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The Effects of the 4-(4-Methylpiperazine)phenyl Group on Nucleosides and Oligonucleotides: Cellular Delivery, Detection, and Stability

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Dedicated to Professor Eiichi Nakamura on the occasion of his 60th birthday

Abstract: As drug candidates, one promising way to improve the cellular delivery efficacy of oligonucleotides is to introduce a cationic group. By introducing a cationic moiety into the oligonucleotide structure, they become capable of approaching the cell surface and also of crossing the cellular membrane. In an effort to develop cell-permeable oligonucleotides, we examined the piperazinephenyl-bearing 2′-deoxyuridine (^{PP}U), which can be not only cationic but also fluorescent as a cationic monomer for cationic oligonucleotides. Several modified DNA oligonucleotides with different numbers of ^{PP}U building blocks were synthesized and evaluated for the effect on thermal stability and conformation by the introduction of ^{PP}U. The cellular delivery of modified oligonucleotides was different depending on the number of ^{PP}U building blocks. Furthermore, these ^{PP}U-modified oligonucleotides had sufficient fluorescence that we were able to identify the delivery results without the use of conventional fluorescent tags. They were predominantly localized in the cell cytoplasm. In addition, they were stable enough after 3 hours in the presence of nuclease. These results showed that a piperazinephenyl moiety that is conjugated with nucleobase is able to deliver and detect the oligonucleotides, which suggests that this concept of 'dual-function oligonucleotides' might be utilized in diagnostics, therapeutics, and as a convenient biological tool for probing the activity of oligonucleotides inside cells.

Keywords: cations · drug delivery · nucleobases · oligonucleotides · stability

Introduction

Despite several decades of research, the potential of gene therapy has been hindered by several unresolved issues.[1–5] One of the main problems involves proper delivery of the therapeutic agent. At present, several strategies utilize oligonucleotides loaded onto specialized carrier systems such as cationic lipids and dendrimers.[6–9] Or the terminal of an oligonucleotide is conjugated to functional compounds and polymers, such as cholesterol and poly(ethylene glycol) (PEG), for better cellular uptake.[10] In addition, the nucleotide monomer itself may be chemically modified. Highly charged, anionic oligonucleotides cannot easily penetrate into cell or endosomal membranes. Therefore, several studies[11] have utilized modified oligonucleotides with cationic moieties to reduce the net negative charge. The most common cationic moieties include guanidinium functionalities[12–19] and several types of amino groups[20–24] that exhibit a net positive charge over a wide pH range and under physiological conditions. The other inherent weakness of therapeutic oligonucleotides is their low resistance to degradation by nuclease (endo- and exonuclease). However, by employing structural variations (such as dumbbell or hairpin shapes)[25–27] or nuclease-resistant modified nucleotide (such as LNA nucleotide)[28], the enzymatic stability of oligonucleotides can be modified.

In the current study, we assess the potential of cationic hairpin-structured oligonucleotides by incorporation of a modified nucleotide in therapeutic applications such as a hairpin oligonucleotide decoy. A new piperazinephenyl-appended nucleoside (^{PP}U) was synthesized by a simple Suzuki coupling reaction and incorporated into oligonucleotides to achieve enhancement of cellular uptake (Scheme 1). This building block (^{PP}U) is designed to have a tertiary amine moiety as the cationically charged part and suitable fluorescence obtained by simultaneous π conjugation between piperazinephenyl and a nucleobase. Herein, we report the characteristic features of ^{PP}U and its oligonucleotides for

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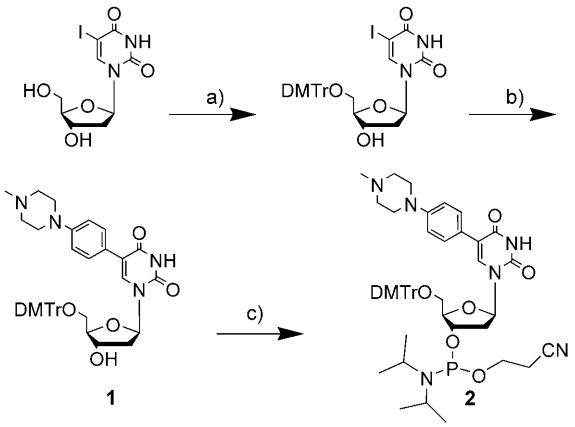

