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Novel DNA Analog for Potential Gene Regulating Agent. A Convenient Synthesis of α-Oligodeoxyribonucleotide Phosphorothioate Bearing 3'-Monophosphate

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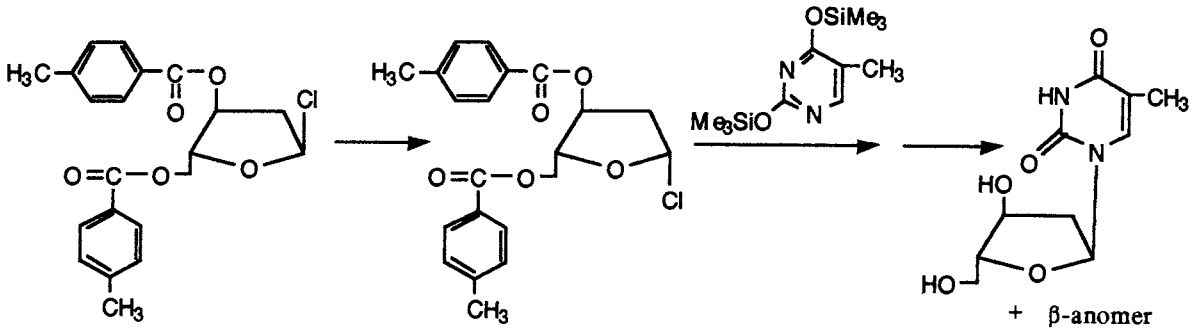
α-2'-Deoxyoctathymydilic acid phosphorothioate analog bearing 3'-monophosphate was conveniently synthesized *via* phosphoramidite method using a riboadenosine attached Teflon-based solid support. The obtained oligomer exhibited enhanced stability toward the digestion by common nucleases.

In recent years, there has been a growing attention to utilize synthetic oligonucleotide analogs as new class of gene regulating agents.[1] Such oligonucleotide analogs are expected to hybridize with their complementary mRNA, thus interfere gene expression(antisense mechanism).[1] A good antisense agent should owe strong nuclease resistant property to maintain its biological activity for substantial period in living organisms. Both β-DNA phosphrothioate analog[1b,1c] and α-DNA,[2] which are chemically synthesized unnatural analogs of DNA, showed high water solubility and enhanced stability toward the action of common nucleases.[3,4] Furthermore, β-DNA phosphorothioate possesses high cell permeability.[1b] We, therefore, expected that a phosphorothioate analog of α-DNA will make a good candidate of a novel gene controlling agent, and developed its convenient synthesis using Tefron-based solid support. Nuclease resistant property of this new DNA analog is also to be reported.

The starting material, α-2'-deoxythymidine[5] was prepared by the modified Hilbert-Johnson method using bis-silylated thymine and 1-chloro-2-deoxy-3,5-di-O-*p*-toluoylribofuranose[6] as shown in Scheme 1.

