Supplementary Information

RNA-binding fluorogenic probes: G-clamp conjugated with a thiazole orange derivative for screening RNA-binding small molecules

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Experimental procedure

Material and methods

General chemicals were purchased from FUJIFILM Wako Pure Chemical, the Tokyo Chemical Industry, Kanto Chemical or Aldrich. Target RNAs were purchased from JBioS (Japan). ^{1}H NMR spectra (400, 500, and 600 MHz) were recorded using Bruker AVANCE III 400, 500, and 600 spectrometers, respectively. ^{13}C NMR spectra (151 MHz) were recorded using Bruker AVANCE III 600 spectrometer. High-resolution electrospray mass analysis was performed using a Bruker MicrOTOF-Q II. HPLC purification was performed with a JASCO HPLC System (CO-631A or CO-2065Plus) using a reverse-phase C_{18} column (COSMOSIL 5C_{18}-AR-II, Nacalai Tesque, 10×250 mm for ligand purification and 4.6×250 mm for analysis).

RNA secondary structure prediction and visualization

The secondary structures of single-stranded and double-stranded RNA were predicted by RNAfold and RNAcofold v. 2.5.1 in the ViennaRNA package,[1] respectively, with the temperature set to 37°C. For SHAPE-guided RNA secondary structure prediction, the structures were predicted along with SHAPE reactivity data by the same software with the SHAPE method set to D. The forna website[2] was used to generate the illustrations of the secondary structures of single-stranded RNA that were predicted.

Fluorescence binding assay

A solution (50 μL) of the binder (0.005 or 0.01 μM for TO-G-clamp(am), 0.005 μM for TO-G-clamp-Me, 0.01 μM for TO-G-clamp-Bn and -Phe, 0.1 μM for TO-PRO-1 and G-clamp-N_3, 1 μM for Dequalinium, and 10 μM for AZ191) in 1× Binding buffer (1 or 5% DMSO (v/v), 20 mM phosphate, 20 mM NaCl and 80 mM KCl) was transferred to a micro quartz cell with a 1-cm path length. Serial aliquots of a concentrated solution of RNA in 1× buffer was added to the binder solution and allowed to equilibrate for 2 min. The excitation wavelength was set at 507 nm for TO-G-clamp(am) and TO-G-clamp-Me, 506 nm for TO-G-clamp-Bn, 512 nm for TO-G-clamp-Phe, 501 nm for TO-PRO-1, 360 nm for G-clamp-N_3, 320 nm for Dequalinium, and 334 nm for AZ191, and the emission was recorded at 20°C. Fluorescence measurements were performed with a JASCO-6500 or JASCO-8300 spectrofluorometer (JASCO, Tokyo, Japan).

The data from the titrations were analyzed according to the independent-site model by non-linear fitting to Equations (1) or (2), in which F_0 is the initial fluorescence intensity in the absence of RNA, Q (= F_{max}/F_0) is the fluorescence enhancement upon saturation, A = K_{Dapp}/C_{ligand} and X = nC_{RNA}/C_{ligand} (n is the putative number of binding sites on RNA, n = 2 was used for the double-stranded sequence; w/o hairpin loop, and n = 1 was used for the others).[3] The parameters Q and X were determined by KaleidaGraph (Synergy Software, PA). The K_{Dapp} values in the main text show the mean ± standard error values of three independent experiments.

$$F/F_0 = 1+(Q-1)/2\{A+1+X-[(X+1+A)^2-4X]^{1/2}\} \quad (1)$$

$$\text{or} \quad \Delta F = F-F_0 = F_0(Q-1)/2\{A+1+X-[(X+1+A)^2-4X]^{1/2}\} \quad (2)$$

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In silico RNA motif extraction

All motifs including human pre-miRNA in the RNA structure library were extracted from miRBase as detailed previously.[4] To design the library, the human pre-miRNA motifs were filtered based on length (< 107 nt), with 1804 species collected in total. Next, we obtained RNA secondary structure datasets as determined by SHAPE-MaP or DMS-MaPseq with structural analysis.[5,6] Predicted structures and conserved elements of SARS-CoV2 were obtained from a published study.[7] From the collected datasets, we divided long continuous RNAs into terminal motifs and defined them as structural units using FOREST.py (https://github.com/KRK13/FOREST2020). In total, 1099 motifs were collected from the transcripts of SARS-CoV2 and Influenza A viruses. As controls, selected RNA structural motifs, aptamers, and defective mutants were collected and loaded into the library.

Design of a template pool of RNA structure library and DNA barcode microarray

Multiplexed single-stranded DNA sequences were used as templates for RNA probes in the library. The extracted RNA motifs were attached with T7 promoter, RNA barcodes, and stabilizing stem sequences for detection and hybridization to the DNA barcode microarray as previously described.[4] The ssDNA templates were synthesized by SurePrint oligonucleotide library synthesis (Agilent technologies). The size of the oligo template was limited to 190 nt for the RNA structure library. After assigning barcodes to RNA structures, the DNA reverse complementary strands of RNA barcodes were used by SureDesign (Agilent technologies), a custom CGH array design service, to synthesize DNA barcode microarrays. Probe Replication Factor was set to 5× and 3×.

3'-Terminal labeling with Cy3

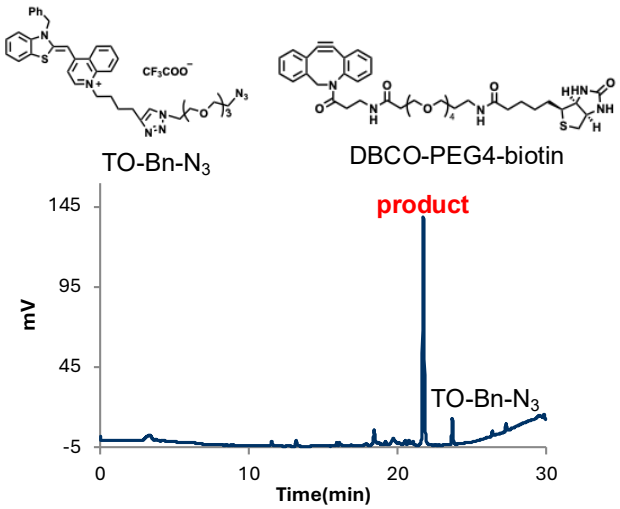
All RNA probes in the RNA structure library were labelled with a fluorescent dye at the 3' end. Seven micromolar RNA structure library, 70 μM pCp-Cy3 (Jena Bioscience), and 0.5 U/μL T4 RNA Ligase (Thermo Fisher Scientific) were mixed in 100 μL of 1× T4 Ligase Buffer (Thermo Fisher Scientific). The mixture was incubated at 16 °C for 48 h on a ThermoMixer (Eppendorf) with ThermoTop (Eppendorf). After incubation, the labelled RNA was purified using Zymo RNA Clean and Concentrator (Zymo Research) and stored at −28 °C until use.

Synthesis of TO-Bn-N_3 for FOREST analysis

The N_3-modified TO-Bn, named TO-Bn-N_3, was synthesized via a copper-catalyzed click reaction using N_3–PEG_3–N_3 as an N_3 linker after preparing the corresponding alkyne intermediate (Schemes S3).

Biotin modification of TO-Bn-N_3 by strain-promoted azide–alkyne cycloaddition (SPAAC)

To a solution of DBCO-PEG4-biotin in DMSO (9 mM, 1 μL), a solution of TO-Bn-N_3 in DMSO (10 mM, 1 μL) was added, and the mixture (4.5 and 5 mM, 2 μL) was incubated at 37 °C. After 60 min, the mixture was analyzed by reverse phased HPLC. HPLC conditions; A: 0.1% TFA in distilled water, B: 0.1% TFA in MeCN; B: 0%→60% (～20 min)→100% (～25 min)→100%(～30 min). Flow rate = 1 mL/min; Temp. = 35°C; UV = 254 nm, C-18 column (Nacalai Tesque: COSMOSIL 5C_{18}-AR-II, 4.6 × 250 mm). The mixture was used in following assays without any purification. TO-Bn-biotin: ESI-HRMS (*m*/*z*): $[M+H]^{2+}$ calcd for $C_{77}H_{95}N_{13}O_{11}S_2^{2+}$, 720.8352; found 720.8364.



HPLC profiles of biotin conjugation of TO-Bn-N_3 by strain-promoted azide-alkyne cycloaddition (SPAAC).

RNA pull-down

The RNA structure library was prepared in 1× Binding buffer (20 mM phosphate pH 7.0, 20 mM NaCl, and 80 mM KCl). For folding, RNA was heated at 95 °C and cooled to 4 °C on ice. During the folding step, 150 pmol of TO-Bn-biotin and 50 µL of Streptavidin Mag Sepharose (Cytiva) were mixed in 900 µL of 1× Binding buffer to prepare the small molecule-conjugated beads. The mixture was vortexed at room temperature for 60 min. The tube was placed on a magnetic rack to remove the supernatant and 500 ng of the refolded RNA structure library in 500 µL of 1× Binding buffer was added. A mixture containing only the beads was prepared as a control for background subtraction. The mixture was vortexed at room temperature for further 60 min. The mixture was washed three times with 200 µL of 1× Binding buffer when the reaction ended. Two hundred microliters of 1× Elution buffer (1% SDS (w/v), 10 mM Tris-HCl pH 8.0, 2 mM EDTA) was added to the magnetic beads and the mixture was heated at 95 °C for 3 min. The bound RNA structures were collected from the supernatant by removing the magnetic beads and purified with phenol-chloroform extraction and ethanol precipitation.

Hybridization and microarray scanning

Eighteen microliters of the bound RNA structures was mixed with 4.5 µL of 10× Blocking Agent (Agilent Technologies) and 22.5 µL of Hi-RPM Hybridization Buffer (Agilent Technologies). The samples were incubated for 5 min in a heat block set at 104 °C, then rapidly cooled and incubated for 5 min in ice water. The samples were applied to an 8× 60 K Agilent microarray gasket slide (Agilent Technologies). The prepared gasket slide and CGH custom array 8× 60 K (Agilent Technologies) were assembled with SureHyb. Hybridization was performed for 20 h at a temperature of 55.5 °C at 20 rpm. The microarray slide was washed for 5 min with Gene Expression Wash Buffer 1 (Agilent Technologies) in a glass container at room temperature following hybridization. The microarray slide was moved to a glass container containing Gene Expression Wash Buffer 2 (Agilent Technologies), which was immersed in a thermostatic bath at 37 °C. The washing step was performed for 5 min. Fluorescence scanning was performed on the microarray and fluorescence image data were acquired using SureScan (Agilent Technologies). The acquired images were converted to numeric fluorescence intensities for each spot by Feature Extraction (Agilent Technologies) and GeneSpringGX (Agilent Technologies).

Calculation of binding intensity

The binding intensities of each RNA structure were calculated by subtracting the fluorescence intensities of the no-

ligand control samples. To alleviate the effect of undesired interactions with the RNA barcode, we calculated the mean fluorescence intensities of each RNA structure from the intensities of three RNA probes that had the same RNA structure but different RNA barcodes. For this reason, we filtered the maximum and minimum values from a set of five intensities.

