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Optimization of the separation of oligodeoxyribonucleoside phosphoramidates and their characterization by circular dichroism spectroscopy

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ABSTRACT

Oligodeoxyribonucleoside phosphoramidates (OPAs) were synthesized via H-phosphonate chemistry. The oxidation of oligodeoxyribonucleoside H-phosphonates was performed in the presence of isopropylamine–carbon tetrachloride (1:4, v/v). Reversed-phase high-performance liquid chromatography was used to separate the diastereoisomers of OPAs for the study of their conformation in solution and their ability to act as antisense molecules. It was found that complete purification of diastereoisomers of OPAs by reversed-phase HPLC could be achieved by a two-step chromatographic approach adopting optimized chromatographic conditions: acetonitrile gradient, column temperature and column length. From the circular dichroism measurements of the tetranucleoside phosphoramidates, it was found that the eight diastereoisomers of the tetranucleotides can be classified into four groups according to their molar ellipticity changes and that the diastereoisomers show different abilities to form hybrids with complementary DNA.

INTRODUCTION

Viral diseases are often fatal in humans because they can rarely be treated by conventional medical care. In particular, infection with human immunodeficiency virus (HIV), completely incurable so far, is rapidly increasing all over the world. One of the most promising treatment approaches is the antisense DNA/RNA method [1,2], in which synthetic oligodeoxyribonucleotides and their analogues are used to bind to the target sequences of DNAs or RNAs and consequently to regulate gene expression in a highly specific manner. To be suitable for trial, there are several requirements in designing the antisense molecules [3]: (1) specificity for the target DNAs or RNAs; (2) stability of the duplex formed with the target DNAs or RNAs; (3) resistance to nucleases; (4) cell membrane permeability; (5) metabolism in body. To satisfy these requirements, modification of the internucleotide phosphate linkages of DNAs or RNAs has been attempted [1,3]. One such group of oligonucleotide analogue is the oligodeoxyribonucleoside phosphoramidates (OPAs) [4]. This type of antisense molecule is resistant to nuclease digestion and is expected to easily penetrate into the cellular membrane because of its high hydrophobicity.

However, when a phosphate linkage of DNA or RNA is modified, the modification results in diastereoisomers, as shown in Fig 1. Therefore, OPAs consist of a number of diastereoisomers. The question arises: Is there a difference in the stability of hybrids between the diastereoisomers and their complementary oligonucleotides [5]? To help answer this question, in the present study, optimization of the reversed-phase HPLC to separate diastereoisomers of di-, tri and tetra-

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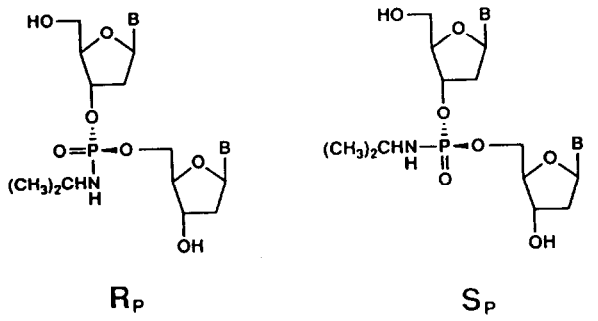

Fig. 1. R_P and S_P configurations of dinucleoside phosphoramidates.

nucleoside phosphoramidate was explored and the diastereoisomers were characterized by circular dichroism (CD) spectroscopy.

MATERIALS AND METHODS

Reagents

Controlled pore glass was loaded with 35–45 μmol/g of the fully protected nucleoside unit. H-Phosphonate nucleosides were purchased from Milligen/Biosearch (Burlington, CA, USA). HPLC-grade acetonitrile was purchased from J.T. Baker (NJ, USA). Distilled water was purchased from Wako (Osaka, Japan), and further purified with a Millipore Milli-QII system. Other reagents were purchased from Nakalai Tesque (Kyoto, Japan).

