JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Communication

pubs.acs.org/JACS

Triplex-Forming Peptide Nucleic Acid Probe Having Thiazole Orange as a Base Surrogate for Fluorescence Sensing of Double-stranded RNA

Takaya Sato, Yusuke Sato, and Seiichi Nishizawa*

Department of Chemistry, Graduate School of Science, Tohoku University, Sendai 980-8578, Japan

Supporting Information

ABSTRACT: We have developed a new fluorescent sensing probe for double-stranded RNA (dsRNA) by integrating thiazole orange (TO) as a base surrogate into triplex-forming PNA. Our probe forms the thermally stable triplex with the target dsRNA at acidic pH; and the triplex formation is accompanied by the remarkable light-up response of the TO unit. The binding of our probe to the target dsRNA proceeds very rapidly, allowing real-time monitoring of the triplex formation. Importantly, we found the TO base surrogate in our probe functions as a universal base for the base pair opposite the TO unit in the triplex formation. Furthermore, the TO unit is significantly more responsive for the fully matched dsRNA sequence compared to the mismatch-containing sequences, which enables the analysis of the target dsRNA sequence at the single-base pair resolution. The binding and sensing functions of our probe are described for the development of fluorescent probes applicable to sensing biologically relevant dsRNA.

Synthetic fluorescent probes capable of binding to RNA structures have been used as powerful tools for the study of RNA functions. Representative examples are genetically encoded probes fused to fluorescent proteins and fluorophore-modified hybridization probes.[1] These probes, in principle, can recognize single-stranded regions of target RNAs, accompanying the fluorescence light-up response. Meanwhile, targeting double-stranded RNA (dsRNA) structures by fluorescent probes has been a challenging task. The main conventional approach relies on the probe binding to the specific secondary structures such as bulges,[2] mismatches,[3] and dangling ends.[4,5] In contrast to the structure-selective binding, there are no reports on fluorescent probes for direct sensing of dsRNA *sequences* because it is difficult to carry out a rational design of the molecular recognition of the dsRNA sequences.

Rozners and co-workers reported that homopyrimidine peptide nucleic acids (PNAs) tightly bind to homopurine tracts within dsRNA by means of sequence-selective Hoogsteen base pairing.[6] While the triplex formation by conventional triplex-forming oligonucleotides (TFOs) generally shows binding preference for dsDNA over dsRNA,[7] triplex-forming PNAs (TFPs) exhibit higher binding affinity for dsRNA.[6] These findings encouraged the development of a novel strategy targeting dsRNA structures in biochemical and therapeutic applications.[8] In this context, much effort has been made to explore new PNA monomers to achieve effective triplex formation under physiological conditions[9,10] as well as to expand the targetable dsRNA sequences.[11] However, following our interest in the design of analytical tools for biologically important RNAs,[4,5] we have sought to develop a new class of TFP-based sensing probes capable of sequence-selective detection of dsRNA.

In this work, we focus on the integration of thiazole orange (TO) as a base surrogate to the middle of the TFP sequence to develop fluorescent probes that exhibit a light-up response upon triplex formation with dsRNA, which we call tFIT (triplex-forming forced intercalation of thiazole orange) probes. As shown by Seitz and co-workers, the TO base surrogate is very useful for designing light-up hybridization probes (FIT probes) to detect single-stranded DNA and RNA.[12−14] Fluorescence "off−on" ability of the TO unit arises from the restriction of rotation around the methine bridge between the two heterocycles when the probes form a duplex.[15] We envision that the TO base surrogate also functions as the light-up unit in our TFP-based probes targeting dsRNA sequences because the TO unit would be forced to intercalate at a chosen site and then experience the rotation-restricted environment in the resulting triplex (Figure 1A). In addition, the fluorescence signaling of the TO base surrogate is expected to be unaffected by guanine quenching through photoinduced charge transfer unlike most fluorophores,[12,16] which is an important photophysical property for the fluorescence analysis of the target purine-rich sequences.

