Syntheses and Properties of Oligothymidylate Analogs Containing Stereoregulated Phosphoromorpholidate and Phosphodiester Linkages in an Alternating Manner

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(Received February 19, 1990)

Syntheses and some properties of undecathymidylate analogs containing stereoregulated phosphoromorpholidate and phosphodiester linkages in an alternating manner are described. These analogs were easily obtained by the phosphorobisamidite approach starting from pre-separated diastereomerically pure dithymidine phosphoromorpholidate derivatives. Both isomers were found to be high resistant to nucleases, in comparison with natural oligothymidylate; the phosphodiester linkages except 3'-end in the isomers were not cleaved by S1 nuclease and both isomers were very slowly degraded by phosphodiesterase I and micrococcal nuclease. The rate of each degradation was dependent on the configuration of phosphoromorpholidate linkage in each isomer. The UV and CD spectral studies show that one of the isomer can interact with poly(dA) to give B-type duplex possessing the same thermal stability as natural oligothymidylate-poly(dA) duplex and the other isomer does not interact with poly(dA) under the comparable conditions.

Synthetic oligonucleotides and their analogs have been noted as regulatory substances for gene expression by the sequence-specific binding to the target DNA/RNA. For example they have been shown to inhibit viral replication in cell culture with human immunodeficiency virus[1,2] and herpes simplex virus type 1.[3] It is considered that the oligonucleotide analogs having modified phosphate groups, such as methylphosphonates, phosphorothioates, and phosphoramidates, have been shown to be useful as inhibitors of protein translation due to their nuclease resistance and their increasing cell membrane permeability compared with normal oligonucleotides having all phosphodiester linkages.[4,5] The modified backbone can be introduced into oligonucleotides through routes based on the phosphotriester,[6] phosphite,[7,8] and hydrogen phosphonate[9] methods of oligonucleotide synthesis. These modifications sometimes influence the stabilities of duplexes. It has been presumed that the influence results from the electronic nature of the substituent groups, their steric hindrance, the disturbance of the spine of the hydration around the modified phosphate groups, and the absolute stereochemistry at phosphorus atoms.[9,10] In particular, Miller and co-workers demonstrated that the absolute stereochemistry at phosphorus atoms in methylphosphonate linkages controls duplex formation.[10] It is therefore speculated that stereochemically pure oligonucleotide analogs would increase the potential as gene regulatory substances.[11] In order to investigate the effects of the modified phosphate groups in oligonucleotide analogs, easy and systematic preparation methods of those analogs are required. We have developed the novel method for generating phosphoromorpholidate linkages by one-pot procedure.[12,13]

In this paper, we described the syntheses of oligothymidylate analogs having stereoregulated phosphoromorpholidate linkages from pre-separated diastereomerically pure dithymidine phosphoromorpholidate derivatives. Resistances of these analogs to nucleases and their abilities to form the complex with complementary poly(dA) were also examined.

Experimental

General Methods High-performance liquid chromatography (HPLC) was carried out on Cosmosil $5C_{18}$ (4.6×150 mm, Nacalai Tesque Co. Ltd., Kyoto, Japan) or DAISO GEL ST-120-5 ODS (6.0×150 mm, Daiso Co. Ltd., Osaka Japan) at 35 °C using Shimadzu LC-6A chromatographic system (Shimadzu Co. Ltd., Kyoto, Japan). For the purification of 5'-*O*-dimethoxytritylated undecathymidylate analogs, a linear gradient of 6—60% acetonitrile (1%/min) in 0.1 M[a] triethylammonium acetate (TEAA) buffer (pH 7.0) was used at a flow rate of 1.0 mL min^{-1} on Cosmosil $5C_{18}$. For the further purification and the check of purities of detritylated undecathymidylic acid and undecathymidylate analogs, a linear gradient of 6—30% acetonitrile (0.5%/min) in 0.1 M TEAA buffer was used at a flow rate of 0.5 mL min^{-1} on Cosmosil $5C_{18}$. To analyze the products obtained from the treatment of the oligomers with nucleases, a linear gradient of 3—24% acetonitrile (1%/min) in 0.1 M TEAA and sequentially an isocratic eluent (24% acetonitrile) in 0.1 M TEAA were used at a flow rate of 1.0 mL min^{-1} on DAISO GEL ST-120-5 ODS column. Thin layer chromatography (TLC) was carried out on Kieselgel $60F_{254}$ (Art. 5554, E. Merck, Darmstadt, F.R.G.) using ethyl acetate/dichloro-

[a] M=mol dm^{-3}

methane/triethylamine (5/4/1, v/v/v) as a solvent. Dichloromethane was refluxed over calcium hydride and distilled prior to use. Acetonitrile was distilled over phosphorus pentoxide, then further distilled over calcium hydride, and stored over Molecular Sieves 4A. Diethylamine was standing over potassium hydroxide and then distilled over calcium hydride. 5-(*p*-Nitrophenyl)tetrazole was prepared by the method reported in the literature.[14] Chlorobis(diethylamino)phosphine was prepared by the method described elsewhere.[15] 5′-*O*-Dimethoxytritylthymidine loaded silica gel support (T-resin) was purchased from Dojindo Laboratories (Kumamoto, Japan). Undecathymidylic acid ($(dT)_{11}$) was synthesized by a phosphoramidite method. Polydeoxyadenylic acid (poly(dA)), Sl nuclease (*Asperillus oryzae*), phosphodiesterase I (*Crotalus adamanteus Venom*), and micrococcal nuclease (*Staphylococcus aureus*) were purchased from Pharmacia LKB Biotechnology (*Uppsala, Sweden*). All other chemicals were reagent grade.