Molecular stability analysis

Photostability assay: A mixture (50 μL) of the indicator and pre-mir-221-motif (10 μM TO-G-clamp-Bn or TO-PRO-1; 10 μM pre-mir-221 motif) in 1× Binding buffer (5% DMSO (v/v), 20 mM phosphate pH 7.0, 20 mM NaCl and 80 mM KCl) was transferred to a micro quartz cell with a 1-cm path length. The excitation wavelength was set at 506 nm for TO-G-clamp-Bn and 501 nm for TO-PRO-1, and the solution was continuously irradiated for 1 ~ 10 minutes (1 min interval) by manually controlling the open/closed state of the excitation shatter. The emissions before and after the irradiation were recorded and the data (Fluorescence intensity value at 539 nm for TO-G-clamp-Bn and 530 nm for TO-PRO-1) was normalized by dividing the obtained fluorescence intensity value for each measurement by the fluorescence intensity value before irradiation (0 min). The irradiation and fluorescence measurement were performed using JASCO-6500 spectrofluorometer (JASCO, Tokyo, Japan).

Stability in cell lysate: To a mixture of 25 μL of 2× Binding buffer (40 mM phosphate pH 7.0, 40 mM NaCl and 160 mM KCl) and 19.5 μL of distilled water, 0.5 μL of DMSO solution (10 mM TO-G-clamp-Bn in DMSO for TO-G-clamp-Bn and TO-G-clamp-Bn + Cell lysate, DMSO for Cell lysate sample) was added. Five microliters of HeLa Whole Cell Lysate (Santa Cruz Biotechnology) or distilled water (HeLa Whole Cell Lysate for TO-G-clamp-Bn + Cell lysate and Cell lysate, distilled water for TO-G-clamp-Bn sample) was added to the mixture and incubated at 37 °C for 1 hour. After incubation,150 μL of DMSO was added, then the solution was filtered through DISMIC 13HP045AN (ADVANTEC). The filter was further washed with 100 μL of DMSO, and the collected solution was analyzed by reverse phase HPLC. HPLC conditions; A: 0.1% TFA in distilled water, B: 0.1% TFA in MeCN; B: 5%→95% (~ 20 min)→100% (~ 25 min)→100% (~ 30 min); Flow rate = 1 mL/min; Temp. = 35 °C; UV = 254 nm, C-18 column (Nacalai Tesque: COSMOSIL $5C_{18}$-AR-Ⅱ, 4.6 × 250 mm).

FID assay

Fluorescence intensities in FID assays were measured with a microplate reader Infinite® 200 PRO (TECAN Group Ltd., Mannedorf, Switzerland) using i-control® and LBS coated Optiplate™-96F as 96-well plates. RNA (pre-mir-221 motif) was diluted to 10 μM in 1× Binding buffer (20 mM phosphate pH 7.0, 20 mM NaCl, and 80 mM KCl), and the solution was heated at 95 °C for 5 min and cooled on ice. 1× Binding buffer (20 mM phosphate pH 7.0, 20 mM NaCl, 80 mM KCl) was added to each well (49 μL for blank, negative control, positive control, and sample wells), followed by the addition of 0.25 μL of DMSO or 20 μM indicator solution (TO-G-clamp-Bn and TO-PRO-1) in DMSO to the blank or negative control, positive control, and sample wells, respectively, to achieve a final concentration of indicator of 0.1 μM. A half of one microliter of 1× Binding buffer or 10 μM folded RNA solution in Binding buffer was dispensed in the blank and negative control or positive control and sample wells, respectively, so that a final concentration of the RNA becomes 0.1 μM. Zero point two five microliter of DMSO or 1 mM compound solution in DMSO (LOPAC® 1280, Sigma-Aldrich) was added to the blank, negative control, and positive control, or sample wells, respectively, to achieve a final concentration of the compound of 5 μM, and mixed with RNA-ligand solutions. Fluorescence intensities of the mixtures were measured after incubating at 25°C for 30 min. The excitation

wavelength was set at 485 nm. Normalized fluorescence intensity (*F*) was calculated using Equation (3) described below:

$$Normalized\ F = \frac{F_{(indicator + RNA + test\ compounds)} - F_{(buffer + indicator)}}{F_{(indicator + RNA)} - F_{(buffer + indicator)}} \qquad (3)$$

Hits were selected based on a reduction of the fluorescence signal by less than two standard deviations (2σ) from the mean. Normalized fluorescence intensities greater than 1.5 were excluded from calculations for the mean and σ.

The Z'-factors of TO-G-clamp(am) and TO-PRO-1 were calculated using Equation (4),

$$\mathrm{Z'-factor} = 1 - \frac{3\left(\sigma_p + \sigma_n\right)}{\left|\mu_p - \mu_n\right|} \qquad (4)$$

where σ_p and σ_n are the standard deviations of the positive and negative controls, respectively, and μ_p and μ_n are the means of the positive and negative controls, respectively. Fluorescence intensities of ten each of blank, negative, and positive control wells were measured to calculate the Z'-factors.

Dose-dependency analysis

The procedure is similar to FID assay described above with minor changes. 1× Binding buffer (20 mM phosphate pH 7.0, 20 mM NaCl, 80 mM KCl) was added to each well (48.75 μL for blank, negative control, positive control, and sample wells), followed by the addition of 0.25 μL of DMSO or 20 μM indicator solution (TO-G-clamp-Bn and TO-PRO-1) in DMSO to the blank or negative control, positive control, and sample wells, respectively, to achieve a final concentration of indicator of 0.1 μM. A half of one microliter of 1× Binding buffer or 10 μM folded RNA solution in Binding buffer was dispensed in the blank and negative control or positive control and sample wells, respectively, so that a final concentration of the RNA becomes 0.1 μM. A half of one microliter of DMSO or the competitive analyte solutions (0.001, 0.01, 0.5, 0.1, 0.5, 1, 5, and 10 mM) in DMSO, each of the competitive analytes was purchased individually, was added to the blank, negative control, and positive control, or sample wells, respectively, to achieve a final concentration of the competitive analyte of 0 to 100 μM, and mixed with the RNA-ligand solutions. Fluorescence intensities of the mixtures were measured after incubating at 25°C for 30 min. The excitation wavelength was set at 485 nm. Normalized fluorescence intensity (*F*) was calculated using Equation (3) described above. The IC_{50} values were calculated using Equation (5),

$$\text{Fluorescence fold change} = \frac{1}{1 + 10^{(\log IC_{50} - \log x) \times slope\ factor}} \qquad (5)$$

where *x* is the concentration of the competitive analyte. Two-tailed Student's T test was made by Scipy v. 1.7.3 in python 3.7.

SPR analysis

Immobilization: 5′-biotinylated RNA (pre-mir-221 motif) was diluted to 1 μM in 1× Binding buffer (20 mM phosphate pH 7.0, 20 mM NaCl, and 80 mM KCl), and the solution was heated at 95 °C for 5 min and cooled on ice. The folded RNAs were injected over a streptavidin-coated sensor chip (Series S Sensor chip SA, Cytiva) at 60 μL/min to reach an immobilized level of 1156 or 1202 RU.

Binding analysis by multi-cycle kinetics: the RNA binder (Dequalinium, AZ191, NF023, and NAV-2729) in 1× Binding buffer (1% DMSO (v/v), 20 mM phosphate pH 7.0, 20 mM NaCl, and 80 mM KCl) was injected at increasing

concentrations (1, 2, 3, 5, 10, 20, 30, 50, and 100 μM), up to their solubility limits, to the RNA-immobilized sensor surface with a regeneration step between each concentration. Each of the RNA binder was injected with a flow rate of 60 μL/min, contact time of 30 s, and dissociation time of 120 s using the running buffer at 25 °C. A regeneration step was conducted with a flow rate of 60 μL/min and contact time of 30 s using 1 M NaCl solution. All sensorgrams were corrected by subtracting the blank flow cell and buffer injection responses. SPR response values at 20 s were used to compute the K_{Dapp} values using the 1:1 binding equation {y = (B_{max}·x) / (K_{Dapp} + x)}, where y is the SPR response, B_{max} is the maximum SPR response, K_{Dapp} is the apparent dissociation constant, and x is the concentration of the added RNA binder.

Binding analysis by single-cycle kinetics: TO-G-clamp-Bn in 1× Binding buffer (1% DMSO (v/v), 20 mM phosphate pH 7.0, 20 mM NaCl, and 80 mM KCl) was injected at increasing concentrations (0.2, 0.4, 0.6, 0.8, 1 μM) to the RNA-immobilized sensor surface without a regeneration step between each concentration. Each of the RNA binder was injected with a flow rate of 60 μL/min, contact time of 30 s, and dissociation time of 120 s using the running buffer at 25 °C. A regeneration step was conducted with a flow rate of 60 μL/min and contact time of 30 s using 1 M NaCl solution. All sensorgrams were corrected by subtracting the blank flow cell and buffer injection responses. K_{Dapp} were obtained by Biacore T200 evaluation software using a two-state reaction model. Three technical replicates were conducted.

SHAPE-MaP

Design of RNA library

As RNA sequences in an RNA library, 47 sequences of pre-mir-RNA, repetitive RNA, and virus motifs were selected from 3,000 RNA structural library in FOREST,[8] including pre-mir-221 and 4520-1 motifs. Additionally, TPP and FMN riboswitches and so on were selected as control sequences. To maintain the secondary structures of the extracted RNAs, we attached the common stem (5'-CGAAGUUUCAGC-3' for 5' end and 5'-GCUGAAGCUUCG-3' for 3' end) to all of the pre-miRNA motifs. For sample preparation after a SHAPE modification step, adapter sequences, which form 3 nt hairpin loops not to be modified by SHAPE reagents, were attached at both of 5' and 3' ends.

RNA library preparation

DNA library (Twist Bioscience) in 1×IDTE buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA) was amplified by the PCR amplification (Platinum SuperFi II DNA Polymerase, Thermofisher). The PCR (25 μL of the reaction volume for 10 cycles) was carried out for the amplification of the DNA using 0.5 μM of forward and reverse primers, followed by purification using Monarch® DNA Cleanup Columns (NEB). The RNA transcript was synthesized by HiScribe® T7 High Yield RNA Synthesis Kit (NEB), purified by RNAClean XP (Beckman), and stored with nuclease-free water at -80°C till further use.

Modification in vitro

For a sample, 3 μL of nuclease-free water was added to 174 ng of the transcribed RNA and denatured by heating at 95°C for 2 min and cooled on ice for 2 min. The denatured RNA was folded using 1.5 μL of 3.3× Reaction buffer (330 mM HEPES, pH 8.0, 330 mM NaCl, 33 mM $MgCl_2$) and incubated at 37°C for 20 min. Four point five microliters of RNA binders in 1× Reaction buffer was added to the folded RNA solution with a final concentration of 10 μM for TO-G-clamp(am), 100 μM for TO-PRO-1, and 200 μM for AZ191, followed by incubation at 37°C for another 60

min. As controls, two DMSO solutions were prepared. To 1 μL of a solution of 500 mM 2A3 in DMSO, 9 μL of the mixture of RNA and RNA binders were added to afford 10 μL of the reaction volume with a final concentration of 2A3 of 50 mM and incubated at 37°C for 10 min. After the incubation, the reaction was quenched by the addition of 10 μL of 500 mM DTT. The modified RNA was purified using 40 μL of RNAclean XP (Beckman) and stored with 10 μL of nuclease-free water at -20°C till further use.

Reverse transcription

Seven microliters of the modified RNA was mixed with 2 μL of dNTPs (10 mM each, NEB) and 1 μL of 2 μM RT primer. The samples were incubated at 85°C for 1 min and 70°C for 10 min followed by cooling at 4°C. To reverse transcribe the RNA, 9 μL of 2.22× PAIR-MaP buffer (111 mM Tris-HCl, pH 8.0, 167 mM KCl, 22.2 mM DTT, 2.22 M betaine, 13.3 mM $MnCl_2$) was added and incubated at room temperature for 2 min. One microliter of SuperScript™ II (Invitrogen) was added to the mixture and reverse transcribed following the condition (25°C for 10 min → 42°C for 90 min → [50°C for 2 min → 42°C for 2 min] ×15 → 72°C for 10 min → 4°C). Five microliters of 1 M NaOH and 0.4 M EDTA solution was added and incubated at 95°C for 3 min to hydrolyze the RNA. The mixture was neutralized by an addition of 1 μL of 5 M HCl solution, then purified using 52 μL of AMPure XP (Beckman) and stored with 10 μL of nuclease-free water at -20°C till further use.

