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SYNTHESIS, ISOLATION AND CHARACTERIZATION OF DIASTEREOCHEMICALLY PURE DITHYMIDINE PHOSPHORMORPHOLIDATE DERIVATIVES

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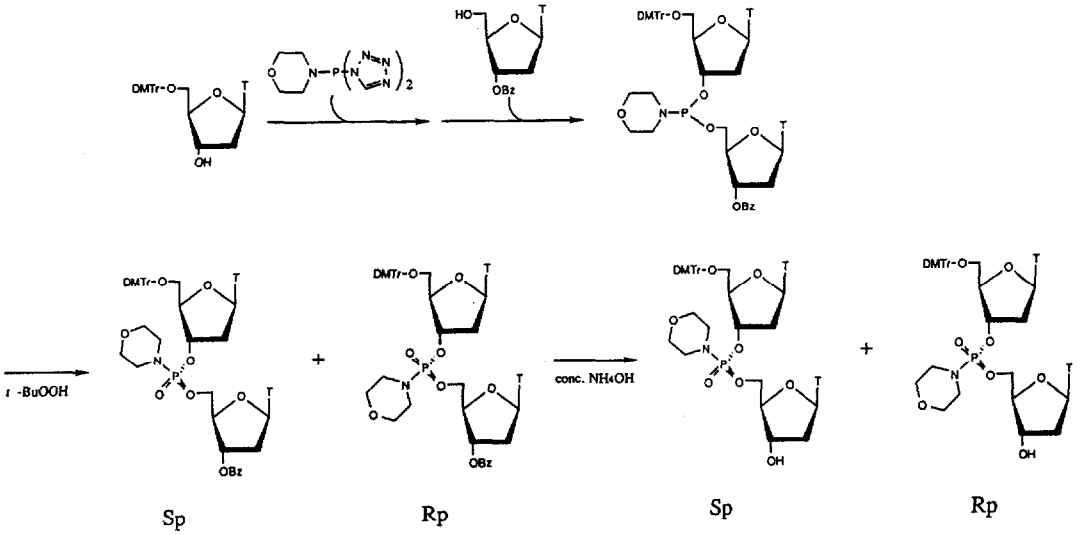
Abstract: 5'-O-Dimethoxytrityl dithymidine phosphormorpholidate has been synthesized by one-pot procedure. The chirally pure dithymidine phosphormorpholidate derivatives were isolated by chromatographic separation. The configuration of each diastereoisomer was tentatively characterized on the basis of proton NMR studies.

Oligonucleotide analogs containing modified backbone such as methylphosphonate[1)], phosphorothioate[2)], and phosphoramidate[3)] have been receiving increasing attention because of their potential as gene regulatory substances[4)]. The modified back-bone can be introduced into oligonucleotides through routes based on the phosphotriester[5)], phosphite[6)] and hydrogen phosphonate[3)] methods of oligonucleotide synthesis. These modifications result in the formation of diastereoisomers that differ in configuration about the phosphorus atom. Several reports have shown that oligonucleotides possessing diastereochemically pure phosphorothioate linkages can be used to study enzymatic cleavage of phosphodiester bonds[7)], and conformational properties of DNA[8)]. However, diastereochemically pure oligonucleotide analogs having phosphoramidate linkages have not been used for these studies. More importantly, it is speculated that oligonucleotide analogs possessing chirally pure modified backbone would have increased potential as gene regulatory substances[4)].

We have initiated the synthesis of such oligodeoxyribonucleotides through dimer couplings of pre-separated diastereochemically pure dideoxyribonucleoside phosphoramidate derivatives. We have already developed the method for generating phosphormorpholidate linkages by the phosphite approach using morpholinophosphorditetrazolide as a selective phosphitylating reagent[9)]. The procedure of our method is quite simple; i)phosphitylation of 5'-O-dimethoxytrityldeoxyribonucleoside with the phosphitylating reagent, ii)coupling of the phosphitylated deoxyribonucleoside with deoxyribonucleoside, and iii)non-aqueous oxidation

of the resulting amidite by using tert-butyl hydroperoxide, which can be carried out in one flask. Additionally, the resulting phosphormorpholidate linkage was found to be stable both under acidic and basic conditions, and therefore, the dideoxyribonucleoside phosphormorpholidate derivatives can be used in the block condensation to obtain oligodeoxyribonucleotides having phosphormorpholidate linkages by a combination of usual phosphoramidite or phosphorbisamidite methods[10].