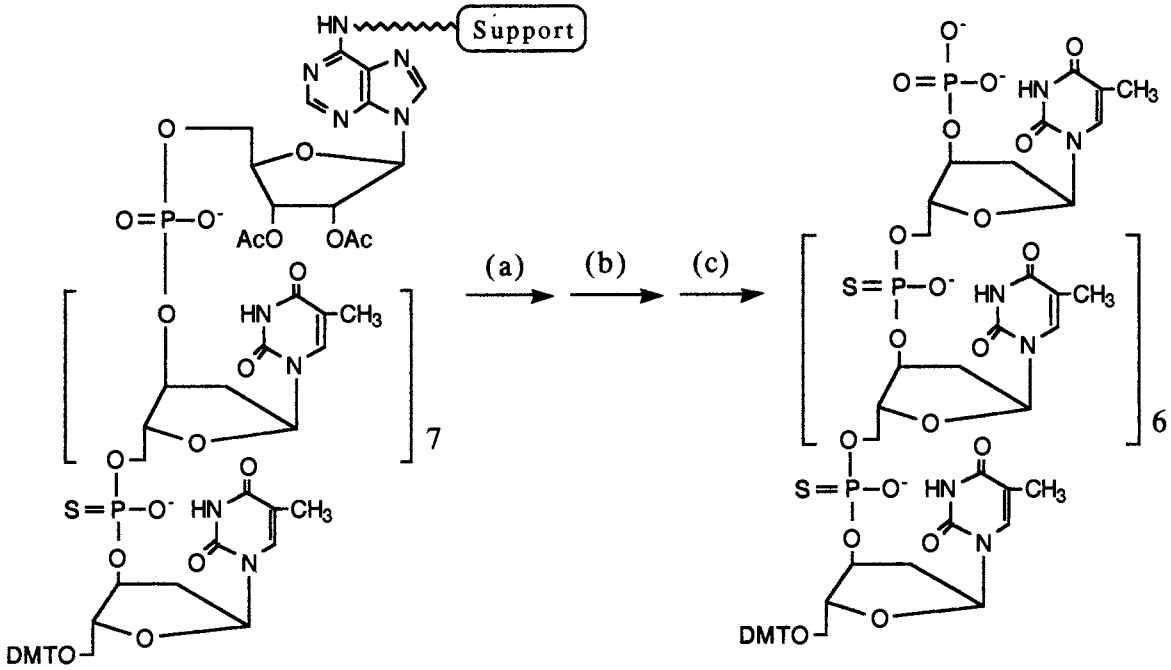


Scheme 1. Preparation of α-2'-deoxythymidine.

The reaction is expected to proceed through SN2 mechanism. Since the chlorine atom of 1-chlorosugar is known to have α-configuration and anomerizes to β-form in a polar solvent, we first examined the effect of solvent on this reaction. Several solvents, such as chloroform, dichloromethane and acetonitrile were tested. In our hand, a mixture of acetonitrile-THF (5 : 1) worked best. Thus, the chlorosugar was dissolved in this mixed solvent and stirred for 30 min at 4 °C under N_2 prior to the reaction. The solution was then mixed with the solution of bis-silylated thymine in the same solvent system as above and was stirred at 4 °C under N_2 for 18 h. The proton NMR peak intensity of the crude product revealed that the ratio of α-2'-deoxythymidine to the β-counterpart was approximately 3:1. Separation of the anomeric mixture by column chromatography (Kiesel gel 60H, chloroform / ethyl acetate = 1 : 1) and the removal of *p*-toluoyl group gave pure α-2'-deoxythymidine in 51.1% yield based on silylated thymine.

After the protection of 5'-OH with dimethoxytrityl (DMT) group in an usual manner, α-2'-deoxythymidine was converted to its 3'-O-methylphosphoramidite derivative[7] by known procedure.[8] The product was obtained in 92% of yield and subjected to the synthesis of oligothymidylate phosphorothioates.

Assembly of the oligomer was performed using automated DNA synthesizer (ABI Model 381-A) and "Oligo Affinity Support",[9] in which 2',3'-di-O-acetyl-5'-dimethoxytrityladenosine is bound to Teflon thread(30-60 μmol / g) through its 6-amino group. The synthetic scale was 0.5 μmol, however, the following major modifications were made on its standard synthetic cycle ; a) the coupling reactions were carried out for 180 s; b) introduction of sulfur atom to trivalent phosphite intermediate was performed using either the mixture of S_8/CS_2/pyridine[4] or tetraethylthiuram disulfide[10] with the reaction period of 900 s in both cases.[11]



Scheme 2. Preparation of α-2'-deoxyoctathymidylate.

The efficiency of the oligomer synthesis was estimated spectrophotometrically by determining the amount of dimethoxytrityl cation released by acidic treatment in an each cycle. The average coupling yield based on the trityl assay was higher than 98.9% in the case of octathymidylate synthesis. After the deprotection of methyl

group from internucleotide phosphates, the product was removed from the support by the following procedure[12] (Scheme 2) ; (a) treatment of conc. ammonium hydroxide (50 °C, 15 h) for the removal of acetyl group from 3'-terminal ribose ring; (b) oxidation of 3'-terminal ribose ring with $NaIO_4$ (20 mM in Na_2HPO_4-NaH_2PO_4 buffer, pH7.2) under the darkness (20 min); (c) the alkaline hydrolysis of 3'-end phosphodiester linkage with 5% of aqueous triethylamine (50 °C, 5 h). The concentration of the reagent and the reaction period for the oxidation step was critical. More severe conditions resulted in the accumulation of possible sulfur-oxidized byproducts, and shorter reaction periods caused insufficient oxidation of ribose ring, therefore lowered the yield of the product. It should be noted that no products were cleaved from the support by the simple treatment of conc. ammonium hydroxide treatment(step a) prior to the oxidation(step b). The 5'-DMT bearing product was purified by reverse phase HPLC. As shown in Fig. 1, the dimer compound exhibited two peaks with equal intensity. This may be due to the formation of diastereomeric isomers by the introduction of sulfur atom to phosphorous. Attempt to separate these isomers by HPLC was not successful. Removal of DMT group was effected by the treatment with aqueous acetic acid (10%, 20 °C, 10 min). The isolated yield of the completely deblocked product was 37.5% for the octamer(α-dTps$_7$Tp) and 45.6% for the dimer(α-dTpsTp). In ^{31}P NMR, phosphorothioate diester bond gave the peak at 55.0-55.4 ppm(referenced to 85% H_3PO_4 external standard in 0.1 M triethylammonium acetate, 50% D_2O, pH 7.0).[13]

