Dramatic Change in the Tertiary Structure of Giant DNA without Distortion of the Secondary Structure Caused by Pteridine–Polyamine Conjugates

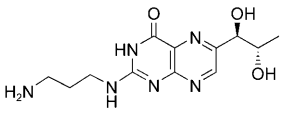
Ning Chen,[a] Shizuaki Murata,*[a] and Kenichi Yoshikawa[b]

Abstract: Pteridine–polyamine conjugates, such as 2-polyamine (1,3-diaminopropane and spermine) substituted 6,7-dimethyl-3*H*-pteridine-4-one, induce a folding transition of a giant DNA molecule more effectively than the corresponding polyamines. However, since neither a DNA high-temperature shift of denaturation (T_m) curve nor distortion of the UV/fluorescence spectra is observed in a mixture of these compounds with DNA, they do not interact with the DNA duplex strongly.

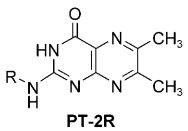
Keywords: denaturation · DNA recognition · DNA structures · polyamines · pteridines

Introduction

Biogenic polyamines, such as putrescine, spermidine and spermine, are known to be biochemical markers for various solid and blood cancers.[1–5] However, because of a significant increase in the ratio of two unconjugated pterins (neopterin/biopterin) in the urine and serum of cancer patients,[6–9] neopterin has been considered as another biochemical marker for human cancers.[10–12] Regarding to these two kinds of nitrogen containing compounds, the new pteridine derivative, oncopterin: 2-(3-aminopropyl)amino-6-[(1′*R*,2′*S*)-1′,2′-dihydroxypropyl]-3*H*-pteridine-4-one, has been found to increase about 70- to 100-fold in malignant lymphoma patients.[13,14] On the other hand, it is known that biogenic polyamines can interact with DNA, leading to charge neutralization and subsequent DNA compaction. In solution, the DNA molecule exists in an elongated coil state, and transformation to a globule state proceeds with more than 10000 times compaction of its effective molecular volume in the presence of various cationic condensing agents.[15,16] As a general rule, the charge of a condensing agent should be at least +3 to enable DNA compaction.[17] DNA compaction is closely related to chromatin-producing DNA folding in vivo. An alternation of the DNA folding structure has been suggested to affect gene expression and suppression.[18] Since the structure of oncopterin is simply a combination of polyamine and pteridine fragments, these pteridine–polyamine conjugates might play an inhibitory or promotional role in cancer cells with a higher gene activity level than in normal cells. To understand the biological activity of pteridine–polyamine conjugates, it is necessary to clarify their influence on the DNA-folding transformation. In the present paper, we discuss the DNA folding transformation induced by designed pteridine–polyamine conjugates.



oncopterin



PT-2R

PT-2DAP, **PT-4DAP**: R = – $(CH_2)_3NH_2$
PT-2SPM, **PT-4SPM**: R = – $(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$

Results and Discussion

Pteridine–polyamine conjugates, which are abbreviated as PT-2DAP and PT-2SPM, were prepared from 2-methylthio pteridine derivative by the substitution reaction with 1,3-di-