PCR for preparation of dsDNA library

The dsDNA libraries were prepared by the PCR amplification of the reverse transcribed cDNA (Platinum SuperFi II DNA Polymerase, Thermofisher). First PCR (25 μL of the reaction volume for 15 cycles) was carried out for the amplification of the cDNA using 0.5 μM of forward and reverse primers. For unique barcoding of the libraries, another PCR was performed (25 μL of the reaction volume for 8 cycles) using 0.5 μM of Illumina primers. Each library was then purified using Monarch® DNA Cleanup Columns (NEB), eluted in 15 μL of elution buffer (Qiagen), and stored at -20°C till sequencing. All of the libraries were pooled and sequenced on an Illumina iSeq instrument following the standard sequencing protocol, outputting 2 × 150 paired-end data sets.

Processing of the sequencing data

The obtained fastq files were processed by Cutadapt (parameters: -discard-untrimmed -e 0.2 -minimum-length 10) to trim the adapter sequences at both edges. The mapping was performed using Bwa-mem2 and sorted by Samtools sort with default parameters. The mutation counts were obtained after counting the sorted data. For analysis of the percentage of mutation, nucleotides with a read count less than 200 or a mutation count is 0 were discarded. The delta mutation rates of each sample were obtained by the subtraction of the mean of the two DMSO controls.

Statistical test for a difference in mutation rates of a given nucleotide

To test statistically significant difference in mutation rates of a given nucleotide between sample 1 and 2 with and without an RNA binder, respectively, we performed the comparison of Two Poisson Counts tests[9,10] to calculate Z-test (Z) values using the equations (6 to 9), where Z_p and Z_n are positive and negative Z, respectively, m_1 and m_2 are the number of modification counts in sample 1 and 2, respectively, n_1 and n_2 are the number of total reads in sample 1 and 2, respectively, p_1 is the proportion of modifications in sample 1. As the sample 2, the mean of the mutation rates that was calculated from the two DMSO controls were utilized.

$$p_1 = \frac{n_1}{n_1 + n_2} \qquad (6)$$

$$Z_p = \frac{m_1 - p_1(m_1 + m_2) + 0.5}{\sqrt{p_1(1 - p_1)(m_1 + m_2)}} \quad (7)$$

$$Z_n = \frac{m_1 - p_1(m_1 + m_2) - 0.5}{\sqrt{p_1(1 - p_1)(m_1 + m_2)}} \quad (8)$$

$$Z = \min(|Z_p|, |Z_n|) \quad (9)$$

Then, the calculated *Z* values were converted into the *p* values using the two-tailed hypothesis of the equation {2(1 – *CDF*(Z))}, where *CDF* is the cumulative distribution function.

To minimize the multiple testing problem, we discarded less differential nucleotides from the statistical test. The absolute delta mutation rates were calculated for each RNA sequence individually, and nucleotides with the delta mutation rates less than 50% of the total sequence were discarded. To further alleviate the multiple testing problem, false discovery rate (FDR) correction was performed using the Benjamini-Hochberg method. The nucleotides with FDR < 5%; *q* value < 0.05 were taken as statistically significant differences.

Table S1. K_{Dapp} values of G-clamp, TO-G-clamp analogs, and TO-PRO-1 (n=3)

Rank (G-clamp-N_3)	Z-score (G-clamp-N_3)	Name	Sequence[c]	K_{Dapp} (μM) (G-clamp)[a]	K_{Dapp} (μM) (TO-G-clamp(am))	K_{Dapp} (μM) (TO-G-clamp-Me)	K_{Dapp} (μM) (TO-G-clamp-Bn)	K_{Dapp} (μM) (TO-G-clamp-Phe)	K_{Dapp} (μM) (TO-PRO-1)
1	4.83	hsa-mir-4520-1	CCAAAUCAGAAAAGGAUUUGG	0.024	0.0053±0.0007	0.028±0.004	0.058±0.009	0.088±0.02	0.22±0.05
-	-	G12A	CCAAAUCA**A**AAAAGGAUUUGG	0.011	<0.005	-	-	-	0.74±0.06
-	-	G17A	CCAAAUCAGAAAA**A**GAUUUGG	15	320±70	-	-	-	0.12±0.01
2	4.26	hsa-mir-6847	GAGCUGGAAGGAGUGGGCCUGGCUC	0.022	-	-	-	-	-
4	3.76	hsa-mir-125a	CUGUGAGGACAUCCAGGGUCACAG	0.14	0.0035±0.0005	0.0085±0.001	0.065±0.01	0.17±0.05	-
13	3.09	hsa-mir-6790	GUUCGGGGGUUCCGACGGCGAC	0.071	-	-	-	-	-
28	2.74	hsa-mir-6850	CGGGGCGGGAGGGGAAGGGACGCCCG	0.19	-	-	-	-	-
38	2.56	G4 (GGGU)6	GGGUGGGUGGGUGGGUGGGUGGGU	0.15	0.0078±0.001	-	-	-	0.23±0.04[d]
43	2.50	r(CUG)16	CUGCUGCUGCUGCUGCUGCUGCUGCUGCUGC UGCUGCUGCUGCUG	13	-	-	-	-	-
83	1.95	hsa-mir-4291	AGCUGGGUGGAGGCAGAGCU	0.56	0.0046±0.0003	0.017±0.0008	0.090±0.01	0.14±0.04	-
111	1.67	hsa-mir-526a-1	UCUGUUGCUUGAAAGAAGAGA	2.3	0.0064±0.0005	-	-	-	-
160	1.44	hsa-mir-105-1	CUGUGGUGGCUGCUCAUGCACCACGG	5.4	0.14±0.006	-	-	-	-
225	1.17	hsa-mir-100	CUUGUGGUAUUAGUCCGCACAAG	1.1	0.0070±0.00009	0.011±0.002	0.19±0.02	0.26±0.03	-
522	0.46	hsa-mir-548ba	UGCUAUUAGAAAGUAAUGGCA	10	0.54±0.1	-	-	-	-
633	0.25	hsa-mir-203b	UCACAAUUGCGCUACAGAACUGUUGA	2.9	-	-	-	-	-
717	0.12	hsa-mir-221	AUUUCUGUGUUCGUUAGGCAACAG	2.7±0.2[b]	0.0093±0.001	0.014±0.004	0.17±0.006	0.046±0.009	0.44±0.09[d]
945	-0.20	hsa-mir-6786	GCGUCACGUGCACCCAAGUGACGC	6.6	0.24±0.03	-	-	-	-
1034	-0.30	hsa-mir-299	CAUACAUUUUGAAUAUGUAUG	16	0.85±0.1	0.65±0.05	2.1±0.2	3.6±0.08	-
1192	-0.48	hsa-mir-4773-1	UCUGCUUUAUUUCAUCAAAGCAGA	>20	2.0±0.2	1.8±0.2	3.4±0.3	>4	0.43±0.04[d]
1775	-1.54	hsa-mir-4282	UUUAGUAUGCCACAUUUCUAAA	>20	4.5±0.2	-	-	-	-

a) Previously reported. The data are mean (n=2). b) The data are corrected in the present study and mean ± SE (n=3). c) The common stems (5'-UCAGC-3' and 5'-GCUGA-3' for pre-mir-221 motif and 5'-AGC-3' and 5'-GCU-3' for the others) were attached at 5' and 3' ends to stabilize the secondary structures. d) Previously reported. The data are mean (n=3).

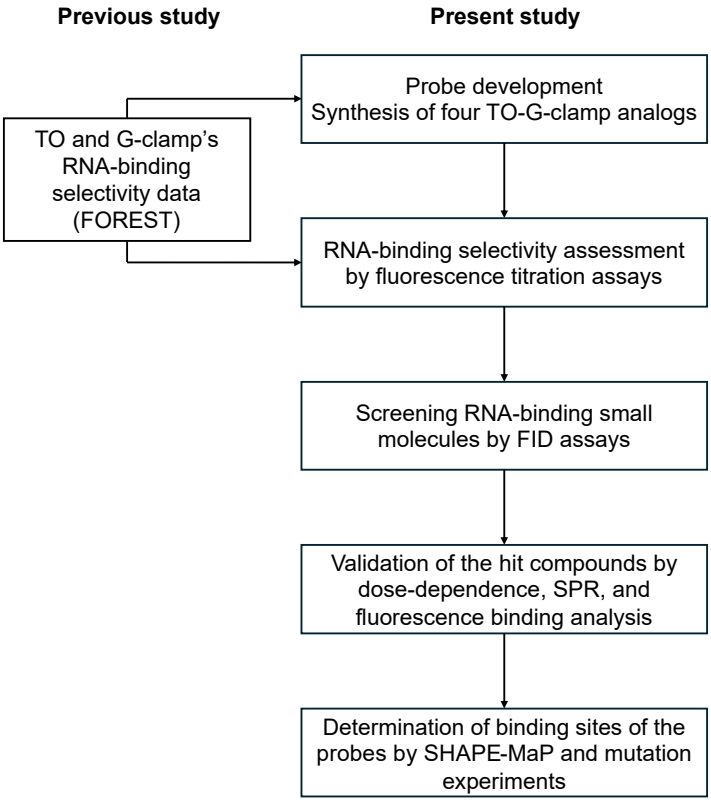


Figure S1. The flowchart of this study.

