Supporting Information for

Lysine linkage in abasic site-binding ligand-thiazole orange conjugates for improved binding affinity to orphan nucleobases in DNA/RNA hybrids

Yusuke Sato,* Hiroki Saito, Daisuke Aoki, Norio Teramae, and Seiichi Nishizawa*

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

Experimental

Reagents: All of the RNA and DNAs were custom-synthesized and HPLC-purified by Sigma Genosys (Hokkaido, Japan) or Nihon Gene Reserach Laboratoriers Inc. (Sendai, Japan). Other reagents were commercially available analytical grade and were used without further purification. The concentrations of DNAs were determined as previously described[1] and those of RNAs were determined from the absorbance at 260 nm measured at 85°C using the molar extinction coefficients[2]. Water was deionized (≥18.0 MΩ cm specific resistance) by an Elix 5 UV Water Purification System and a Milli-Q Synthesis A10 system (Millipore Corp., Bedford, MA), followed by filtration through a BioPak filter (Millipore Corp., Bedford, MA) in order to remove RNase.

DML-Lys-TO was easily dissolved in water to prepare the stock solution (31 μM). The stock solution (21 μM) of alkyl linker-containing conjugate (**DML-TO**), however contained some amount of ethanol due to poor water solubility. Final ethanol concentrations in sample solutions containing **DML-TO** were below 1.0 % (v/v). No organic solvents was required to prepare the stock solution of **DP-Lys-TO** (21 μM).

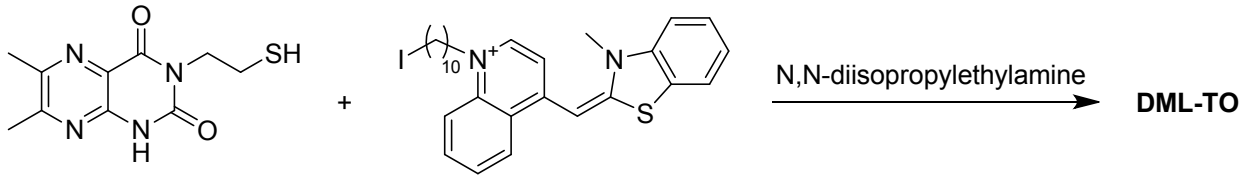
All measurements were performed in 10 mM sodium cacodylate buffer solutions (pH 7.0) containing 100 mM NaCl and 1.0 mM EDTA. Before measurements, the sample solutions were annealed in order to assure the formation of DNA/RNA hybrids. Annealing conditions were as follows: heated at 75°C for 10 min, and gradually cooled to 5°C (3°C/min), after which the solution temperature was raised again to 20°C (1°C/min).

UV-visible spectra and fluorescence spectra measurements: Absorption and fluorescence spectra were measured at 20°C with a JASCO model V-570 UV–vis spectrophotometer and FP-6500 spectrofluorophotometer (Japan Spectroscopic Co. Ltd., Tokyo, Japan), respectively. Both instruments were equipped with thermoelectrically temperature-controlled cell holders. Measurements of absorption and fluorescence spectra were done using a 1.0 × 10 mm quartz cell (optical path length: 10 mm) and a 3 × 3 mm quartz cell, respectively. Excitation wavelegth was set at the excitation maximum of the TO unit of conjugates bound to target A opposite an AP site in the DNA/RNA hybrids.

Temperature dependence of the binding affinity: The fluorescence titration experiments were carried out at 5, 10, 15, and 20°C, and apparent 1:1 binding constants (K_{11}) were obtained at each temperature. The obtained

temperature dependence of K_{11} was used for determination of free binding free energy (ΔG), the binding enthalpy (ΔG) and the binding entropy (ΔS) using the linear van't Hoff analysis ($\ln K_{11} = -\Delta H/RT + \Delta S/R$).

Synthesis of DML-TO: A mercaptoethyl group was introduced to the N-3 position of 6,7-dimethyl lumazine (DML) according to the literature procedure,[3] and the resulting DML derivative (**1**) was then coupled to the TO unit having a decanyl spacer terminated with an iodo group[4] (**2**) to afford **DML-TO**.



