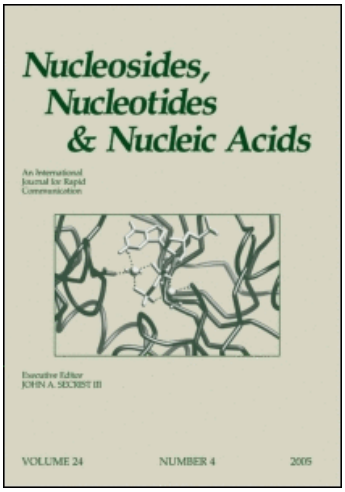

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PREPARATION OF STEREOREGULATED ANTISENSE OLIGODEOXYRIBONUCLEOSIDE PHOSHOROTHIOATE AND INTERACTION WITH ITS COMPLEMENTARY DNA AND RNA

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ABSTRACT: Diastereoisomeric specificity of oligodeoxyribonucleoside phosphorothioate (OPT) in DNA/OPT and RNA/OPT hybrid formation was investigated. The difference in the configuration between RRRR and SSSS was reflected in the conformation and the stability of the DNA/OPT and RNA/OPT hybrids. Therefore, findings of this report rationalize the antisense effect by non-stereoregulated OPT and the difference of diastereoisomerism in susceptibility to RNase H.

INTRODUCTION

Nearly three decades have passed since synthetic oligonucleotide analogs were used to control cellular functions by specifically interacting with certain RNA in cells.[1] After the report, oligodeoxyribonucleoside phosphodiesters (ODN) were used for controlling the RNA expression of Rous sarcoma virus.[2,3] In 1980's, the surprising observation that endogenous RNAs regulate gene expression in eucalyotic cells was reported.[4] The mechanism of gene regulation above mentioned was later named as 'antisense mechanism', and was regarded as a new concept for the drug innovation.[5] Since then, the concept has

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This paper is dedicated to the memory of Professor Tsujiaki Hata.

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been examined to be effective for various diseases such as HIV, cancers, and other incurable diseases.[6-8] In the early stage of the studies, the structure of antisense molecules was the main concern of the research because ODNs are easily hydrolyzed in human bodies.[9] Oligodeoxyribonucleoside phosphorothioate (OPT) was thus chosen and has been intensively examined.[10] Actually, OPT is the only compound that is under clinical trials in Phase III status.[11,12] Following these situations, the focus of antisense gene therapy has moved on toward the actual aspects of antisense mechanism.

In fact, to realize the antisense therapy, there are numbers of problems to be studied.[13-17] They are, for example, clarification of mechanism of antisense effect, development of drug delivery system, and pharmacological and chemical characterization of antisense molecules. Recently, there arise two major concerns as to OPT. The one is the diastereoisomerism due to the thiophosphate linkages.[18] The diastereoisomerism may be crucial for the expression of antisense effects in terms of the stability of the hybrid. Another is the interaction of OPT with biological substances other than nucleic acids.[19-21] It has been demonstrated that OPT interacts with biologically important proteins such as thrombin, CD4, NFκB, RNase H and so on.[22] These findings suggest that some of the interactions reported by now should be re-evaluated. Furthermore, as to the diastereoisomerism concerning with this matter, a single report using transcriptional factors as a sort of protein was published indicating that there was no stereospecificity in the interaction. Thus, characterization of the diastereoisomerism of modified oligonucleotides is a sober but an important research theme for antisense therapy.[18,23-25]

We describe here that the evaluation of diastereoisomerism of OPT in terms of the conformation and the stability of ODN/OPT and oligoribonucleoside phosphodiesters (ORN)/OPT hybrids. We tried to obtain diastereoisomerically pure OPT by use of reversed-phase HPLC,[25] because there have existed many difficulties on either the stereoselective[18] or stereospecific synthesis.[26] The difficulties have been almost overcome by Stec's group,[27] and, however, chromatographic procedures can serve an alternative and convenient method. This is the first paper showing that the diastereoisomers of OPT-pentamer are effectively

separated using reversed-phase HPLC. We also suggest that the diastereoisomerism is one of the essential factors for the antisense effects of OPT by CD spectroscopy.

