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## Synthesis and Configurational Analysis of Diastereomers of 5'-O-(2'-Deoxyadenosyl)-3'-O-(2'-deoxyadenosyl)-Phosphorothioate

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A procedure is described for the synthesis of the title compound *via* phosphotriester intermediates. The preparation of *R<sub>p</sub>* and *S<sub>p</sub>* diastereomeric dinucleotide of d[Ap(S)A] was performed by the condensation of protected deoxyadenosine, 2,5-dichlorophenylphosphorodichloridothioate and 1-hydroxybenzotriazole in THF. Their designation of configuration at phosphorus as *R<sub>p</sub>* and *S<sub>p</sub>* follows from analysis of <sup>31</sup>P-NMR spectroscopy and reversed-phase HPLC and the stereospecificity in the hydrolysis catalyzed by nuclease P1.

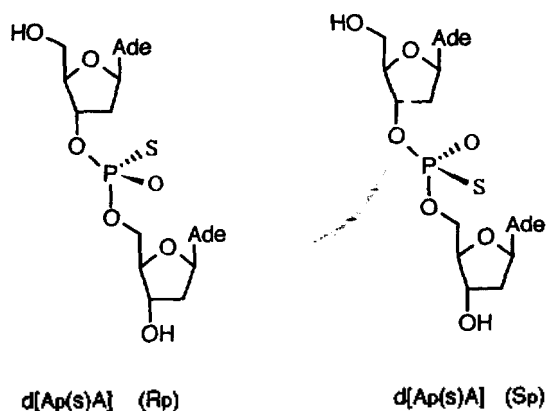
### Introduction

Restriction endonuclease catalyzed the cleavage of double-strand DNA specific sites. Although these enzyme are immensely important in genetic engineering, little mechanistic information is available.<sup>1</sup> Diastereomeric phosphorothioate analogues of nucleotides in which a nonbridging oxygen atom of a phosphate group is replaced by sulfur atom are important tools for the investigation of the stereospecificity as well as of the stereochemistry of action of these enzymes. For instance, the stereochemical course of action of EcoRI has been established by an oligonucleotide containing the appropriate recognition sequence with a phosphorothioate internucleotidic linkage of known absolute configuration.<sup>2,3</sup> In a studying for mechanism of action of restriction endonuclease Hind III, we need the oligonucleotide d[Ap(s)AGCTT] which is the recognition sequence for Hind III and contains a phosphorothioate group at the cleavage site.

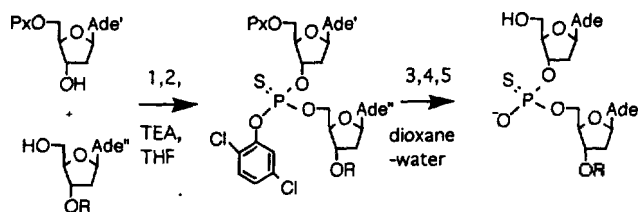
In this paper we described the synthesis, separation and configurational analysis of diastereomeric dinucleotide phosphorothioates, 5'-O-(2'-deoxyadenosyl)-3'-O-(2'-deoxyadenosyl)-phosphorothioate (d[Ap(s)A]) (Figure 1).

### Result and Discussion

The desired phosphorylating agent, 2,5-dichlorophenylphosphorodichloridothioate was prepared following modifications of the reported procedure<sup>4,5</sup> in 72% yield. Both diastereomers of phosphorothioate-containing d[Ap(s)A] dimer were prepared by a modification of Kemal's procedure<sup>6</sup> which uses phosphotriester approach leads to high yield. The d[Ap(s)A] dimer was prepared by condensing 6-N-pivaloyl-5'-O-pivaloyl-2'-deoxyadenosine and 6-N-pivaloyl-3'-O-[(*p*-chlorophenoxy)acetyl]-2'-deoxyadenosine using 2,5-dichlorophenylphosphorodichloridothioate and 1-hydroxybenzotriazole as condensing agent in THF (Scheme 1). A brief treatment with ammonia