1 **2**

To a solution of **1** (30 mg, 0.12 mmol) and **2** (18 mg, 0.03 mmol) in 15 mL ethanol, 2mL *N*, *N*-diisopropylamine was added and the mixture was stirred at room temperature for 20 h under the nitrogen atmosphere. After evaporation of the solvents, the residue was purified by MPLC (CH_3Cl/MeOH), leading to **DML-TO** as a red powder (17 mg, 0.025 mmol, 83%).

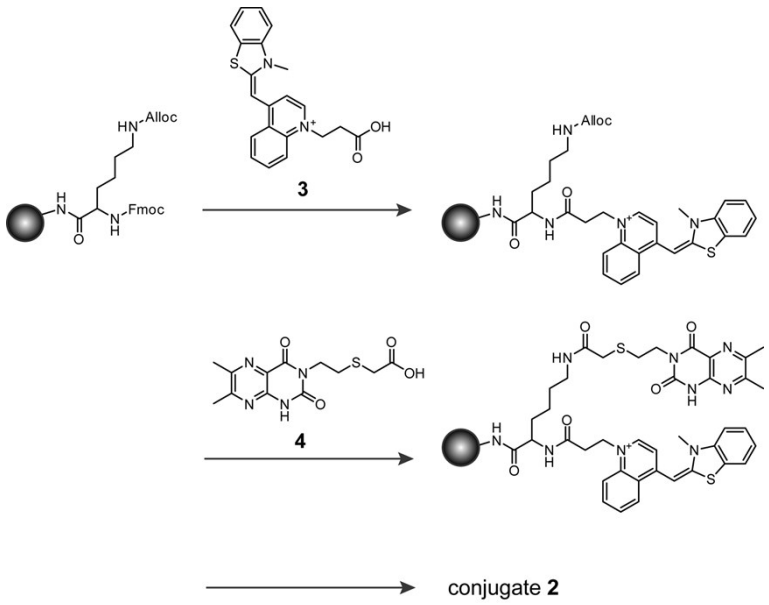
^{1}H NMR (CD_3OD; Fig.S1) : δ(ppm) = 8.52 (d, 1H), 8.37 (d, 1H), 7.94 (t, 1H), 7.79 (d, 1H), 7.69 (t, 1H), 7.49 (m, 2H), 7.31 (m, 1H), 7.23 (d, 1H), 6.71 (s, 1H), 4.51 (t, 2H), 3.95 (t, 2H), 3.89 (s, 3H), 2.61 (t, 2H), 2.49 (t, 2H), 2.37 (s, 3H), 2.35 (s, 3H),1.93 (m, 2H), 1.50 (m, 2H), 1.46-1.24 (m, 12H)

HRMS (ESI; Fig. S2) calcd for $C_{38}H_{45}N_6O_2S_2^+$ 681.3040, found 681.3183.

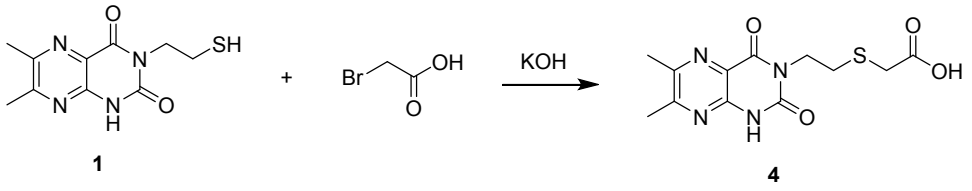
Synthesis of conjugate DML-Lys-TO: DML-Lys-TO was synthesized by a solid-phase method using Endeavor 90 peptide synthesizer (Aapptec, Louisville, KY, USA). All coupling reactions were carried out using DIC/HOBt activation. The completion of the coupling reactions was confirmed by the Kaiser test. Fmoc-Lys-(Alloc) was first loaded onto Rink Amide AM resin using DIC/HOBt activation. After the Fmoc group on the lysine was removed by piperidine, the TO unit having an ethyl spacer terminated with a carboxylate group (**3**)[5] was coupled to the main chain of lysine on the resin. Subsequently, the alloc group of the side chain in the lysine was deprotected by treating with tetrakis(triphenylphosphine) palladium (0) and dimethylamine borane in dichloromethane, followed by coupling with carboxylate-terminated ethyl spacer-containing DML derivative (**4**, synthesis scheme is shown below) to the ε-amino group of the lysine side chain. Cleavage from the resin was performed using a mixture of trifluoroacetic acid (TFA)/triisopropylsilane/ water (95/2.5/2.5 v/v). After the resulting solution was filtered to remove the resin, the probes were then precipitated in cold diethyl ether. The crude products were purified using a reverse-phase HPLC system (pump; PU-2086 Plus x2, mixer; MX 2080-32, column oven; CO-1565 (40°C), detector; UV-2070 plus and UV-1570M: Japan Spectroscopic Co. Ltd., Tokyo, Japan) equipped with a C18 column (Inertsil ODS3: GL Sciences, Tokyo, Japan) using a gradient of water/acetonitrile containing 0.1% TFA.