RESULTS

Separation and Purification of Diastereoisomers

In order to prepare diastereoisomerically pure OPT, the two-step chromatographic approach utilizing reversed-phase HPLC was used.[24, 25, 28-30] FIG. 1 is the chromatogram of the optimized first step separation of 5'- DMT-dCpsApsTpsCpsG-3' (DMT-OPT-AS). Four well-separated peaks appeared when TEAA was used as the buffering salt. When other salts such as ammonium formate and ammonium acetate were used, the baseline separation could not be achieved (data not shown). The four fractions, which were designated to be A to D in order of elution as shown in FIG. 1, were pooled. FIG. 2 shows the second step separation of the four peaks of FIG. 1 and revealed that all 16 diastereoisomers of OPT-AS arising from Rp/Sp thiophosphate chirality at the four internucleotide linkages were successfully separated. In this case, ammonium formate was found to be most effective compared with other salts such as TEAA and ammonium acetate.

Configurational Analysis of Diastereoisomers

The configuration of the 16 diastereoisomers of OPT-AS was determined by the combination of enzymatic digestion and the reversed-phase HPLC analysis as described previously.[25] A thiophosphate linkage with Sp configuration is a substrate for nuclease P1 [31] but not hydolyzed by svPDE.[32] Using nuclease P1, one peak which was completely hydrolyzed to give five substances corresponding to dC, dCMPS, dGMPS, dTMPS, and dAMPS could be identified (SSSS)-OPT-AS, and another peak which was not hydrolyzed at all could be identified (RRRR)-OPT-AS. Other peaks were partially hydrolyzed by nuclease P1 and the resulting substances were further hydrolyzed by svPDE, which hydrolyzes only Rp-configuration. On the basis of this manner, the absolute configuration of 16 diastereoisomers was determined as shown in FIG. 2.

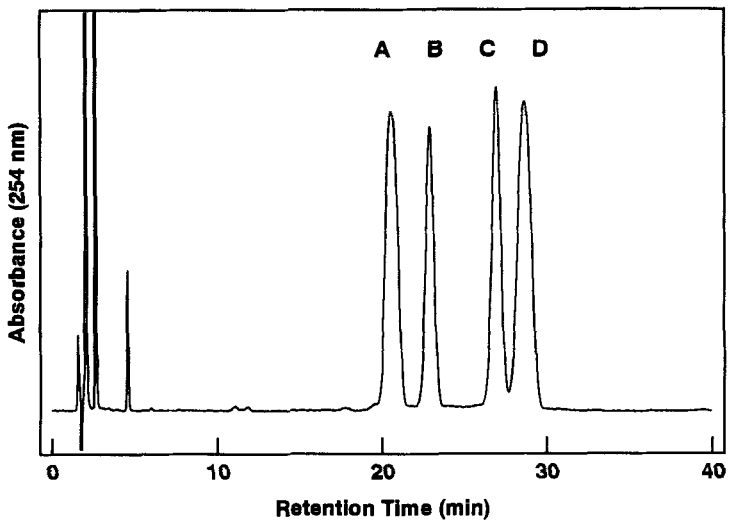

FIG. 1. Reverse-phase HPLC analysis of the diastereomers of 5'-DMT-OPT-AS. Column: ULTRON VX-Nucleotide (5 mm, 4.6x150 mm). Eluents: **a** 0.1 M TEAA (pH 7.0), **b** 50 % CH_3CN in 0.1 M TEAA (pH 7.0); 50 % **b** for 5min, 50-90 % **b** in 40 min linear, flow-rate, 1 ml/min; 254 nm at 40°C.