Oligodeoxyribonucleotide synthesis

Oligodeoxyribonucleoside phosphodiesters (ODNs) were synthesized at the micromolar scale on a Milligen Cyclon Plus DNA synthesizer (Milligen/Biosearch) via phosphoramidite chemistry [4,6]. Oligodeoxyribonucleoside phosphoramidates (OPAs) were synthesized at the 10 μmol scale in a gas-tight syringe via H-phosphonate chemistry, and the oxidation of oligodeoxyribonucleoside H-phosphonates was carried out in the presence of isopropylamine–carbon tetrachloride (1:4, v/v) [4]. Deprotection of oligodeoxyribonucleotides except for the 5′-dimethoxytrityl (DMT) group was carried out with fresh ammonium hydroxide (28%) at 55°C for 8 h.

Sample preparation for HPLC

The ammonium hydroxide solution (5 ml) containing OPAs was evaporated to one-half of its initial volume, and 0.1 *M* triethylammonium acetate (TEAA, pH 7.0, 2.5 ml) was added. The solution was applied to a C_{18} Sep-Pak cartridge (Waters, Milford, MA, USA). After sequential elution with 0.1 *M* TEAA (pH 7.0) and 0.1 *M* TEAA (pH 7.0)–acetonitrile (85:15), the cartridge was eluted with 0.1 *M* TEAA (pH 7.0)–acetonitrile (50:50). After evaporation to dryness, the residue was dissolved in 0.1 *M* TEAA (pH 7.0)–acetonitrile (70:30) and used for HPLC purification [7].

HPLC separation and purification

Oligonucleotides (ODNs and OPAs) were analysed by reversed-phase HPLC using a Shimadzu LC-6A chromatography system (Shimadzu, Kyoto, Japan). The column temperature was controlled by a CTO-6A column oven (Shimadzu). Reversed-phase columns used were as follows: for the separation of di- and trinucleoside phosphoramidates, a Superspher RP-18(e) (4 μm, 4 × 125 mm, Merck, Darmstadt, Germany); for the separation and the purification of 5′-DMT-dAp(iPr)Tp(iPr)Cp-(iPr)G (iPr = isopropyl), a Superspher RP-18(e) (4 μm, 4 × 250 mm, Merck); for the purification of the diastereoisomers of dAp(iPr)Tp(iPr)Cp-(iPr)G, an ODS-NC_{18} (5 μm, 20 × 250 mm, Shinwa, Kyoto, Japan). The chromatographic conditions are described in the captions to the figures.

Sample preparation for circular dichroism (CD) measurement

Solutions of single strands were prepared by using a buffer consisting of 10 m*M* sodium phosphate, adjusted to pH 7.0, and for hybrids 10 m*M* sodium phosphate and 3 *M* NaCl, adjusted to pH 7.0. Oligonucleotide concentrations were determined spectroscopically by measuring the absorbance at 260 nm. The extinction coefficients for the single-stranded oligodeoxyribonucleotides at 25°C were calculated by the nearest-neighbour approximation [8]. Extinction coefficients in units of $10^{-4}/M^4$/cm at 25°C

are as follows: dApTpCpG, $4.09 \cdot 10^4$; and dAp(iPr)Tp(iPr)Cp(iPr)G, $4.09 \cdot 10^4$.

Single-stranded samples for CD melting studies were prepared at $2.5 \cdot 10^{-5}$ *M* concentration in a buffer containing 10 m*M* sodium phosphate, adjusted to pH 7.0. Hybrid samples for CD melting studies were prepared at $2.5 \cdot 10^{-4}$ *M* concentration in total oligonucleotide strands and annealed in a buffer containing 10 m*M* sodium phosphate, 3.0 *M* NaCl, adjusted to pH 7.0, and incubated at −8°C for 1 h.