DML-Lys-TO was verified by MALDI-TOF-MS (4800 Plus MALDI TOF/TOF analyzer: AB Sciex, Tokyo, Japan) and its purity was > 96% (Fig. S3).

MALDI-TOF MS for $C_{39}H_{44}N_9O_5S_2$ $[M]^+$; calcd 782.2901, found 782.4515 (Fig. S4).



Synthesis of 4: To a solution of **1** (0.19 g, 0.67 mmol) in 30 mL ethanol and 1.3 mL KOH (1.0 M), 2-bromoacetic acid in 3 mL ethanol was added and the mixture was stirred at room temperature for 5h under the nitrogen atmosphere. Neutralization with HCl resulted in the precipitate formation, followed by filtration. The filtrate was evaporated and dissolved with methanol. The filtrate of the methanol solution was evaporated. The obtained mixture contained **4** as the main product, verified by ESI-MS, and it was then subjected to the coupling with lysine on the resin in the above-mentioned scheme.



Synthesis of DP-Lys-TO: 2,4-diamino-5-chlorobenzoic acid was utilized for the conjugation with the ε-amino group of the lysine on the resin. The conjugate was prepared by the same synthesis protocol as that for **DML-Lys-TO**. The purity of the conjugate was > 95% (Fig. S3).

MALDI-TOF MS for $C_{32}H_{35}Cl\ N_9O_3S$ $[M]^+$; calcd 660.2266, found 660.3546 (Fig. S4).