We designed a 9-mer homopyrimidine PNA containing a TO base surrogate at the central position (H_2N-Lys-tctc(TO)tccc-$CONH_2$). The probe sequence is complementary to the homopurine sequence within RNA1 (5′-AGA GGA GGG-3′/3′-UCU CCU CCC-5′, Figure 1B), which carries the G−C base pair at the opposite position of the TO unit when the triplex is formed. The probe was synthesized by a divergent solid-phase synthesis,[17] purified by reverse phase HPLC, and characterized by MALDI-TOF-MS (see the SI, Figures S1−S5 and Table S1).

We first examined the triplex formation of the probe with dsRNA by UV melting experiments in 10 mM sodium acetate (pH 5.5) or sodium phosphate (pH 7.0) buffer solutions containing 100 mM NaCl and 1.0 mM EDTA. As shown in the melting curve monitored at 275 nm (Figure 2A, red), two sequential steps were observed for RNA1 in the presence of the probe at pH 5.5, which was consistent with the triplex melting

Received: May 31, 2016
Published: July 21, 2016

DOI: 10.1021/jacs.6b05554
J. Am. Chem. Soc. 2016, 138, 9397−9400

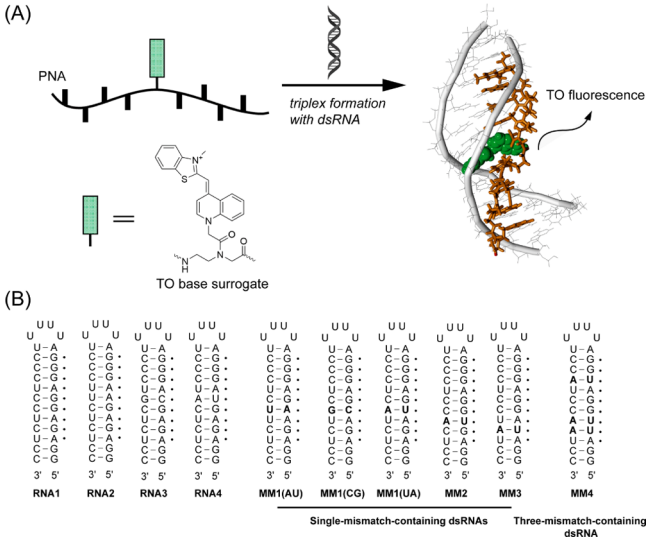

Figure 1. (A) Schematic illustration of fluorescence sensing of dsRNA by triplex-forming PNA having a TO base surrogate. (B) DsRNA sequences used in this study. Target purine rich sequences are indicated by dots. Mismatched base pairs are written in bold.

behavior. A higher transition (melting temperature (T_m) = 97 °C) resulted from the melting of the RNA1 hairpin structure into a random coil as in the case of the melting curve of RNA1 only (Figure 2A, black). A lower transition (T_m = 80 °C) was thus attributed to the dissociation of the probe from RNA1. As for this triplex-to-duplex transition, we also observed the hypochromicity at 300 nm, most likely due to deprotonation of N3 protonated PNA cytosines (C^+) involved in Hoogsteen base pairs with G−C (C^+·G−C triplets) in the triplex structures.[18] However, the broad, weak transition around 30 °C only at 275 nm, which might result from an unstable probe-dsRNA triplex, was observed at pH 7.0 (Figure 2A, blue) since hardly any PNA cytosines are protonated. These results clearly indicate our probe formed the thermally stable triplex with dsRNA at acidic pH. This was further supported by circular dichroism (CD) measurements in which we observed a similar CD spectral change for the probe binding to RNA1 (Figure S6) as that of PNA−dsRNA triplexes in the literature.[6] Meanwhile, the TO unit of our probe was found to cooperatively bind to dsRNA as indicated by the sigmoidal absorbance change at 520 nm along with the triplex-to-duplex transition at both 275 and 300 nm. In addition, when mismatches were located in the target sequences, the thermal stability of the probe-dsRNA triplexes decreased (Figure S7 and Table S2). Even a single mismatch (MM1−MM3; Figure 1B) led to a remarkable decrease in the T_m values (ΔT_m = −26 to −44 °C). We observed no triplex formation for the target sequence containing three mismatches (MM4). These results lend strong support for the sequence-selective triplex formation by our probe.