Circular dichroism (CD) spectroscopy

CD spectra were recorded using a Jasco Model J-720 spectropolarimeter interfaced with an NEC PC-9801 RX microcomputer. The sample temperature was maintained by placing the sample in a 1 mm or 10 mm path length jacketed cylindrical cell equipped with a Neslab RTE-100 bath circulator. Each spectrum was scanned from 320 to 220 nm, and corresponds to an average of eight scans minus the averaged spectra of the buffer alone. CD melting profiles were recorded by measuring the change in molar ellipticity ([θ]) at 254 nm while the temperature was scanned.

RESULTS AND DISCUSSION

Synthesis of oligodeoxyribonucleoside phosphoramidates

Preparation of OPAs was carried out according to previously reported procedures [4,6] and the averaged coupling yields were estimated to be more than 98% by reversed-phase HPLC.

HPLC separation

In order to characterize the diastereoisomerism of OPAs, dinucleoside phosphoramidates [dAp(iPr)T, dTp(iPr)C and dCp(iPr)G] with a 5′-DMT group were first separated with reversed-phase HPLC [9,10]. The results represented in Fig. 2 show the appearance of two distinct peaks (I and II) and baseline separation in all cases. The two separated peaks correspond to the diastereoisomers due to the phosphoramidate linkage. It was found that the ratios of the yielded diastereoisomers were not equal, as suggested in a previous paper [11]. On the other hand, when the 5′-DMT group was removed by acid treatment, the diastereoisomers of dAp(iPr)T could not be completely separated. It should be noted that, when peak II of dTp(iPr)C, for example, was treated with 80% acetic acid, peaks other than dTp(iPr)C appeared (data not shown). Therefore, it is suggested that the purification of OPAs by reversed-phase HPLC is required to perform the two-step purification, *i.e.* as a first step separation of 5′-DMT-on-OPA and as a second step the separation of 5′-DMT-off-OPA.

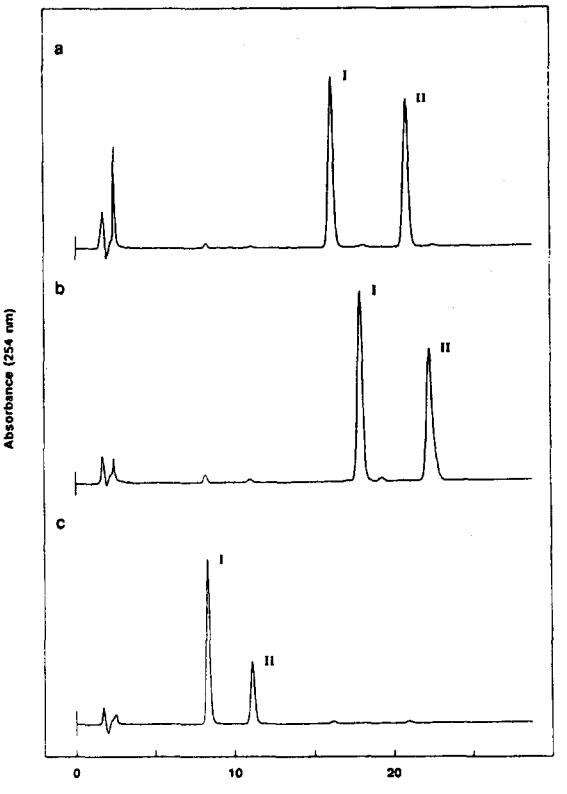

Fig. 2. HPLC analysis of diastereoisomers of (a) 5′-DMT-dAp(iPr)T, (b) 5′-DMT-dTp(iPr)C and (c) 5′-DMT-dCp(iPr)G. Column: Superspher RP-18(e) (4 μm, 4 × 125 mm). Eluents: (A) 0.1 *M* TEAA (pH 7.0) and (B) 0.1 *M* TEAA–acetonitrile (50:50), linear gradient from 75 to 95% B in 40 min. Flow-rate: 0.5 ml/min. Temperature: 30°C. For peak identification, see text.