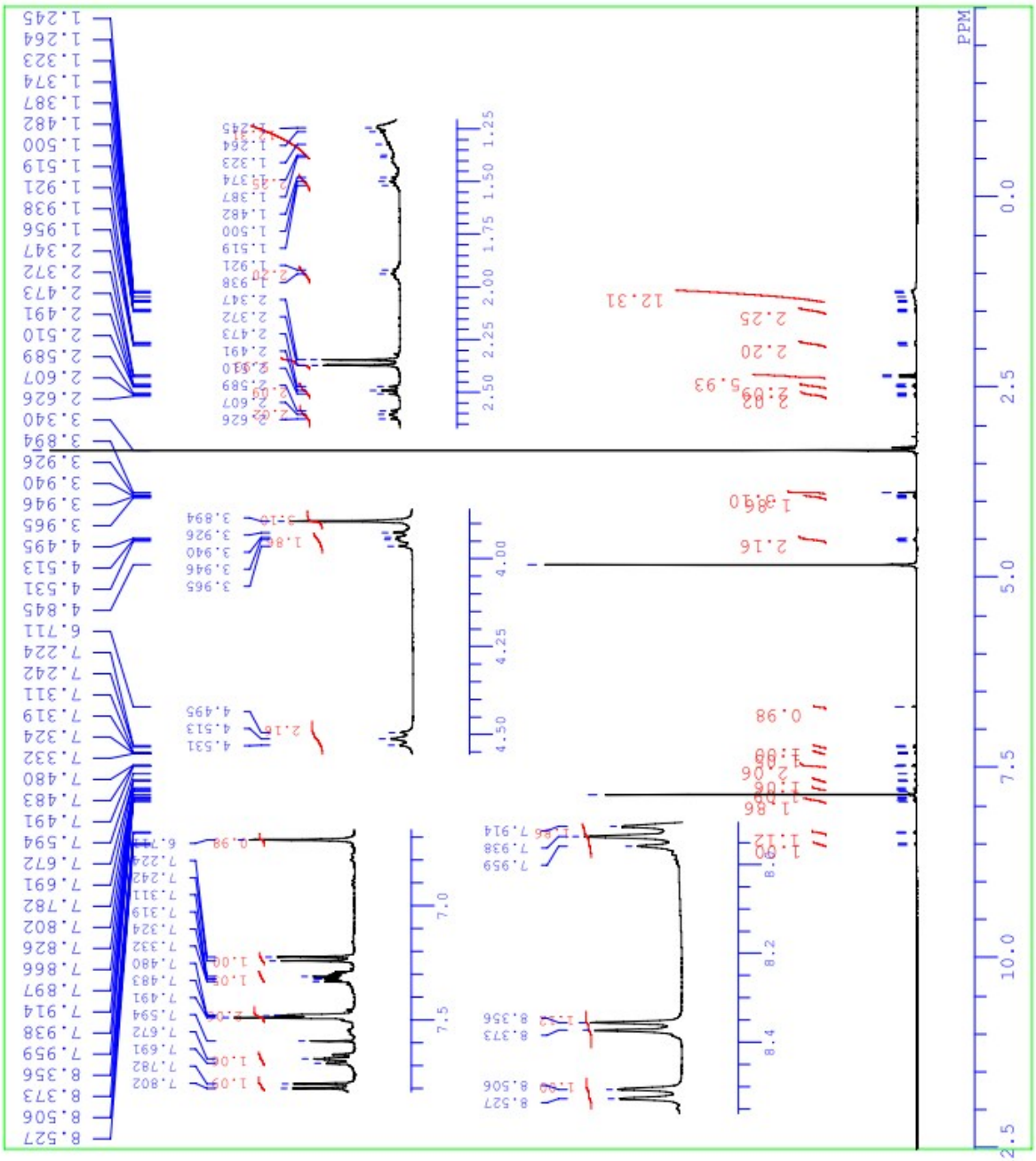
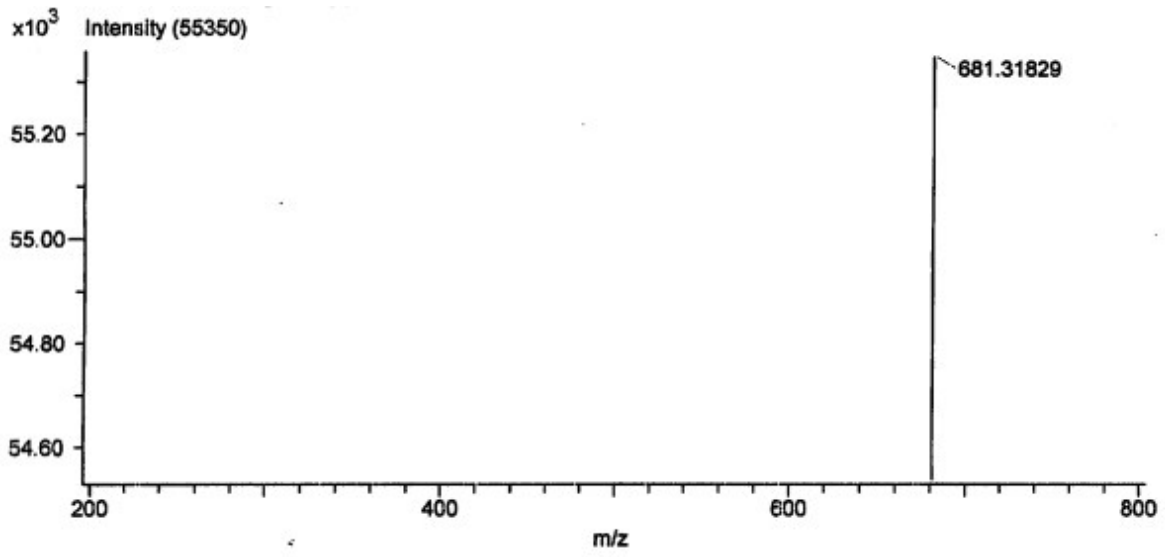


Fig. S1. ^{1}H NMR of **DML-TO**.



Fig, S2. ESI-MS spectra of **DML-TO**.