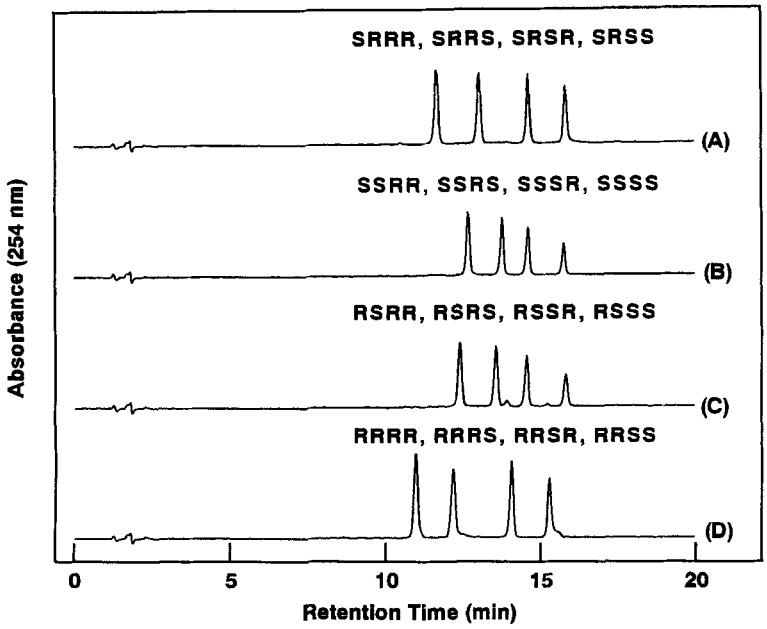

FIG. 2. Reversed-phase HPLC analysis of the diastereomers of (A) OPT-AS from A, (B) from B, (C) from C, (D) from D of FIG. 1. Column: ULTRON VX-Nucleotide (5 mm, 4.6x150 mm). Eluents: **a** 50 mM Ammonium Formate (pH 6.8), **b** 50 % CH_3CN in 50 mM Ammonium Formate (pH 6.8); 10 % **b** for 5 min, 10-50 % **b** in 40 min linear; flow-rate, 1 ml/min; 254 nm at 40°C.

CD Spectra of Single-Stranded DNA

Temperature-dependent CD spectra were measured for single-stranded dCpApTpCpG (ODN-AS), (RRRR)-OPT-AS, and (SSSS)-OPT-AS. The spectra of (RRRR)-OPT-AS and (SSSS)-OPT-AS were similar to that of ODN-AS, although the intensity of these two OPT-AS were smaller than that of ODN-AS: The order of the CD intensities, which corresponding to the extent of the base stacking, was ODN-AS > (RRRR)-OPT-AS > (SSSS)-OPT-AS (data not shown).

CD Spectra of ODN/OPT Hybrids

To analyze the conformation and the stability of the hybrids such as dCpGpApTpG/dCpApTpCpG (ODN-S/ODN-AS) and dCpGpApTpG/dCpsApsTpsCpsG (ODN-S/OPT-AS), temperature-dependent CD spectra were recorded for each 4 K rise in temperature from -4 to 44 °C. FIG. 3A shows the temperature-dependent CD spectra of ODN-S/ODN-AS (FIG. 3A-a), ODN-S/(Mixture)-OPT-AS (FIG. 3A-b), ODN-S/(RRRR)-OPT-AS (FIG. 3A-c) and ODN-S/(SSSS)-OPT-AS (FIG. 3A-d). These spectra contained a negative band at 290 nm that was characteristic of short duplexes.[33] The spectrum of the ODN-S/ODN-AS at -4°C showed a conservative CD spectrum with a positive ellipticity at 273 nm and a negative one at 252 nm, indicating B-form conformation.[34] The spectra of ODN-S/(Mixture)-OPT-AS and ODN-S/(RRRR)-OPT-AS at -4°C were similar to that of ODN-S/ODN-AS, although their intensities of the positive and the negative peaks were smaller than that of ODN-S/ODN-AS. On the other hand, the spectrum of ODN-S/(SSSS)-OPT-AS at -4°C had the smaller positive and the larger negative peaks than that of ODN-S/ODN-AS. Furthermore, to investigate the stability of ODN-S/ODN-AS and ODN-S/OPT-AS, the melting profiles by CD spectroscopy was studied. As the temperature increased, the molar ellipticity at 254 nm decreased in characteristic manners as shown in FIG. 4A. The ellipticity at 254 nm was plotted against the temperature, and the sigmoidal curves depicted in FIG. 4A allowed us to determine the melting temperature (Tm).[35] Tm for these compounds are listed in TABLE 1.