Syntheses of Undecathymidylate Analogs. 5′-*O*-Dimethoxytrityldithymidine phosphoromorpholidate, **1**, was converted into their phosphorobisamidite derivative by chlorobis(diethylamino)phosphine. Compound **1** (0.1 mmol) was reacted with chlorobis(diethylamino)phosphine (0.1 mmol) in dry dichloromethane (0.5 mL) containing diethylamine (0.3 mmol) at room temperature for 15 min. After extraction with saturated aqueous sodium chloride, the organic layer was dried over sodium sulfate. After removal of the solvent in vacuo, the residue was redissolved in dry acetonitrile (1 mL) and used to oligomer syntheses. The purity of the resulting phosphorobisamidite, **2**, was checked by TLC.

The synthetic cycle of undecathymidylate analogs on T-resin (20 mg, 5′-*O*-dimethoxytritylthymidine; 0.05 mmol g^{-1}) is as follows; 1) washing with dichloromethane (3×0.3 mL), 2) detritylation with 3% dichloroacetic acid in dichloromethane (0.5 mL, 2 min), 3) washing with dry acetonitrile (5×0.3 mL), 4) coupling with **2** (20 equiv) using 5-(*p*-nitrophenyl)tetrazole (40 equiv) in dry acetonitrile (0.4 mL, 10 min), 5) hydrolysis with 0.5 M tetrazole in acetonitrile/water (4/1, v/v, 0.5 mL, 5 min), 6) washing with acetonitrile (3×0.3 mL). After the last cycle, the resin was treated with 0.1 M iodine solution in water/2,6-lutidine/THF (1/2/2, v/v/v) for 10 min. The undecathymidylate analogs were removed from the resin by the treatment with concd aq ammonia (room temperature, 2 h). After evaporation, the products were purified by reversed-phase HPLC. Appropriate fractions were collected, evaporated and coevaporated with water. The residues were detritylated by 80% acetic acid at room temperature for 30 min. After evaporation, the products were again purified by reversed-phase HPLC.

To confirm the length of these analogs, they were treated with 10% isopentyl nitrite in water/acetic acid/ethanol,(1/4/5, v/v/v) at 45 °C for 45 h to remove a morpholine moiety[9] and the products were analyzed by HPLC.

Measurements of UV Melting Behaviors. UV spectra were measured by Shimadzu MPS-2000 spectrophotometer (Shimadzu, Kyoto, Japan) equipped with Neslab EX-100 bath circulator and FTC-350A flowthru cooler (Neslab Instruments Inc., N.H., U.S.A). The temperature of the cell was measured by Yokogawa Model 2455 digital thermometer (Yokogawa Electric Co., Tokyo, Japan). Condensation of moisture at low temperature was prevented by passing dry nitrogen. The absorbance of the samples was monitored by increasing the temperature at total nucleotide concentration of 4×10^{-5} M. Relative absorbance at 260 nm is calculated by the following equation; Relative absorbance (260 nm)= Abs(observed)/Abs(observed at 80 °C). Molar extinction coefficients used were 8.7×10^3 (266 nm)[16] for oligothmidylic acid and its analogs and 8.6×10^3 (257 nm)[17] for poly(dA).

Measurements of CD Spectra. Circular dichroism spectra (CD) were measured by JASCO J-600 spectropolarimeter (JASCO, Tokyo, Japan) equipped with Neslab EX-100 bath circulator and FTC-350A flowthru cooler. The spectra were measured in 10 mM phosphate buffer (pH 7.2) containing 150 mM sodium chloride at total nucleotide concentration of 2×10^{-5} M for undecathymidylate analogs and 4×10^{-5} M for the mixtures of undecathymidylate analogs and poly(dA).

Enzymatic Digestion of Undecathymidylate Analogs. Digestion of undecathymidylate analogs (0.1 $O.D._{266}$) was carried out at 37 °C in the following solution. Sl nuclease; enzyme (ca. 380 unit) solution (200 μL) in 0.05 M ammonium acetate buffer (pH 5.0) containing 0.05 M sodium chloride and 1 mM zinc acetate. Phosphodiesterase I; enzyme (1 unit) solution (300 μL) in 0.02 M Tris-HCL buffer (pH 8.8) containing 0.02 M magnesium chloride. Micrococcal nuclease; enzyme (30 unit) solution (200 μL) in 0.05 M sodium borate (pH 8.8) containing 2.5 mM calcium chloride. Aliquots were taken out at appropriate time intervals, heated at 80 °C and analyzed by reversed-phase HPLC. Under those conditions, $(dT)_{11}$ was completely hydrolyzed within 5 min.

