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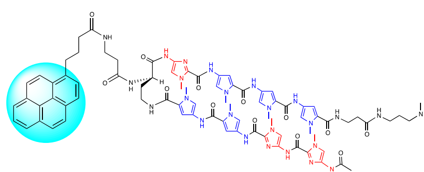
Thangavel Vaijayanthi[a,c], Toshikazu Bando[a], Kaori Hashiya[a], Ganesh N. Pandian[b] and Hiroshi Sugiyama[a,b,c,*]
[a]*Department of Chemistry, Graduate School of Science, Kyoto University, Sakyo, Kyoto 606-8501, Japan*
[b]*Institute for Integrated Cell-Material Sciences (WPI-iCeMS), Kyoto University, Sakyo, Kyoto 606-8502, Japan*
[c]*International Research Unit of Integrated Complex System Science (IRU-ICSS), Kyoto University, Sakyo, Kyoto 606-8501, Japan*



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Design of a New Fluorescent Probe: Pyrrole/Imidazole Hairpin Polyamides with Pyrene Conjugation at their γ-Turn

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[a]*Department of Chemistry, Graduate School of Science, Kyoto University, Sakyo, Kyoto 606-8501, Japan*
[b]*Institute for Integrated Cell-Material Sciences (WPI-iCeMS), Kyoto University, Sakyo, Kyoto 606-8502, Japan*
[c]*International Research Unit of Integrated Complex System Science (IRU-ICSS), Kyoto University, Sakyo, Kyoto 606-8501, Japan*
*Corresponding author. Tel.: +8175753 4002; Fax: +81 75 753 3670; Email address: hs@kuchem.kyoto-u.ac.jp

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ABSTRACT

*Fluorophores that are conjugated with N-methylpyrrole-N-methylimidazole (Py–Im) polyamides postulates versatile applications in biological and physicochemical studies. Here, we show the design and synthesis of new types of pyrene-conjugated hairpin Py-Im polyamides (**1–5**). We evaluated the steady state fluorescence of the synthesized conjugates (**1–5**) in the presence and absence of oligodeoxynucleotides 5′-CGTATGGACTCGG-3′ (ODN 1) and 5′-CCGAGTCCATACG-3′ (ODN 2) and observed a distinct increase in emission at 386 nm with conjugates **4** and **5**. Notably, conjugate **5** that contains a β-alanine linker had a stronger binding affinity (K_D = 1.73 × 10^{-8} M) than that of conjugate **4** (K_D = 1.74 × 10^{-6} M). Our data suggests that Py–Im polyamides containing pyrene fluorophore with a β-alanine linker at the γ-turn NH_2 position can be developed as the competent fluorescent DNA-binding probes.*

1. Introduction

In recent years, development of methods to diagnose the hereditary diseases in their early stages is of increasing demand. DNA carries the genetic information specifying the structure and function of the cells across generations. Hence, diagnostic molecules that could directly detect specific double-stranded DNA (dsDNA) sequences obviating the need for denaturation, hybridization, washing, or labeling of the DNA samples have been gaining immense interest owing to their potential clinical applications.[1–4] Accordingly, molecular beacons and DNA-binding molecules have been demonstrated as powerful tools to probe for the specific DNA sequences.

Among the DNA-binding molecules, *N*-methylpyrrole (Py)-*N*-methylimidazole (Im) polyamides have been extensively studied for their ability to recognize each of the four Watson–Crick base pairs.[5,6] Moreover, Py-Im polyamides could be altered to achieve sequence-specific DNA bindings and could be conjugated to a wide variety of functional groups.[7,8] Accordingly, Dervan and co-workers explored Py–Im polyamide–fluorophore conjugates[1,2,9] and novel fluorescent scaffolds of Py–Im polyamides without dye molecules as fluorescent probes.[10] We have also been developing various types of sequence-specific alkylating Py–Im polyamides and have investigated their chemical and biological properties.[11–13] In addition to such basic applications pertaining to gene regulation, the sequence-specific binding properties of Py–Im polyamides have been explored to detect precise sequences of duplex DNA for potential diagnostic applications.[9,14,15] Previously, we have demonstrated recognition of the target and CAG-repeated DNA sequences by Py–Im polyamide-pyrene and perylene fluorophores.[16–18] Pyrene-based fluorophores are attractive molecules that could be developed into efficient DNA binding probes. Because it is known that pyrene-based fluorescence exhibits large extinction coefficients, excellent quantum yields, and good stability in aqueous solution.

