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Synthesis of novel cationic spermine-conjugated phosphotriester oligonucleotide for improvement of cell membrane permeability

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ABSTRACT

A spermine-conjugated ethyl phosphotriester oligonucleotide was obtained by solid-phase synthesis based on phosphoramidite chemistry. The ethyl phosphotriester linkage was robust to exonuclease digestion and stable in fetal bovine serum. Cell membrane permeability of the spermine-conjugated ethyl phosphotriester oligonucleotide was studied by fluorescence experiments. The effective cell penetrating potency of the spermine-conjugated ethyl phosphotriester oligonucleotide was determined by confocal laser scanning microscopy and measurement of intracellular fluorescence intensity.

Oligonucleotide (ON) therapy based on antisense effects is an important therapeutic tool for various diseases.[1] Antisense ON binding to target mRNA results in the inhibition of specific RNA processing or translation. However, the use of ONs as a therapeutic tool is limited as ONs are rapidly digested by nucleases in vivo and the negative charge of the sugar-phosphate backbone is incompatible with cell membrane permeability. Phosphotriester (PTE) ON is one of the phosphate-backbone-modified ONs developed in the 1970s.[2,3] PTE ON is a promising candidate for effective antisense ON as the phosphate modification increases resistance to nuclease digestion in cell culture[4] and improves cell membrane permeability due to the absence of negative charges on the phosphate moieties.[5] More recently, bioreversible PTE linkage-contained RNA were developed as effective RNAi prodrugs.[6] However, neutral ONs, such as methylphosphonate and PTE ONs, exhibit poor solubility in water due to the modification.[7] This indicates that the conventional purification by reversed-phase and anion-exchange HPLC is often difficult in PTE ON synthesis.

Cationic ONs have been studied in an effort to improve the cell membrane permeability of ONs.[8,9] Cationic ONs, which are conjugates of natural phosphodiester (PDE) ON with polycation moieties, such as peptides[10] and polyamines,[11] have been reported. Recently, it was reported that spermine-conjugated PDE ON has higher affinity to complementary strands than natural PDE ON due to the decrease of electrostatic repulsion,[12,13] and also exhibits effective cell penetrating potency by electrostatically anchoring to the cell surface.[14] However, a large polycation moiety is required to neutralize the large number of negative charges on a long ON and the large polycation moiety exhibits cytotoxicity in living cells.[15]

In this study, we designed spermine-conjugated PTE ON (Fig. 1) as a novel cationic ON. The conjugation of PTE ON, which has no negative charge, with spermine may resolve issues surrounding both PTE ON and the polycation moiety, as the positive charges on the spermine moiety may increase the solubility of PTE in water, and a large polycation moiety is unnecessary for PTE ON to be cationic. We present herein the solid-phase syntheses of spermine-conjugated PTE ONs and their effective cell membrane permeability.

Generally, PTE linkages are unstable under harsh basic conditions, such as concentrated aqueous ammonia employed to remove the *N*-acyl protective groups.[16] In order to confirm the stability of PTE ON under deprotection conditions, model PTE thymidine dimers (**5a**, **b**) were synthesized. As shown in Scheme 1, methyl (**3a**) and ethyl (**3b**) phosphoramidite units were synthesized from 5′-*O*-protected thymidine (**1**) in two steps in 57% and 56% yields, respectively. Those amidite units were subjected to the reaction with 3′-*O*-protected thymidine in the presence of 1*H*-tetrazole as an activator, followed by oxidation with 2-butanone peroxide in toluene solution and the removal of 3′,5′-*O*-protections with 80% AcOH, to afford methyl (**5a**) and ethyl (**5b**) PTE dimers in 40% and 32% overall yields in six steps from thymidine, respectively.