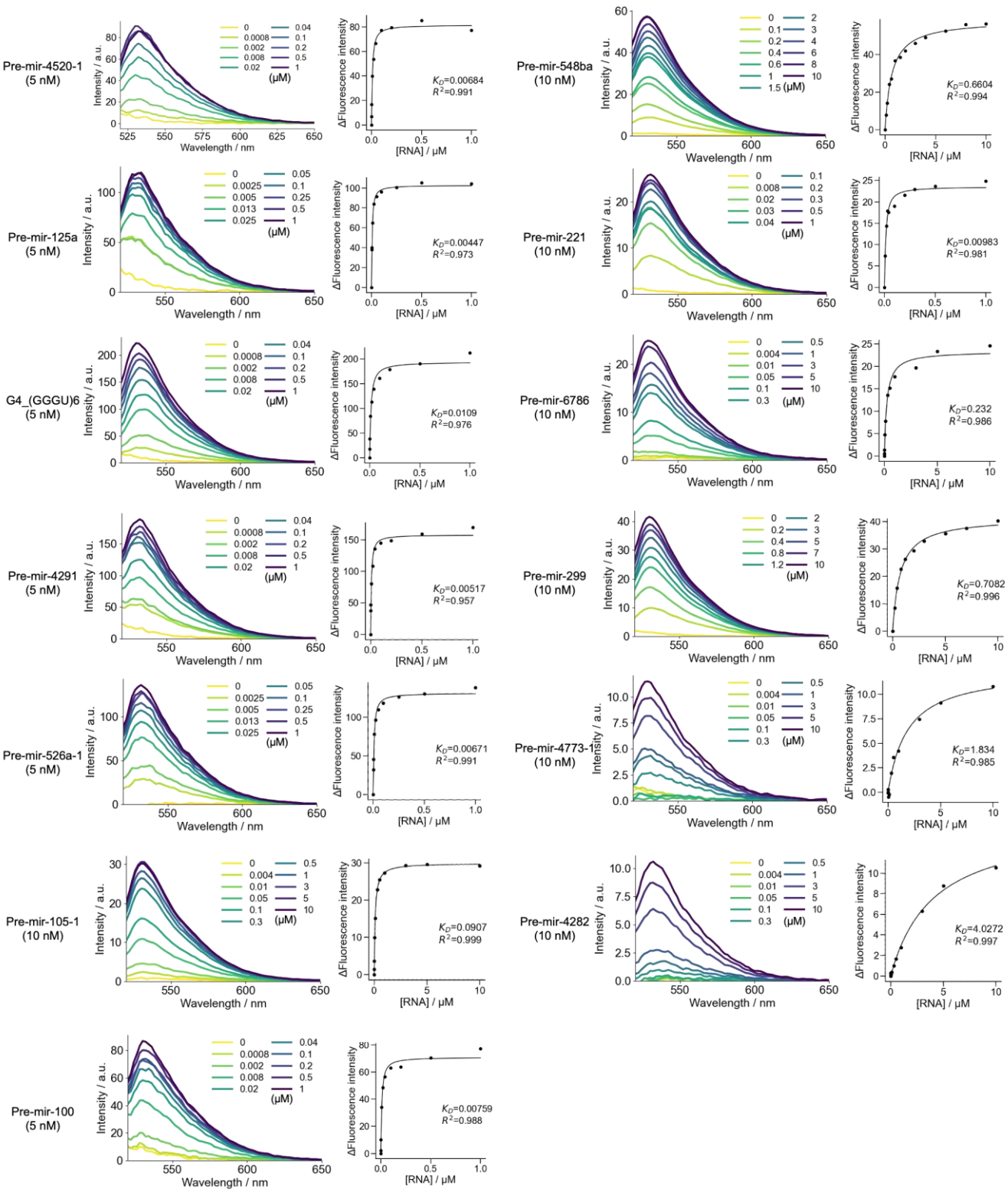


Figure S2. Fluorescence titrations to determine the apparent dissociation constants (K_{Dapp}) of TO-G-clamp(am). Fluorescence titration spectra were measured using TO-G-clamp(am) (5 or 10 nM) upon addition of RNA (0-1 or 10 μM) in Binding buffer (pH 7.0) including 5% DMSO. Conditions: λex: 507 nm, λem: 530 nm. The representative data and K_{Dapp} values are shown. Each of the concentration of TO-G-clamp(am) is shown below the name of RNA.

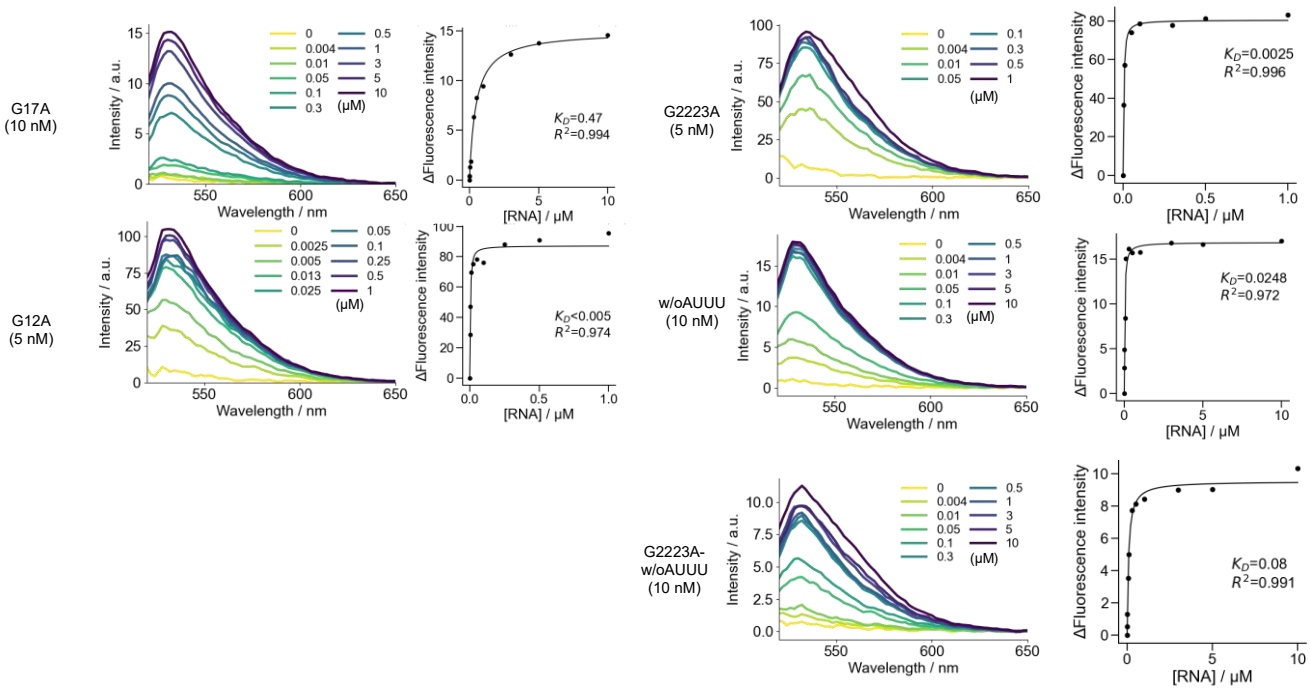


Figure S3. Mutation experiments using fluorescence titrations to determine the apparent dissociation constants (K_{Dapp}) of TO-G-clamp(am). Fluorescence titration spectra were measured using TO-G-clamp(am) (5 or 10 nM) upon addition of RNA (0-1 or 10 μM) in Binding buffer (pH 7.0) including 5% DMSO. Conditions: λex: 507 nm, λem: 530 nm. The representative data and K_{Dapp} values are shown. Each of the concentration of TO-G-clamp(am) is shown below the name of RNA.

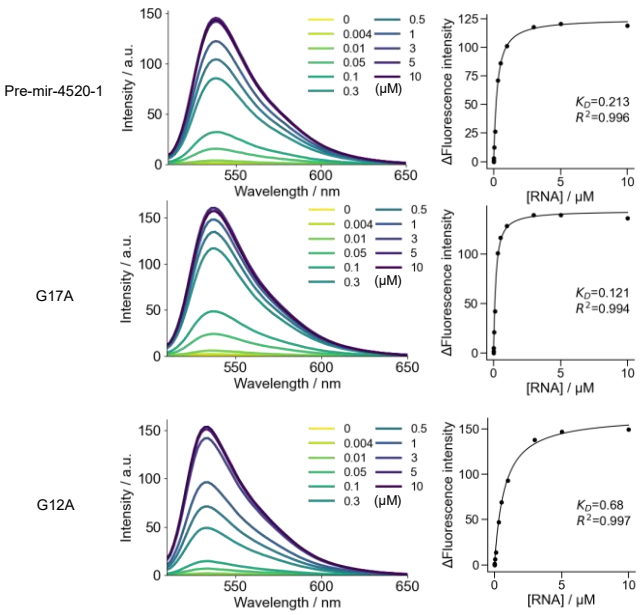


Figure S4. Mutation experiments using fluorescence titrations to determine the apparent dissociation constants (K_{Dapp}) of TO-PRO-1 against pre-mir-4520-1 mutant motifs. Fluorescence titration spectra were measured using TO-PRO-1 (0.1 μM) upon addition of RNA (0-10 μM) in phosphate buffer (pH 7.0) including 1% DMSO. Conditions: λex: 501 nm, λem: 530 nm. The representative data and K_{Dapp} values are shown.

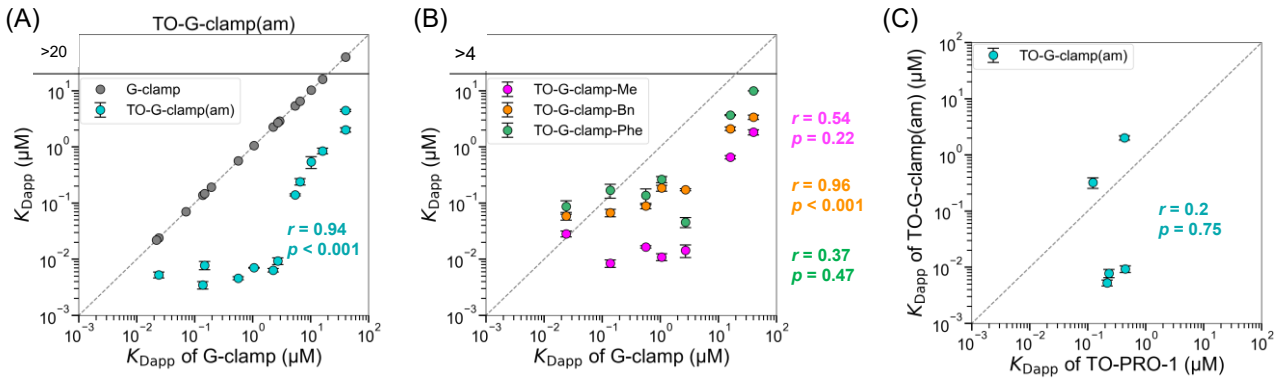


Figure S5. Comparison between K_{Dapp} of G-clamp and (A) TO-G-clamp(am) and (B) TO-G-clamp analogs. Data of TO-G-clamp(am) and TO-G-clamp analogs are mean ± standard error (n=3). Data of G-clamp are mean (n=2). (C) Comparison between K_{Dapp} of TO-PRO-1 and TO-G-clamp(am). *r* represents Spearman's correlation coefficient. Tests of no correlation were performed to calculate *p* values. One outlier (CUG repeat) was eliminated from this graph.

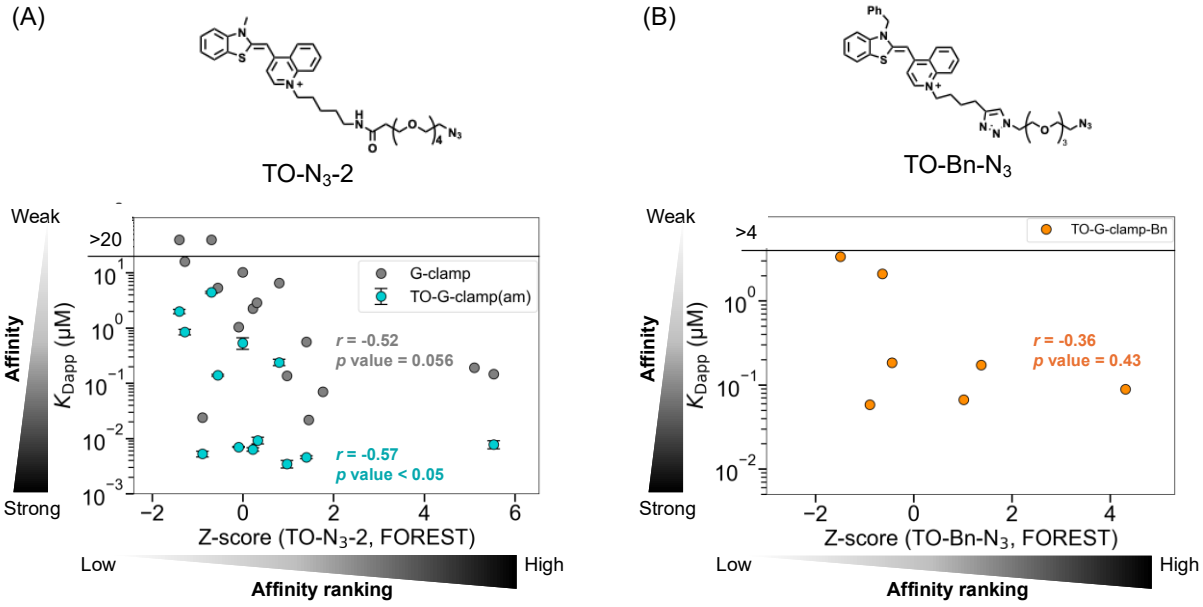


Figure S6. Correlation between TO-G-clamp analogs and TO moieties. (A) Chemical structure of TO-N_3-2 (top). Correlation graph between large-scale affinity score, named Z-score, of TO-N_3-2 and K_{Dapp} of G-clamp in grey and TO-G-clamp(am) in cyan (bottom). The Z-score of TO-N_3-2 and K_{Dapp} of G-clamp were obtained previously. (B) Chemical structure of TO-Bn-N_3 (top). Correlation graph between Z-score of TO-Bn-N_3 and K_{Dapp} of TO-G-clamp-Bn in orange (bottom). Data of TO-G-clamp(am) are mean ± standard error from independent triplicated experiments. *r* represents Spearman's correlation coefficient. Tests of no correlation were performed to calculate *p* values. One outlier (CUG repeat) was eliminated from the graph (A).