**Figure 1.** Configuration of the  $S_p$  and  $R_p$  diastereomers of the dinucleoside phosphorothioates.

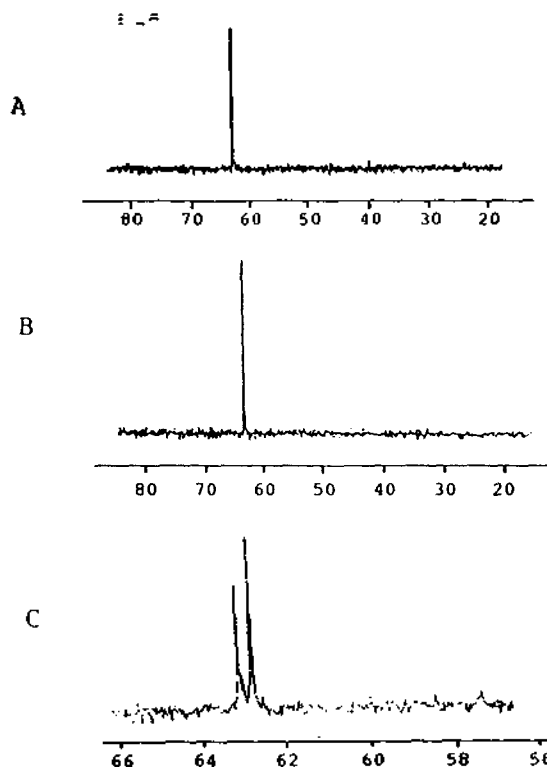


(1) 2,5-dichlorophenylphosphorodichloridithioate, (2) 1-hydroxybenzotriazole, (3) phenyldihydrogenphosphate, (4) *syn*-2-nitrobenzaloxime, (5) tetramethylguanidine, Ade': 6-N-pivaloyladenine, Ade'': 6-N-benzoyladenine.

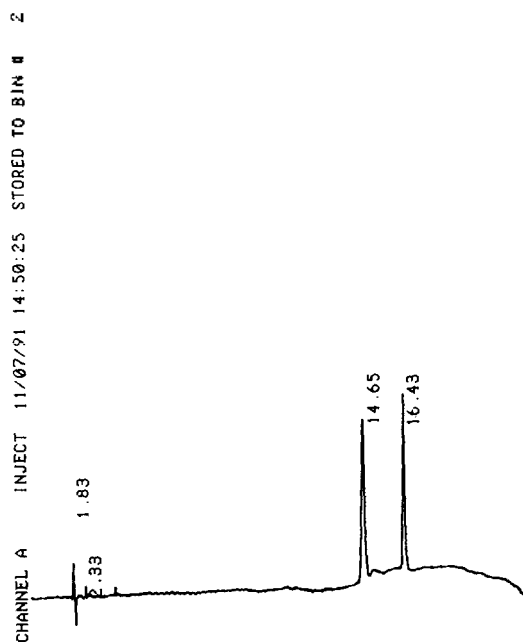
**Scheme 1.** Synthesis of protected diastereomeric dinucleoside phosphorothioate.

then removed the (*p*-chlorophenoxy)acetyl protecting group. Purification and separation of diastereomers were simultaneously achieved by short column chromatography over silica gel. It is important to note that before column chromatography removal of the (*p*-chlorophenoxy)acetyl protecting group was required. Otherwise separation of diastereomers was impractical. It is believed that the large and very hydrophobic character of the (*p*-chlorophenoxy)acetyl protecting group makes the separation difficult. The diastereomers were checked with high performance thin layer chromatography (HPTLC) plates because the difference in  $R_f$  values of two diastereomers were too small to be separated with regular TLC plates. Short column chromatography afforded the pure higher  $R_f$  [0.350,  $CHCl_3$ -EtOH(95:5 v/v)] diastereomer in 27% isolated yield and the pure lower  $R_f$  (0.345) diastereomer in 18% isolated yield.  $^{31}P$ -NMR spectroscopy of the diastereomers at 25°C in  $CDCl_3$  showed resonance at 63.07 and 62.76 ppm, respectively (Figure 2).

The absolute configuration at phosphorus of the two fractions was established by  $^{31}P$ -NMR spectroscopy and reversed-phase HPLC analysis after the complete deblocking of two diastereomers. The diastereomers were treated first with *syn*-2-nitrobenzaloxime and  $N^1, N^1, N^3, N^3$ -tetramethylguanidine in dioxane-water at room temperature to unblock the internucleotide linkage and then with aqueous ammonia to remove pivaloyl groups.  $^{31}P$ -NMR spectroscopy of the resulting fully unblocked  $d[Ap(s)A]$  showed resonance at 56.26 and 55.68 ppm, respectively. Since it is known that the  $S_p$ ,



**Figure 2.**  $^{31}P$ -NMR spectra of the diastereomers of  $d[Ap(s)A]$ : (A)  $(R_p)$ - $d[Ap(s)A]$  (63.07 ppm), (B)  $(S_p)$ - $d[Ap(s)A]$  (62.76 ppm) and (C) a mixture of  $(R_p)$ - and  $(S_p)$ - $d[Ap(s)A]$ .



