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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_p and S_p 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_p and S_p follows from analysis of ^{31}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.[1] Diasteromeric 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.[2,3] 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 cofigurational 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 phophorylating agent, 2,5-dichlorophenylphosphorodichloridothioate was prepared following modifications of the reported procedure[4,5] in 72% yield. Both diastereomers of phosphorothioate-containing d[Ap(s)A] dimer were prepared by a modification of Kemal's procedure[6] which uses phosphotriester approach leads to high yield. The d[Ap(s)A] dimer was prepared by condensing 6-N-pivaloyl-5′-O-pixyl-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

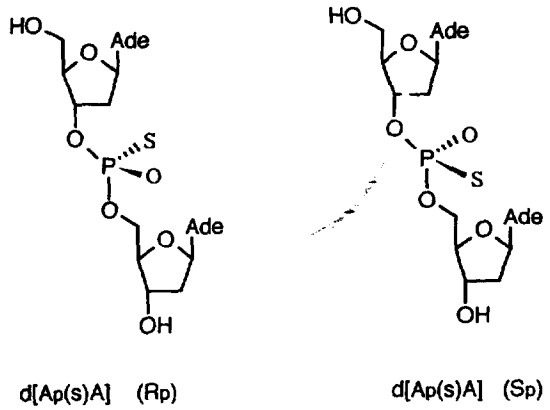
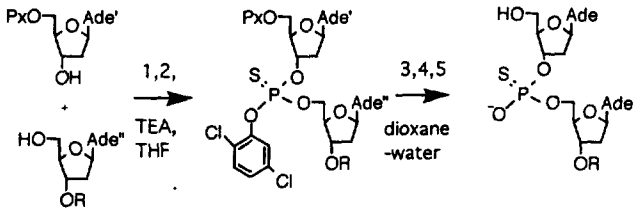


Figure 1. Configuration of the S_p and R_p diastereomers of the dinucleoside phosphorothioates.



(1) 2,5-dichlorophenylphosphorodichloridothioate, (2) 1-hydroxybenzotriazole, (3) phenyldihydrogenphosphate, (4) syn-2-nitrobenzaldoxime, (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 removel 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℃ 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 diasteremers. The diastereomers were treated first with syn-2-nitrobenzaldoxime 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 diastereomer of dinucleoside phosphorothioates resonates at higher field than the R_p diastereomer,[7,8] 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

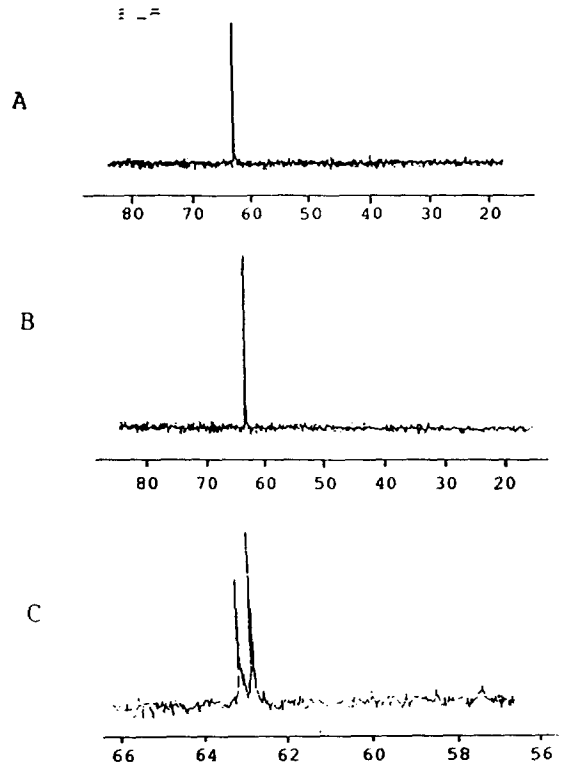


Figure 2. ^{31}P-NMR spectra of the diastereomers of d[Ap(s)A]: (A) (R_p)-d[Ap(s)A] (63.07 ppm), (B) (S_p)-d[Ap(s)A] (62.76 ppm) and (C) a mixture of (R_p)- and (S_p)-d[Ap(s)A].