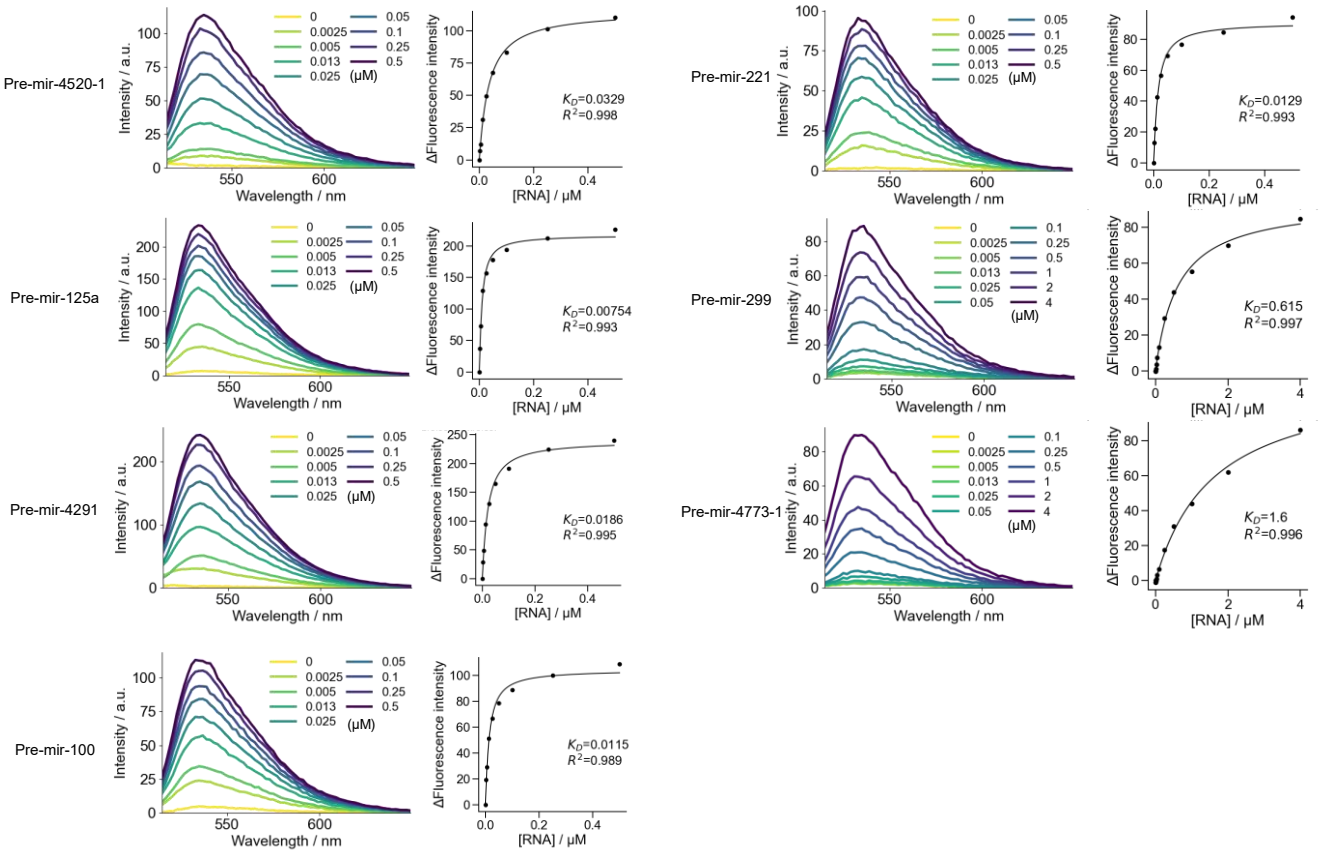


Figure S7. Fluorescence titrations to determine the apparent dissociation constants (K_{Dapp}) of TO-G-clamp-Me. Fluorescence titration spectra were measured using TO-G-clamp-Me (5 nM) upon addition of RNA (0-0.5 or 4 μM) in phosphate buffer (pH 7.0) including 5% DMSO. Conditions: λex: 507 nm, λem: 533 nm. The representative data and K_{Dapp} values are shown.

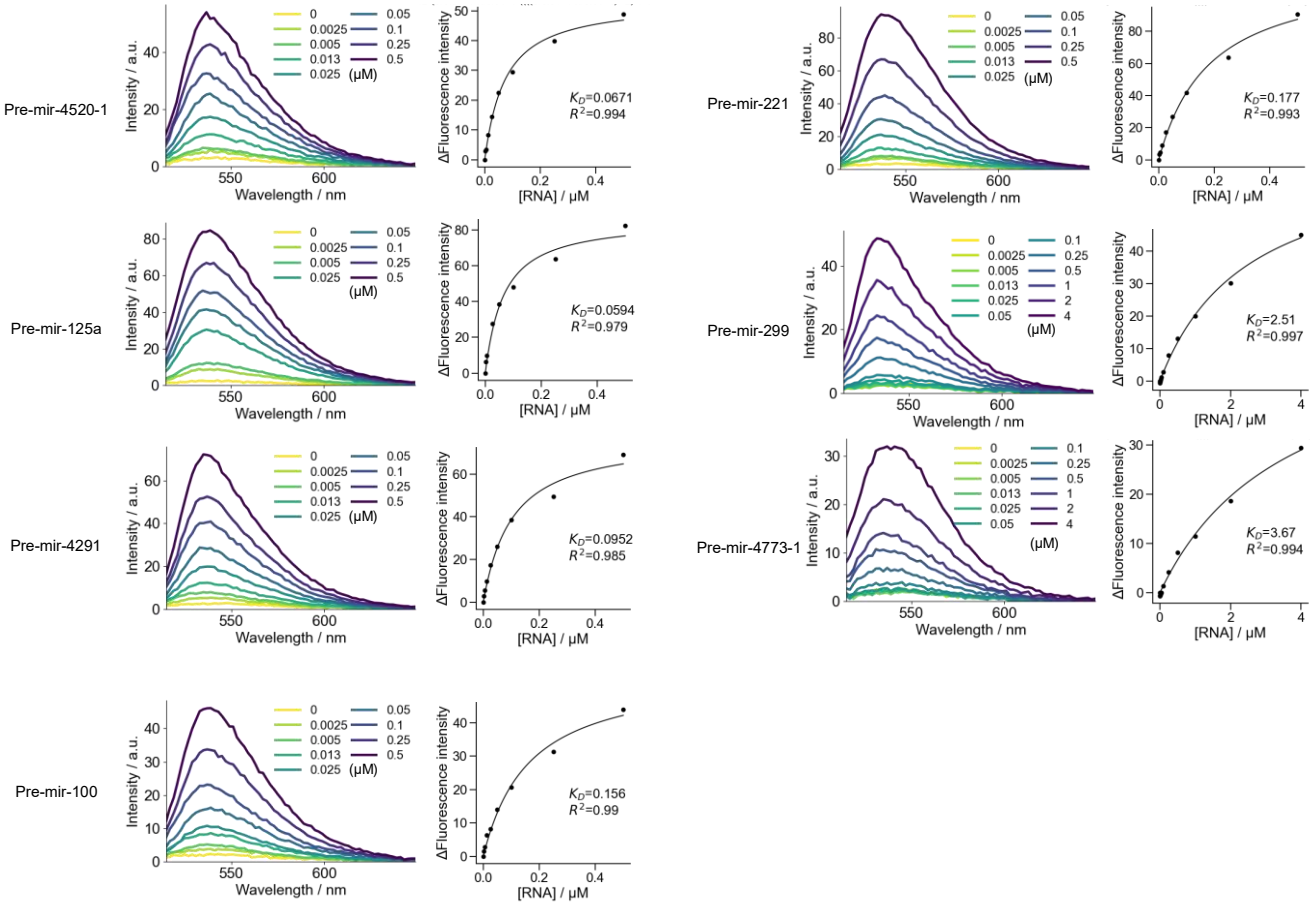


Figure S8. Fluorescence titrations to determine the apparent dissociation constants (K_{Dapp}) of TO-G-clamp-Bn. Fluorescence titration spectra were measured using TO-G-clamp-Bn (10 nM) upon addition of RNA (0-0.5 or 4 μM) in phosphate buffer (pH 7.0) including 5% DMSO. Conditions: λex: 506 nm, λem: 539 nm. The representative data and K_{Dapp} values are shown.