Results and Discussion

Syntheses of Undecathymidylate analogs. A pair of undecathymidylate analogs having stereoregulated phosphoromorpholidate and phosphodiester linkages in an alternating manner were synthesized by phosphorobisamidite approach[18] from diastereomerically pure dithymidine phosphoromorpholidate derivatives. The preparation of 5′-*O*-dimethoxytrityldithymidine phosphoromorpholidate, **1**, by our reported method[12] gave a mixture of isomers with *R* and *S* configurations at phosphorus atom, Rp and Sp. These two diastereomers could be easily separated on a reversed-phase column, as shown in Fig. 1. The diastereomers are named as **1-I** and **1-II** in their elution order. The configurations of **1-I** and **1-II** were tentatively assigned to Rp- and Sp-forms by proton NMR study, respectively.[13]

Scheme 1 shows the synthetic route of stereoregular oligothymidylate analogs. Diastereomerically pure **1-I** or **1-II** was converted into phosphorobisamidate derivative by chlorobis(diethylamino)phosphine. The phosphorobisamidite **2** prepared in situ was used to synthesize the oligothymidylate analog by block condensation on solid support using syringe technique.[19] The average coupling yield, estimated from the trityl assay, was ca. 89%. The diastereomers of **2**, **2-I** and **2-II**, showed no difference in the yield of the block condensation. The oligothymidylate analogs were purified by reversed-phase HPLC before and after detritylation. The undecathymidylate analog

having phosphoromorpholidate linkages is abbreviated to (Tp(mor)Tp)$_5$T, as shown in Scheme 1. The diastereomers of undecathymidylate analogs are named as **4-I** synthesized from **1-I** and as **4-II** synthesized from **1-II**.

Figure 2 shows the chromatograms of **4-I**, **4-II**, and (dT)$_{11}$. Retention times of those analogs on reversed-phase HPLC were as follows: **3-I**, 32.4 min; **3-II**, 34.4 min under the purification conditions for 5′-*O*-dimethoxytritylated undecathymidylate analogs; **4-I**, 33.6 min; **4-II**, 33.4 min, where **3-I** was 5′-*O*-dimethoxytritylated undecathymidylate analog synthesized from **1-I** and **3-II** was that from **1-II**. Both **4-I** and **4-II** were eluted much slower than (dT)$_{11}$ and **4-I** was eluted slower than **4-II**. Interestingly, the elution order of undecathymidylate analogs was reversed by the deprotection of the 5′-*O*-dimethoxytrityl group. This reversal of the elution order was also observed in dithymidine phosphoromorpholidate[13] and oligonucleotide analogs bearing phosphorothioate linkages.[20] Removals of the morpholine moieties from the analogs gave the compound comigrating with (dT)$_{11}$ and therefore the length of **4-I** and **4-II** was confirmed.

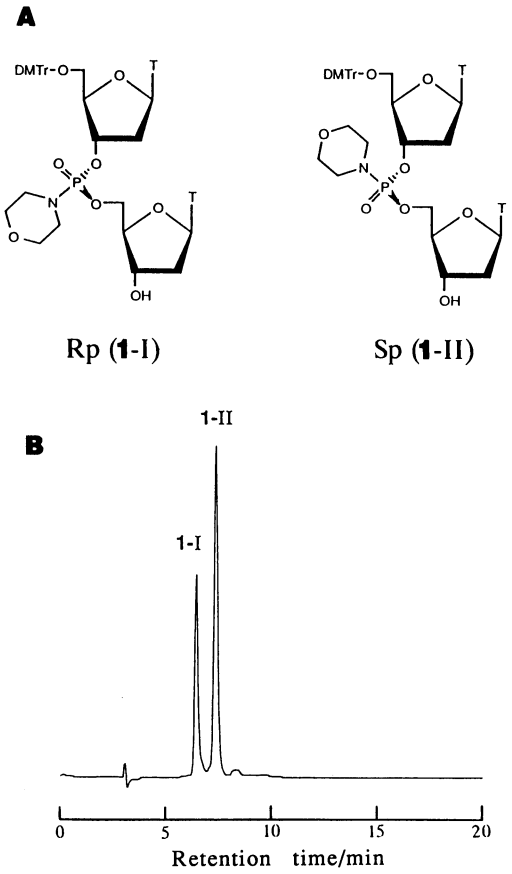


Fig. 1. (A) Absolute configurations of 5′-*O*-dimethoxytrityldithymidine phosphoromorpholidates. (B) HPLC profile of the mixture of **1-I** and **1-II** on DAISO ODS column using 80% methanol in water as an eluent at a flow rate of 1 mL min^{-1}.