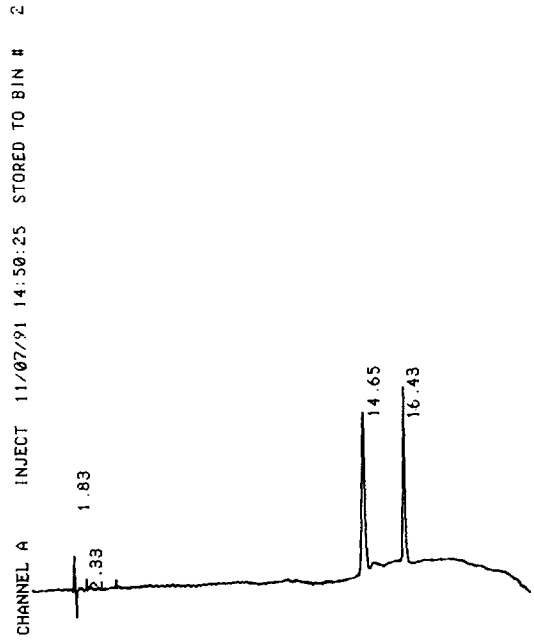


Figure 3. HPLC analysis of a mixture of dinucleoside phosphorothioates: (R_p)-d[Ap(s)A] (R_T, 14.65 min) and (S_p)-d[Ap(s)A] (R_T, 16.43 min).

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

	^{31}P-NMR (ppm)	R_f[c]	R_T (min)[d]	nuclease P1d igestion	configuration
protected *d*[Ap(s)A]	63.07[a]	0.35	-	-	R_p
protected *d*[Ap(s)A]	62.76[a]	0.34	-	-	S_p
d[Ap(s)A]	56.24[b]		14.62	not cleaved	R_p
d[Ap(s)A]	55.68[b]		16.40	cleaved	S_p
d[Gp(s)C][e]	55.59		11.2	not cleaved	R_p
d[Gp(s)C][e]	55.13		12.7	cleaved	S_p

[a]Measured in $CDCl_3$. [b]Measured in 1 M TEAB buffer. [c]HPTLC plate, solvent, 5% Methanol in $CHCl_3$. [d]HPLC retention time in gradient I. [e]Data taken from reference 11.

analysis of the unblocked mixture in which the higher R_f diastereomer elutes before the lower one (Figure 3). Again the R_p diastereomer of dinucleoside phosphorothioates is known to elute before the S_p in reversed-phase HPLC system.[7,8] The above results were confirmed by studying the hydrolysis of these diastereomers catalyzed by nuclease P1, whcih is known to hydrolyzes dinucleoside phosphorothioate of the S_p but not the R_p configuration.[9] The lower R_f diastereomer in the HPLC was completely digested by nuclease P1 and higher R_f one was not hydrolyzed by this enzyme. The results presented in Table 1 clearly demonstrate that the fast-moving isomer (higher R_f) of triester corresponds to the dinucleoside phosphorothioate of the R_p configuration, and the slow-moving (lower R_f) to that of the S_p configuration (Figure 1). The protected optically pure (R_p) 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-Dichlophenol, PCl_3 and $PSCl_3$ 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 ^{1}H-NMR and ^{31}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 m*l*/min) consisting of 0.1 M triethylammonium acetate (TEAA), pH 7.0 (A) and 0.1 M TEAA, pH 7.0, containing 60% CH_3CN (B) was used (t=0 min, 14% B; t=20 min, 40%) (gradient I). To resolve nuclease P1 digestion products of the diastereomers, linear gradient (flow rate 1.5 m*l*/min) prepared from 50 mM KH_2PO_4, pH 6.0 (A) and 50 mM KH_2PO_4, pH 6.0, containing 30% CH_3CN (B) was used (t=0 min, 0% B; t=15 min, 50% B) (gradient II).