Scheme 1. Synthesis of 4-(4-methylpiperazine)phenyl-appended nucleoside. Reagents and conditions: a) 4,4′-dimethoxytrityl chloride (DMTrCl), Et_3N, pyridine, RT, 5 h, 90%; b) 1-methyl-4-[4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl]piperazine, [$Pd(PPh_3)_4$], NaOH, THF/H_2O/MeOH (2:2:1), 70–75°C, 6 h, 82%; c) 2-cyanoethyl diisopropylchlorophosphoramidite, 4-methylmorpholine, CH_2Cl_2, RT, 1 h, 95%.

cellular delivery and detection. By using a piperazine moiety, we have demonstrated that oligonucleotides that contain ^{PP}**U** have increased thermal stability relative to unmodified ones and can be delivered to the inside of a cell, cytoplasm in particular. In addition, their presence in cytoplasm can be identified without fluorescent labeling by using a confocal microscope. Finally, we have demonstrated that the ^{PP}**U**-modified oligonucleotides are more stable than natural ones against enzymatic degradation.

Results and Discussion

Methylpiperazine, a tertiary amine, was employed as a cationic modifier due to its affordable gene-transfer effect[29] and basicity. The conjugate acid of methylpiperazine exhibits a relatively high pK_a value ($\approx$10) relative to other amines. To introduce the piperazine moiety, 4-(4-methylpiperazine)phenyl boronic acid was used to modify the C5 position of 2′-deoxyuridine by means of Suzuki coupling[30] to yield 5-[4-(4-methylpiperazine)phenyl]-2′-deoxyuridine (^{PP}**U**) in high yield. With this simple synthetic procedure, ^{PP}**U** is able to have fluorescence by π conjugation between the phenyl ring and uracil.

The new cationic monomer, ^{PP}**U**, was fluorescent. This effect was likely due to delocalization of the nitrogen lone-pair electrons through the π-conjugated system of *para*-phenylene and uracil.[31] Therefore, ^{PP}**U** served as a "dual-function oligonucleotide," thus facilitating both delivery and detection. Prior to the oligonucleotide synthesis, the spectroscopic characteristics of ^{PP}**U** were investigated by UV absorption and fluorescence measurements. Absorption peaks were observed at 258 nm and 298 nm, which originated from the nucleobase and the π-conjugated system of the (methylpiperazine)phenyl moiety with the nucleobase, respectively. By using the information obtained from the UV absorption spectra (i.e., the excitation maxima), a fluorescence spectrum was obtained. Two emission peaks at 389 nm and 502 nm were observed, but the major is the latter one. As observed by the emission spectrum, the green color of ^{PP}**U** was detected under UV illumination. The quantum yield of ^{PP}**U** in several selected solvents was almost 0.05 ($CHCl_3$ in neutral pH), and the fluorescence intensity was low relative to conventional fluorophore-appended 2′-deoxyuridine, for example, ^{FL}**U** (2′-deoxy-5-(2-ethynylfluorenyl)uridine; Figure 1).[32–34] As the pH of solvent was decreased from 7 (neutral) to 4 (acidic), fluorescence quenching of ^{PP}**U** was observed and was presumably due to the protonation of the piperazine moiety (see the Supporting information).