Resistance to Nuclease. Resistances of **4-I** and **4-II** to nucleases were investigated by use of S1 nuclease, phosphodiesterase I, and micrococcal nuclease. Both diastereomers of dithymidine phosphoromorpholidate were not degraded by the treatment with these nucleases, though the same conditions led to hydrolysis of dithymidine monophosphate. This results indicate that the phosphoromorpholidate linkage is resistant to nucleases. The resistance of phosphoramidate linkages to nucleases is in agreement with other reports.[7,21] Undecathymidylic acid was completely degraded within 5 min by the treatment with these nucleases.

When **4-I** was incubated with S1 nuclease, thymidine 5′-monophosphate (5′-TMP) and one major peak in HPLC were obtained. The mojor peak was eluted a little later than **4-I** and was not degraded by the further incubation. The major porduct is therefore assumed to be the compound which was obtained by the cleavage of mononucleotide at 3′-end of **4-I**, (Tp(mor)-Tp)$_4$Tp(mor)T. Thus, the phosphodiester linkages in the resulting (Tp(mor)Tp)$_4$Tp(mor)T were not hydrolyzed by the further incubation. This resistances to enzymatic degradation may result from the decrease of negative charges or the steric hindrance of morpholino

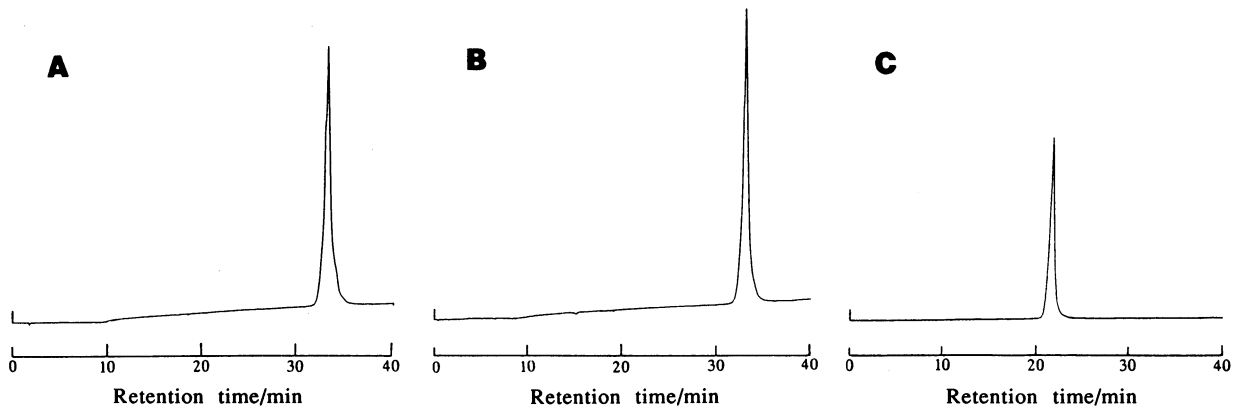
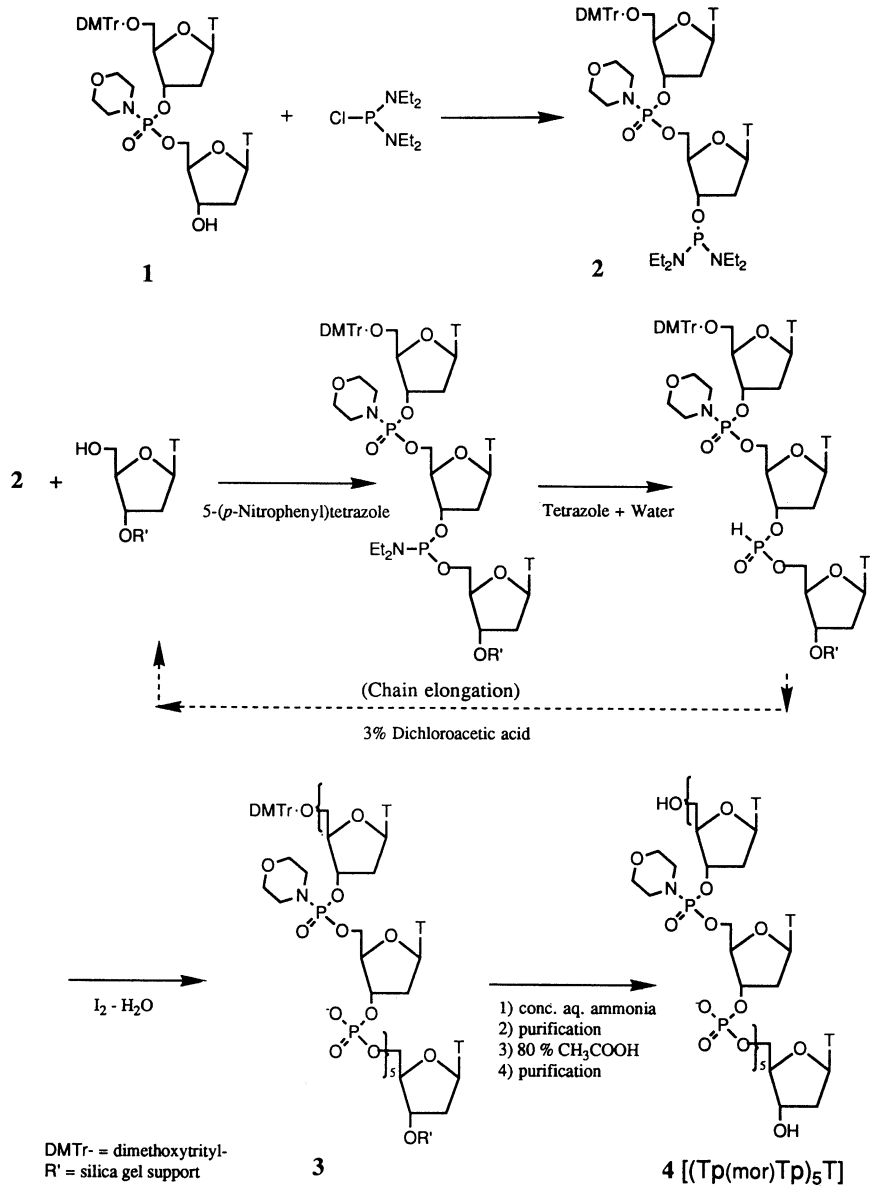