Next, we examined the fluorescence response of the TO base surrogate in the probe at pH 5.5 (Figure 2B). The probe (50 nM) showed negligible fluorescence in the absence of RNA1 because of free rotation in the TO unit causing nonradiative energy decay (Φ = 0.0098). The addition of the equimolar RNA1 caused an increase in the TO fluorescence intensity by as much as 120-fold (Φ = 0.12). The fluorescence response of the probe significantly increased in an RNA concentration-dependent manner (Figure 2B, inset). In contrast, almost no response was found at pH 7.0 due to no triplex formation (Figure 2A). The fluorescence Job's

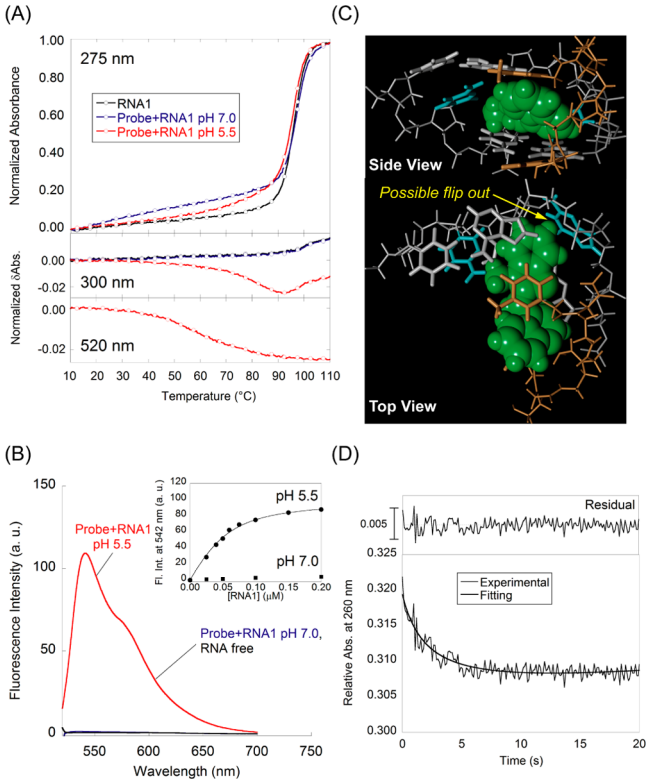

Figure 2. (A) UV melting curves of RNA1 in the presence of the probe recorded at 275, 300, and 520 nm at pH 5.5 (red); at pH 7.0 (blue); in the absence of the probe at pH 5.5 (black). (B) Fluorescence spectra of the probe (50 nM) in the presence of the equimolar RNA1 at pH 5.5 (red) or at pH 7.0 (blue), and in the absence of RNA1 at pH 5.5 (black). Inset: Fluorescence titration curves for the probe (50 nM) binding to RNA1 (0−200 nM). Excitation: 514 nm. (C) Molecular modeling of the possible binding mode of the TO unit in the probe-dsRNA triplex: PNA (orange); TO (green); RNA G−C base pair opposite the TO unit (light blue); RNA (gray). (D) Association kinetics trace of the absorbance at 260 nm for the probe (1.0 μM) binding to the equimolar RNA1. The fitting curve is the bold line, and the corresponding residual plot for the fit is presented above the kinetics trace.