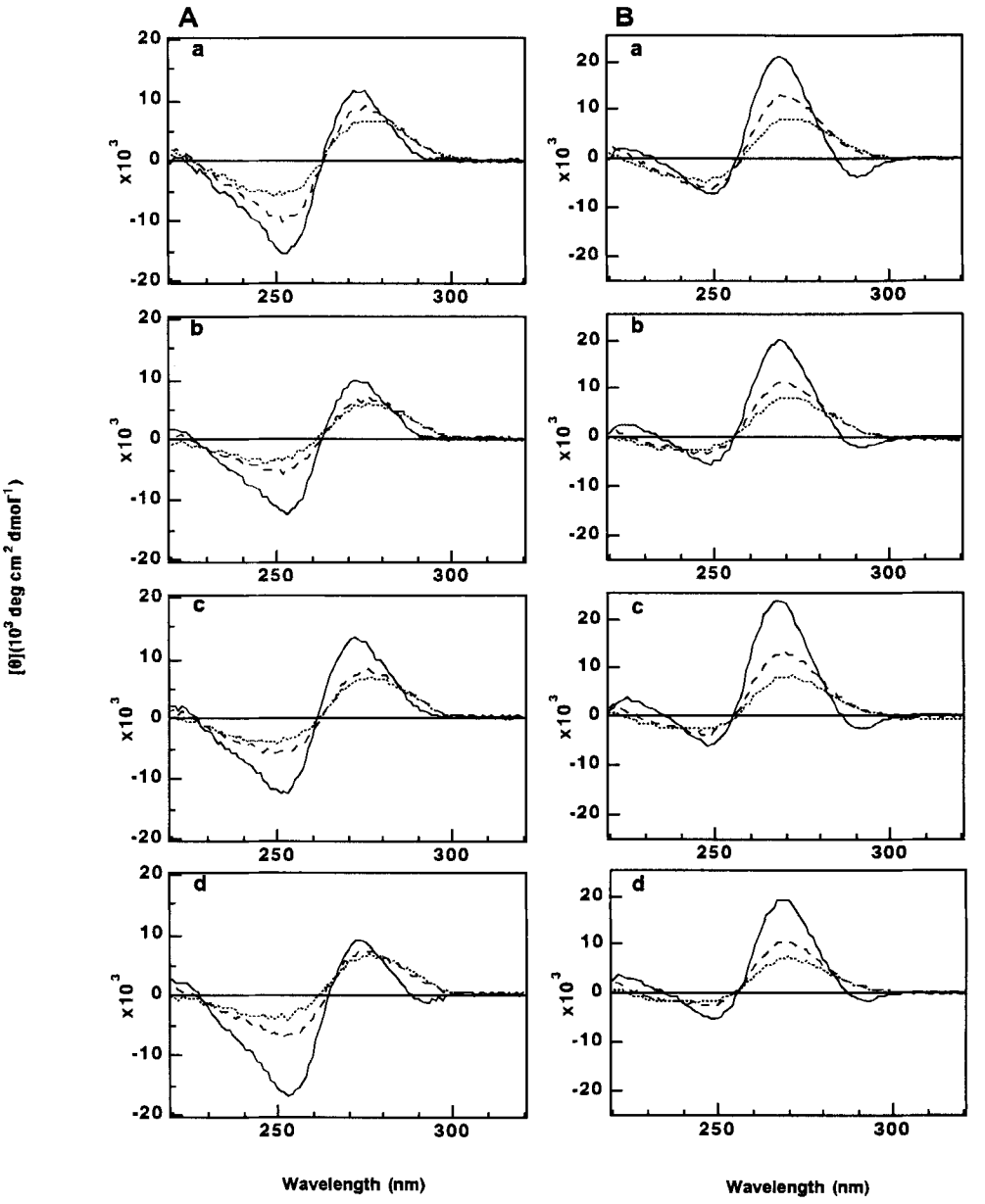

FIG. 3. A Temperature dependent CD spectra of a ODN-S/ODN-AS, b ODN-S/(Mixture)-OPT-AS, c ODN-S/(RRRR)-OPT-AS and d ODN-S/(SSSS)-OPT-AS, B temperature dependent CD spectra of a ORN-S/ORN-AS,b ORN-S/(Mixture)-OPT-AS, c ORN-S/(RRRR)-OPT-AS and d ORN-S/(SSSS)-OPT-AS, in 10 mM sodium phosphate (pH 7.0), 1 M NaCl. The total nucleotides concentration is 2.0×10^{-4} M. Solid line: -4°C, dotted line: 20°C, dashed line: 44°C.