**Figure 3.** HPLC analysis of a mixture of dinucleoside phosphorothioates:  $(R_p)$ - $d[Ap(s)A]$  ( $R_f$ , 14.65 min) and  $(S_p)$ - $d[Ap(s)A]$  ( $R_f$ , 16.43 min).

diastereomer of dinucleoside phosphorothioates resonates at higher field than the  $R_p$  diastereomer,<sup>7,8</sup> this established the higher  $R_f$  contained the diastereomer with the  $S_p$  configuration and the lower  $R_f$  the one with  $R_p$  configuration. Confirmation of this results comes from reversed-phase HPLC

**Table 1.** Analytical Data on the Protected and Unprotected Dinucleoside Phosphorothioate

	<sup>31</sup> P-NMR (ppm)	R <sub>f</sub>	R <sub>T</sub> (min) <sup>d</sup>	nuclease P1d igestion	configu- ration
protected d[Ap(s)A]	63.07 <sup>a</sup>	0.35	-	-	R <sub>p</sub>
protected d[Ap(s)A]	62.76 <sup>a</sup>	0.34	-	-	S <sub>p</sub>
d[Ap(s)A]	56.24 <sup>b</sup>		14.62	not cleaved	R <sub>p</sub>
d[Ap(s)A]	55.68 <sup>b</sup>		16.40	cleaved	S <sub>p</sub>
d[Gp(s)C] <sup>c</sup>	55.59		11.2	not cleaved	R <sub>p</sub>
d[Gp(s)C] <sup>c</sup>	55.13		12.7	cleaved	S <sub>p</sub>

<sup>a</sup>Measured in CDCl<sub>3</sub>. <sup>b</sup>Measured in 1 M TEAB buffer. <sup>c</sup>HPTLC plate, solvent, 5% Methanol in CHCl<sub>3</sub>. <sup>d</sup>HPLC retention time in gradient I. <sup>e</sup>Data taken from reference 11.

analysis of the unblocked mixture in which the higher R<sub>f</sub> diastereomer elutes before the lower one (Figure 3). Again the R<sub>p</sub> diastereomer of dinucleoside phosphorothioates is known to elute before the S<sub>p</sub> in reversed-phase HPLC system.<sup>7,8</sup> The above results were confirmed by studying the hydrolysis of these diastereomers catalyzed by nuclease P1, which is known to hydrolyzes dinucleoside phosphorothioate of the S<sub>p</sub> but not the R<sub>p</sub> configuration.<sup>9</sup> The lower R<sub>f</sub> diastereomer in the HPLC was completely digested by nuclease P1 and higher R<sub>f</sub> one was not hydrolyzed by this enzyme. The results presented in Table 1 clearly demonstrate that the fast-moving isomer (higher R<sub>f</sub>) of triester corresponds to the dinucleoside phosphorothioate of the R<sub>p</sub> configuration, and the slow-moving (lower R<sub>f</sub>) to that of the S<sub>p</sub> configuration (Figure 1). The protected optically pure (R<sub>p</sub>) diastereomer was used to synthesis of hexanucleotide d[Ap(s)AGCTT] which is the recognition sequence for Hind III. The synthesis and characterization of the d[Ap(s)AGCTT] will be reported separately.

## Experimentals

Nucleosides were obtained from Sigma Chemical company. Pyridine, triethylamine and tetrahydrofuran were distilled over calcium hydride. Dioxane was distilled from Na. 2,5-Dichlorophenol, PCl<sub>3</sub> and PSCl<sub>3</sub> were purchased from Fluka Chemical Co. (Switzerland). Nuclease P1 (from penicillium citrinum, 300 units/mg) was purchased from Boehringer Mannheim (Germany). Thin layer chromatography was performed with Merck HPTLC plates that were eluted with chloroform methanol mixture. Merck Kiesel 60 H was used for column chromatography. The <sup>1</sup>H-NMR and <sup>31</sup>P-NMR spectra were measured with a Bruker 300-MHz spectrometer. An Applied Biosystem HPLC system equipped with an absorbance detector (model 783A) and with gradient pump system (model 400) was employed. In all cases, the reverse-phase Jones APEX ODS column (250×4.6 mm) was utilized. Two buffer systems were used. To purify completely deblocked diastereomers, a linear gradient (flow rate 1.5 ml/min) consisting of 0.1 M triethylammonium acetate (TEAA), pH 7.0 (A) and 0.1 M TEAA, pH 7.0, containing 60% CH<sub>3</sub>CN (B) was used (t=0 min, 14% B; t=20 min, 40%) (gradient D). To resolve nuclease P1 digestion products of the diastereomers, linear gradient (flow rate 1.5 ml/min) prepared

from 50 mM KH<sub>2</sub>PO<sub>4</sub>, pH 6.0 (A) and 50 mM KH<sub>2</sub>PO<sub>4</sub>, pH 6.0, containing 30% CH<sub>3</sub>CN (B) was used (t=0 min, 0% B; t=15 min, 50% B) (gradient II).

