C(3′), H–C(4′), $CH_2(5')$); 3.75 (*s*, $(MeO)_2Tr$); 2.20 (br., $CH_2(2')$); 1.52–0.97 (*m*, 4 Me of piperidinyl, 3 Me of Et_3NH^+). FAB-MS: 992.2 ($[M + 2H]^+$), 1092.2 ($[M + Et_3NH]^+$), 1093.2 ($[M + Et_3NH + H]^+$).

5. *Oligomer Synthesis* was performed with a *Cruachem* semi-manual DNA synthesis module using the phosphotriester method [25]. The synthesis was usually carried out on a 1-µmol scale with 25 mg of glass bead. For

the cleavage of the spin-labeled oligonucleotides from the support and for the deprotection, two approaches (*Methods I* and *II*) were selected.

Method I: 'syn'-4-Nitrobenzaldehyde oxime (14 mg) and 1,1,3,3-tetramethylguanidine (10 µl) in 1 ml of pyridine were added to the support. The mixture was sealed and left at r.t. for 16 h. After removal of the supernatant, *ca.* 1 ml of conc. NH_3 was added to the support and kept for an additional 3 h. The support was then washed with EtOH/H_2O 1:1 (3 × 1 ml). After combining all the solns., the solvents were concentrated to *ca.* 1 ml before immediate desalting on a *Bio Gel P-2* column.

Method II: Conc. NH_3 (1.5 ml) was added to the support and the mixture kept at r.t. for 5 h. After removal of the supernatant, the support was washed with EtOH/H_2O 1:1 (3 × 1 ml) and the combined solns. evaporated with a *Speed-Vac* and desalted on a *Bio Gel P-2* column.

For the detritylation, the dry residue was incubated with 0.5 ml of 75% AcOH for 3 min, neutralized with 15% NH_3 soln. at 0°, and then desalted on a *Bio Gel P-2* column.

Purification. A linear gradient of NaCl was used with the *Mono Q HR 5/5* anion-exchange column to purify the spin-labeled oligonucleotides by FPLC. The last major peak was determined to contain the desired material. Partial evaporation of this peak to *ca.* 2 ml with a *Speed-Vac* was carried out before final desalting on a *Bio Gel P-2* column. The purity of the spin-labeled oligomers was verified by anal. FPLC. The amount of final product was determined by UV absorption at 260 nm and was depending on the oligomer size: 3–8 *OD* for a 1-µmol synthesis (see *Table*).

6. *Enzymatic Hydrolyses.* The base composition of the spin-labeled product was verified by enzyme digestion and subsequent HPLC analysis: 0.1 OD_{260} of FPLC-purificd spin-labeled oligomer was digested with 0.02 units of phosphodiesterase for 16 h at r.t. and then with 0.1 units of alkaline phosphatase in 50 mM *Tris*-HCl (pH 8.5)/10 mM $MgCl_2$ in a total volume of 150 µl for 1 h. An aliquot of the product mixture was analyzed by HPLC (*Bioanalytical Systems* instrument *Delta-Pak C-18* column (*Waters, Inc.*), elution conditions given in *Fig. 5*).

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