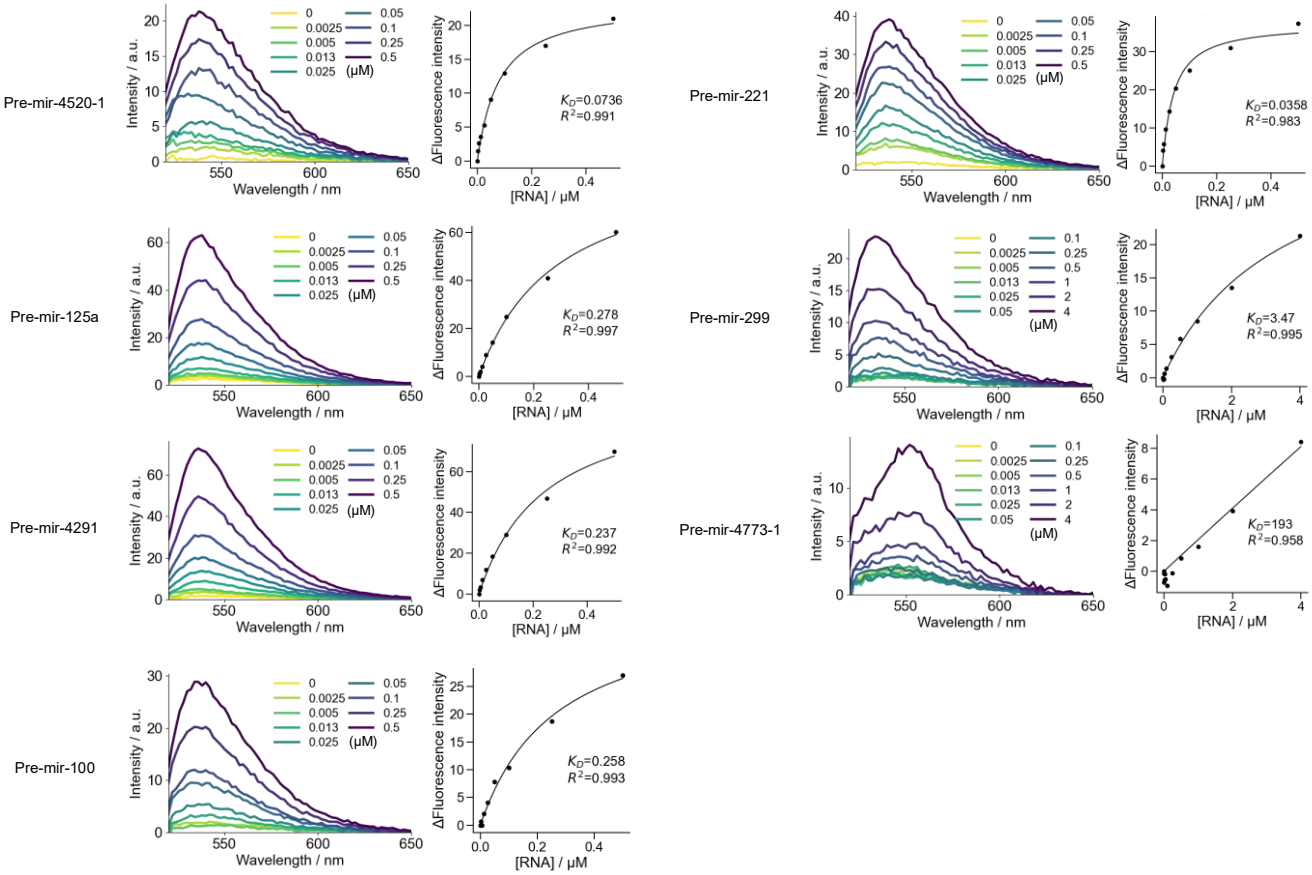


Figure S9. Fluorescence titrations to determine the apparent dissociation constants (K_{Dapp}) of TO-G-clamp-Phe. Fluorescence titration spectra were measured using TO-G-clamp-Phe (10 nM) upon addition of RNA (0-0.5 or 4 μM) in phosphate buffer (pH 7.0) including 5% DMSO. Conditions: λex: 512 nm, λem: 538 nm. The representative data and K_{Dapp} values are shown.

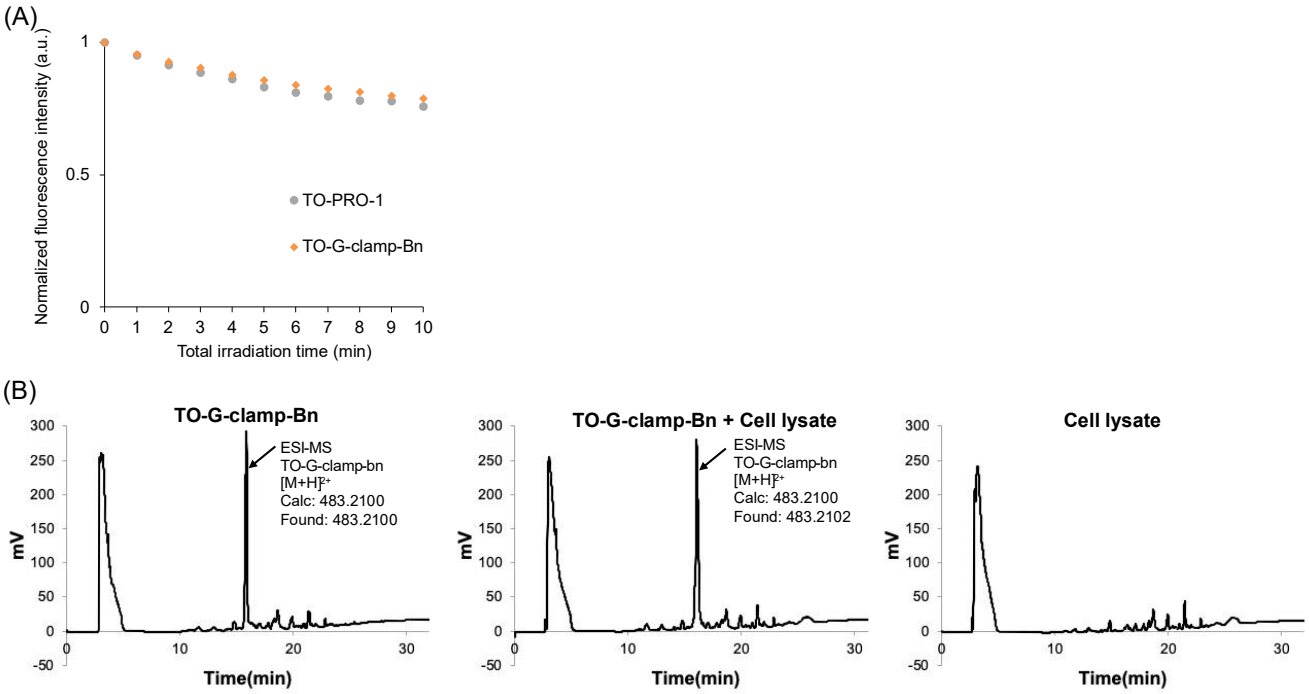


Figure S10. Molecular stability of TO-G-clamp-Bn. (A) Photostability assay with TO-G-clamp-Bn and TO-PRO-1 in the presence of pre-mir-221 motif. Conditions; 0.1 μM probe, 0.1 μM RNA, 501 and 506 nm light for TO-G-clamp-Bn and TO-PRO-1, respectively. (B) Stability of TO-G-clamp-Bn in cell lysate. HPLC chromatogram of samples (TO-G-clamp-Bn; 100 μM TO-G-clamp-Bn, TO-G-clamp-Bn + cell lysate; 100 μM TO-G-clamp-Bn in 10% cell lysate, Cell lysate; 10% cell lysate).

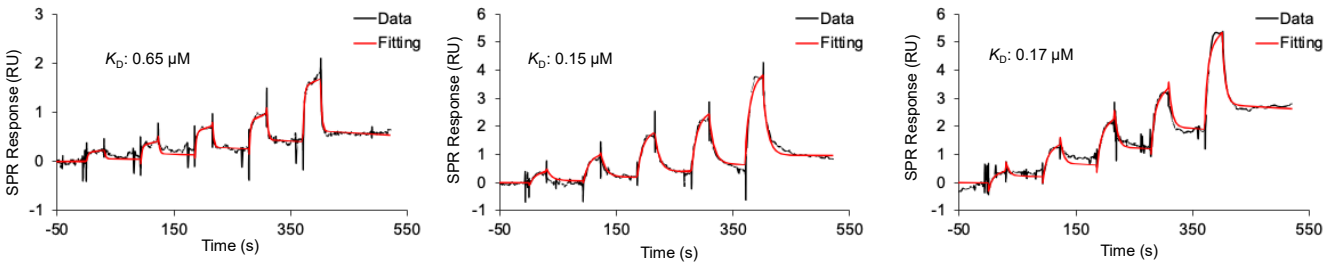


Figure S11. Surface plasmon resonance (SPR) analysis to validate the binding of TO-G-clamp-Bn. The SPR sensorgrams of TO-G-clamp-Bn (200, 400, 600, 800, and 1000 nM) with 5'-biotinylated pre-mir-221 motif are shown. The binding kinetics were determined in single-cycle kinetic mode using two-state binding model. Three technical replicates were conducted.

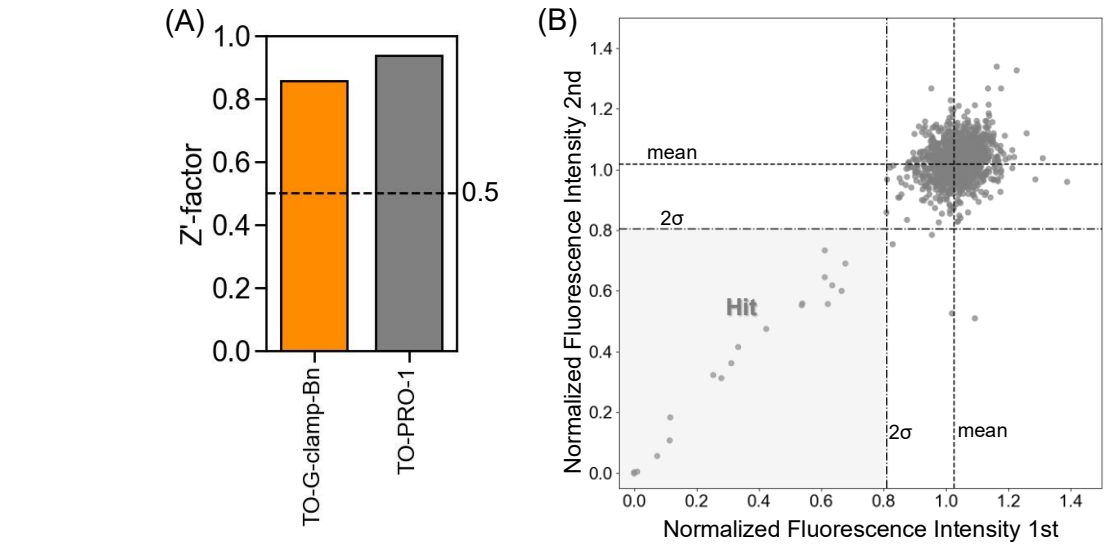


Figure S12. FID assays. (A) Z'-factor of TO-G-clamp-Bn in orange and TO-PRO-1 in grey. (B) Normalized fluorescence intensities in FID assays using TO-PRO-1. Hit compounds are defined as compounds that meet hit threshold (mean -2σ) in both assays.

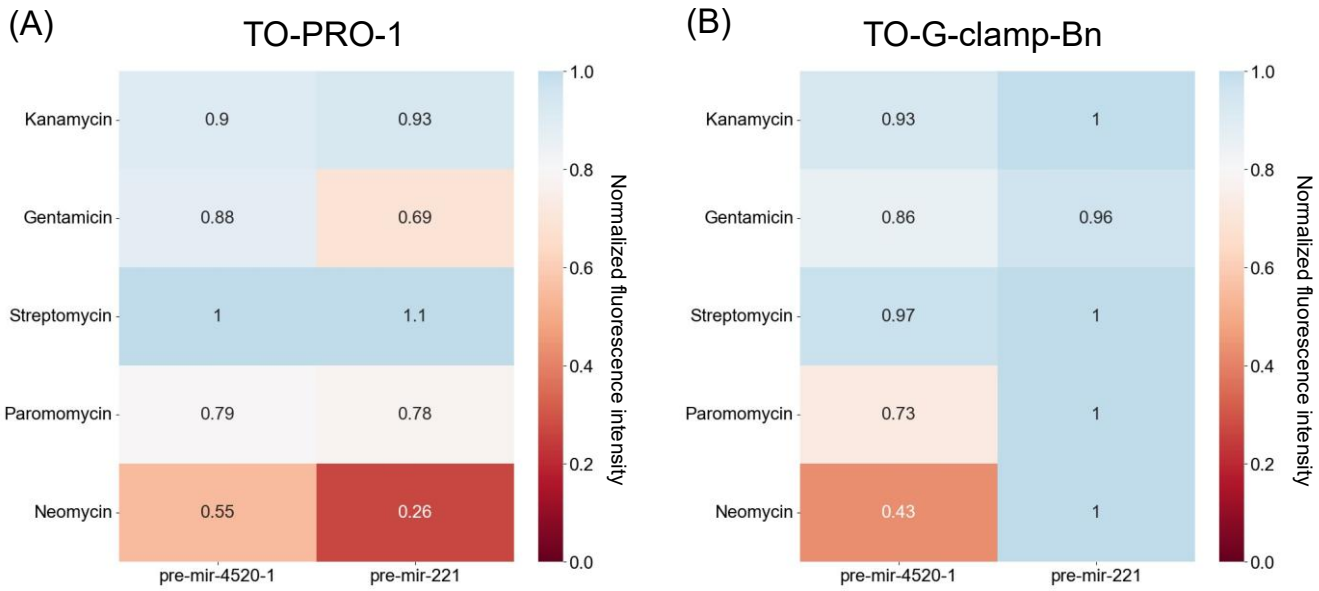


Figure S13. The heatmaps of the FID assay using aminoglycosides and (A) TO-PRO-1 and (B) TO-G-clamp-Bn. Normalized fluorescence intensities in the FID assays are shown. The data are mean (n=2). Conditions: 0.1 μM RNA, 0.1 μM TO-G-clamp-Bn or TO-PRO-1, 5 μM the aminoglycosides; λ(Ex): 485 nm, λ(Em): 535 nm; buffer: Binding buffer (pH 7.0) including 1.5% DMSO, Incubation Time : 30 min.