plot at pH 5.5 suggested 1:1 binding stoichiometry between the probe and RNA1 (Figure S8). These results indicate that the TO base surrogate in tFIT probe exhibits the light-up response upon triplex formation with the target dsRNA. The remarkable light-up response was mainly attributed to the low background emission from the probe. The obtained concentration dependence of the fluorescence response was then used to assess the binding affinity of the probe. The intensity plot at 542 nm could be well fitted by a 1:1 binding isotherm (Figure 2B, inset), which gave the dissociation constant (K_d) of 23 ± 0.5 nM (n = 3). It is highly likely that the light-up response observed here was derived from the restricted rotation of the TO unit by stacking interactions in the resulting triplex structure. This was supported by the observation of a large red shift in the absorption of the TO unit as well as a negative induced CD (ICD) upon triplex formation (Figure S9). The molecular modeling study also suggested the π-stacking interaction between the TO unit and the flanking base pairs (Figure 2C). This might accompany the flipping of the bases opposite the TO unit and/or the formation of a local bulge structure, as similarly suggested for the TO base surrogate inside PNA−DNA duplexes.[12]

It should be noted that the triplex formation between our probe and dsRNA proceeds very rapidly. Fluorescence response

DOI: 10.1021/jacs.6b05554
J. Am. Chem. Soc. 2016, 138, 9397−9400

of the probe upon binding to RNA1 was found to reach a plateau in less than 180 s (Figure S10). This binding kinetics was superior to the conventional TFO probes that required more than an 8 h incubation for the response maximization.[19,20] We further carried out stopped-flow experiments to quantitatively determine the binding kinetics of the probe (Figure 2D). The association rate constant (k_{assoc}) was estimated as 3.8×10^5 M^{-1} s^{-1} from monitoring the decrease in the absorbance at 260 nm upon mixing the probe and RNA1 solutions. Significantly, this value was at least 2 orders of magnitude higher than association rate constants of conventional TFOs.[21] We found that such fast association kinetics resulted from the intrinsic nature of the PNA scaffold in our probe, given the fact that the control TFP without the TO unit showed the comparable k_{assoc} value (2.0×10^5 M^{-1} s^{-1}, Figures S1 and S11). In addition to the short length, the neutral charge of the PNA backbone would make a large contribution to the observed fast association as suggested for PNA−dsDNA triplex formation.[22] The association kinetics of our probe was even comparable to the PNA−RNA duplex formation kinetics.[23] The kinetics was also examined by monitoring the increase in TO fluorescence upon triplex formation. This gave the k_{assc} of 3.2×10^5 M^{-1} s^{-1} (Figure S12), which was consistent with that obtained from the absorbance change (Figure 2D). Therefore, the fluorescence of the TO base surrogate does probe the triplex formation reaction in real time unlike the fluorophores tethered to the terminus of TFOs.[19,24]

We found that the TO base surrogate in our probe can serve as a universal base that nondiscriminatorily binds with all four kinds of base pairs in the triplex. The probe showed the light-up response for RNA2, RNA3, and RNA4 carrying an A−U, C−G, and U−A base pair, respectively, which the TO unit faces in the resulting triplexes as in the case for a G−C base pair in RNA1 (Figures 2B and 3). From the fluorescence titration experiments,

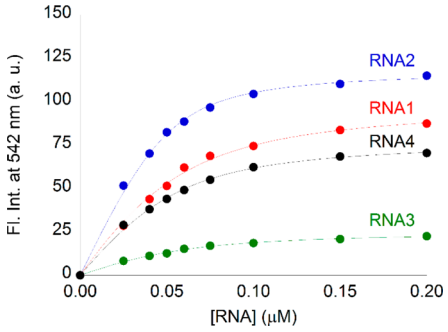

Figure 3. Fluorescence titration curves for the probe (50 nM) binding to 0−200 nM RNA1 (red), RNA2 (blue), RNA3 (green), and RNA4 (black).