In this communication, we describe the synthesis, isolation and characterization of diastereochemically pure 5'-O-dimethoxytrityl dithymidine phosphormorpholidate.



A dithymidine phosphormorpholidate derivative was synthesized from 5'-O-dimethoxytritylthymidine (DMTr-T) and 3'-O-benzoylthymidine (Tbz) by the previously described procedure[9] as shown in the scheme. A solution of DMTr-T (1.089 g, 2 mmol) in dry CH_2Cl_2 was added dropwise to morpholino-phosphorditetrazolide (2.2 mmol) in dry CH_2Cl_2-pyridine at 0°C under Ar atmosphere. After the reaction mixture was stirred for 35 min, a solution of Tbz (0.693 g, 2 mmol) in dry CH_2Cl_2-pyridine containing diisopropyl-ethylamine (4.5 mmol) was added to the reaction mixture. The mixture was stirred for an additional 100 min. The reaction mixture was oxidized with 3 M tert-butyl hydroperoxide in 2,2,4-trimethylpentane for 10 min at 0°C and then evaporated to dryness. Two diastereoisomer can be separated by silica gel column chromatography (eluent; ethyl acetate-methanol, 97:3, v/v). Rf on silica gel TLC (ethyl acetate-methanol, 95:5, v/v); 0.34 and 0.42. The benzoyl group of the isolated product was removed using conc. NH_4OH (8 hr., room temperature). Each deprotected product was purified by silica gel

column chromatography (chloroform-methanol-triethylamine, 89:10:1, v/v/v). Isolated yields: isomer I, 260 mg (14.2 %); isomer II, 392 mg (21.4 %). Retention time on reversed-phase HPLC (Cosmosil 5C18, 70% methanol in water, 1mL/min): isomer I, 6.4 min; isomer II, 7.9 min. Isomer I was faster eluted in reversed-phase HPLC and isomer II was later eluted. Interestingly the elution order was reversed by detritylation (80 % acetic acid, room temperature, 1.5 hr.) of the each isomer. Retention time on reversed-phase HPLC (Cosmosil 5C18, 12% acetonitrile in 0.1 M triethylammonium acetate aq. solution (pH 7.0), 1.0 mL/min): detritylated isomer I, 6.15 min; detritylated isomer II, 5.80 min. This property is the same as oligonucleotide analogs having phosphorothioate linkages[11].