Design and synthesis of novel Py-Im conjugates that causes notable shift in the fluorescence upon binding to the minor groove of duplex DNA could lead to the development of effective sequence-specific fluorescence-based probe(s). Here, we report the molecular design of new types of pyrene-based hairpin Py–Im conjugates **1–5**, synthesized by Fmoc solid-phase methods. Also, through studies with the steady state fluorescence we suggest the vital role of β-alanine linker at the γ-turn NH_2 position in improving the DNA binding affinity. Our data could be used in the field of chemical biosensors for dsDNA.

2. Results and discussion

*2.1. Synthesis of pyrene conjugates (**1–5**)*

The pyrene-conjugated Py–Im polyamides **1–5** were synthesized following the previously reported procedures (Figure 1).[19–21] The corresponding Py–Im polyamide units were synthesized using an Fmoc solid-phase synthesis method using HCTU as the coupling reagent. For polyamide **9**, *R*-Boc-Dab(Fmoc)-OH was used instead of γ-butyric acid at the position of the γ-turn. On completion of the solid-phase synthesis, Boc-protected polyamides **6**, **7**, and **9** were obtained by cleaving from their corresponding β-wang resins using aminolysis with *N*,*N*-

dimethyl-1,3-propanediamine (Dp). **8** was obtained by treatment with methyl di(aminpropyl)amine. The desired conjugates **1**–**4** were produced by the deprotection of Boc with TFA and subsequent coupling with 1-pyrene butyric acid *N*-hydroxy succinimide ester (Pyrb-OSu). Conjugate **5** was obtained by coupling with Pyb-β-CO_2H, which was synthesized by the coupling of Pyb-OSu with ethyl β-alanine, followed by hydrolysis. HPLC purification yielded the polyamide conjugates **1**–**5** as pale-yellow powders with > 95% purity. The purity and identity of pyrene conjugates **1**–**5** were verified by reversed-phase analytical HPLC and electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS).

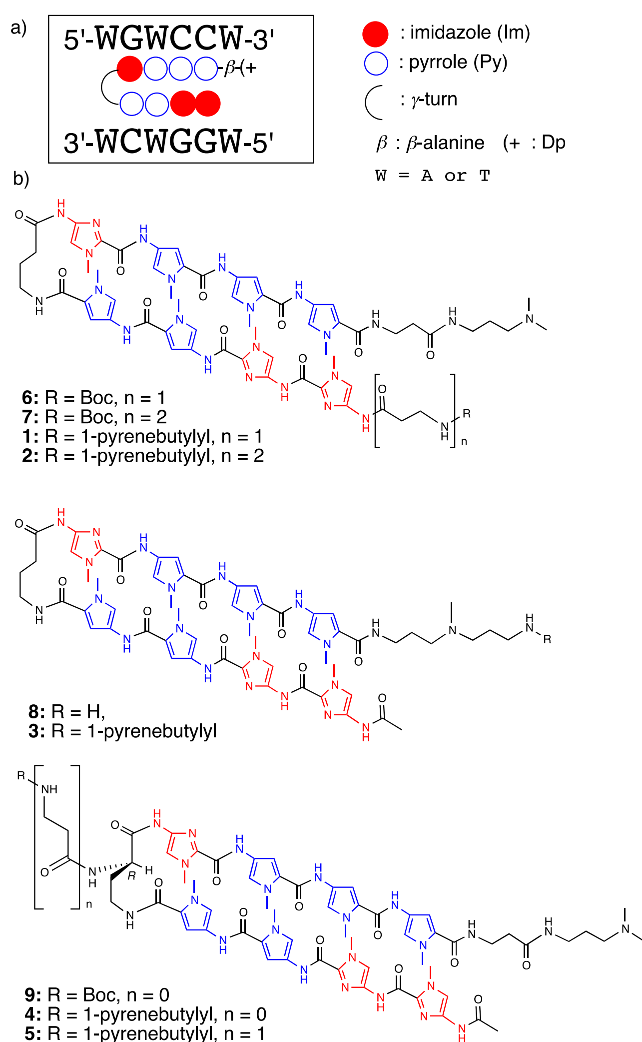

Figure 1. a) Schematic representation of sequence-specific DNA binding by Py–Im polyamide. b) Chemical structures of compounds **1**–**9**.