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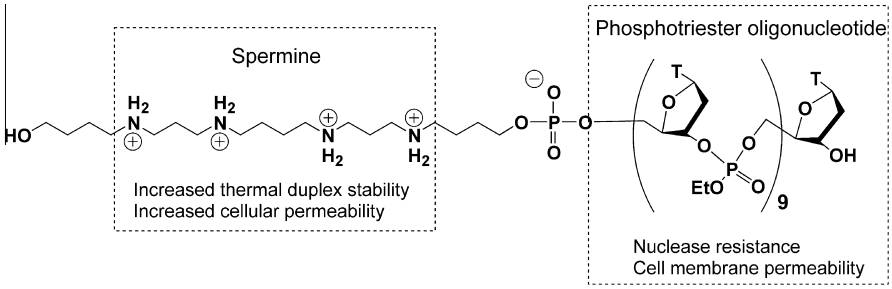
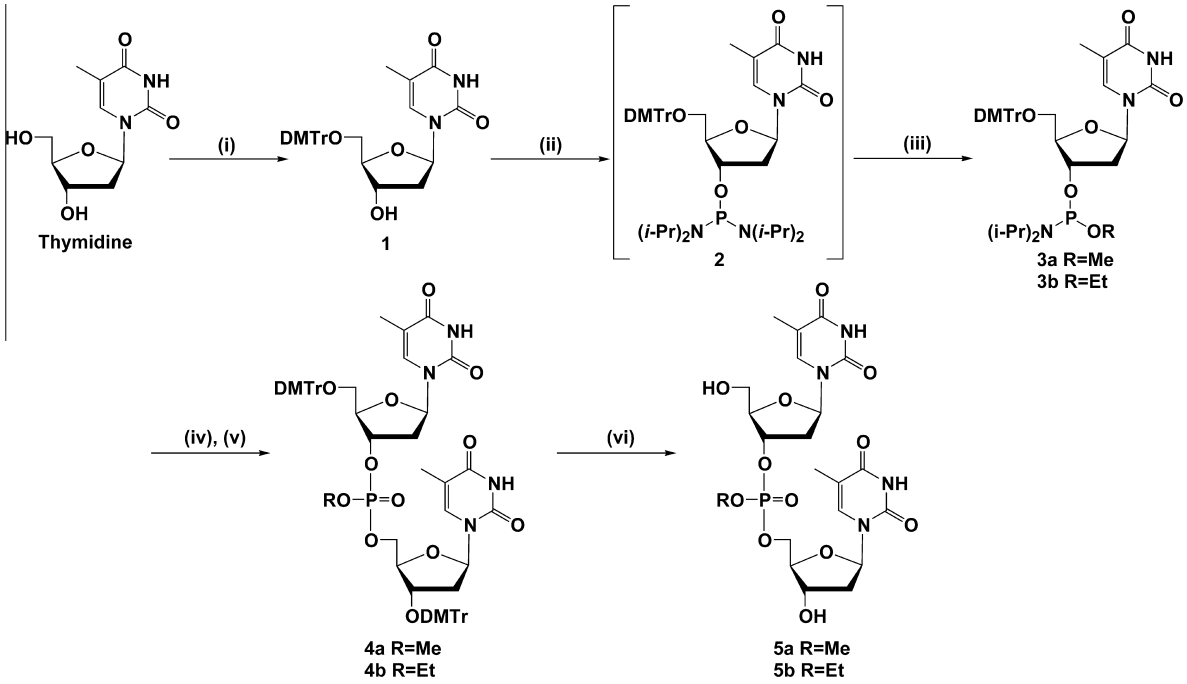


Figure 1. Spermine-conjugated PTE T_{10}.



Scheme 1. Synthesis of phosphotriester (PTE) dimer **5**. (i) 4,4′-dimethoxytrityl chloride (DMTrCl), pyridine, rt, 2.5 h; (ii) $[(i\text{-Pr})_2N]_2PCl$, *N*,*N*-diisopropylethylamine, CH_2Cl_2, rt, 2 h; (iii) ROH, 1*H*-tetrazole, CH_2Cl_2, rt, 2 h; (iv) 3′-*O*-DMTr thymidine, 1*H*-tetrazole, CH_2Cl_2, rt, 3 h; (v) 6.7% 2-butanone peroxide in toluene, rt, 5 min; (vi) 80% AcOH aq, rt, 1.5 h.

The structures of dimers were characterized by ^{31}P NMR and mass spectrometry.[17]

The stability of PTE dimers **5a** and **5b** under standard (conc. NH_3 aq at 55 °C for 8 h) and mild (conc. NH_3 aq at rt for 2 h) deprotection conditions was studied. The reactions were analyzed by reversed-phase HPLC. Diastereomeric peaks were found in the HPLC chromatograms of untreated dimers **5a** and **5b** (Fig. 2A and D). After treatment with conc. NH_3 aq at 55 °C for 8 h, methyl PTE dimer **5a** was completely degraded into PDE dimer d(TpT), whereas ethyl PTE dimer **5b** was partially degraded (Fig. 2B and E). On the other hand, after treatment with conc. NH_3 aq at rt for 2 h, ethyl PTE **5b** was stable, whereas methyl PTE **5a** was almost completely degraded (Fig. 2C and F). The results indicate that labile protective groups, such as phenoxyacetyl (Pac) for dA, isopropylphenoxyacetyl (*i*-PrPac) for dG, and acetyl (Ac) for dC groups, are suitable for the synthesis of ethyl PTE ON.

To confirm the stability of the ethyl PTE dimer against exonuclease (snake venom phosphodiesterase, SVPDE) and in serum (fetal bovine serum, FBS), ethyl PTE d(CpT), which is synthesized according to the above procedure using allyoxycarbonyl (Alloc) protection for dC,[18,19] and parent PDE d(CpT) dimers were incubated under enzymatic digestive conditions.[20] The reactions were analyzed by reversed-phase HPLC and the peak areas of the intact dimers in the chromatograms were calculated. After incubation for 180 min, natural parent PDE dimer was rapidly hydrolyzed by SVPDE, whereas ethyl PTE dimer was extremely stable (Fig. 3). Similar results were obtained in 10% FBS. The results suggest that the PTE linkage is sufficiently stable in vitro and in vivo.

Next, we synthesized spermine phosphoramidite unit **10** according to literature procedure[21] with slight modifications, enabling shorter step synthesis (Scheme 2). The amino groups of spermine tetrahydrochloride were protected by trifluoroacetyl (TFA) groups, and subsequently the protected terminal amide groups were reacted with *tert*-butyl(4-iodobutoxy)dimethylsilane ($TBDMSO(CH_2)_4I$) in the presence of sodium hydride (NaH) to afford **7**. Removal of the TBDMS group with tetrabutylammonium fluoride (TBAF) afforded compound **8**. One terminal hydroxyl group of **8** was protected by a 4,4′-dimethoxytrityl (DMTr) group and finally, the other hydroxyl group was phosphitylated to afford spermine phosphoramidite unit **10**. The overall yield of **10** obtained from spermine tetrahydrochloride was 11%.