Fig. 2. The elution profiles of **4-I** (A), **4-II** (B), and (dT)$_{11}$ (C). Conditions are described in Experimental Section.



Scheme 1.

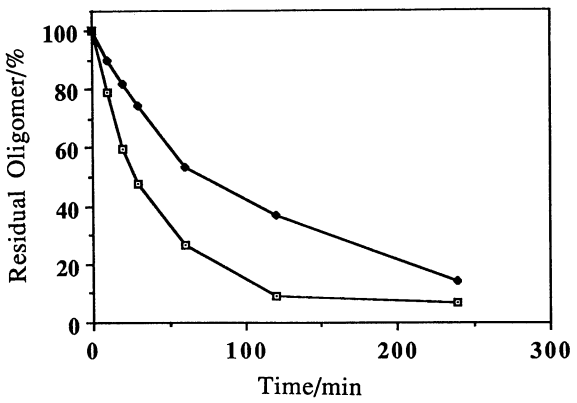


Fig. 3. Degradation of **4-I** (-□-) and **4-II** (-◆-) by S1 nuclease. Residual oligomer shows amounts of remaining **4-I** or **4-II**. Conditions are described in Experimental Section.

groups. The treatment of **4-II** with S1 nuclease led to a similar result as that of **4-I** except the rate of hydrolysis. The rate to hydrolyze the 3′ terminal phosphodiester linkage of **4-I** was faster than that of **4-II** (Fig. 3). The difference of the hydrolysis rate suggests that S1 nuclease recognizes the difference of the configuration around the 3′-end.

Compounds **4-I** and **4-II** were degraded by the treatment with phosphodiesterase I within 5 min to give several products containing 5′-TMP. The products except 5′-TMP were slowly degraded by further treatment. Figure 4A shows the HPLC profile of the products obtained by the treatment of **4-I** with phosphodiesterase I for 20 min. Peak 1 was assigned to be 5′-TMP and peak 3 was assigned to be dithymidine phosphoromorpholidate. Since peak 2 was converted