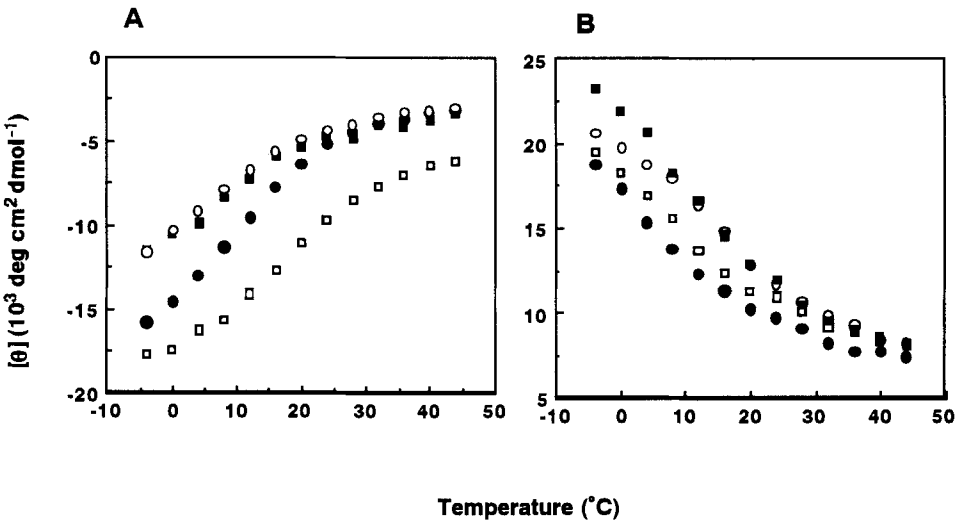

FIG. 4. A CD melting curves of ODN-S/ODN-AS (open squares), ODN-S/(Mixture)-OPT-AS (open circles), ODN-S/(RRRR)-OPT-AS (closed squares) and ODN-S/(SSSS)-OPT-AS (closed circles), **B** ORN-S/ORN-AS (open circles), ORN-S/(Mixture)-OPT-AS (open squares), ORN-S/(RRRR)-OPT-AS (closed squares) and ORN-S/(SSSS)-OPT-AS (closed circles), in 10 mM sodium phosphate (pH 7.0), 1 M NaCl. The total nucleotides concentration is 2.0×10^{-4} M.

TABLE 1. Thermal stabilities of ODN-S or ORN-S/Antisense DNA Hybrids.

ODN-S or ORN-S	Antisense DNA	Tm (°C)
ODN-S	ODN-AS	18
	(Mixture)-OPT-AS	N.D.
	(RRRR)-OPT-AS	N.D.
	(SSSS)-OPT-AS	12
ORN-S	ODN-AS	16
	(Mixture)-OPT-AS	8
	(RRRR)-OPT-AS	12
	(SSSS)-OPT-AS	8