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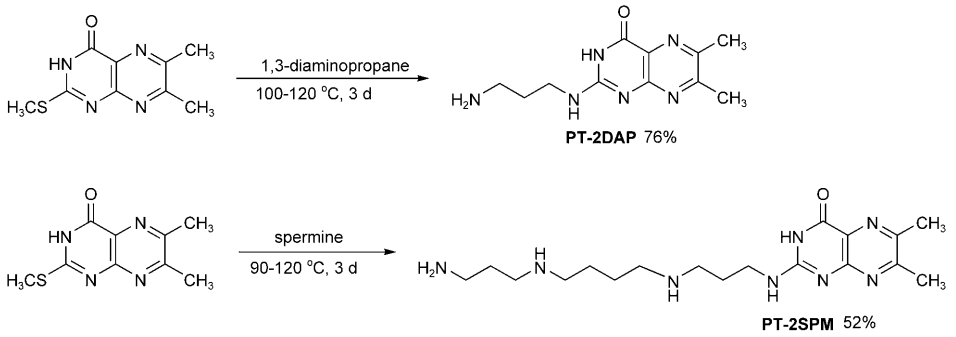
aminopropane (DAP) and spermine (SPM), respectively,[19] as illustrated in Scheme 1. To understand the basicity of the individual nitrogen atoms in the pteridine–polyamine conjugates, the pK_a values was measured by using a UV spectroscopic method. Two pK_a values (ca. 2.3 and 7.9) for 2-amino-3*H*-pteridine-4-one (abbreviated as PT-H) have been reported thus far.[20,21] The smaller value corresponds to the proton dissociation of ammonium ion of the $H_2NC(2)$ group: $[PT\text{-}H_2]^+ \rightarrow PT\text{-}H + H^+$; however, for this p$K_a$ value, the DNA phosphate groups are not affected because the ammonium ion is more acidic than the DNA phosphoric acid (pK_a 1.0). The larger pK_a value corresponds to deprotonation in a basic solution: $PT\text{-}H \rightarrow [PT]^- + H^+$. Consequently, the basicity of pteridine itself does not lead to the interaction with the DNA phosphate group under neutral conditions (pH 7.6). Similar pairs of pK_a values, 1.72/8.17 and 1.72/8.14, were observed in PT-2DAP and PT-2SPM, respectively. It should be noted that these measurements are applicable only for protonation and deprotonation equilibrium of the UV-active pteridine part. The amino nitrogen (HNC(2)) bound to the pteridine ring is not basic enough to neutralize phosphoric acid due to strongly electron-withdrawing influence of pteridine, although it originally belonged to 1,3-diaminopropane or spermine. Therefore, one and three aliphatic amino groups remain in PT-2DAP and PT-2SPM, respectively, and these compounds act as +1 or +3 charged ions when they interact with DNA.

The influence of the pteridine–polyamine conjugates on the conformational behavior of giant T4 DNA (166 kbp, 57 μm full length) in an aqueous solution was investigated by using fluorescent microscopy (FM), which is a powerful tool for observing the fluorescent profiles of long DNA molecules at the single-molecule level.[22,23] Typical FM images of DNA in coil, coil–globule coexistence, and globule states are shown in Figure 1a. In the absence or at lower concentrations of the pteridine–polyamine conjugate, all DNA molecules were detected as elongated coils appearing as hazy images with a 3.5 μm mean long-axis length (L). With an increase in the concentration, DNA compaction occurred at the single molecular level to give a globule state appearing as a bright particle with $L < 0.8$ μm, and the coil–globule coexistence state was observed. Finally, at higher concentrations, all DNA molecules were converted into the globule state. Figure 1b and c represent DNA compaction by conjugates and corresponding polyamines as the dependences of the DNA coil fraction on the concentration of the compaction agents. In the case of PT-2DAP, the minimum concentrations to initiate and complete the coil–globule transformation were 0.3 and 2.0 mM, respectively, while 1,3-diamopropane itself did not complete DNA compaction even at 5 mM. On the other hand, T4 DNA compaction was promoted by PT-2SPM at a minimum concentration of 7 μM, and was completed at 9 μM. The concentration range of the coil–globule coexisting region (7–9 μM) induced by PT-2SPM was $^1/_4$ of that induced by spermine (30–40 μM), and the results shown in Figure 1b and c clearly indicate that PT-2DAP and PT-2SPM are con-



Scheme 1. Synthesis of PT-2DAP and PT-2SPM pteridine–polyamines conjugates.