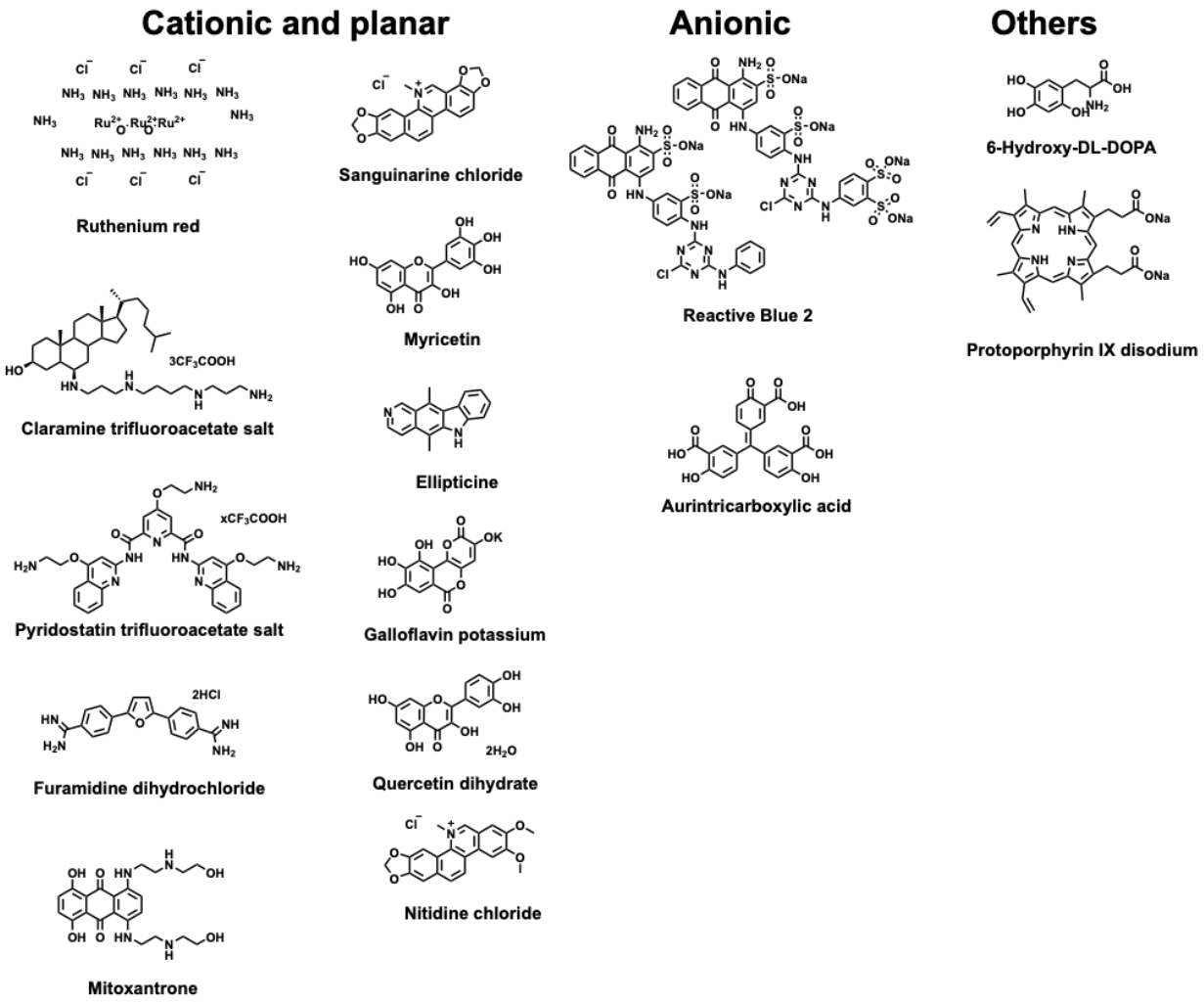


Figure S14. Chemical structures of the common hit compounds found by TO-G-clamp-Bn and TO-PRO-1 in FID assays. The hit compounds can be mainly classified into three, cationic and anionic compounds and intercalators.

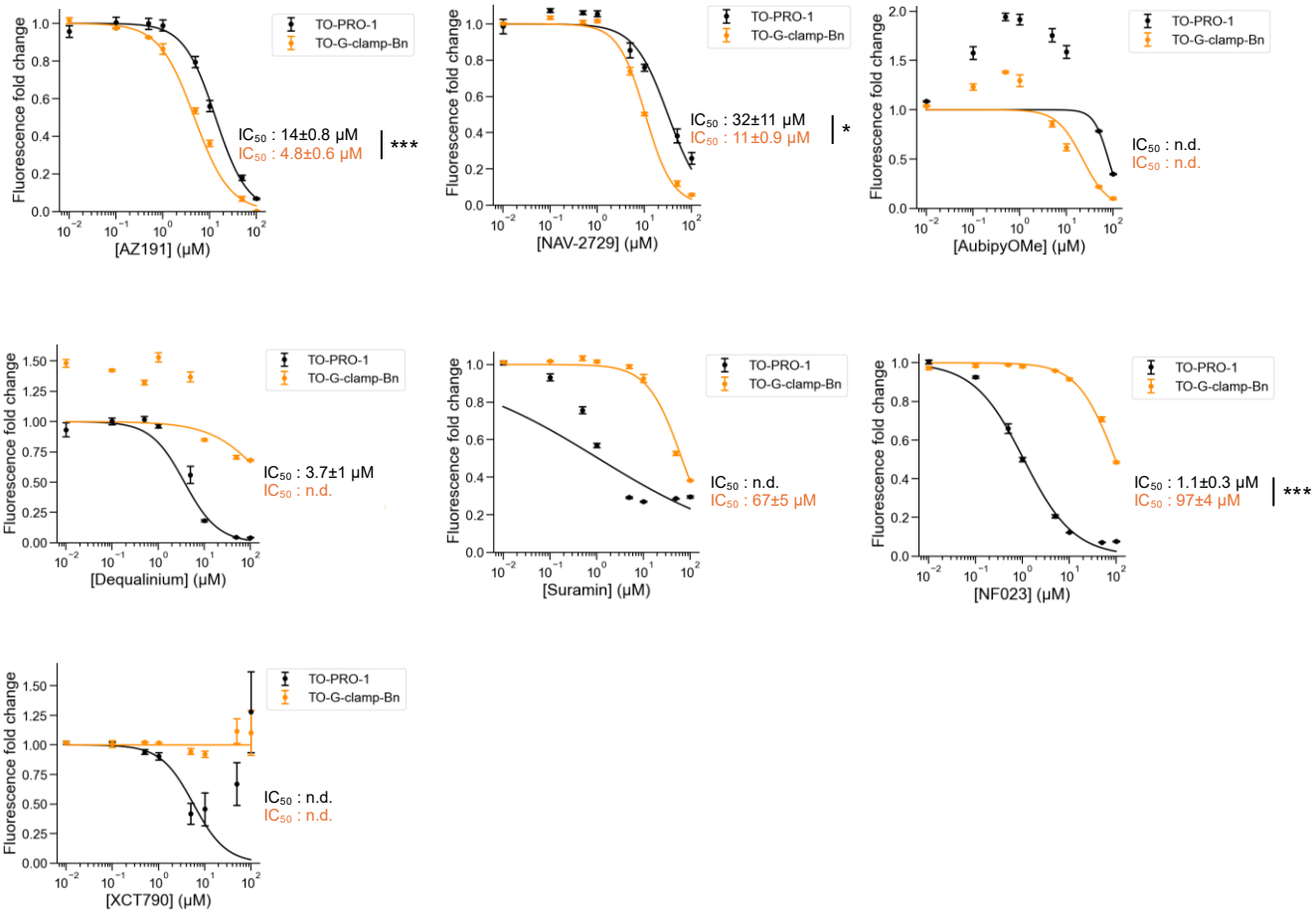


Figure S15. Dose-response curves for the unique hit compounds. N=3 for all concentrations. The data are mean ± SD. The *p* values were calculated by two-tailed Student's T test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Conditions: 0.1 μM pre-mir-221, 0.1 μM TO-G-clamp-Bn or TO-PRO-1, 0.01-100 μM each of the unique compound; λ(Ex): 485 nm, λ(Em): 535 nm; buffer: Binding buffer (pH 7.0) including 1.5% DMSO, Incubation Time : 30 min. n.d. represents not determined.

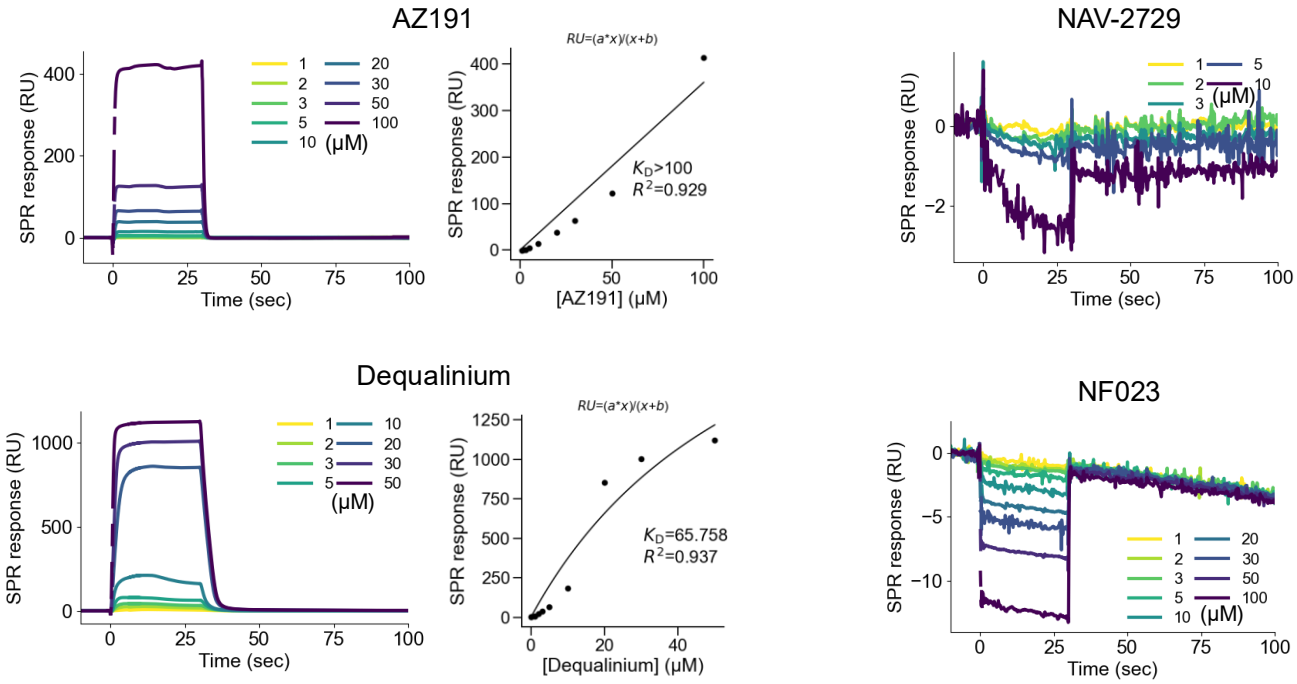


Figure S16. Hit validation by SPR. Representative sensorgrams of hit compounds that showed dose-responses. Hit compounds were added to 0 to 10, 50, or 100 μM depending on their solubility. The data were corrected twice as technical replicates and showed similar results.