the dissociation constants for these three targets were found to be comparable to that for RNA1, which shows the binding affinity of the probe was unaffected by the kind of base pair opposite the TO unit (K_d/nM; RNA2, 13 ± 1.0; RNA3, 20 ± 1.5; RNA4, 16 ± 0.1, Figure 3). However, the kind of base pair strongly influenced the fluorescence intensity of the probe. These results indicate the TO base surrogate in tFIT probe possesses the characteristics of a universal base while maintaining the intrinsic light-up property. We consider that such nondiscriminatory binding resulted from the flipping of a base opposite the TO unit into an extrahelical position (Figure 2C) although further structural studies such as NMR analysis are needed to clarify this issue. To the best of our knowledge, this is the first report on the universal base that works for triplex formation with dsRNA, although several aromatic molecules were reported as universal bases targeting dsDNA sequences.[25−27] This property of the TO unit should be useful to overcome the limitation of target dsRNA sequences that can be recognized based on triplex formation. It is well-known that even a single pyrimidine−purine inversion in target sequences severely impairs triplex formation. In fact, C−G (RNA3) or U−A inversion (RNA4) in the target sequence resulted in the drastic decrease in thermal stability of the triplex with the control TFP having no TO unit ($\Delta T_m < -22$ °C; Figure S13 and Table S3). By contrast, our probe did not suffer from these inversions and exhibited higher T_m values for these RNAs relative to those of the control TFP (ΔT_m = +10 to 22 °C; Figure S13). Therefore, the TO base surrogate used in tFIT probe can provide a useful and powerful approach to expand targetable dsRNA sequences by the triplex formation strategy.

Another point of significance in dsRNA sensing by our probe is that the fluorescence signaling of the TO unit allows for selective analysis of target dsRNA sequences at the resolution of a single base pair. Figure 4A shows the fluorescence intensity of the TO

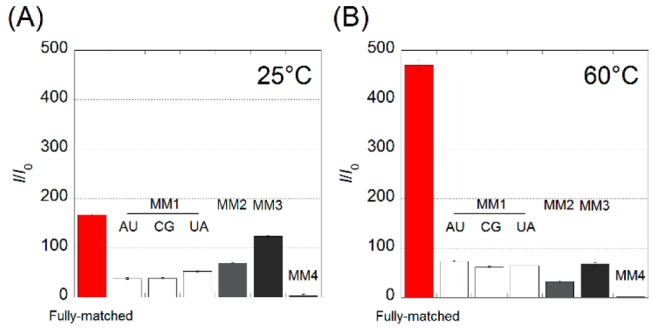

Figure 4. Fluorescence response of the TO unit in the probe (1.0 μM) at 542 nm in the presence of the equimolar fully matched dsRNA (RNA1), single-mismatch-containing dsRNAs (MM1−MM3), and three-mismatch-containing dsRNA (MM4) at 25 °C (A) and 60 °C (B). Errors are the standard deviations obtained from three independent experiments. I and I_0 represent the fluorescence intensity in the presence and absence of RNA1, respectively.

unit in the presence of the target fully matched (RNA1), single-mismatch-containing (MM1−MM3) or three-mismatch-containing (MM4) dsRNAs at 25 °C. It is clearly seen that our probe exhibited the larger light-up response for the fully matched dsRNA than the mismatch-containing dsRNAs. Almost no response was observed for MM4 because of no triplex formation (Figure S7). The examination for MM1−MM3 revealed the strong influence of the position of the single mismatch within the target dsRNA on the light-up response of the TO unit, where a single mismatch adjacent to the TO unit (MM1) resulted in the weakest response. Given that triplexes with these RNAs are formed at 25 °C, we reason the observed small response for the single mismatch-containing dsRNAs resulted from the increase in local flexibility of the TO unit in the triplex structures. This is supported by the fact that the fluorescence response increased as the single mismatch was located apart from the intercalation site of the TO unit (MM1 < MM2 < MM3). At elevated temperature (60 °C) above the T_ms of the mismatched triplexes, the sequence selectivity significantly improved (Figure 4B). Besides the local flexibility of the TO unit, the thermal instability of the formed triplexes was responsible for the observed high selectivity of our probe. It is noteworthy that the light-up factor (I/I_0) of the

DOI: 10.1021/jacs.6b05554
J. Am. Chem. Soc. 2016, 138, 9397−9400

probe-dsRNA was 470 at 60 °C, which is the highest to date as an RNA-binding light-up probe carrying a single fluorophore.