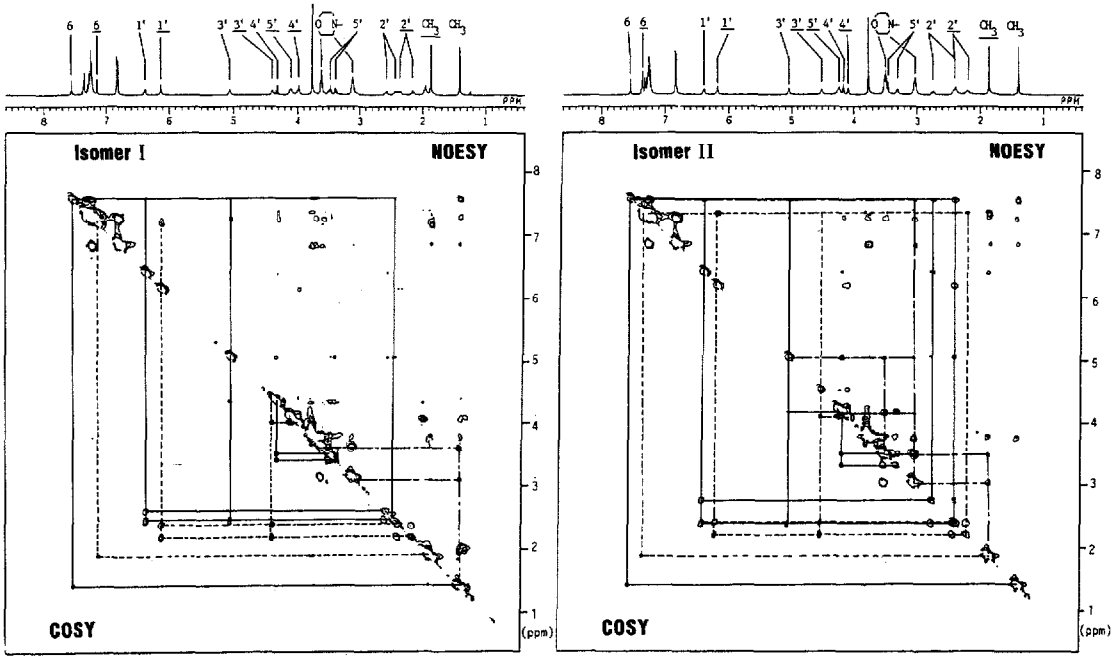


Figure 400 MHz 1D, 2D-COSY and NOESY proton NMR spectra of isomers of 5'-O-dimethoxytrityl dithymidine phosphormorpholidate in $CDCl_3$. The underlined protons were contained in 3'-end thymidine. Mixing time of NOESY measurement was 800 ms.

Chirally pure 5'-O-dimethoxytrityl dithymidine phosphormorpholidates (DMTr-T_3'p_5'T), which were further purified by reversed-phase HPLC, were characterized by proton NMR study. Proton NMR spectra of two isomers were depicted in the Figure. The assignment of each proton resonance in two diastereoisomer was carried out by COSY and NOESY techniques[12]. The spin systems of the deoxyriboses (H_1', H_2', H_3', H_4', and H_5') and thymine (H_6 and methyl) were identified by spin-spin couplings with the COSY cross peaks. Sequential resonance assignments within the individual spin systems

were completely achieved by NOESY, that is to say, NOESY cross peaks between deoxyribose proton ($H_{1'}$) and base proton (H_6) or deoxyribose protons ($H_{3'}$ and $H_{4'}$) and aromatic protons of dimethoxytrityl group. The results of assignments are shown in the Figure.

A syn/anti orientation about the glycosyl bond was investigated by the NOESY experiments. In isomer I, H_6 in DMTr-T_3'p- part interacts to $H_{1'}$, $H_{2'}$, and $H_{3'}$ in equal intensity. This result suggests that the thymidine unit (DMTr-T_3'p-) has anti conformation. Since $H_{6'}$ in -p_5'T component show the stronger correlation with $H_{1'}$ in -p_5'T, syn conformation dominates over anti one. On the other hand, the same analysis for isomer II suggests that the conformations about the glycosyl bond in both thymidines are anti form rather than syn one since the correlation of H_6 with $H_{1'}$, $H_{2'}$ and $H_{3'}$ in each nucleoside component was seen.

Additionally the NOESY cross peaks between morpholino proton and CH_3- on DMTr-T_3'p- component were seen in isomer I. However, another isomer (isomer II) did not show the same NOESY cross peaks. In the NOESY analysis of isomer II, the proton of morpholino group were correlated with aromatic protons, H_6, $H_{3'}$ and $H_{4'}$ in DMTr-T_3'p- component and CH_3- in -p_5'T. Based on the above NMR data, Corey-Pauling-Koltun (CPK) models were built[13]. These results may suggest that the absolute configurations of isomer I and isomer II are Rp- and Sp-configuration, respectively. In order to confirm the absolute configurations of those isomers, further studies involving X-ray crystallographic analysis are in progress. The investigation of chemical and biochemical properties of oligodeoxyribonucleotides possessing stereoregular phosphormorpholidate linkage in connection with gene regulatory substances is also under way in our laboratory.

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