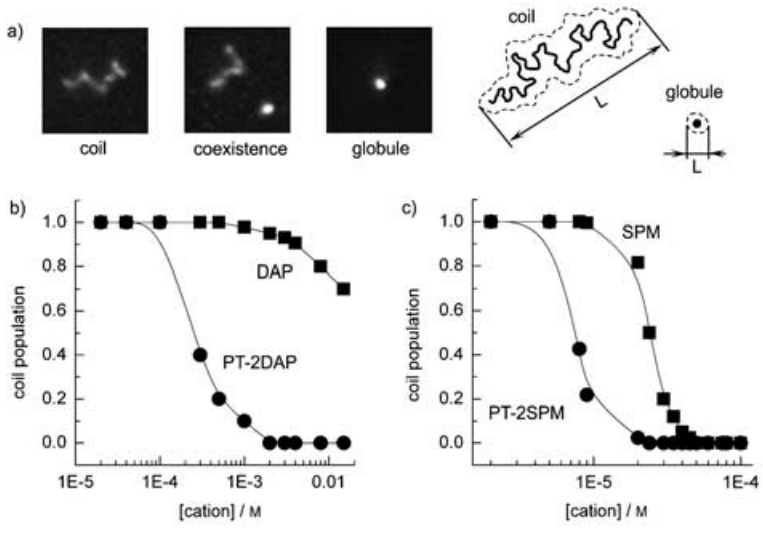


Figure 1. a) Fluorescent images of T4 DNA in coil, coil-globule coexistence, and globule states and their schematic illustrations; b) the dependence of the fraction of T4 DNA molecules in a coil state on the concentration of 1,3-diaminopropane and PT-2DAP. The fraction of DNA in a coil state was obtained based on analysis of at least 100 DNA individual molecules; c) the dependence of the fraction of T4 DNA molecules in a coil state on the concentration of spermine and PT-4SPM.

siderably more effective in DNA compaction than the corresponding polyamines. In addition, PT-2SPM is obviously able to perform a sharp on/off type DNA-folding transition as is clear from the narrow coil–globule coexisting region. Taking into account the lower charge values of PT-2DAP and PT-2SPM compared with the corresponding polyamine as discussed in the pK_a study, 1,3-diaminopropane and spermine are less charged but possess stronger DNA compacting potential when they are connected to the pteridine ring. It is well known that polyamine with a larger positive charge value has greater efficiency in DNA compaction. For example, the DNA compaction efficiencies (CE_{50}) of spermine (+4), spermidine (+3) and 1,3-diaminopropane (+2), which are defined as concentrations affording 50% DNA compaction, under the same conditions are 9.5, 90, and 900 μM, respectively.[24] Furthermore, it is recognized that +1 charged compounds never provoke DNA compaction in aqueous media,[25] since they are unable to neutralize the necessary value (90%) of whole negative charges on the DNA chain.[17] In contrast, monocationic PT-2DAP induces DNA compaction, and its DNA compaction potential (CE_{50} = 0.25 mM) is even more higher than that of dicationic 1,3-diaminopropane. On the other hand, pteridine itself, that is, PT-H and 2-amino-6,7-dimethyl-3*H*-pteridine-4-one, can not compact DNA due to the zero effective charge value according to the pK_a values. Therefore, in pteridine–polyamine conjugates, the polyamine part plays the crucial role in providing a strong DNA compacting efficiency by modifying some physico-chemical factors other than the charge value.

DNA double helix melting (T_m) measurements in the presence of PT-2DAP and PT-2SPM were carried out to understand the DNA-binding potential of the pteridine–polyamine conjugates. Since the melting temperature of long T4 DNA was too high to observe the full melting state, relatively short lambda DNA (ca. 48.5 kbp) was employed. Dissociation of the double strand (T_m) of lambda DNA started at 50°C and was completed at 65°C. The presence of DNA-binding chemicals usually stabilizes DNA double helix against thermal denaturation, and this phenomenon is observed as an increase in T_m. Indeed, in the presence of 50 μM 1,3-diaminopropane, the T_m curve shifted to the range of 65–90°C. In contrast, it is noteworthy that PT-2DAP did not affect the T_m curve significantly at the same concentration. On the other hand, spermine was found to provide a T_m shift similar to 1,3-diaminopropane at a $^1/_{10}$ concentration, and PT-2SPM slightly shifted the T_m curve to 55–75°C at 5 μM concentration. T_m curves shown in Figure 2 indicate that the binding character of the pteridine–polyamine conjugate to DNA is weaker than that of the corresponding polyamine. These results also support the seemingly contradictory conclusion that weakly interacting with DNA PT-2SPM and PT-2DAP conjugates can condense DNA at lower concentrations. It should be mentioned that the concentration of polyamines or conjugates in the DNA melting experiments was below the values necessary for DNA compaction.