Under the same conditions, trinucleoside phosphoramidates [dAp(iPr)Tp(iPr)C and dTp(iPr)Cp(iPr)G] with a 5′-DMT group, which consist of four diastereoisomers, were separated.

As depicted in Fig. 3, four distinct, well separated peaks (I, II, III and IV) and baseline separations were achieved in each case. However, when the same chromatographic conditions were applied to the separation of eight diastereoisomers of dAp(iPr)Tp(iPr)Cp(iPr)G with a 5′-DMT group, the eight diastereoisomers could not be separated. For the optimization of the separation, the acetonitrile gradient profile was studied according to the procedure proposed previously [12,13]. The resulting chromatogram is shown in Fig. 4a, indicating that the separation of the diastereoisomers was not complete and that the baseline separation was not achieved. When the same sample was detritylated, six peaks appeared, as shown in Fig. 4b.

To separate all diastereoisomers, improvements in the chromatographic conditions were

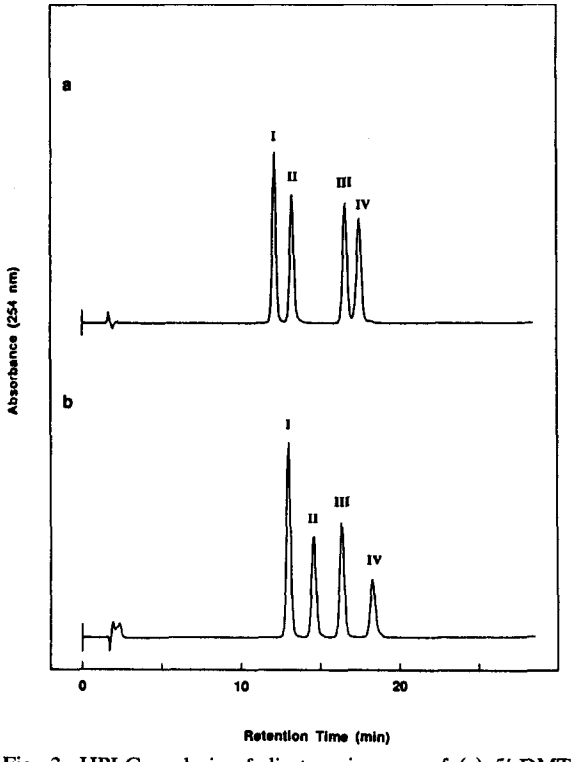

Fig. 3. HPLC analysis of diastereoisomers of (a) 5′-DMT-dAp(iPr)Tp(iPr)C and (b) 5′-DMT-dTp(iPr)Cp(iPr)G. Column: Superspher RP-18(e) (4 μm, 4 × 125 mm). Eluents: (A) 0.1 *M* TEAA (pH 7.0) and (B) 0.1 *M* TEAA–acetonitrile (50:50), linear gradient from 75 to 95% B in 40 min. Flow-rate: 0.5 ml/min. Temperature: 30°C. For peak identification, see text.