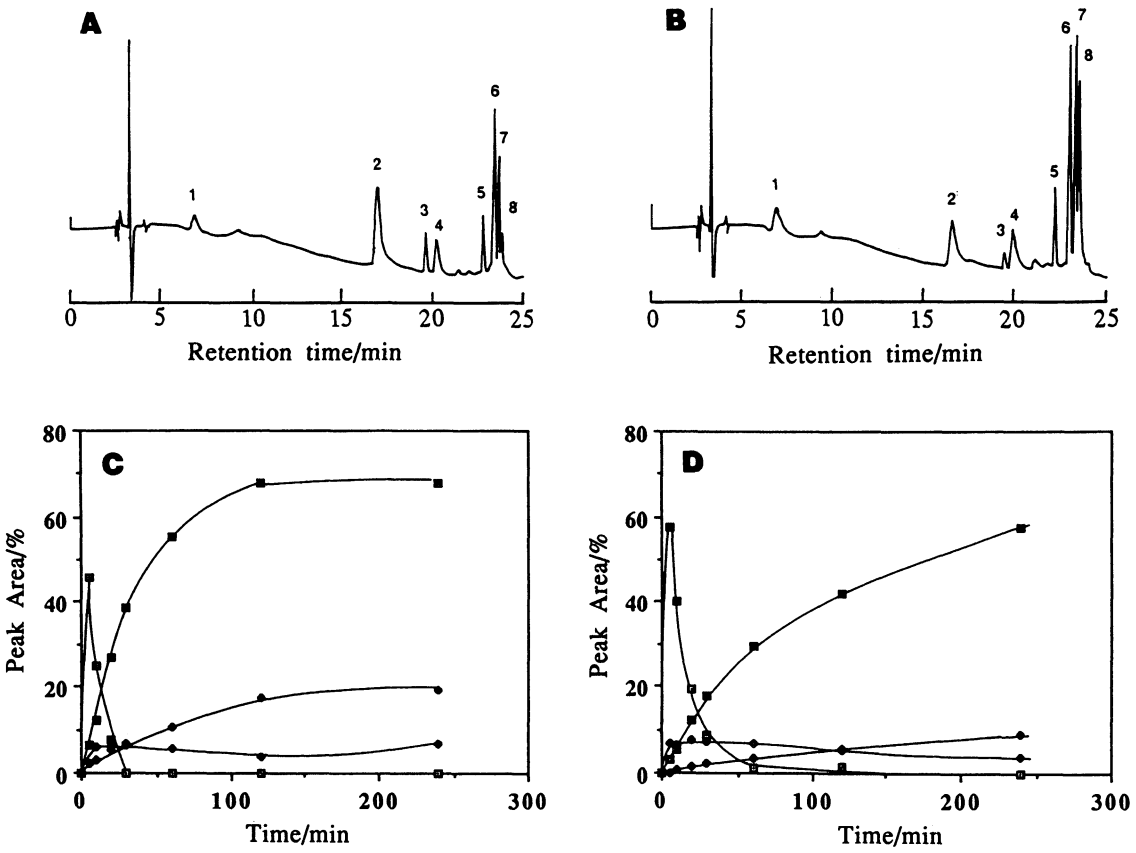


Fig. 4. HPLC profiles of the products obtained by the treatment of **4-I** (A) and **4-II** (B) with phosphodiesterase **I** for 20 min and time courses of their products obtained from **4-I** (C) and **4-II** (D). -◇-, peak 1; -■-, peak 2; -◆-, peak 3; -⊡-, peak 8. Conditions are described in Experimental Section.

to dithymidine phosphoromorpholidate by the treatment with alkaline phosphatase, this compound was assigned to be phosphorylated dithymidine phosphoromorpholidate. Peak 8 was in accord with the product obtained by the treatment of **4-I** with S1 nuclease, (Tp(mor)Tp)$_4$Tp(mor)T. The time conversion curves of peaks 1, 2, 3, and 8 were shown in Fig. 4C. The amount of (Tp(mor)Tp)$_4$Tp(mor)T (peak 8) decreased and those of dithymidine phosphoromorpholidate derivatives (peaks 2 and 3) increased. Also, the amount of 5′-TMP was almost constant after 5 min. Amounts of peaks 4 to 7 reached maximum and then diminished. Although these compounds are not identified, they are presumably the short oligomers resulted by the hydrolysis of phosphodiester linkages in **4-I**. The treatment of the final products (peaks 1, 2, and 3) with alkaline phosphatase gave thymidine and dithymidine phosphoromorpholidate in molar ratio of 1:4.7. The similar results were obtained by the treatment of **4-II** with phosphodiesterase I, as shown in Figs. 4B and D. These results suggest that these internucleotide phosphodiester linkages of **4-I** and **4-II** are slowly cleaved by phosphodiesterase I. Similar results were obtained by Miller and co-workers for oligothymidylate analogs containing stereoregulated methylphosphonate and phosphodiester linkages.[10] Moreover, Figs. 4C and D show that the phosphodiester linkages of **4-I** are cleaved more repidly than those of **4-II**. This difference of the cleaving rates may result from the different configurations of the site recognized by this nuclease.

Compounds **4-I** and **4-II** were very slowly degraded by the treatment with micrococcal nuclease compared to phosphodiesterase I and **4-I** was degraded much slower than **4-II**, as shown in Fig. 5. The difference of the hydrolysis rates between **4-I** and **4-II** suggests that the configuration of internucleotide phosphoromorpholidate linkages affects the binding of the oligonucleotide analog to nucleases and/or the cleaving of that with nucleases.

UV Melting Behaviors. Complex formations between undecathymidylate analogs, **4-I** and **4-II**, with poly(dA) were investigated by the melting behavior using UV spectrophotometer, respectively. The experiments were carried out at an equal nucleotide concentration of either **4-I** or **4-II** and poly(dA), 2×10^{-5} M. Figure 6 shows the melting curves of **4-I**, **4-II**, or (dT)$_{11}$ with poly(dA), and Table 1 summarizes

melting temperatures (T_m) for the complexes of **4-I**, **4-II** or $(dT)_{11}$ with poly(dA) at different salt concentrations. Little change in absorbance was observed for **4-I**, **4-II**, or $(dT)_{11}$ over the temperature range investigated. The complex of **4-II** with poly(dA) showed distinct T_m whereas the complex of **4-I** with poly(dA) did not, indicating that **4-II** has a favorable configuration around the phosphoromorpholidate linkage in forming the complex with poly(dA). This consideration was also supported by the CD sepctra described below. The melting temperature of the complex of **4-II** with poly(dA) was similar to that of $(dT)_{11}$ with poly(dA), while the effect of ionic strength on the stability of the complex was different. The T_m change of **4-II** and poly(dA) by decreasing ionic strength was smaller than that of $(dT)_{11}$ and poly(dA). This result may be attributed to the reduction of repulsive force by the decrease of negative charges in the backbone of **4-II** in the complex formation with poly(dA).