In order to confirm that pteridine–polyamine conjugates do not bind strongly with DNA double helix, we also investigated the changes of the UV and fluorescent spectra of the compacting agents in the presence of DNA. Since naturally occurring pteridine derivatives such as biopterin and folic acid are metabolites of guanosine triphosphate (GTP), the guanosine-like structure of the pteridine can produce hydrogen-bond pairs with cytosine. On the other hand, some aromatic heterocyclic compounds such as YOYO have the potential to bind to DNA through intercalation or in a groove-binding manner.[26] These specific DNA-binding mechanisms bring the heteroaromatic ring into a neighboring position to the DNA double helix, and, the UV and fluorescent spectra of the heterocycle would thus be greatly influenced by electronic interactions. However, no significant distortion was observed in the UV or fluorescent spectra of PT-2SPM when it was mixed with lambda DNA. The UV spectra at the range of 300–400 nm assigned to the absorption of pteridine chromophore, and the fluorescence spectra are shown in Figure 3a and b, respectively. These results suggest that DNA bases and the pteridine ring compacting agent do not interact with each other. According to the T_m and spectroscopic investigations, the pteridine–polyamine conjugates did not occupy any neighboring site of the DNA duplex. The fact that fluorescent quenching was not recognized in a mixture of PT-2SPM with DNA suggests the utility of PT-2SPM as a fluorescent dye for FM observation instead of YOYO. Indeed, DNA globules with high optical density became visible on FM observation when DNA compaction was observed in the absence of fluorescent dye, while DNA coil was not detectable due to low contrast against the background.

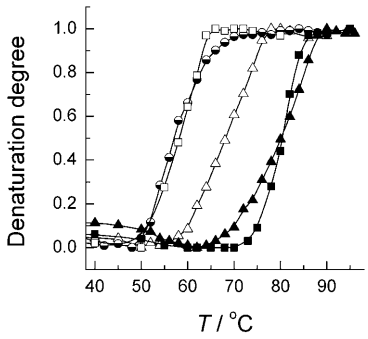


Figure 2. Lambda DNA melting curves in 1 mM NaCl solution in the presence of 50 μM DAP (■), 50 μM PT-2DAP (□), 5 μM SPM (▲), 5 μM PT-2SPM (△); and control melting curve of DNA only (◒).

To understand the macro-scale conformational transformation of DNA induced by the pteridine–polyamine conjugates, it is useful to consider the conformation changes in the micro scale. Circular dichroism (CD) spectra have been employed as a highly sensitive monitoring tool for the DNA secondary structure change caused by the mutual location of DNA nucleotides.[27] CD spectra of lambda DNA in the presence of different concentrations of PT-2SPM are shown in Figure 3c. The addition of PT-2SPM to a DNA solution leads to a decrease in positive shoulder at 277 nm with no changes in negative shoulder at 257 nm. The behavior

shown in Figure 3c resembles to the previously reported evolution of DNA CD spectra induced by spermine itself.[28,29] Because of this similarity, the change of DNA secondary structure is assumed to be provided entirely by the polyamine part.

It is well known that pteridine derivatives can be associated by intermolecular hydrogen bonds, and these strong associations have been employed in the construction of self-assembled supramolecular systems.[30,31] In the particular case of the pteridine–polyamine conjugates, the hydrogen-bonding assembly of several molecules, examples of which are illustrated in Figure 4, affords bulky multicationic species. The bulkiness prevents the polycations from accessing the double strand (i.e., interaction of pteridine rings with DNA bases), but their large positive charge value allows them to eliminate intercharge repulsion on the DNA surface and induce DNA compaction with high efficiency.