CD Spectra of ORN/OPT Hybrids

In common with the analysis of ODN-S/ODN-AS and ODN-S/OPT hybrids, temperature-dependent CD measurements were carried out for rCpGpApUpG/dCpApTpCpG (ORN-S/ODN-AS) and rCpGpApUpG/dCpsApsTpsCpsG (ORN-S/OPT-AS). FIG. 3B shows the temperature-dependent CD spectra of ORN-S/ODN-AS (FIG. 3B-a), ORN-S/(Mixture)-OPT-AS (FIG. 3B-b), ORN-S/(RRRR)-OPT-AS (FIG. 3B-c) and ORN-S/(SSSS)-OPT-AS (FIG. 3B-d). Similar to the results of ODN-S/ODN-AS and ODN-S/OPT-AS, a negative band at 290 nm that was characteristic of short duplexes was observed. The CD spectrum of ORN-S/ODN-AS at -4° C showed a non-conservative CD spectrum with a larger positive ellipticity at 280 nm and a small negative one at 240 nm, indicating A-like conformation.[33,34] The CD spectra of ORN-S/OPT-AS hybrids at -4° C were similar to that of ORN-S/ODN-AS, although the molar ellipticities were different each other. The order of the CD intensity of the duplexes at 270 nm is ORN-S/(RRRR)-OPT-AS > ORN-S/ODN-AS > ORN-S/(SSSS)-OPT-AS = ORN-S/(Mixture)-OPT-AS. As the temperature increased, the molar ellipticities at 270 nm decreased in characteristic manners as shown in FIG. 4B.[35] In this case, these four hybrids could have their individual Tm, as listed in TABLE 1.

DISCUSSION

Characterization of diastereoisomerism of OPT can lead us the certain concept to design antisense molecules of the next generation. In parallel to the stereospecific synthesis of OPT by Stec's group,[18,27] we adopted the chromatographic separation of OPT to obtain diastereoisomerically pure OPT.[25] As this method is applicable to any sequences and is not time-consuming, it is worth being tried for the study of the antisense effect in terms of diastereoisomerism. The CD measurement was, therefore, performed to analyze the hybrid conformation and the hybrid stability between stereoregulated OPT diastereoisomers and ODN or ORN.

Separation of Diastereoisomers and Their Conformation in Single-stranded Form

To obtain the diastereoisomerically pure OPT, it was found that commercially available reversed-phase HPLC packings could be applicable for the purpose. However, a

single-step separation was not sufficient to obtain well-qualified OPT diastereoisomers.[18] We adopted the two-step chromatographic approach on the separation,[24, 25, 28-30] and could separate sixteen diastereoisomers of OPT-AS. The trial can be applicable to another OPT-pentamer. However, the trial to pursue similar experiments using OPT-hexamers has not been successful by now (data not shown), and it may be due to the limited ability of reversed-phase HPLC packings.

After configurational analyses, it was found that the separation pattern showed the characteristic manner. The components of each peak in FIG. 1 had two common contiguous configurations at the 5'-terminus; the diastereoisomers contained in peak A, B, C and D in FIG. 1 had 5'-SR**, 5'-SS**, 5'-RS** and 5'-RR** configuration, respectively. Although the detailed mechanisms of these ordered separations have not been elucidated, the hydrophobic core arising from the 5'-DMT group and two common contiguous configurations at the 5'-terminus may predominates the elution order of these diastereoisomers. Contrarily, the elution order of OPT diastereoisomers seemed to be governed by two contiguous thiophosphate linkages at 3'-terminus. For example, four fractions of Peak A were eluted in the order of **RR-3', **RS-3', **SR-3', and **SS-3', and this order was consistent in other fractions as shown in FIG. 2. Though the detail mechanisms of these separations have not been elucidated, it is considered that the difference of hydrophobicity among diastereoisomers predominates the elution order as we mentioned in a previous paper.[25]

To support above assumption, preliminary molecular modeling calculation was performed for single-stranded diastereoisomers (data not shown) and the result was compared with the chromatographic separation. As Jaroszewski et al. reported,[36] the molecular modeling calculation showed that the sulfur atom of single-stranded Sp diastereoisomer is located in the vicinity of the bases and the sugar rings, which makes it difficult to form a stable base stacking. In contrast, the location of the sulfur atom of Rp diastereoisomer facilitate the base stacking. The magnitude of the CD rotation depends upon the angle between the dipole moment vector of bases.[37] Taking these results into account, it can be presumed that (RRRR)-OPT-AS might have a greatly diminished rotation that permits a

larger base-base overlapping and that it might form a compact hydrophobic core. As to Sp diastereoisomer, it is considered that those effects are not dominant. This presumption is consistent with the results obtained for the single-stranded molecules in the previous report,[25] and this report, that is (RRRR)-OPT-AS > (SSSS)-OPT-AS (data not shown). Using this relationship, it is explained that the elution order of the diastereoisomers could be affected by the degree of the hydrophobic interaction between the diastereoisomers and the stationary phase: Faster eluting diastereoisomer ((RRRR)-OPT-AS) should have the more compact hydrophobic core than the more slowly eluting one ((SSSS)-OPT-AS).