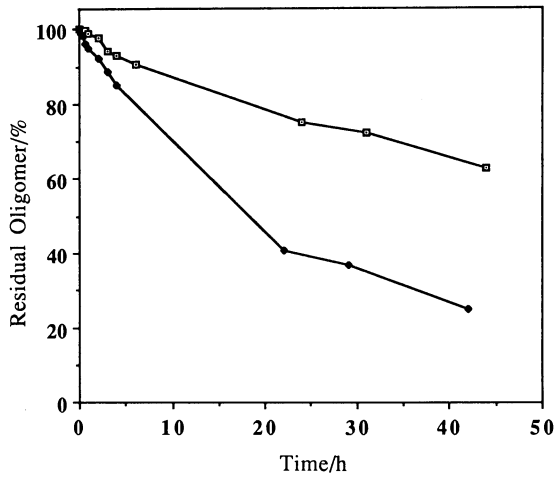


Fig. 5. Degradation of **4-I** (-□-) and **4-II** (-◆-) by micrococcal nuclease. Residual oligomer shows amounts of remaining **4-I** or **4-II**. Conditions are described in Experimental Section.

CD spectra. The conformations of **4-I** or **4-II** and their complexes with poly(dA) were examined by CD

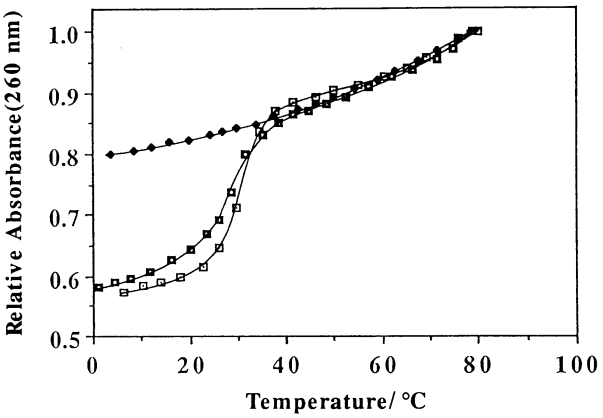


Fig. 6. Absorbance profiles of the complexes of **4-I** (-◆-), **4-II** (-■-), or $(dT)_{11}$ (-□-) with poly(dA) in 10 mM phosphate buffer (pH 7.2) containing 150 mM sodium chloride at total nucleotide concentrations of 4×10^{-5} M.

Table 1. Melting Temperatures (/°C) of the Complexes Formed by **4-I**, **4-II**, or $(dT)_{11}$ with Poly(dA)[a]

NaCl concn/mM	150	15
4-I	n.d.[b]	n.d.[b]
4-II	28	19
$(dT)_{11}$	30	15

a) 10 mM Sodium phosphate buffers were used. b) n.d. indicates that melting temperature was not detected.

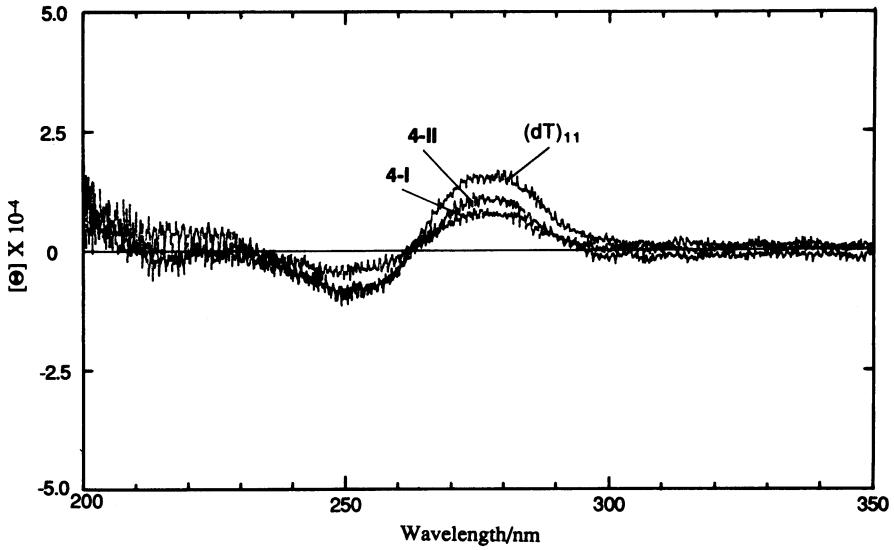


Fig. 7. CD spectra of **4-I**, **4-II**, and $(dT)_{11}$ in 10 mM phosphate buffer (pH 7.2) containing 150 mM sodium chloride at 10°C.