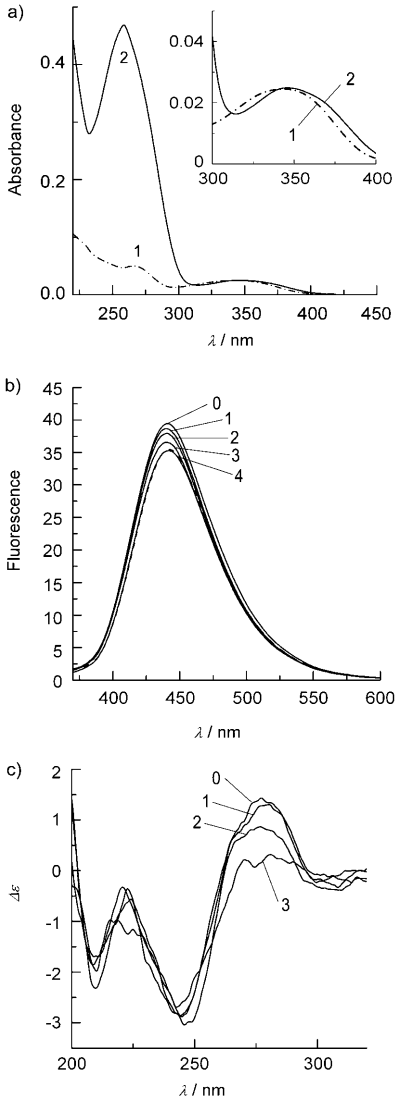


Figure 3. a) UV spectra of 5 μM PT-2SPM (1), and 5 μM PT-2SPM with 50 μM lambda DNA (2); b) fluorescence spectra of 50 nM PT-2SPM (0) and 50 nM PT-2SPM in the presence of lambda DNA with concentrations of 0.1 nM (1), 1 nM (2), 10 nM (3), and 100 nM (4); c) circular dichroism spectra of 50 μM lambda DNA (0) and 50 μM lambda DNA in the presence of 5 μM (1), 10 μM (2), and 25 μM (3) of PT-2SPM.

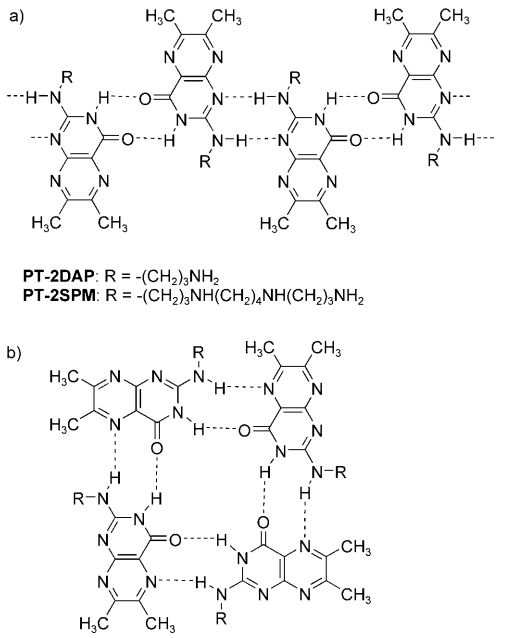


Figure 4. Examples of molecular assemblies, which can be realized by pteridine–polyamine conjugates: a) linear ribbon-like aggregation, b) cyclic disk-like aggregation.

There are two major DNA interaction modes concerning DNA compaction: long-distance charge neutralization and close binding to the double strand. Nevertheless, many known DNA compacting compounds have both short- and long-distance interaction modes such as spermine, and generally the former is stronger. Closely DNA-binding spermine effectively neutralizes all anionic charge of DNA phosphates to afford tightly compacted DNA, while long-range neutralization of the DNA surface charge induced by the bulky PT-2SPM assembly is not effective in eliminating inner charge repulsion of the DNA chain, and performs loose DNA compaction. Consequently, DNA chains are bundled loosely to give a particle with lower degree of condensation. The molecular design on the polyamine described herein distinguishes the long-distance interaction from the direct DNA duplex-binding interactions; the weaker interaction is clearly crucial for the DNA compaction induced by surface charge neutralization. Typical transmission electron microscope (TEM) images of the DNA globule states created by PT-2SPM and spermine are shown in Figure 5. The difference between the two images is obvious: PT-2SPM creates a loosely folded thicker toroidal nanoparticle (large outer and small inner diameters) with lower density.