2.2. Fluorescence emission of conjugates (*1*–*5*) in the presence of ODNs 1/2

To demonstrate the ability of the synthesized conjugates **1**–**5** to precisely bind to the target sequences, their fluorescence emission was examined in both the absence (**I**) and presence (**II**) of 13-mer DNA: 5′-CGTATGGACTCGG-3′ (ODN 1) and 5′-CCGAGTCCATACG-3′ (ODN 2), as shown in Figure 2. Conjugates **1** and **2** showed about a threefold increase in fluorescence intensity emission centered at ~430 nm in the presence of ODNs 1/2 (Figure 2a, Dark blue and Orange lines respectively). DNA interaction of the pyrene moiety located at the *N*-terminus position of conjugates **1** and **2** may have caused this increase in emission. The increase in fluorescence emission with a bathochromic shift of ~45 nm of conjugates **1** and **2** might have been caused by the aqueous phase charge/electron transfer from pyrrole to pyrene. Hence, conjugates **1** and **2** with an incorporation of the pyrene fluorophore at the *N*-terminal position did not have suitable properties to act as DNA-binding probes. In contrast, a weak increase in fluorescence emission by using conjugate **3** was observed at 385 nm in the presence of ODNs 1 and 2 (Figure 2a, Red line).

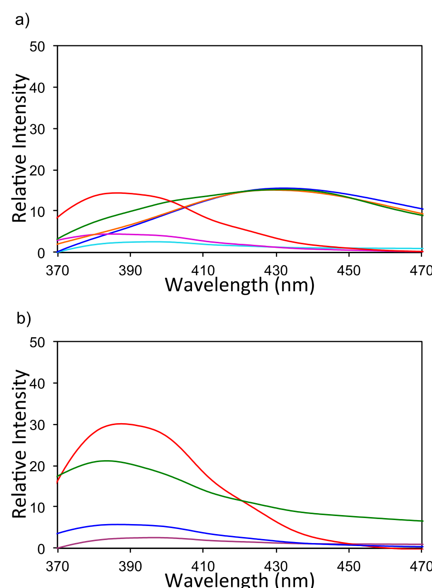

Figure 2. Steady state fluorescence spectra of conjugates. a) **1**–**3** and b) **4-5**; **I**: absence of ODNs 1 and 2; **II**: presence of ODNs 1 and 2 (2 μM). **1-I** (light/pale blue); **1-II** (Dark blue); **2-I** (Pink line); **2-II** (Orange line); **3-I** (Green line); **3-II** (Red line); **4-I** (purple line); **4-II** (Red line); **5-I** (Blue line); **5-II** (Green line). 200 nM of conjugates (**1**–**5**); buffer: 5 mM Na phosphate buffer, pH 7.0, 1% v/v DMF; λ_{ex}: 345 nm and λ_{em}: 386 nm.

To design Py–Im polyamides as efficient fluorescent probes, pyrene moieties were attached to the NH_2 at the γ-turn. Interestingly, FITC and BoFl-polyamides linked at the γ-turn were reported to have better nuclear uptake profiles than *C*-tail-linked polyamides.[22] In our results, conjugate **4** showed ~7-fold increase in fluorescence emission compared with **5**, which showed ~4-fold increase in fluorescence emission (Figure 2b, Red and Green lines respectively). It is important to note here that Py-Im polyamides conjugated with the fluorophores such as Bis-Pyrene, Perylene, Tetramethyl rhodamine and Thiazole orange were known to have an extremely weak or almost no fluorescence emission in the presence of the mismatched ODNs even at higher concentrations.[1, 9, 16-18] Hence, it is reasonable to assume that our conjugates (**1-5**) might have poorer binding affinity with mismatched ODNs and give weak fluorescence emission.