### 2,5-Dichlorophenylphosphorodichloridothioate.

Following the reported procedure,<sup>4,5</sup> the desired phosphorylating agent, 2,5-dichlorophenylphosphorodichloridothioate was prepared by heating 2,5-dichlorophenylphosphorodichloridite, thiophosphoryl chloride, and sulfur in the presence of activated charcoal in 72% yield: bp. 160-200°C at 100 mmHg; <sup>31</sup>P-NMR (CDCl<sub>3</sub>), 51.6 ppm.

**Protected 2'-Deoxyadenosine.** 6-N-Trimethylacetyl-2'-deoxyadenosine and 6-N-benzoyl-2'-deoxyadenosine were synthesized using a modification of the reported procedure.<sup>9</sup> 5'-O-(9-Phenyl-9-H-xanthen-9-yl)-6-N-trimethylacetyl-2'-deoxyadenosine was synthesized followed the reported procedure.<sup>10</sup> 6-N-Benzoyl-3'-O[(p-chlorophenoxy)acetyl]-2'-deoxyadenosine was synthesized followed the reported procedure.<sup>2</sup>

**Synthesis of Protected R<sub>p</sub>- and S<sub>p</sub>- Diastereomers of d[Ap(s)A].** Following the reported procedure,<sup>5</sup> the phosphorylating agent (3 mmol) was first allowed to react with 1-hydroxybenzotriazole (6.5 mmol) and triethylamine (6 mmol) in tetrahydrofuran (4.8 ml) at room temperature. After 20 min, 5'-O-pixyl-6-N-trimethylacetyl-2'-deoxyadenosine (2 mmol) and dry pyridine (4 ml) were added to the resulting products. After a further period of 75 min, 3'-(4-chlorophenoxy)acetyl-6-N-benzoyl-2'-deoxyadenosine (2 mmol) and pyridine (4 ml) were added and ensuing reaction was allowed to proceed for 8 h before it was worked up. The saturated NaHCO<sub>3</sub> solution (4 ml) was added to the reaction mixture and the mixture was stirred for 10 min and extracted with chloroform (2×70 ml). Chloroform layer was washed with triethylammonium bicarbonate (TEAB) buffer solution (0.1 M, pH 7.5). The organic layer was dried (MgSO<sub>4</sub>) and evaporated under reduced pressure. The 3'-O-(4-chlorophenoxy)acetyl protecting group was removed by dissolving the product in dioxane (50 ml) and 25% aqueous ammonium solution (15 ml) and by stirring at room temperature for 70 min. The final product was purified by short column chromatography over silica gel eluting with ethanol in chloroform. Fractions of 10 ml were collected and analyzed by HPTLC plates. Fractions of 45-65 (high R<sub>f</sub> diastereomer) and 68-85 (low R<sub>f</sub> diastereomer) were pooled. Both diastereomers appeared >95% in HPTLC. <sup>1</sup>H-NMR (DMSO-d<sub>6</sub>) δ 1.28 (m, 9H, (CH<sub>3</sub>)<sub>3</sub>C), 2.65-2.76 (m, 1H, 2'H), 2.88-2.98 (m, 1H, 2'H), 3.02-3.08 (m, 1H, 2'H), 3.22-3.40 (m, 1H, 2'H), 4.17-4.23 (m, 1H, 4'H), 4.30-4.36 (m, 1H, 4'H), 4.47-4.56 (m, 1H, 3'H), 4.58-4.69 (m, 1H, 3'H), 5.58 (d, 1H, OH), 6.45 (t, 1H, 1'H), 6.57 (t, 1H, 1'H), 6.86-8.50 (m, 21H), 8.45 (s, 2H, 8H), 8.57 (s, 1H, 2H), 8.67 (s, 1H, 2H), 10.02 (bs, 1H, NH), 11.06 (bs, 1H, NH). Anal. Calcd for C<sub>50</sub>H<sub>51</sub>N<sub>10</sub>O<sub>10</sub>P<sub>1</sub>S<sub>1</sub>C<sub>12</sub>. 2H<sub>2</sub>O: C, 57.23; H, 4.64; N, 11.71. Found: C, 57.74; H, 4.52; N, 11.95.