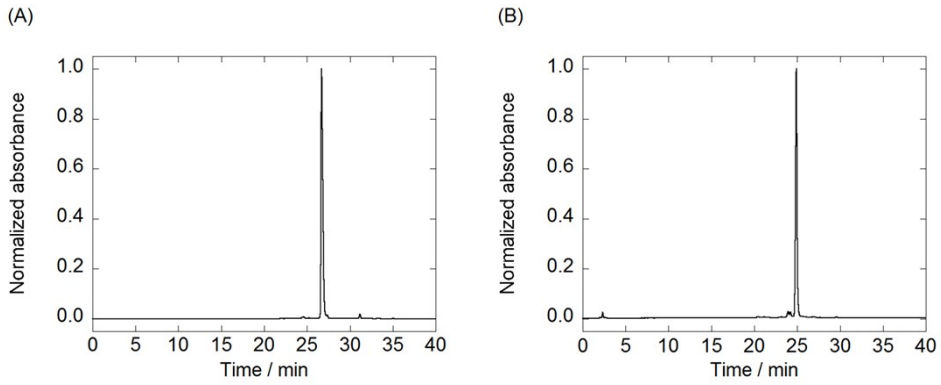


Fig. S3. HPLC profile of (A) **DML-Lys-TO** and (B) **DP-Lys-TO**. Absorbance was monitored at 500 nm. Gradient conditions: (A) 10-50% CH_3CN (0.1% TFA) in H_2O (0.1% TFA) in 40 min, (B) 20-50% CH_3CN (0.1% TFA) in H_2O (0.1% TFA) in 40 min.

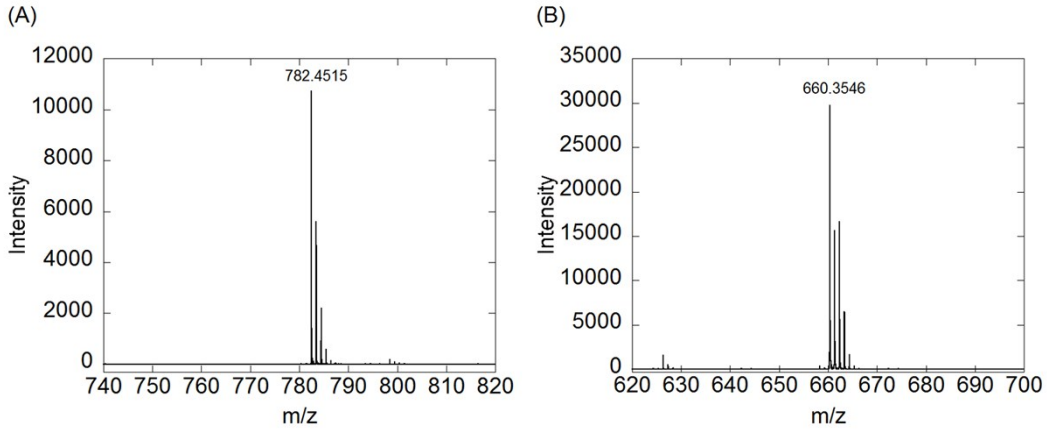


Fig. S4. MALDI-TOF-MS spectra of (A) **DML-Lys-TO** and (B) **DP-Lys-TO**.

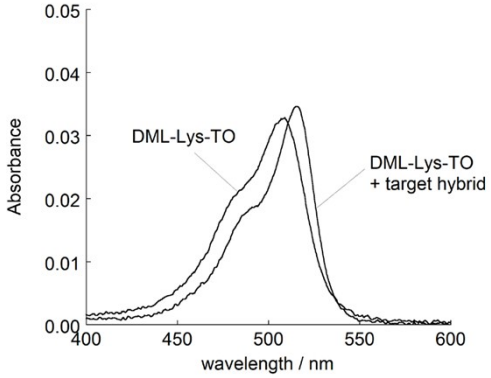


Fig. S5. UV-visible spectra of the TO unit in **DML-Lys-TO** (1.6 μM) in the absence and presence of AP site-containing probe DNA/target RNA hybrids having the target A (2.0 μM; 5'-d(GGG GAA GGA AXA GAA GGA AAA)-3'/ 3'-r(CCC CUU CCU UAU CUU CCU UUU)-5', X = AP site (spacer C3)). Other solution conditions were the same as those given in Figure 1B in the main text.