Furthermore, our probe exhibited a marked binding preference for dsRNA to the corresponding dsDNA (Figure S14). From the fluorescence titration experiments, the binding affinity was found to be 37-times lower than that for dsRNA (K_d = 870 ± 77 nM). The observed binding selectivity for dsRNA over dsDNA may be a result of the geometric compatibility of TFP and the favorable backbone−backbone interaction in the major groove of the dsRNA.[10]

In summary, we described the novel ability of the TFP-based probe containing a TO base surrogate, tFIT probe, for fluorescence sensing of dsRNA sequences. Our probe demonstrated the remarkable light-up response of the TO unit upon PNA−dsRNA triplex formation through Hoogsteen base pairing at acidic pH. The association kinetics is so fast that the probe facilitates real-time monitoring of the triplex formation reaction. The TO base surrogate in our probe functions as a universal base capable of binding to all combinations of base pairs opposite the TO unit with similar binding affinity in a nondiscriminatory fashion. In addition, our probe can discriminate the fully matched sequences from the mismatched dsRNA sequences, which allows the fluorescence detection of a single-base pair mismatch. The results obtained here should provide valuable insights into the molecular basis for further development of fluorescence sensing probes for targeting dsRNA sequences.

It is commonly found that biologically relevant RNA duplexes such as bacterial/viral RNA, rRNA, and precursors of small noncoding RNAs have more than eight contiguous purines interrupted by a few pyrimidines[9,28,29] or by unpaired bases within bulge and loop structures. Considering the fact that such purine-rich sequences need a double-stranded form, we expect a specific dsRNA target will be reasonably selected and targeted for *in vitro* applications. For example, fluorescence *in situ* hybridization (FISH) using our probe may be a unique application in fixed cells as hybridization at acidic pH condition and washing procedure under stringent conditions can be used. If necessary, conjugation to our tFIT probe with hybridization probe for single-stranded region[4,30] or small molecules binding to secondary structures,[2,3] which are often formed nearby the stem, would further increase the binding selectivity. However, pH limitation to triplex formation as well as competitive binding to the complementary single-stranded RNA (Figure S15) seem to pose a problem for *in vivo* applications. We envision incorporation of cytosine base analogues such as 2-aminopyridine[9] and thio-pseudoisocytosine[10] in place of cytosine, which would achieve binding ability at neutrality but also suppress competitive binding to single-stranded RNA. We are continuing studies in these directions.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b05554.

Experimental details and supplementary Figures 1−15 (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*nishi@m.tohoku.ac.jp

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by the Grants-in-Aid for Scientific Research (B) (No. 16H04159) from the Ministry of Education, Culture, Sports, Science and Technology, and by a grant from the Nakatani Foundation.