2.3. Binding affinity of the conjugates (*4*–*5*)

To acquire additional insight into the interactions of conjugates **4** and **5** with target binding sequences, their binding affinities were investigated using SPR methods and biotinylated hairpin DNA (Figures 3a and 3b). The dissociation equilibrium constant (K_D) was obtained by fitting the resulting sensorgrams

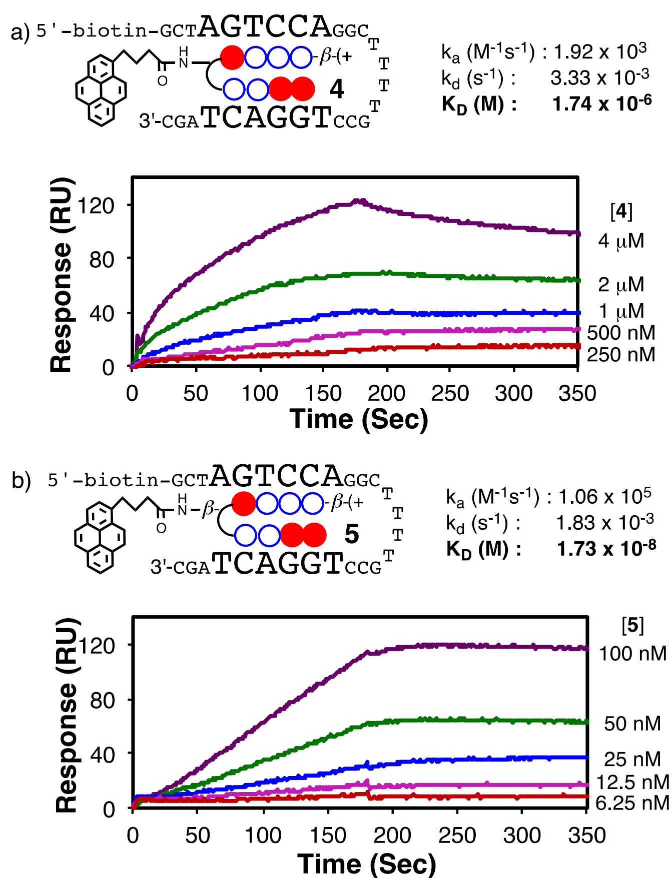

Figure 3. SPR sensorgrams for the interaction: a) conjugate **4**, b) conjugate **5** with hairpin DNAs immobilized on the surface of a sensor chip SA. All the experiments were performed in HBS-EP buffer (0.01 M HEPES, pH 7, 0.15 M NaCl, 3 mM EDTA, 0.005% surfactant P20) with 0.1% DMSO (v/v) at 25 °C, [**4**] = 250 nM – 4 μM, [**5**] = 6.25 nM –100 nM.

to a theoretical model. The values of K_D for **4** and **5** were determined to be 1.74 × 10^{-6} (M) and 1.73 × 10^{-8} (M), respectively. In contrast, conjugates **1–3** were observed to have weak binding affinity, > 1.0 × 10^{-5} (M), because of the hindrances by the pyrene moiety against the DNA minor groove (unpublished data).

Conjugate **5** has approximately 10^2 times higher DNA-binding affinity than conjugate **4**, whereas the K_D of AcImImPyPy-*γ*-ImPyPyPy-*β*-Dp was 5.8 × 10^{-9} (M), reported by SPR methods using the same biotinylated hairpin DNA.[23] Therefore, conjugate **5** has approximately one third of the DNA-binding affinity of nonlabeled polyamides; however, similar difficulties in binding polyamide conjugates have been reported. For example, Rucker *et al.* showed that the binding affinities of polyamides with TMR fluorophore at DNA match sites were reduced by a factor of 10–50 from the nonlabeled polyamides.[1] Fujimoto *et al.* also observed that the binding affinity of polyamides linked with two pyrene molecules at pyrrole rings was about ~60-times decreased when compared with nonlabeled polyamides.[16,17] Introducing the planar pyrene fluorophore at the *γ*-NH_2 position would probably adjust the appropriate space for the binding with DNA, and thereby the electron/charge transfer that leads to greater fluorescence emission. The pyrene fluorophore linked through the flexible *β*-alanine would be positioned beside the target sequence out of the minor groove. These observed results imply the potential utility of conjugate **5** as a fluorescent probe to recognize specific DNA sequences.