TO unit showed the increase in the absorption intensity and a red shift by 7.5 nm upon binding to the hybrid, which is consistent with the intercalation-induced spectral change of the parent TO (ref. 5a in the main text).

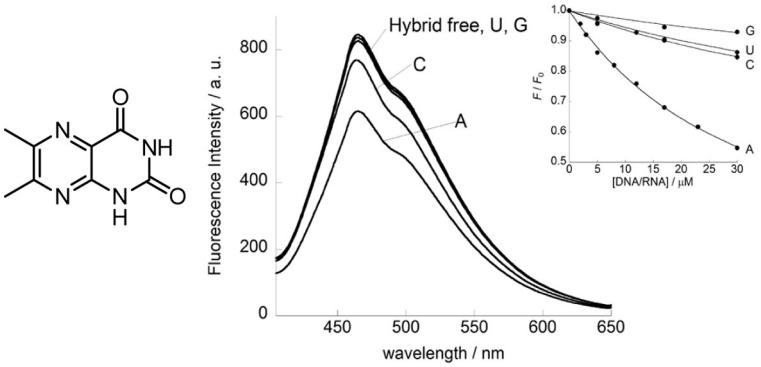


Fig. S6 (left) Chemical structure of DML monomer. (right) Fluorescence spectra of DML monomer (5.0 μM) in the absence and presence of AP site-containing probe DNA/target RNA hybrids (15 μM; 5'-d(GGG GAA GGA AXA GAA GGA AAA)-3'/ 3'-r(CCC CUU CCU UNU CUU CCU UUU)-5', X = AP site (spacer C3), N = target nucleobase (A, C, U, or G)). Inset: Fluorescence titration curve for the binding of DML monomer (5.0 μM) to the target nucleobases in the hybrid (0-30 μM). Other solution conditions were the same as those given in Figure 1 in the main text. Temperature, 5°C. Excitation, 330 nm. Analysis, 465 nm.

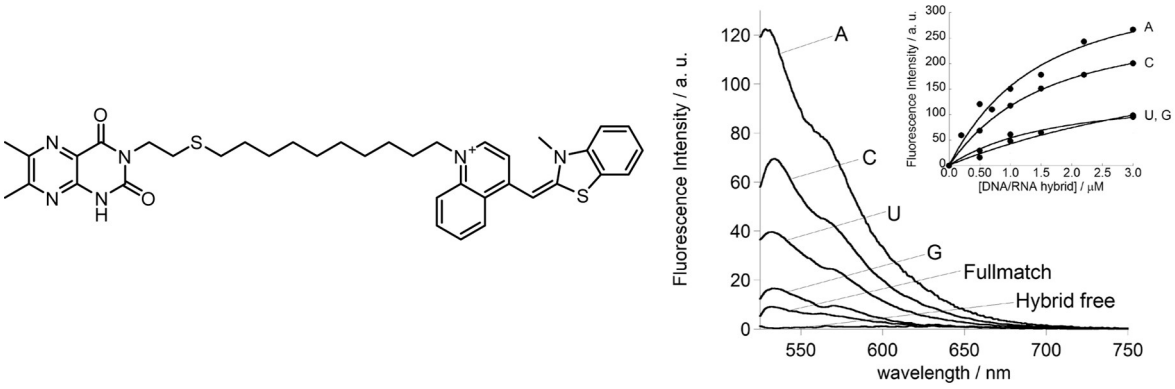


Fig. S7 (left) Chemical structure of **DML-TO**. (right) Fluorescence spectra of **DML-TO** (0.5 μM) in the absence and presence of AP site-containing probe DNA/target RNA hybrids (0.5 μM; target nucleobase = A, C, U, or G) or a fully-matched hybrid (0.5 μM). Inset: Fluorescence titration curve for the binding of DML-TO (0.5 μM) to the target nucleobases in the hybrid (0-3.0 μM). Other solution conditions were the same as those given in Figure 1 in the main text. Temperature, 5°C. Excitation, 512 nm. Analysis, 528 nm.