Diastereoisomerical Specificity for ODN/OPT and ORN/OPT Hybrids Structure

Information about the global conformation of ODN-S/OPT-AS and ORN-S/OPT-AS hybrids may be obtained by the CD measurements. As shown in FIG. 3, the spectrum of ODN-S/(RRRR)-OPT-AS was similar to that of B-form DNA, although both the positive and negative intensities were smaller than those for ODN-S/ODN-AS. In contrast, ODN-S/(SSSS)-OPT-AS produced the spectrum rather indicative of C-form like conformation. These results suggest that configurations at the thiophosphate linkage affect the conformation of ODN-S/OPT-AS hybrid. On the other hand, the spectra of ORN-S/OPT-AS hybrids were similar to that of an ORN-S/ODN-AS which is A-form like conformation.[38] The extent of the CD intensity at 270 nm for the duplexes was ORN-S/(RRRR)-OPT-AS > ORN-S/ODN-AS > ORN-S/(SSSS)-OPT-AS. This result suggests (RRRR)-OPT-AS forms the more stable A-form hybrids with ORN than (SSSS)-OPT-AS and may explain the RNase H susceptibility to the diastereoisomers of OPT.[39-41] Recently, Koziolkiewicz et al. reported [42] that ORN/(All-R)-OPT was found to be more susceptible to RNase H-dependent degradation compared with ORN/(all-S)-OPT or ORN/(mixture)-OPT. As RNase H seems to recognize stable A-form hybrids,[43] our results may support their conclusion from the conformational aspect.

Melting profiles of ODN-S/OPT-AS and ORN-S/OPT-AS hybrids monitored by CD spectroscopy show the influences of diastereoisomerism on the stability of these hybrids. The order of the thermal stability obtained for the ODN-S/OPT-AS hybrids agreed with

results reported by Miller et al.[23] that the open stacked diastereoisomers (in the present study, (SSSS)-OPT-AS) can make more stable hybrids than the highly stacked diastereoisomers (in the present study, (RRRR)-OPT-AS). Contrary to the ODN-S/OPT-AS hybrids, the order of the thermal stability of ORN-S/OPT-AS hybrids was ORN-S/(RRRR)-OPT-AS > ORN-S/(SSSS)-OPT-AS. One of the reasons for this result might be lying on the character of the structure of ORN/ODN hybrids.[44-46] Salazar et al. have reported [38] that an ORN strand in ORN/ODN hybrid not only retains its N-type structure, but also exerts an influence on the conformation of DNA strand where the sugar packering changes from O4'-endo to C2'-endo allowing stable hybrid formation. We presume that the diastereoisomer with Sp configuration that has the sulfur atom in closer proximity to the sugar rings hardly adopt such conformational change in the OPT's sugar packering. Therefore, it is assumed that (SSSS)-OPT-AS could not form a stable hybrid with ORN-S.[47,48]

CONCLUSION

The procedure described above provides the most effective method for the separation of the diastereoisomers of OPT up to pentamer by means of reversed-phase HPLC.[25,49] Followed by the success of the separation, it was demonstrated that the diastereoisomerism of OPT is responsible for the hybrid formation of OPT diastereoisomers with its complementary ODN and ORN. Consequently, it was suggested that OPT with Rp configuration produced the stable conformation that may be preferably recognized by RNase H. Findings of this report rationalize the antisense effect by non-stereoregulated OPT and explain the different susceptibility of stereoregulated OPT to RNase H.

EXPERIMENTAL

Oligonucleotides Synthesis.