2,5-Dichlorophenylphosphorodichloridothioate. Following the reported procedure,[4,5] 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℃ at 100 mmHg; ^{31}P-NMR ($CDCl_3$), 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.[9] 5'-O-(9-Phenyl-9-H-xanthen-9-yl)-6-N-trimethylacetyl-2'-deoxyadenosine was synthesized followed the reported procedure.[10] 6-N-Benzoyl-3'-O[(p-chlorophenoxy)acetyl]-2'-deoxyadenosine was synthesized followed the reported procedure.[2]

Synthesis of Protected R_p- and S_p- Diasteromers of d[Ap(s)A]. Following the reported procedure,[5] the phosphorylating agent (3 mmol) was first allowed to react with 1-hydroxybenzotriazole (6.5 mmol) and triethylamine (6 mmol) in tetrahydrofuran (4.8 m*l*) at room temperature. After 20 min, 5'-O-pixyl-6-N-trimethylacetyl-2'-deoxyadenosine (2 mmol) and dry pyridine (4 m*l*) 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 m*l*) were added and ensuing reaction was allowed to proceed for 8 h before it was worked up. The saturated $NaHCO_3$ solution (4 m*l*) was added to the reaction mixture and the mixture was stirred for 10 min and extracted with chloroform (2×70 m*l*). Chloroform layer was washed with triethylammonium bicarbonate (TEAB) buffer solution (0.1 M, pH 7.5). The organic layer was dried ($MgSO_4$) and evaporated under reduced pressure. The 3'-O-(4-chlorophenoxy)acetyl protecting group was removed by dissolving the product in dioxane (50 m*l*) and 25% aqueous ammonium solution (15 m*l*) 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 m*l* were collected and analyzed by HPTLC plates. Fractions of 45-65 (high R_f diastereomer) and 68-85 (low R_f diastereomer) were pooled. Both diastereomers appeared >95% in HPTLC. ^{1}H-NMR (DMSO-d_6) δ 1.28 (m, 9H, $(CH_3)_3C$), 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 $C_{50}H_{51}N_{10}O_{10}P_1S_1C_{12}$. $2H_2O$: 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-nitrobenzaldoxime (0.033 g, 0.20 mmol) and N^1,N^1,N^3,N^3-tetramethylguanidine (0.023 m*l*, 0.18 mmol) in dioxane-water (1 : 1 v/v, 0.6 m*l*) was stirred at room temperature.[6,12]. After 18 h, the reaction mixture was concentrated under reduced pressure and the residue was redissolved in aqueous amonia (*d*, 0.88, 5 m*l*) and stirred overnight. After evaporation to film, 10 m*l* of water was added to it and bubbled CO_2 to adjust pH to 5. Aqueous solu-

tion was washed with $CHCl_3$ 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 m*l*) and solution of nuclease Pl (3.5 μg) in the same buffer (0.02 m*l*) were added to a solution of the diastereomer (0.2 mg) in water (0.01 m*l*). The resulting solution was maintained for 2 h at 37℃. 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 $AlPO_4$-5 Molecular Sieves

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In order to determine the cobalt substituting site in $AlPO_4$-5 framework, ASED-MO theory has been used. The substitution of cobalt for aluminum is energetically more favorable than that for phasphorous. 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 $AlPO_4$-5 and $CoAlPO_4$-5 systems. Then, twe 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 ($A^{III}B^{V}O_4$) are industrially important as acid site, reagent for separation, ion exchanger, catalyst and catalyst support.[1,2]

Aluminophosphate frameworks[3-6] 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,[7] Shiralkar and coworkers[8] studied on some of the porous aluminophosphates that contain isomorphous substitution in framework of M^{2+} for Al^{3+}. $AlPO_4$-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-oxigen ring system. It possesses a hexagonal crystal symmetry with $a \simeq 13.7$ Å and $c \simeq 8.5$ Å.

The isomorphous substitution[8] in $AlPO_4$-5 framework investigated 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.[8]

This paper investigates the substituting site of Co for Al or P and calculate the net charge of Al, P, Co, and O in $AlPO_4$-5 and $CoAlPO_4$-5 framework. The net charge were calculated using the atom superposition and electron delocalization molecular orbital (ASED-MO) theory[10-15] and backbone model.

Theoretical Method

The atom superposition and electron delocalization molecular orbital theory (ASED-MO) used in past studies[10-15] 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.