**Unblocking and Purification of the Fully Protected Diastereomers.** A solution of the protected diastereomer (0.0183 g, 0.02 mmol), syn-2-nitrobenzaldehyde (0.033 g, 0.20 mmol) and N<sup>1</sup>,N<sup>1</sup>,N<sup>3</sup>,N<sup>3</sup>-tetramethylguanidine (0.023 ml, 0.18 mmol) in dioxane-water (1:1 v/v, 0.6 ml) was stirred at room temperature.<sup>6,12</sup> After 18 h, the reaction mixture was concentrated under reduced pressure and the residue was redissolved in aqueous ammonia (d, 0.88, 5 ml) and stirred overnight. After evaporation to film, 10 ml of water was added to it and bubbled CO<sub>2</sub> to adjust pH to 5. Aqueous solu-

tion was washed with  $\text{CHCl}_3$  and ether. The aqueous layer was evaporated. The unblocked material was passed through a Sephadex G-15 and was chromatographed on DEAE-Sephadex A-25. The Column was eluted with TEAB buffer (pH 7.5, linear gradient 0.001-1.0 M). Analytical details are given in Table 1.

**Enzymatic hydrolysis.** 0.025 M Tris-HCl buffer (pH 7.0, 0.2 ml) and solution of nuclease PI (3.5  $\mu\text{g}$ ) in the same buffer (0.02 ml) were added to a solution of the diastereomer (0.2 mg) in water (0.01 ml). The resulting solution was maintained for 2 h at 37°C. The products were analyzed by reversed-phase HPLC (gradient II).

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## Theoretical Study of the Cobalt Substituting Site in the Framework of $\text{AlPO}_4\text{-5}$ Molecular Sieves

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In order to determine the cobalt substituting site in  $\text{AlPO}_4\text{-5}$  framework, ASED-MO theory has been used. The substitution of cobalt for aluminum is energetically more favorable than that for phosphorous. The stabilized energy of the former is 51 eV lower than that of the latter. The calculated net charge was +1.27 for Al, +0.85 for P, and +1.56 for Co, respectively. The valence electron population (VEP), reduced overlap population (ROP) and net charge for the charged cluster models were compared for  $\text{AlPO}_4\text{-5}$  and  $\text{CoAlPO}_4\text{-5}$  systems. Then, we find that the covalency of P-O bond was greater than that of Al-O bond.

### Introduction

The crystalline molecular sieves having porous frameworks of zeolite type ( $\text{A}^{\text{III}}\text{B}^{\text{V}}\text{O}_4$ ) are industrially important as acid site, reagent for separation, ion exchanger, catalyst and catalyst support.<sup>1,2</sup>

Aluminophosphate frameworks<sup>3-6</sup> were synthesized by Wilson and coworkers. They thought that their frameworks are strict alternation of phosphorus and aluminum tetrahedra. Since many of the industrially important hydrocarbon conversion reactions require acidic catalysts,<sup>7</sup> Shiralkar and coworkers<sup>8</sup> studied on some of the porous aluminophosphates that contain isomorphous substitution in framework of  $\text{M}^{2+}$  for  $\text{Al}^{3+}$ .  $\text{AlPO}_4\text{-5}$  structure identified among aluminophosphate molecular sieves has a unidirectional pore system consisting of cylindrical channels with large pore opening of 8 Å, bounded by a 12 membered-oxygen ring system. It possesses a hexagonal crystal symmetry with  $a \approx 13.7$  Å and  $c \approx 8.5$  Å.

The isomorphous substitution<sup>8</sup> in  $\text{AlPO}_4\text{-5}$  framework inve-

stigated by X-ray diffraction, scanning electron microscopy, Mössbauer spectroscopy and Fourier transform infrared spectroscopy. However, it is uncertain whether the substitution of cobalt takes place in aluminum or phosphorus site.<sup>8</sup>

This paper investigates the substituting site of Co for Al or P and calculate the net charge of Al, P, Co, and O in  $\text{AlPO}_4\text{-5}$  and  $\text{CoAlPO}_4\text{-5}$  framework. The net charge were calculated using the atom superposition and electron delocalization molecular orbital (ASED-MO) theory<sup>10-15</sup> and backbone model.

### Theoretical Method

The atom superposition and electron delocalization molecular orbital theory (ASED-MO) used in past studies<sup>10-15</sup> is a semi-empirical theory for deriving molecular structures, force constants, bond strengths, electronic spectra and orbitals starting with experimental atomic valence ionization potentials and corresponding Slater orbitals. This theory identifies two energy components for the chemical bond formation.