Oligodeoxyribonucleoside (ODN) and oligoribonucleoside (ORN) phosphodiesters and oligodeoxyribonucleoside phosphorothioates (OPT) were synthesized on the 1 μmol scale on an ABI Model 391 DNA synthesizer (Applied Biosystems, Inc., CA, USA)

employing ß-cyanoethyl phosphoramidite chemistry. For the OPTs synthesis, the iodine oxidation step was replaced by the sulfurization with tetraethylthiuram disulfide (TETD). Except for the dimethoxytrityl (DMT) group deprotection of oligonucleotides was carried out with fresh concentrated ammonium hydroxide (28%) for 8 hr at 55°C. The DMT group of ODN and OPT was removed with 80% CH_3COOH for 30 min at ambient temperature. Both the DMT and 2' protecting groups of ORN were removed with 10 mM HCl (pH 2.0) for 15 hr at 30°C. Deoxyribonucleoside 5'-phosphorothioates (dCMPS, dGMPS, dTMPS and dAMPS), which were used as standard references for the HPLC peak assignment, were prepared by introducing a thiophosphate group into 5'-end of the monomer linked on the CPG supports using Phosphate-ON (Perkin Elmer) and TETD.

Separation and Purification of Oligonucleotides.

Oligonucleotides (ODN, ORN and OPT) were analyzed by reversed-phase HPLC using a Shimadzu LC-6A chromatography system equipped with a CTO-6A column oven (Shimadzu Corpo, Kyoto, Japan). Reversed-phase HPLC columns used were analytical ULTRON VX-Nucleotide (5 μm, 4.6x150 mm, Shinwa Chemical Industries, Co., Ltd., Kyoto, Japan) and preparative ULTRON VX-Nucleotide (5 μm, 20x250 mm, Shinwa). In the case of the analysis of the enzyme hydrolyzate, a guard column (Guardpak, μ Bondapak C_{18}, Waters, MA, USA) was connected to the head of the analytical column. The mobile phase consisted of aqueous 100 mM triethylammonium acetate (TEAA, pH 7.0)/CH_3CN or 50 mM ammonium formate (pH 6.8)/CH_3CN. Various acetonitrile gradients were examined to optimize each separation. The final chromatographic conditions are described in the figure legends.

Configurational Analysis of Diastereoisomers.

For the configurational assignment, nuclease P1 digestion was used first to hydrolyze Sp-thiophospate linkage.[31] To each HPLC fraction containing an OPT diastereoisomer (0.1 $OD_{260\,nm}$) which was dissolved in 40 μl of 50 mM sodium acetate buffer (pH 5.3), nuclease P1 (8 unit/40 μl) and zinc sulfate (10 mM, 4 μl) was added . After 1 hr incubation at 37°C, the hydrolyzate was analyzed and fractionated by reversed-phase HPLC. Every single peak was collected, evaporated to dryness, and dissolved in 25 μl of 0.5 M Tris-HCl (pH 8.5) and snake venom phosphodiesterase (svPDE, 0.1 unit/ 25 μl).[32] After 1 hr incubation at 37°C, the hydrolyzate was analyzed by reversed-phase HPLC.

Circular Dichroism (CD) Spectroscopy.

CD spectra were recorded using a Model J-720 spectropolarimeter (JASCO, Tokyo) interfaced with an NEC PC-9801 RX microcomputer. The sample temperature was maintained by placing the sample in a 1 mm path length jacketed cylindrical cell connected

to a Neslab RTE-100 thermo-bath circulator. Each spectrum (220-320 nm) was obtained by averaging ten scans after subtracting that of the buffers alone. Measured intensities were converted to the molecular ellipticity based on the base pair concentration. Samples ($2.0x10^{-4}$ M) for CD melting studies were prepared in 10 mM sodium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1 M NaCl. Oligonucleotide concentrations were determined spectroscopically by measuring the absorbance at 260 nm. The extinction coefficients ($10^{-4}M^{-4}cm^{-1}$) for the single-stranded oligonucleotides at 25° C were calculated by the nearest-neighbor approximation,[50] as follows: dCpGpApTpG, $5.1x10^4$, rCpGpApUpG, $4.3x10^4$, dCpApTpCpG, $4.7x10^4$, and dCpsApsTpsCpsG, $4.7x10^4$.

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