To investigate the effect of ^{PP}**U** in the oligonucleotides, hairpin-type DNA oligonucleotides (18-mer) were prepared

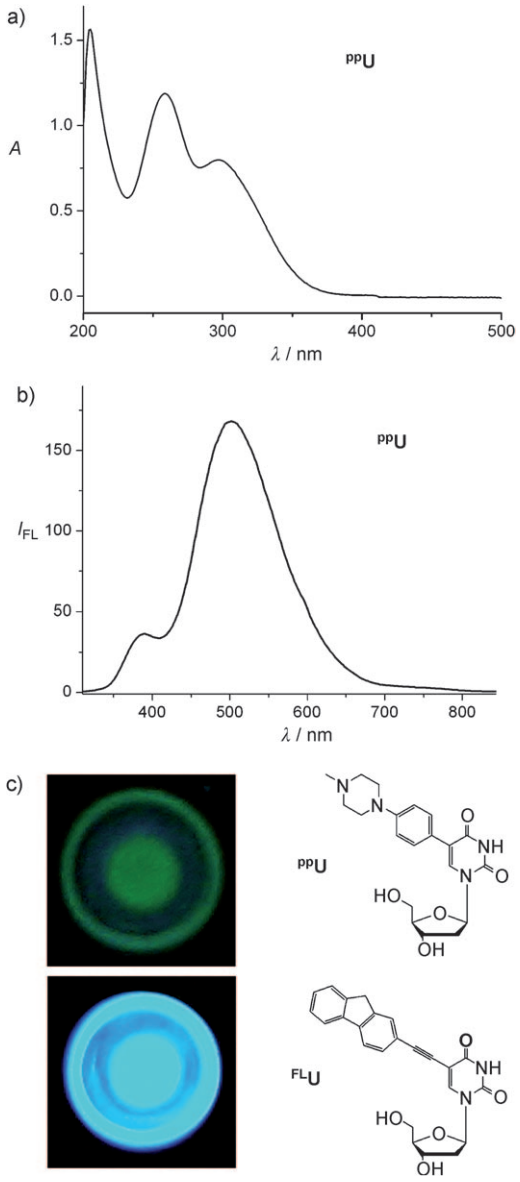

Figure 1. a) UV spectrum of ^{PP}**U**. b) Emission spectrum of ^{PP}**U**. c) The photo image and structure of ^{PP}**U** and ^{FL}**U**.

with varying numbers of ^{PP}**U** (**X**) and compared against a natural unmodified sequence: natural 5′-dATA-TACGTTTTCGTATAT-3′, $(\mathbf{X})_6$ 5′-dATA**X**ACG**XXXX**-CG**X**ATAT-3′, $(\mathbf{X})_8$ 5′-dA**X**A**X**ACG-**XXXX**CG**X**A**X**AT-3′. To identify the effect of ^{PP}**U** on hybridization, the melting temperature (T_m) of synthesized oligonucleotides was measured by UV/Vis absorption in pH 7 Tris buffer (Figure 2a).

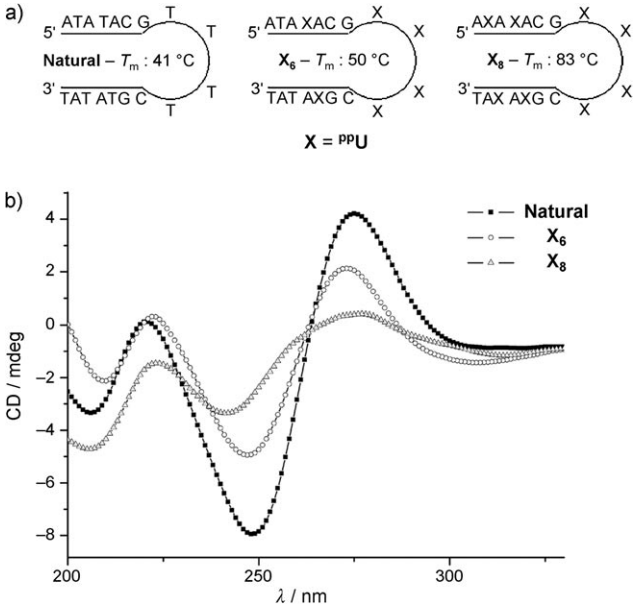

Figure 2. a) Synthesized oligonucleotides and their T_m values. b) CD spectra of ODN natural, $(\mathbf{X})_6$, and $(\mathbf{X})_8$.