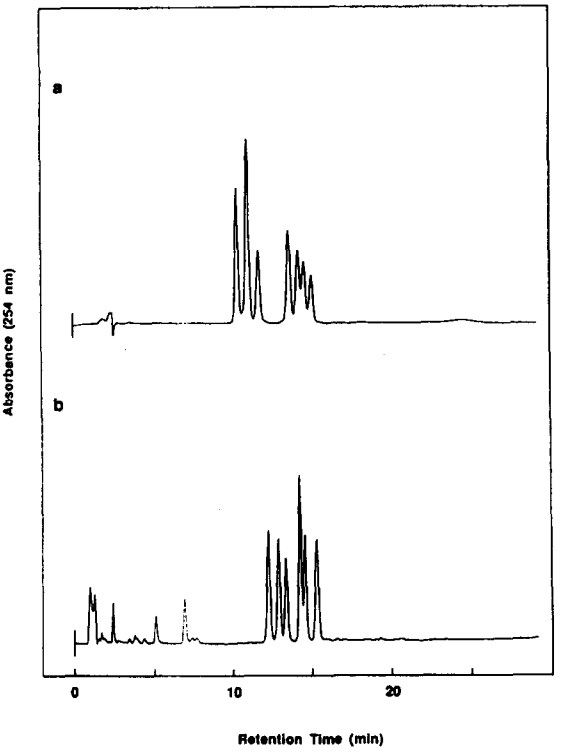

Fig. 4. HPLC analysis of diastereoisomers of (a) 5′-DMT-dAp(iPr)Tp(iPr)Cp(iPr)G, linear gradient from 75 to 95% B in 40 min at a flow-rate of 0.5 ml/min, and (b) dAp(iPr)Tp(iPr)Cp(iPr)G, linear gradient from 35 to 65% B in 30 min at a flow-rate of 1 ml/min. Column: Superspher RP-18(e) (4 μm, 4 × 125 mm). Eluents (A) 0.1 *M* TEAA (pH 7.0) and (B) 0.1 *M* TEAA–acetonitrile (50:50). Temperature: 30°C.

made by changing the column temperature from 30°C to 40°C (Fig. 5a and b) [14] and the column length from 125 mm to 250 mm (Fig. 5b and c). The chromatograms in Fig. 5 show that the separation was largely improved. However, in spite of such improvement, all eight diastereoisomers could not be separated, and baseline separation was not completely achieved. Based on these results, to isolate all eight diastereoisomers, the following protocol was used. First, the sample with a 5′-DMT group was loaded on a Superspher RP-18(e) column (4 μm, 4 × 250 mm) and the seven peaks, which were designated A to G in order of elution, as shown in Fig. 5c, were fractionated. Subsequently each fraction was treated with 80% acetic acid for detritylation, and applied to the second separa-

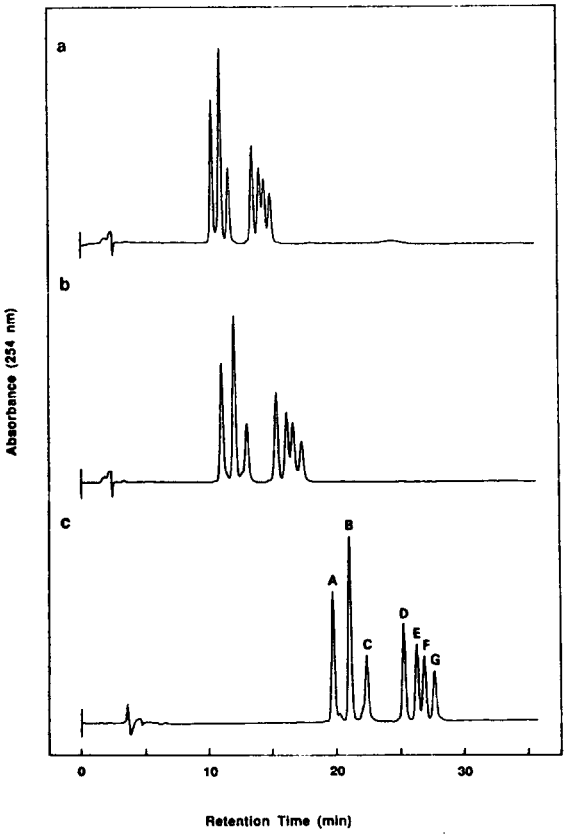

Fig. 5. HPLC analysis of diastereoisomers of (a) 5′-DMT-dAp(iPr)Tp(iPr)Cp(iPr)G on a Superspher RP-18(e) (4 μm, 4 × 125 mm) column and temperature 30°C, (b) 5′-DMT-dAp(iPr)Tp(iPr)Cp(iPr)G on a Superspher RP-18(e) (4 μm, 4 × 125 mm) column and temperature 40°C and (c) 5′-DMT-dAp(iPr)Tp(iPr)Cp(iPr)G on a Superspher RP-18(e) (4 μm, 4 × 250 mm) column and temperature 40°C. Eluents (A) 0.1 *M* TEAA (pH 7.0) and (B) 0.1 *M* TEAA–acetonitrile (50:50), linear gradient from 75 to 95% B in 40 min. Flow-rate: 0.5 ml/min. For peak identification, see text.