3. Conclusions

Cell permeable and nuclear localizing hairpin Py-Im polyamides have been successfully employed in gene regulation to selectively switch \`On\` and \`OFF\` the gene(s) of interest.[24-28] Biologically active hairpin Py-Im polyamides could also be employed to accumulate a huge wealth of biological information when they could be conjugated with effective fluorescent dyes. Pyrene is known to possess unique spectral properties that have been exploited in wide range of biological applications. Hence, we have conjugated our hairpin polyamides with pyrene to synthesize a new type of pyrene-conjugated hairpin Py-Im polyamide (**1–5**) using Fmoc solid-phase synthesis. Steady state fluorescence studies carried out to investigate the photophysical properties of the conjugates **1–5** showed that the fluorescence emission of Py–Im polyamides (possessing a pyrenylbutyl group at the *γ*-NH_2 position in the hairpin) **4** and **5** is dramatically increased in the presence of ODNs 1 and 2. This result clearly demonstrates that the Py and Im moieties present in the polyamide successfully induce the pyrene moiety to generate fluorescence emissions. Also, it is shown that the presence of *β*-alanine linker in conjugate **5** could enable its good affinity for binding with target sequences. Our results suggest the critical role of *γ*-turn position and *β*-alanine linker in improving the sequence-specificity which encourages us to explore and develop compounds harboring them as a new type of DNA minor-groove binding molecule. Efficient recognition of the selective sequences could contribute to the development of potent diagnostic tools for detecting genetic diseases.

4. Experimental

4.1. Materials

Reagents and solvents were purchased from standard suppliers and used without further purification. Abbreviations of some reagents: DMF, *N*,*N*-dimethylformamide; DCM, dichloromethane; TFA, trifluoroacetic acid, CH_3CN, acetonitrile, PyBOP, (Benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate; DIEA, diisopropylethylamine; Pyb-OSu, 1-Pyrenebutyric acid *N*-hydroxysuccinimide ester. Pyb-*β*-CO_2H was synthesized by the coupling with Pyb-OSu and Ethyl *β*-alanine then hydrolysis. Electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS) was performed on BioTOF II (Bruker Daltonics) mass spectrometers. DNA oligonucleotides were purchased from Sigma–Aldrich Co. Fluorescence measurements were performed on Spectrofluorometer FP-6300 (JASCO). All machine-assisted polyamide syntheses were performed on PSSM-8 peptide synthesizer (Shimadzu) with a computer-assisted operation system. HPLC purification was performed with a Chemcobond 5-ODS-H reversed-phase column (10 × 150 mm) in 0.1% TFA with acetonitrile as eluent at a flow rate of 1.0 mL/ min, appropriate gradient elution conditions, and detection at 254 nm.

*4.2. Synthesis of Conjugates (**1-5**)*

Pyrb-(*β*)□-ImImPyPy-*γ*-ImPyPyPy-*β*-Dp [**1** (n=1) and **2** (n=2)]: Boc-(*β*)□-ImImPyPy-*γ*-ImPyPyPy-*β*-wang resin (n=1 and 2) were programmed to synthesize in a stepwise reaction by Fmoc solid-phase protocol. Each resin was cleaved with 500 μL of Dp at 55 °C for 4 h. The resin was removed by filtration,

washed with DCM, concentrated *in vacuo*, and then triturated by diethyl ether. These crude Boc-protected PI polyamides **6**, **7** were used in the next step without further purification. After deprotection of Boc with TFA, the crude polyamides were coupled with Pyrb-OSu and DIEA in DMF at room temperature to yield pyrene conjugates **1** and **2** and were purified by HPLC using a Chemcobond 5-ODS-H column (0.1% TFA/CH_3CN 0-100% linear gradient, 0-20 min, 254 nm). **1**: ESI-TOF-MS (positive) m/z calcd for $C_{80}H_{90}N_{24}O_{12}^{2+}$ $[M+2H]^{2+}$ 790.36; found 790.38, **2**: ESI-TOF-MS (positive) m/z calcd for $C_{83}H_{95}N_{25}O_{13}^{2+}$ $[M+2H]^{2+}$ 825.88; found 825.90.