A higher T_m was observed in DNA that contained ^{PP}**U** (in the case of $(\mathbf{X})_8$ or $(^{PP}\mathbf{U})_8$, it showed another transition at 28 °C), thereby signifying a stabilization of the DNA duplex, presumably due to electrostatic interactions between the piperazine component and the phosphate backbone in the stem region of the hairpin.[19,20] The DNA oligonucleotides used in this study showed somewhat higher T_m and, in the case of $(\mathbf{X})_8$, the sharpness of the melting curve decreased. In general, hairpin-type DNA oligonucleotide has a higher melting temperature than a simple duplex one. Even though the results showed a more enhanced and broader range of melting temperature, the thermal stability caused by the electrostatic interaction with the number of ^{PP}**U** units on oligonucleotides is demonstrated (see the Supporting information for additional T_m of modified DNA or RNA duplex with ^{PP}**U**). The thermal stability of synthesized oligonucleotides was either much higher or was not determined when they were measured in the presence of additional salt (NaCl, KCl, and sodium cacodylate). The modified oligonucleotides were further characterized by circular dichroism measurements (CD). These results showed that the modified oligonucleotides exhibited a conventional B-form DNA (Figure 2b).[35] Therefore, the nucleic acid analogue of ^{PP}**U** does not generate significant perturbation of the DNA duplex structure. In contrast, electrostatic effects due to the base modification stabilized DNA duplex hybridization.

The cellular uptake of ^{PP}**U**-modified oligonucleotide by HeLa cells was evaluated under confocal microscopy. The ^{PP}**U** modifier is slightly fluorescent, and confocal microscopy studies were performed without the use of fluorescent tags. The conditions of cellular uptake were optimized by monitoring the HeLa cell response to three different concentrations of oligonucleotide: 200, 400, and 1000 nM. Natural, $(\mathbf{X})_6$, and $(\mathbf{X})_8$ were tested with and without a transfection agent (Oligofectamine, Invitrogen, Carlsbad, CA, USA). Cells incubated with $(\mathbf{X})_6$ exhibited a relatively stronger fluorescence in the cytoplasm than others at the 200 nM level, independent of transfection agent (Figure 3 and the Supporting information). However, $(\mathbf{X})_8$ showed weakened fluorescence intensity without a transfection agent. Different numbers of ^{PP}**U** modifier ($(^{PP}\mathbf{U})_n$, in which $n=2$, 4, 6, 8) were also evaluated for cellular uptake; $(\mathbf{X})_6$ was optimal. Therefore, the lowest concentration of the modified oligonucleotide, $(\mathbf{X})_6$, induced the higher fluorescence when taken in by the cell. The efficiency of cellular uptake was expected to decrease with increasing amounts of modifier and concentration due to aggregation, as suggested by related polypeptide studies.[36–38]

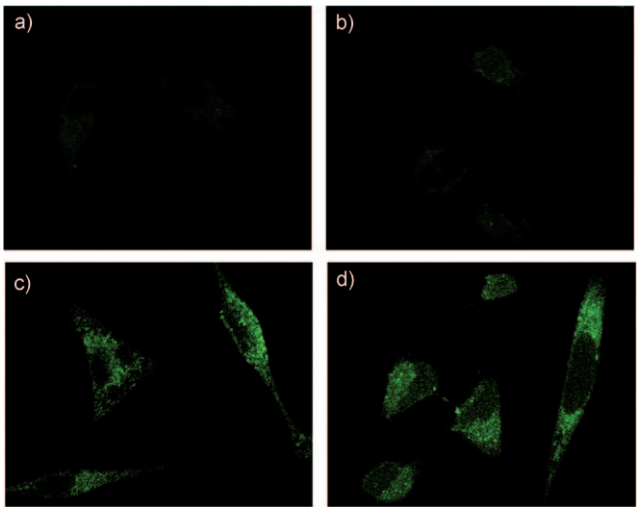

Figure 3. Confocal images of a) HeLa cells. b) HeLa cells incubated with a natural oligonucleotide. c) HeLa cells incubated with $\mathbf{X}_6$ with transfection agent. d) HeLa cells incubated with $\mathbf{X}_6$ without transfection agent.