In conclusion, we have demonstrated that in spite of the lower charge on pteridine–polyamine conjugates than on corresponding polyamines and weaker effect on DNA secondary structure, pteridine–polyamine conjugates efficiently compact DNA in a very sharp on/off manner into loosely folded DNA toroidal condensate. Since it is known that

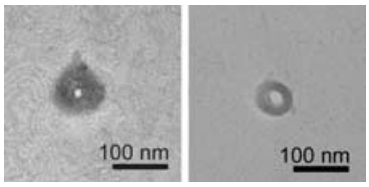


Figure 5. Transmission electron microscopy images of T4 DNA (1 μM) compacted by PT-2SPM (20 μM, left image) and spermine (20 μM, right image).

loosely compacted DNA chain is potentially effective for gene expression in transgenic technology,[32] the idea of the PT-2R type pteridine–polyamine conjugate is expected to be useful in a wide variety of applications in both molecular biology and gene technology.

Experimental Section

Materials and reagents: T4 phage DNA of 166 kbp with a contour length of 57 μm and lambda DNA (48.5 kbp) were purchased from Nippon Gene Co., Ltd. and Takara Bio Inc., respectively. The fluorescent dye, 1,1′-[1,3-propanediylbis[(dimethyliminio)-3,1-propanediyl]]bis[4-[(3-methyl-2(3*H*)-benzoxazolylidene)methyl]]quinolinium tetraiodide (YOYO-1, λ_{max} (ex) = 508 nm) was obtained from Molecular Probes, Inc. 1,3-Diaminopropane and spermine were obtained from Tokyo Kasei Kogyo Co., Ltd. and Sigma-Aldrich Co. Reproduction, respectively. Doubly distilled water, Milli-Q grade, was used and is identified simply as water in this study.

2-(3-Aminopropyl)amino-3*H*-4-one-6,7-dimethylpteridine (PT-2DAP): A mixture of 4-hydroxy-2-methylthio-6,7-dimethylpteridine (0.82 g, 3.7 mmol)[33,34] and 1,3-diaminoprpane (3 mL) was heated at 120 °C for 3 d. Dry diethyl ether (20 mL) was added to the mixture, and precipitates were separated by decantation. Washing the precipitates with dry diethyl ether (5 × 20 mL) afforded the title compound as a yellow powder (0.69 g, 76 %). Analytical samples were prepared by recrystallization from water; m.p. 280 °C (decomp); ^{1}H NMR (400 Hz, mono acetate salts, D_2O, 25 °C): δ = 2.24 (m, 2H; CH_2), 2.68 (s, 3H; CH_3), 2.70 (s, 3H; CH_3), 3.34 (t, J = 7.3 Hz, 2H; CH_2), 3.73 (t, J = 6.6 Hz, 2H; CH_2); ^{13}C NMR (100 Hz, mono acetate salts, D_2O, 25 °C): δ = 21.46, 22.69, 22.92, 27.59, 37.81, 38.38, 125.36, 150.53, 153.34, 161.52, 179.59; UV/Vis at pH 10.11 phosphate buffer: λ_{max} ($\log\varepsilon_{max}$) = 364 (3.94), 258 (4.34); UV at pH 6.63 phosphate buffer: λ_{max} ($\log\varepsilon_{max}$) = 350 (4.21), 276 (4.25); UV at pH 1.58 phosphate buffer: λ_{max} ($\log\varepsilon_{max}$) = 324 (3.87), 288 (4.05); fluorescent spectra (H_2O, $c = 10^{-6}$ mol dm^{-3}): λ_{ex} = 360 nm, λ_{em} (I_{max}) = 447 nm (124); elemental analysis calcd (%) for $C_{11}N_6OH_{16}$ (248.28): C 53.21, H 6.50, N 33.85; found C 53.20, H 6.48, N 33.76.