tion under the chromatographic conditions set for the detritylated samples. It was found that peak B in Fig. 5 contained two diastereoisomers (B′-1 and B′-2 in Fig. 6). Using this protocol, all eight diastereoisomers could be completely separated, as shown in Fig. 6, in which the peaks corresponding to A to G in Fig. 5c are designated A′ to G′, respectively. The reason why the diastereoisomers could be effectively separated is unclear. One possible explanation of the mechanism could be the effects due to the configuration and conformation of OPAs. The isopropyl moiety introduced into internucleotide linkages is hydrophobic and accounts for the hydrophobicity of OPAs. Also, the direction in which the alkyl moiety points might affect the conformation of OPAs. These effects may together affect the affinity of OPAs for the column packing.

CD measurement

The conformations of the eight diastereoisomers and their hybrids with their complementary ODNs were studied by CD spectroscopy [9,15].

We measured the temperature-dependent CD spectra of ODN (dApTpCpG) and eight diastereoisomers of dAp(iPr)Tp(iPr)Cp(iPr)G. Fig. 7 shows the CD spectra of eight diastereoisomers of dAp(iPr)Tp(iPr)Cp(iPr)G together with their parent ODNs (dApTpCpG) at 20°C. The CD spectra of the diastereoisomers were characteristic of right-handed DNAs and, in comparison with their parent ODNs, the intensities of molar ellipticity were small and different in each diastereoisomer. It was also observed that eight diastereoisomers can be classified into four groups (A′ and B′-1, B′-2 and C′, D′ and F′, E′ and G′).

Fig. 8 shows CD melting curves for the sense ODN (dCpGpApT)–antisense ODN (dApTpCpG) hybrid, the sense ODN–diastereoisomer A′ hybrid and the sense ODN–diastereoisomer G′ hybrid. The melting curve of the sense ODN–diastereoisomer A′ hybrid is similar in shape to that of the sense ODN–Antisense ODN hybrid, but the melting curve of the sense ODN–diastereoisomer G′ hybrid was broader than that of the sense ODN–Antisense ODN hybrid [16]. The shapes of the molar ellipticity *versus* temperature profiles allowed us to measure the transition temperature. The data clearly show that some diastereoisomers showed distinct transition temperatures and that some did not. Though the detailed mechanism is now under investigation, it is considered that alkyl moieties might stabilize or disrupt the hybrid of OPAs with ODNs depending on their configurations.

CONCLUSIONS

It has been found conclusively that diastereoisomers of OPAs up to tetramers can be