Further experiments were carried out to identify the exact localization of ^{PP}**U**-modified oligonucleotide inside of the cell in a simple single-strand system. Linear oligonucleotides that had been treated with fluorescein, Flu-$(\mathbf{TTT})_6$ and Flu-$(\mathbf{TXT})_6$, were prepared and incubated with HeLa cells as described before. To address the localization, the nucleus was stained by Hoechst 33258 and a significant green fluorescence was noticed from the cytoplasm in the case of Flu-$(\mathbf{TXT})_6$. When the fluorescence signals were merged together to confirm the localization, an overlapped signal was not

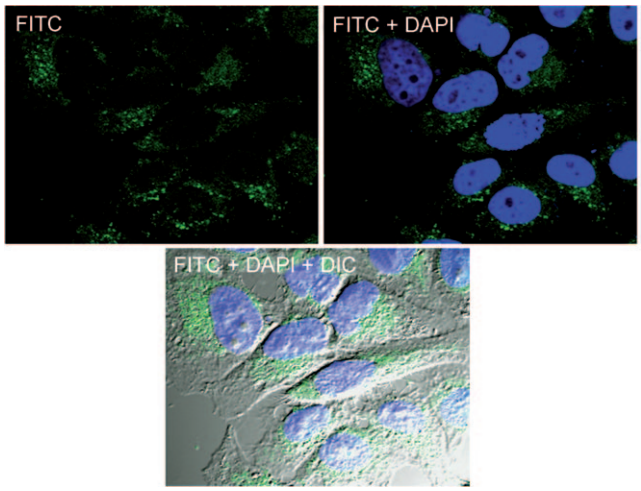

Figure 4. The localization of modified oligonucleotides Flu-(**TXT**)$_6$ in the HeLa cells (FITC = fluorescein isothiocyanate, DAPI = 4′,6-diamidino-2-phenylindole).

observed (Figure 4). However, Flu-(**TTT**)$_6$ did not show any fluorescence except the nucleus stained by Hoechst 33258. Therefore, the cellular uptake of ^{PP}**U**-modified oligonucleotides (**X**)$_6$ and Flu-(**TXT**)$_6$ occurs predominantly in the endosome or whole cytoplasm regardless of their structure and this would be expected for cationic driven internalization.

The sensitivity of oligonucleotides to enzymatic degradation is one of their major problems as a drug candidate. Compared to the natural oligonucleotides, the stability of the modified oligonucleotides was enhanced against the activity of nuclease (Exonuclease III; Promega, Madison, WI, USA).[39–41] Undigested, modified oligonucleotide (**X**)$_6$ still remained after 3 h, whereas the natural oligonucleotide was completely digested (Supporting information). Undigested residue of (**X**)$_6$ was identified by UV visualizer without dye because of its fluorescence. Therefore, the introduction of a ^{PP}**U** monomer could considerably improve the enzymatic stability of ^{PP}**U**-modified oligonucleotides. The increased enzymatic stability of ^{PP}**U**-modified oligonucleotides would be more beneficial for biomedical applications.

Conclusion

In summary, 5-[4-(4-methylpiperazine)phenyl]-2′-deoxyuridine (^{PP}**U**) was synthesized by a simple Suzuki coupling reaction. ^{PP}**U** was amenable to cellular uptake in HeLa cells and exhibited an intrinsic fluorescence, thereby affording simultaneous delivery and detection. Although the experiments performed here did not cover a wide range of conditions and give effective results, the results are still meaningful since cells inoculated in 200 nM **X**$_6$ exhibited similar fluorescent intensities regardless of the addition of a transfection agent. In addition, ^{PP}**U**-modified oligonucleotides showed more enhanced enzymatic stability than natural oligonucleotides. Based on these results, it is assumed that the improved cellular delivery of ^{PP}**U**-modified oligonucleotides originates from not only their cationic character but also enzymatic stability. Currently, more-potent modifiers are required to induce cellular uptake of gene therapeutic agents, but these modifiers must also be easy to implement. The results reported here demonstrate that the modified ^{PP}**U** could easily and readily be used as a modifier to enable intracellular oligonucleotide delivery without fluorescent tagging.