2-(4,9,13-Triazatridecyl)amino-3*H*-4-one-6,7-dimethylpteridine (PT-2SPM): A mixture of 4-hydroxy-2-methylthio-6,7-dimethylpteridine (1.01 g, 4.5 mmol) and spermine (3.27 g, 16.2 mmol) was heated under an argon atmosphere at 100 °C for 3 d. The precipitates were washed with chloroform (20 mL) and diethyl ether (5 × 20 mL), and dried under vacuum conditions. The title product was obtained as an orange powder (0.80 g, 52 %). Analytical samples were obtained by recrystallization from methanol; m.p. 190 °C (decomp); ^{1}H NMR (400 Hz, D_2O, 25 °C): δ = 1.50 (m, 2H; CH_2), 1.67 (m, 4H; CH_2,CH_2), 1.96 (m, 2H; CH_2), 2.50 (s, 3H; CH_3), 2.51 (s, 3H; CH_3), 2.56 (t, J = 7.3 Hz, 2H; CH_2), 2.65(t, J=7.3 Hz, 2H; CH_2), 2.78 (t, J = 7.3 Hz, 2H; CH_2), 2.82 (t, J = 7.1 Hz, 2H; CH_2), 2.96 (t, J = 6.3 Hz, 2H; CH_2), 3.51 (t, J = 5.9 Hz, 2H; CH_2); ^{13}C NMR (100 Hz, D_2O, 25 °C): δ = 21.21, 22.39, 24.45, 25,65, 27.67, 28.66, 37.82, 38.66, 45.06, 46.25, 47.74, 48.28, 126.42, 147.96, 155.50, 158.93, 163.34, 173.18; UV at pH 11.59 phosphate buffer: λ_{max} ($\log\varepsilon_{max}$) = 366 (3.85), 260 (4.26); UV at pH 6.53 phosphate buffer: λ_{max} ($\log\varepsilon_{max}$) = 350 (4.13), 276 (4.18); UV at pH 0.60 phosphate buffer: λ_{max} ($\log\varepsilon_{max}$) = 324 (3.86), 290 (3.84); fluorescent spectrum (H_2O, $c = 10^{-6}$ mol dm^{-3}): λ_{ex} = 360 nm, λ_{em} (I_{max}) = 448 nm (127).

Instruments: The UV spectra of conjugates were recorded on a Jasco U-550 UV/VIS spectrophotometer with a Jasco ETC-505T temperature controller. Fluorescence spectra, CD, and NMR spectra were recorded on a Jasco FP-6600 spectrofluorimeter, a Jasco J-790 spectropolarimeter, and a JEOL A-400 spectrometers, respectively. TEM observations were performed at room temperature using a JEM-1200EX microscope (JEOL) at an acceleration voltage of 100 kV with carbon-coated grids of 300 μm mesh size.

The pK_a values were determined by using a UV spectroscopic method previously described.[35] Fluorescence images of DNA molecules were observed with a Zeiss Axiovert 135 TV microscope equipped with a 100× oil-immersed lens, and the images were recorded by a Hamamatsu SIT TV camera. T4 phage DNA was dissolved in 10 mM Tris/HCl buffer (pH 7.6) with 0.04 μM YOYO-1 and 4 % (v/v) 2-mercaptoethanol. To avoid intermolecular DNA aggregation, all measurements were conducted at low DNA concentrations: 0.2 μM in nucleotide units. After 30 min, an aqueous solution of pteridine–polyamine conjugates (PT-2DAP or PT-2SPM) was added and the sample was gently mixed. Samples ready for observation were additionally held for approximately 30 min to 1 h at room temperature. The solution for electron microscopic observation was prepared by mixing 1 μM of T4 DNA with 20 μM of PT-2SPM or 20 μM spermine in a 0.01 M Tris-HCl buffer solution. Uranyl acetate (1 % in water) was used for negative staining prior to TEM observation. Absorbance (220–310 nm) of the 50 μM lambda DNA in 1 mM sodium chloride solution containing varying concentrations of condensing agents was recorded with steps of 2 to 5 °C step. The degree of DNA denaturation was obtained by the equation $(A-A_0)/(A_{max}-A_0)$, where A_0 and A_{max} are the DNA absorption at room temperature and DNA absorption at the point of complete denaturation, respectively.

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