spectroscopy. Figure 7 shows CD spectra of **4-I**, **4-II**, and $(dT)_{11}$. They had different molecular ellipticities. The values of molecular ellipticities at 277 nm are 0.74×10^4 for **4-I** and 1.12×10^4 for **4-II**. The spectrum of **4-II** was more similar to that of $(dT)_{11}$ than that of **4-I**. The spectra of these molecules scarcely changed for temperature change at a range of 10—40 °C.

The CD spectra of the complexes of **4-I**, **4-II**, or $(dT)_{11}$ with poly(dA) at different temperatures are shown in Fig. 8. The spectra obtained from the complex of **4-II** and poly(dA) largely changed with the temperature in analogy with the complex of $(dT)_{11}$ and poly(dA), while the spectra obtained from the mixture of **4-I** and poly(dA) did not change with the temperature. These temperature dependencies reflect the stability of these complexes. It is reported that the complex of

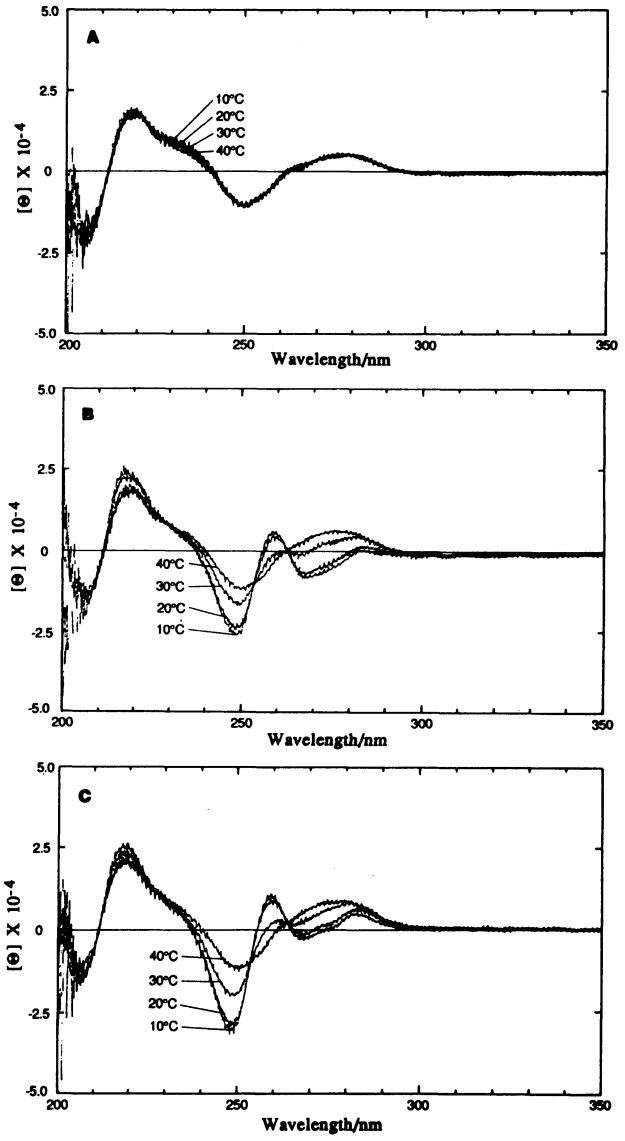


Fig. 8. CD spectra of the complexes of **4-I** (A), **4-II** (B), or $(dT)_{11}$ (C) with poly(dA) at different temperatures in 10 mM phosphate buffer (pH 7.2) containing 150 mM sodium chloride.

oligo(dT) and poly(dA) was assumed to be a B-type geometry.[10] As CD spectra of the complex of **4-II** and poly(dA) are aimilar to that of $(dT)_{11}$ and poly(dA), a geometry of the complex of **4-II** and poly(dA) was assumed to be B-type. CD spectrum obtained from **4-I** and poly(dA) agreed with a sum of CD spectrum of **4-I** and CD spectrum of poly(dA), indicating that **4-I** does not interact with poly(dA) under the experimental conditions. This result agrees with that of UV melting behavior as described.

The results demonstrated here indicate that the configuration of the phosphoromorpholidate linkages in oligonucleotide analogs must be regulated for the stable duplex formation with complementary strand and that the introduction of phosphoromorpholidate linkages into oligonucleotide analogs in greatly increases their resistance to nucleases. The present method for introduction of stereoregulated phosphoromorpholidate linkages into oligonucleotides provides an effective way for increasing the potential of this type of analogs as regulatory substances for gene expression.

We would like to thank Dr. Yasuhisa Kuroda of Kyoto University for the use of CD spectropolarimeter.

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