Experimental Section

General Methods of Chemical Synthesis

All chemicals were obtained from Aldrich Chemical Company or the specified individual chemical companies and were used without further purification. Each reaction was performed under an inert atmosphere of dry argon and by using glassware that was flame-dried under vacuum. Flash chromatography was performed on silica gel 60 (230–400 mesh; ASTM). Melting points are uncorrected and were obtained with an Electrothermal 1A 9000 series apparatus. FTIR spectra were recorded with a Bruker FTIR PS55+ spectrometer. Low-resolution FAB+ mass spectra were obtained with a JEOL JMS-AX505WA (FAB) spectrometer.

^{1}H and ^{13}C NMR spectra were recorded with a Bruker Aspect 300 NMR spectrometer. Chemical shifts (δ) of these spectra are reported in parts per million (ppm) downfield relative to the internal standard, tetramethylsilane (TMS). Coupling constants are reported in Hertz (Hz). Spectral splitting patterns are designed as s (singlet), d (double), dd (double doublet), dt (distorted triplet), t (triplet), m (multiplet), and br (broad).

Synthesis of Cationic Modified Nucleosides

1: 5-Iodo-5′-DMT-2′-deoxyuridine (300 mg, 0.46 mmol) was dissolved in THF/H_2O/MeOH (2:2:1; 25 mL), and then [Pd(PPh_3)$_4$] (53 mg, 0.046 mmol), 1-methyl-4-[4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-phenyl]piperazine (152 mg, 0.506 mmol), and NaOH (366 mg, 9.2 mmol) were added. The reaction mixture was heated at reflux for 6 h at 70–75 °C. The reaction mixture was concentrated under reduced pressure. The residue was purified by chromatography through a short column of silica gel (CH_2Cl_2/MeOH, 70:1) to yield **1** (264 mg, 82 %). m.p. 140–143 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.62 (s, 1H; NH), 7.33–7.397 (m, 2H; Ar–H), 7.16–7.26 (m, 9H; Ar–H), 6.73–6.74 (m, 4H; Ar–H), 6.61–6.64 (m, 2H; Ar–H), 6.37 (t, J = 6.77 Hz, 1H; C1), 4.51–4.53 (m, 1H; C3), 4.07–4.08 (m, 1H; C4), 3.75 (s, 6H; OCH_3), 3.33–336 (m, 2H; C5), 2.52–2.61(m, 10H; piperazine+C2), 2.34 ppm (s, 3H; CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 162.2, 158.8, 151.1, 149.9, 135.5 130.2, 129.1, 128.1, 127.2, 115.8, 113.4, 86.9, 86.2, 85.3, 72.6, 63.7, 55.4, 48.9, 46.4, 46.3, 41.2 ppm; IR (NaCl): $\tilde{\nu}$ = 3460, 3281, 3058, 2941, 2835, 2563, 1703, 1609, 1508, 1510, 1460, 1378, 1294, 1177, 1034, 1008, 922, 827, 791 cm^{-1}; HRMS (FAB): m/z: calcd for $C_{41}H_{45}N_4O_7$: 705.3288 $[M+H]^+$; found: 705.3287.