■ REFERENCES

(1) Tyagi, S. *Nat. Methods* **2009**, *6*, 331.
(2) Zhang, J.; Umemoto, S.; Nakatani, K. *J. Am. Chem. Soc.* **2010**, *132*, 3660.
(3) Arambula, J. F.; Ramisetty, S. R.; Baranger, A. M.; Zimmerman, S. C. *Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106*, 16068.
(4) Sato, T.; Sato, Y.; Iwai, K.; Kuge, S.; Nishizawa, S.; Teramae, N. *Chem. Commun.* **2015**, *51*, 1421.
(5) Sato, T.; Sato, Y.; Iwai, K.; Kuge, S.; Teramae, N.; Nishizawa, S. *Anal. Sci.* **2015**, *31*, 315.
(6) Li, M.; Zengeya, T.; Rozners, E. *J. Am. Chem. Soc.* **2010**, *132*, 8676.
(7) Roberts, R. W.; Crothers, D. M. *Science* **1992**, *258*, 1463.
(8) Endoh, T.; Hnedzko, D.; Rozners, E.; Sugimoto, N. *Angew. Chem., Int. Ed.* **2016**, *55*, 899.
(9) Zengeya, T.; Gupta, P.; Rozners, E. *Angew. Chem., Int. Ed.* **2012**, *51*, 12593.
(10) Devi, G.; Yuan, Z.; Lu, Y.; Zhao, Y.; Chen, G. *Nucleic Acids Res.* **2014**, *42*, 4008.
(11) Gupta, P.; Zengeya, T.; Rozners, E. *Chem. Commun.* **2011**, *47*, 11125.
(12) Köhler, O.; Jarikote, D. V.; Seitz, O. *ChemBioChem* **2005**, *6*, 69.
(13) Hövelmann, F.; Gaspar, I.; Ephrussi, A.; Seitz, O. *J. Am. Chem. Soc.* **2013**, *135*, 19025.
(14) Hövelmann, F.; Seitz, O. *Acc. Chem. Res.* **2016**, *49*, 714.
(15) Karunakaran, V.; Lustres, J. L. P.; Zhao, L.; Ernsting, N. P.; Seitz, O. *J. Am. Chem. Soc.* **2006**, *128*, 2954.
(16) Netzel, T. L.; Nafisi, K.; Zhao, M. *J. Phys. Chem.* **1995**, *99*, 17936.
(17) Jarikote, D. V.; Köhler, O.; Socher, E.; Seitz, O. *Eur. J. Org. Chem.* **2005**, *2005*, 3187.
(18) Sun, B.-W.; Babu, B. R.; Sørensen, M. D.; Zakrzewska, K.; Wengel, J.; Sun, J. S. *Biochemistry* **2004**, *43*, 4160.
(19) Grimm, G. N.; Boutorine, A. S.; Lincoln, P.; Nordén, B.; Hélène, C. *ChemBioChem* **2002**, *3*, 324.
(20) Ikeda, S.; Yanagisawa, H.; Yuki, M.; Okamoto, A. *Artif. DNA* **2013**, *4*, 19.
(21) Torigoe, H.; Hari, Y.; Sekiguchi, M.; Obika, S.; Imanishi, T. *J. Biol. Chem.* **2001**, *276*, 2354.
(22) Bentin, T.; Hansen, G. I.; Nielsen, P. E. *Nucleic Acids Res.* **2006**, *34*, 5790.
(23) Jensen, K. K.; Ørum, H.; Nielsen, P. E.; Nordén, B. *Biochemistry* **1997**, *36*, 5072.
(24) Robles, J.; McLaughlin, L. W. *J. Am. Chem. Soc.* **1997**, *119*, 6014.
(25) Durland, R. H.; Rao, T. S.; Bodepudi, V.; Seth, D. M.; Jayaraman, K.; Revankar, G. R. *Nucleic Acids Res.* **1995**, *23*, 647.
(26) Walczak, K.; Wamberg, M.; Pedersen, E. B. *Helv. Chim. Acta* **2004**, *87*, 469.
(27) Wachowius, F.; Rettig, M.; Palm, G.; Weisz, K. *Tetrahedron Lett.* **2008**, *49*, 7264.
(28) Muse, O.; Zengeya, T.; Mwaura, J.; Hnedzko, D.; McGee, D. W.; Grewer, C. T.; Rozners, E. *ACS Chem. Biol.* **2013**, *8*, 1683.
(29) Devi, G.; Zhou, Y.; Zhong, Z.; Toh, D.-F. K.; Chen, G. *WIREs RNA* **2015**, *6*, 111.
(30) Moses, A. C.; Schepartz, A. *J. Am. Chem. Soc.* **1996**, *118*, 10896.

DOI: 10.1021/jacs.6b05554
J. Am. Chem. Soc. 2016, 138, 9397−9400