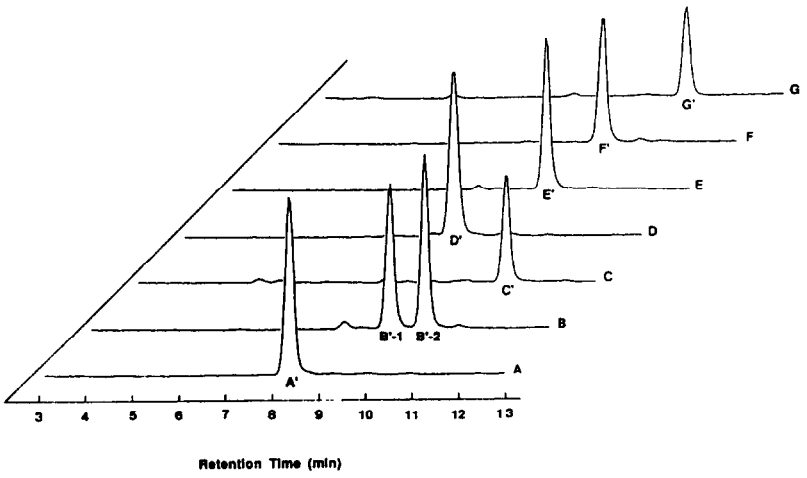

Fig. 6. HPLC analysis of diastereoisomers of d(Ap(iPr)Tp(iPr)Cp(iPr)G). Column: Superspher RP-18(e) (4 μm, 4 × 125 mm). Eluents (A) 0.1 *M* TEAA (pH 7.0) and (B) 0.1 *M* TEAA–acetonitrile (50:50), linear gradient from 35 to 65% B in 30 min. Flow-rate: 1.0 ml/min. Temperature: 40°C. For peak identification, see text.

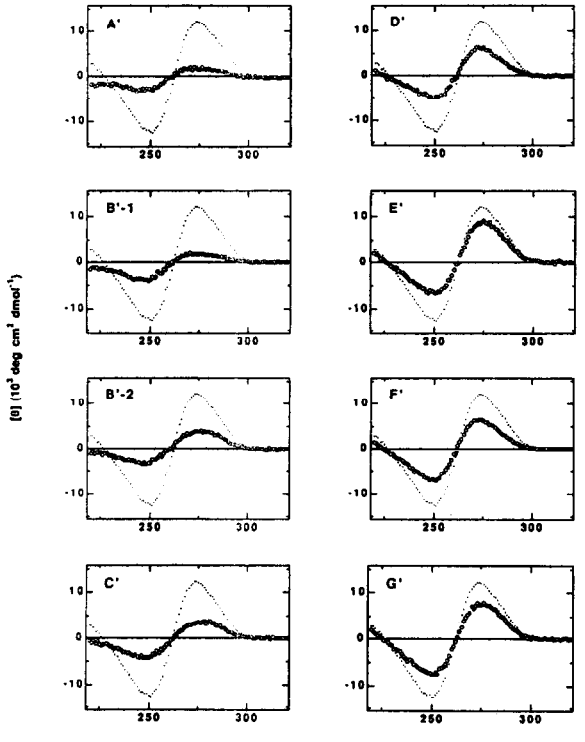

Fig. 7. CD spectra of eight diastereoisomers of dAp(iPr)Tp(iPr)Cp(iPr)G (open circles) and their parent ODN (dApTpCpG) (closed circles) in 10 m*M* sodium phosphate (pH 7.0) at 20°C. The nucleotide concentration is $2.5 \cdot 10^{-5}$ *M*. For peak identification, see text.

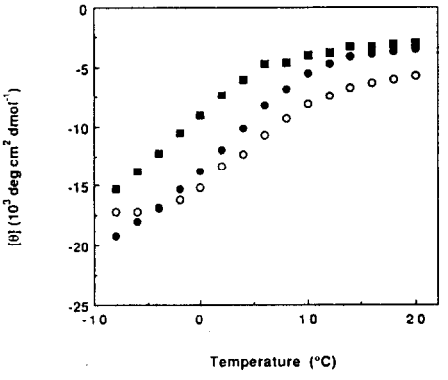

Fig. 8. CD melting curves of sense ODN–antisense ODN hybrid (open circles), sense ODN–diastereoisomer A' hybrid (closed circles), and sense ODN–diastereoisomer G' hybrid (closed squares) in 10 m*M* sodium phosphate (pH 7.0), 3 *M* NaCl. The total nucleotide concentration is $2.5 \cdot 10^{-4}$ *M*.

separated by employing the optimized chromatographic conditions of reversed-phase HPLC. Also, from the CD measurements of the diastereoisomers, it was found that each diastereoisomer shows different conformation in solution and different abilities to form hybrids as antisense DNA.

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