2: 2-Cyanoethyl diisopropylchlorophosphoramidite (120 μL, 0.537 mmol) was added dropwise to a solution of compound **1** (301 mg, 0. 419 mmol) and 4-methylmorpholine (140 μL, 1.27 mmol) in CH_2Cl_2 (12 mL) at room temperature. After the reaction reached completion (1 h), the mixture was concentrated in vacuo and purified by chromatography through a short column of silica gel (CH_2Cl_2/MeOH, 70:1) to yield **2** (360 mg, 95 %). m.p. 78–80 °C (decomp.); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.67 (d, J = 12.3 Hz, 1H), 7.34–7.37 (m, 2H; Ar–H), 6.69–6.73 (m, 4H; Ar–H), 6.55–6.59 (m, 2H; Ar–H), 6.37–6.43 (m, 2H; H-1′), 4.61 (m, 1H; H-3′), 4.15–4.20 (m, 1H; H-4′), 3.73 (s, 6H; OCH_3), 3.56–3.66 (m, 2H; H-5′), 3.34–3.44 (m, 1H; –NCH), 3.24–3.29 (m, 1H; –NCH), 3.13 (s, 4H; piperazine-H), 2.59–2.61 (m, 6H; CH_2 and piperazine-H), 2.41 (t, J = 6.5 Hz, 2H; CH_2), 2.31 (s, 3H; CH_3), 1.06–1.43 ppm (m, 12H; $NCHCH_3$); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 162.6, 158.7, 150.8, 150.2, 144.5, 135.6, 130.1, 129.2, 128.2, 127.1, 123.2, 116.0, 113.3, 85.8, 85.2, 63.3, 55.3, 54.9, 48.7, 46.0, 43.5, 40.3, 24.8, 20.3, 14.4 ppm; ^{31}P NMR (121 MHz, $CDCl_3$): δ = 150.2, 149.7 ppm; IR (NaCl): $\tilde{\nu}$ = 3037, 2935, 2874, 2837, 2800, 2751, 1705, 1685, 1609, 1511, 1458, 1249, 1155, 1080, 1034, 920, 864, 730 cm^{-1};

HRMS (FAB): *m/z*: calcd for $C_{50}H_{62}N_6O_8P$: 905.4367 $[M+H]^+$; found: 905.4370.

Oligonucleotide Synthesis

All reagents for oligonucleotide synthesis were purchase from Glen Research. Natural and modified oligonucleotides were synthesized by means of the standard solid-phase β-cyanoethylphosphoramidite method with a PolyGen 12 column DNA synthesizer (Germany) and purified under standard conditions. The concentration of oligonucleotides was determined by ultraviolet (UV) absorption. Oligonucleotide sequences used for experiments have been listed in Table 1.

Table 1. DNA sequences used in this study.

Oligonucleotide (**X**=^{PP}**U**)	Sequence (5′→3′)	MS (*m/z*)	
		calcd	found
natural	d(ATATACGTTTTCGTATAT)	5479.9	5480.7
$(\mathbf{X})_6$	d(ATAXACGXXXXCGXATAT)	6441.4	6441.4
$(\mathbf{X})_8$	d(AXAXACGXXXXCGXAXAT)	6761.9	6764.1
Flu-$(\mathbf{TTT})_6$	fluorescein-d(TTTTTTTTTTTTTTTTTT)	5950.4	5946.1
Flu-$(\mathbf{TXT})_6$	fluorescein-d(TXTTXTTXTTXTTXTXT)	6911.9	6920.8

Photophysical Properties of ^{PP}U

The absorption and emission wavelength of ^{PP}**U** were determined with a Cary 100 Conc UV/Vis spectrophotometer and Cary Eclipse Fluorescence spectrophotometer. The absorbance of ^{PP}**U** (75 μM in $CHCl_3$) was monitored at room temperature. Emission spectra and quantum yield were obtained using quinine sulfate as a standard for nucleoside ^{PP}**U**. The quantum yield of ^{PP}**U** was estimated using Equation (1):

$$\Phi_{F(X)} = (A_S/A_X)(F_X/F_S)(n_X/n_S)^2\Phi_{F(S)}$$

for which S is standard, X is unknown; A is the absorbance at the excitation wavelength, F is the fluorescence intensity, n is the refractive index of the solvent, and Φ is the quantum yield).