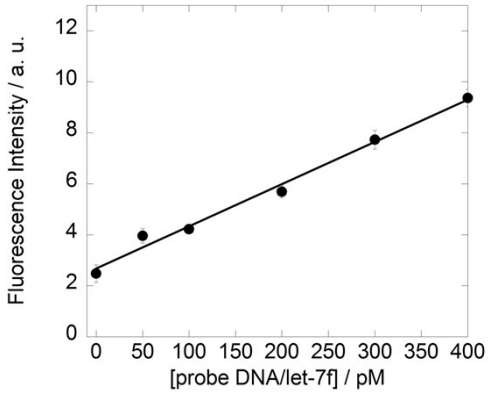


Fig. S8 Determination of the limit of detection (LOD) for let-7f based on the binding-induced fluorescence response of **DML-Lys-TO** (50 nM) for probe DNA/let-7f hybrid. The obtained equation by the linear fitting was y = 0.017 x +2.5 (r^2 = 0.9850). The standard deviation of the fluorescence intensity of **DML-Lys-TO** in the absence of hybrids was determined as 0.34 from three independent experiments. LOD was then estimated as 60 pM based on three times the standard deviations of the fluorescence intensity of **DML-Lys-TO** in the absence of hybrids, obtained from three independent experiments, divided by the slope of the plot.

Solution conditions were the same as those given in Fig. 1B in the main text. Temperature, 5°C. Excitation, 515 nm. Analysis, 532 nm.

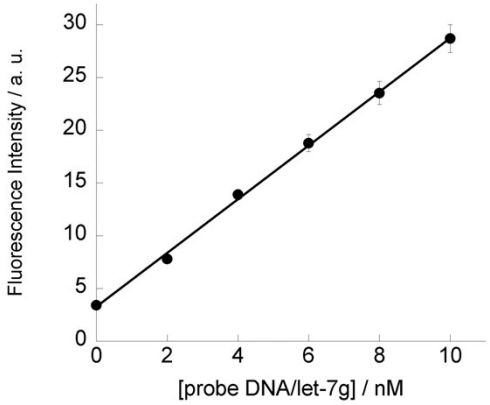


Fig. S9 Determination of the limit of detection (LOD) for let-7g based on the binding-induced fluorescence response of **DP-Lys-TO** (100 nM) for probe DNA/let-7g hybrid. Probe DNA sequence is as follows: 5'-TACAA XCTAC TACCT CA-3' (X = AP site (spacer C3)). The obtained equation by the linear fitting was y = 2.55 x + 3.27 (r^2 = 0.9994). The standard deviation of the fluorescence intensity of **DP-Lys-TO** in the absence of hybrids was determined as 0.162 from three independent experiments. LOD was then estimated as 190 pM based on three

times the standard deviations of the fluorescence intensity of **DP-Lys-TO** in the absence of hybrids, obtained from three independent experiments, divided by the slope of the plot.

Solution conditions were the same as those given in Fig. 3B in the main text. Temperature, 5°C. Excitation, 515 nm. Analysis, 532 nm.

Table S1. Dissociation constants (K_d) of **DML-Lys-TO**, DML monomer, and **DP-Lys-TO** to target nucleobases in the DNA/RNA hybrids.

K_d / nM	**DML-Lys-TO**	DML monomer	**DP-Lys-TO**
A	51 ± 8	28000 ± 4000	781± 321
C	180 ± 20	58000 ± 25000	330 ± 30
U	700 ± 360	> 65000	37 ± 1.9
G	> 1000	> 89000	> 1000

Measurement conditions were the same as those given in Fig. 1B in the main text, Fig. S6, and Fig. 4B for DML-Lys-TO, DML monomer, and **DP-Lys-TO**, respectively.

References

1. Sato, Y., Nishizawa, S., Yoshimoto, K., Seino, T., Ichihashi, T., Morita, K. and Teramae, N., *Nucleic Acids Res.*, **2009**, 37, 1411-1422.
2. Puglisi, J. D. and Tinocco, I., *Methods Enzymol.*, **1989**, 180, 304-325.
3. Falch, E., *Acta Chem. Scand. B*, **1977**, 31, 167-172.
4. Privat, E. and Asseline, U., *Bioconjugate Chem.*, **2001**, 12, 757-769.
5. Ikeda, S. and Okamoto, A., *Photochem. Photobiol. Sci.*, **2007**, 6, 1197-1201.