AcImImPyPy-γ-ImPyPyPy-β-Dp-Pyrb (**3**): AcImImPyPy-γ-ImPyPyPy-β-wang resin was programmed to be synthesized in a stepwise reaction by Fmoc solid-phase protocol. The resin was cleaved with 500 μL of methyl di(aminpropyl)amine at 55 °C for 4 h. The resin was removed by filtration, washed with DCM, concentrated *in vacuo*, and then triturated by diethyl ether and DCM. The crude polyamide **8** was used in the next step without further purification. The crude polyamide was coupled with Pyrb-OSu and DIEA in DMF at room temperature to yield pyrene conjugate **3**, was purified by HPLC using a Chemcobond 5-ODS-H column (0.1% TFA/CH_3CN 0-100% linear gradient, 0-20 min, 254 nm). **3**: ESI-TOF-MS (positive) m/z calcd for $C_{81}H_{92}N_{24}O_{12}^{2+}$ $[M+2H]^{2+}$ 797.38; found 797.45.

AcImImPyPy-[Pyrb-(β)□□□□□□□]-ImPyPyPy-β-Dp [**4** (n=0) and **5** (n=1)]: AcImImPyPy-[Boc□□□□□□]-ImPyPyPy-β-wang resin was programmed to synthesize in a stepwise reaction by Fmoc solid-phase protocol. Each resin was cleaved with 500 μL of Dp at 55 °C for 4 h. The resin was removed by filtration, washed with DCM, concentrated *in vacuo*, and then triturated by diethyl ether. Boc-protected PI polyamide **9** was used in the next step without further purification. After deprotection of Boc with TFA, the ammino-crude was coupled with Pyrb-OSu and DIEA in DMF at rt to yield **4**. Similarly, ammino-crude was coupled with Pyb-β-CO_2H and PyBOP, DIEA in DMF at room temperature to yield **5**. These conjugates were purified by HPLC using a Chemcobond 5-ODS-H column (0.1% TFA/CH_3CN 0-100% linear gradient, 0-20 min, 254 nm). **4**: ESI-TOF-MS (positive) m/z calcd for $C_{79}H_{88}N_{24}O_{12}^{2+}$ $[M+2H]^{2+}$ 783.35; found 783.39. **5**: ESI-TOF-MS (positive) m/z calcd for $C_{82}H_{93}N_{25}O_{13}^{2+}$ $[M+2H]^{2+}$ 818.87; found 818.93. The identity and purity of each conjugate was verified by analytical HPLC, UV-visible spectroscopy and ESI-TOF-MS.

4.3. Steady state fluorescence spectroscopy experiments

Steady–state fluorescence spectra were obtained on a Spectrofluorometer FP-6300 (JASCO) with an excitation wavelength of 345 nm in hybridization buffer, and oligonucleotides at corresponding concentrations of conjugates **1**–**5**. No special efforts were made to remove oxygen from the reaction solution at room temperature.

4.4. SPR assays

SPR experiments were performed on a Biacore X instrument at 25 °C. Biotinylated hairpin DNA, 5`-biotin- labeled GCTAGTCCAGGCTTTTGCCTGGACTAGC-3` was obtained from Sigma–Aldrich Co. Streptavidin-functionalized SA sensor chips were purchased from Biacore. After immobilization of hairpin DNAs onto the sensor chips, measurements of binding curves of polyamides to the hairpin DNA, and data processing ATwere performed according to following procedure: different concentrations of polyamide solutions in HBS-EP buffer with 0.1% DMSO were prepared by dilution from 4 mM stock solutions in DMSO. All binding experiments were carried out using HBS-EP buffer with 0.1% DMSO as running buffer, and the running buffer or 10 mM glycine–HCl (pH 1.5) was used as the regeneration solution. Typically, the buffer was injected at a flow rate of 5 μL/min as a blank control after a stable baseline was obtained, and then samples were injected under conditions identical to those of buffer injection at concentrations ranging from 250 nM to 4 μM for **4**, from 6.25 nM to 100 nM for **5**. Kinetic information was obtained by global fitting of the response units versus time using a model with 1:1 drifting baseline using the BIA evaluation 4.1 program.

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