Thermal Stability

UV melting-temperature experiments were performed with a Cary 100 Conc UV/Vis spectrophotometer on 18-mer DNA hairpin-type oligonucleotides. The absorbance of the 0.5 OD (optical density) sample in 5 mM Tris buffer (pH 7) was monitored at 260 nm from 3 to 91 °C at a heating rate of 1.0 °C min^{-1}. T_m values were determined by means of the maximum value of the first derivate plots of absorbance versus temperature.

Circular Dichroism (CD) Measurements

CD spectra were collected with a JASCO J-810 spectropolarimeter with a temperature controller. The cell holder was flushed with dry nitrogen gas. For each sample, five spectrum scans were accumulated over the 200–500 nm wavelength range at room temperature. The 0.5 OD of oligonucleotides were used in 5 mM Tris buffer (pH 7).

Cell Culture

HeLa cells were cultured in Dulbecco's modified Eagle medium (DMEM; HyClone), supplemented with 10% fetal bovine serum (FBS; HyClone), 100 μg mL^{-1} of streptomycin, and penicillin (100 U mL^{-1}) at 37 °C in 5% CO_2. Cells were split by using trypsin/ethylenediaminetetraacetic acid (EDTA) medium when almost confluent.

Transfection of Oligonucleotides and Microscopic Studies

At first, cover glasses (1.13 cm^2, Deckglaser) were put into a six-well plate, and the plate was coated with 0.2% gelatin. HeLa cells were seeded at a density of 2.5×10^5 cells per well, each well of which contained 10% FBS-supplemented DMEM (2 mL), and were incubated for 4 h.

HeLa cells were transfected in the absence of serum with 200 nM modified oligonucleotides using oligofectamine (Invitrogen), or without any transfection reagent. The cells were allowed to incubate at 37 °C in the presence of oligonucleotides for 6 h in a CO_2 incubator followed by replacement of DMEM (2 mL) that contained 10% FBS.

For preparation, 24 h after transfection, cells were washed with Dulbecco's phosphate buffered saline (DPBS), and then cover glasses were detached from the bottom of the plate. The cells on the cover glass were fixed with 3.5% paraformaldehyde solution. After additional washing with DPBS, the cover glasses were transferred onto slide glass. Confocal images were obtained from the fixed and nonfixed cell with an Olympus FluoView FV1000 confocal microscope (Olympus Optical Co., Ltd., Tokyo).

Hoechst 33258 Nuclear Staining

Each fluorescein isothiocyanate (FITC)-labeled oligonucleotide was diluted in serum-free OptiMEM media to a final concentration of 1000 nM. Then the cells were incubated with oligonucleotide solutions for 3 h. After incubation, the cells were washed three times with PBS and then fixed with 3.5% (w/v) paraformaldehyde (Sigma) at room temperature for 12 min.

After washing three times with PBS, the nucleus of cells was stained with Hochest 33258 dyes diluted in 1% BSA (bovine serum albumin). Then the coverslips were placed on a glass slide and then sealed. The fluorescent confocal images were captured with an Olympus FluoView FV1000 confocal microscope (Olympus Optical Co., Ltd., Tokyo).

Digestion Experiment of Oligonucleotides with Exonuclease III

Prepared oligonucleotides (0.23 OD) were mixed with 10× Exonuclease III buffer (3 μL; final concentration: 66 mM Tris-HCl, pH 8.0, 0.66 mM $MgCl_2$) and the final volume was adjusted with H_2O (30 μL). Exonuclease III (Promega, 200U) was added at room temperature and each aliquot (5 μL) was taken at certain time intervals (10, 30, 60, 120, 180, and 240 min). The enzyme activity of aliquots was quenched by 0.5 M EDTA (2 μL) and PAGE loading buffer (formamide; 5 μL). Using the 15% PAGE (7 M urea, 140 V), the digested samples by Exonuclease III were analyzed. The gel of the natural oligonucleotide was stained by StainsAll (Sigma), but the $(\mathbf{X})_6$ gel images were obtained with UV visualizer without dye.

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