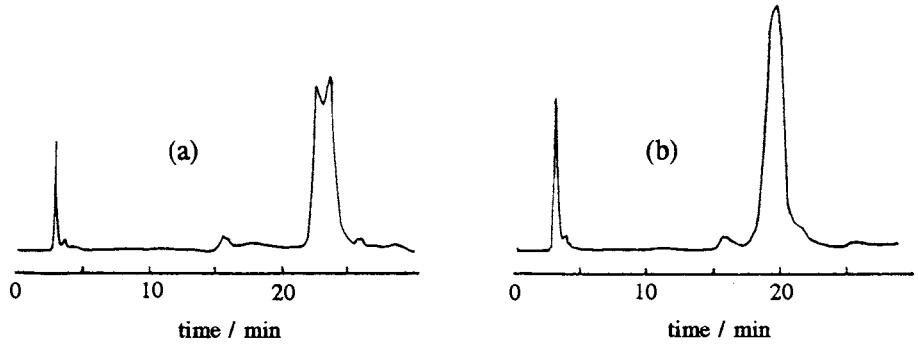


Fig. 1. Reverse phase HPLC analysis of the crude mixture of α-2'-deoxyoligothymidylate phsphorothioate dimer (a) and octamer (b) using PRP-1 column. The products were eluted with linear gradient of 0.1M triethyl ammonium acetate buffer(pH7.0) - acetonitrile(20%, increased to 40% at 20 min).

Table 1. Time course of the cleavage of α- or β-2'-deoxyoctathymidylate by nucleases at 37 °C[a]

	Nuclease S1 (1 unit) time / min			SVP (0.5 unit) time / min			Nuclease P1 (0.1 unit) time / min		
	0	15	30	0	20	60	0	30	180
β-dTp$_7$Tp	100	26.5	8.8	100	8.4	0	100	0[b]	0
α-dTp$_7$Tp	100	99.5	99.3	100	94.0	74.2	100	27.3	10.7
α-dTps$_7$Tp	100	100	99.4	100	98.5	96.5	100	77.9	70.0

a) Each samples contained 0.5 ODunit of the oligomer and the numbers indicate remaining ratio(%) of the starting oligomers after indicated time of incubation. b) The oligomer, β-dTp$_7$Tp, was completely digested within 10 min under this condition.

Nuclease susceptibility of the obtained oligomer was examined by reverse phase HPLC and the results are summarized in Table 1. By the action of nuclease S1, for example, β-2'-deoxyoctathymidylate(β-dTp$_7$Tp) was almost completely digested within 30 min. On the other hand, the corresponding α-oligomer(α-dTp$_7$Tp) and its phosphorothioate analog(α-dTps$_7$Tp) remained almost intact under the same condition. Using snake venom phosphodiesterase, α-2'-deoxyoctathymidylate(α-dTp$_7$Tp) was digested in higher rate than the α-dTps$_7$Tp. Essentially same results were obtained from the reaction with nuclease P1. These results indicate that α-dTps$_7$Tp possess high stability toward the action of common nucleases and are consistent with the properties observed with α-DNA and β-DNA phosphorothioate analogs.[3,4] However, the resistance of α-oligoDNA phosphorothioate towards the action of nuclease was in the greater extent compared to simple α-oligoDNA. Such enhanced stability would make phosphorothioate analogs of α-DNA a promising candidate as a potential antisense agent.

The present method allows simple preparation of novel nuclease-resistant oligonucleotide analogs bearing normal phosphate monoester at their 3'-end, which can be used to introduce other functional moiety by known procedures.[14] Further investigation of biochemical properties of the analogs are progressing.

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