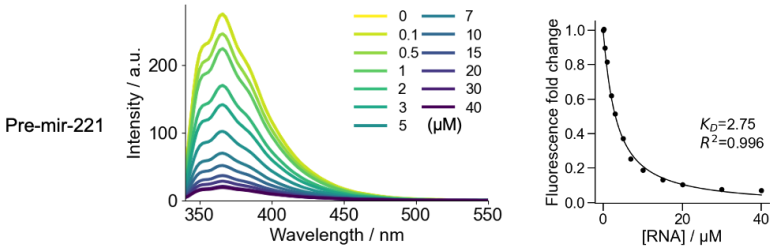


Figure S17. Fluorescence titrations to determine the apparent dissociation constants (K_{Dapp}) of Dequalinium. Fluorescence titration spectra were measured using Dequalinium (1 μM) upon addition of RNA (0-40 μM) in phosphate buffer (pH 7.0) including 1% DMSO. Conditions: λex: 320 nm, λem: 366 nm. The representative data and K_{Dapp} values are shown.

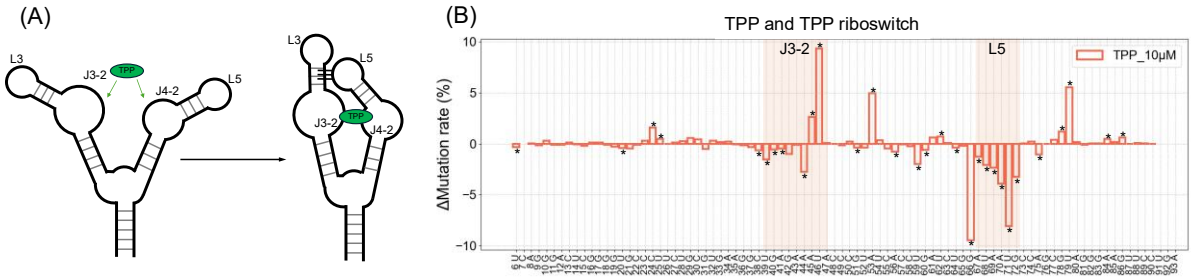


Figure S18. Validation of SHAPE-MaP using the positive control, TPP and TPP riboswitch. (A) Schematic view of the conformational change of TPP riboswitch upon TPP binding. (B) The mutational profiles of TPP riboswitch with 10 μM of TPP. Both of J3-2 and L5 regions in orange were reported that their mutation rates were significantly changed upon TPP binding previously. [11] Two-Poisson counts tests and Benjamini-Hochberg method were conducted to the sample and the DMSO control, and the stars on the mutational profiles represent statistically significant mutational changes (false discovery rate < 5%).

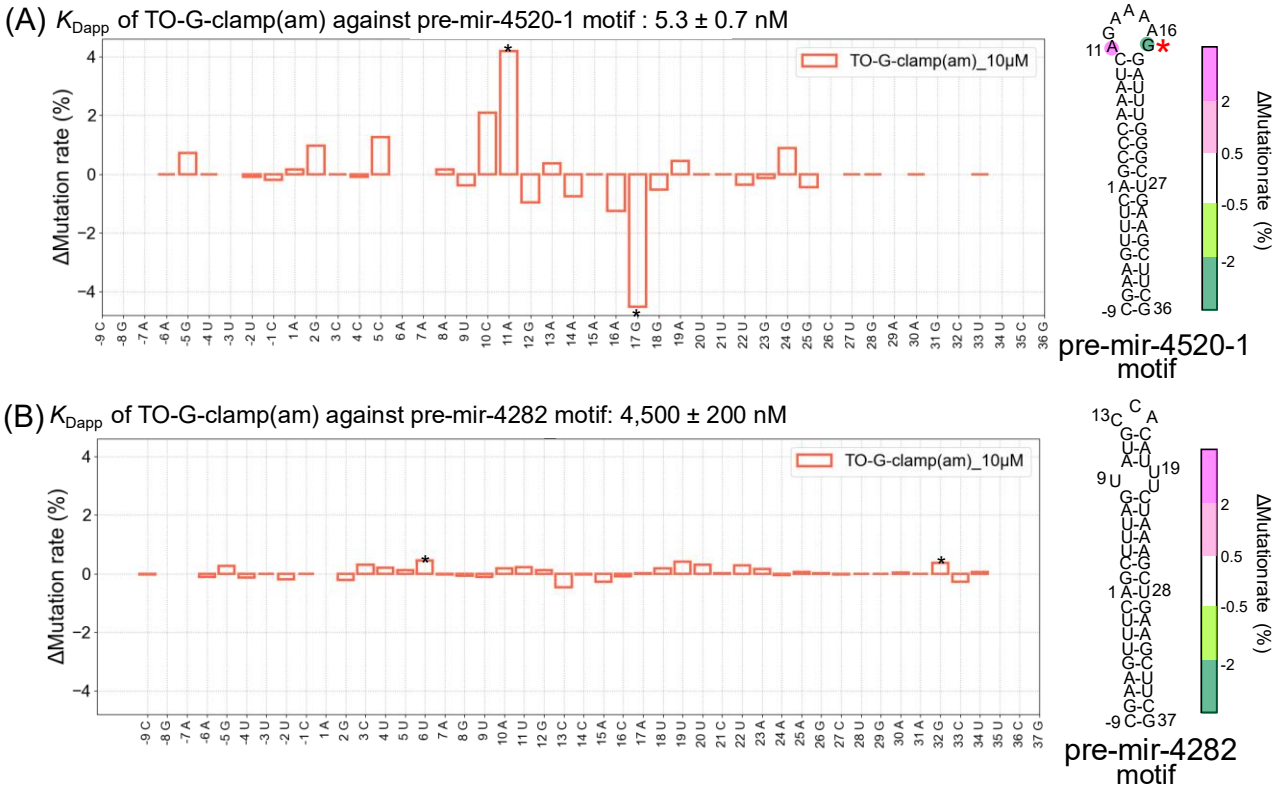


Figure S19. Binding site identification by SHAPE-MaP using TO-G-clamp(am). (A, B) The mutational profiles of (A) pre-mir-4520-1 and (B) pre-mir-4282 motif with TO-G-clamp(am). The delta mutation rates of each sample were mapped onto their corresponding secondary structure of the RNA motifs for SHAPE-MaP (right). Two-Poisson counts tests and Benjamini-Hochberg method were conducted to each of the TO-G-clamp(am)-RNA samples and the DMSO control, and the stars on the mutational profiles represent statistically significant mutational changes (false discovery rate < 5%). The statistically significant mutational changes were visualized in the secondary structures, predicted by RNAfold.

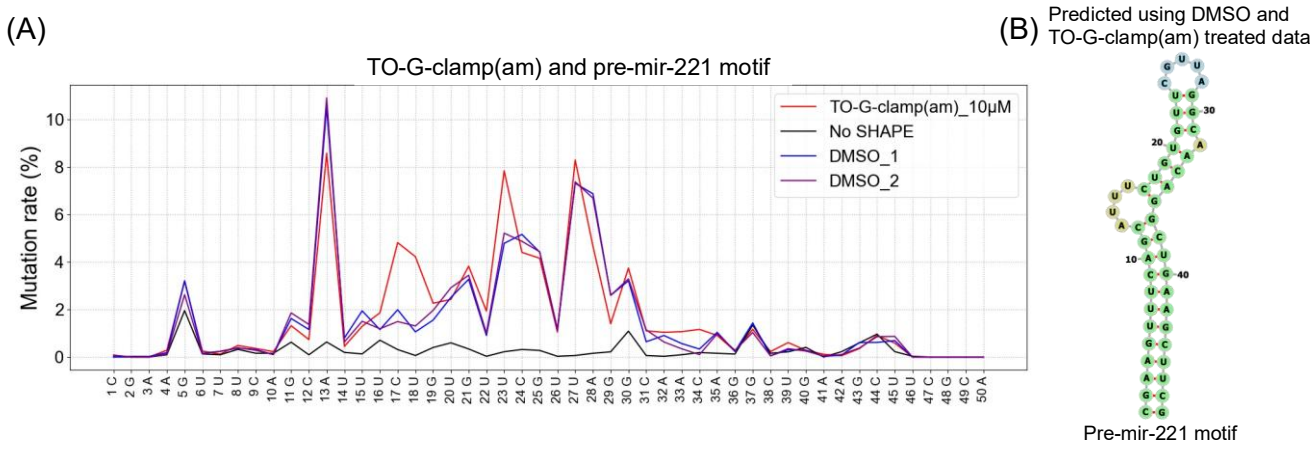
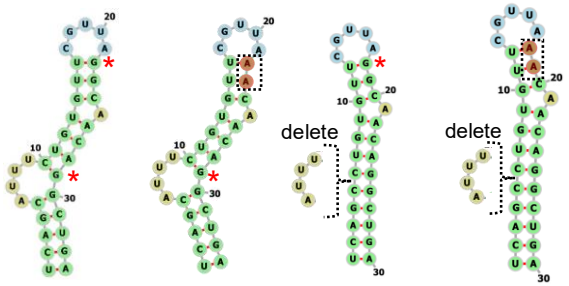


Figure S20. SHAPE-guided secondary structure prediction of RNA. (A) The mutational profile of 10 μM of TO-G-clamp(am) (red), DMSO controls (blue and purple), and no SHAPE reagent (black) samples. (B) The secondary structure of pre-mir-221 motif predicted by RNAfold using the SHAPE data (--T 37 --shapeMethod=D). Both of TO-G-clamp(am) treated and DMSO treated sample provided the same secondary structure. The mean (n=2) was used for the DMSO sample in the secondary structure prediction.

(A)



	Pre-mir-221 motif	G2223A	w/o AUUU	G2223A-w/o AUUU
(B) K_{Dapp} (μM)				
TO-G-clamp	0.0093 ± 0.001	< 0.005	0.022 ± 0.003	0.12 ± 0.02
G-clamp-N_3	2.7 ± 0.2	2.6 ± 0.5	8.9 ± 0.4	17 ± 3

Figure S21. Mutation experiments with TO-G-clamp(am) and G-clamp-N_3. (A) Secondary structure of pre-mir-221 and its mutant motifs, predicted by RNAfold (-T 37). Nucleotides in dark red in dotted rectangles are mutated. Red stars indicate putative binding sites of G-clamp moiety on pre-mir-221 motif. (B) K_{Dapp} values of TO-G-clamp(am) and G-clamp-N_3 in mutation experiments with pre-mir-221 analogs. The data are mean ± standard error from three independent experiments.

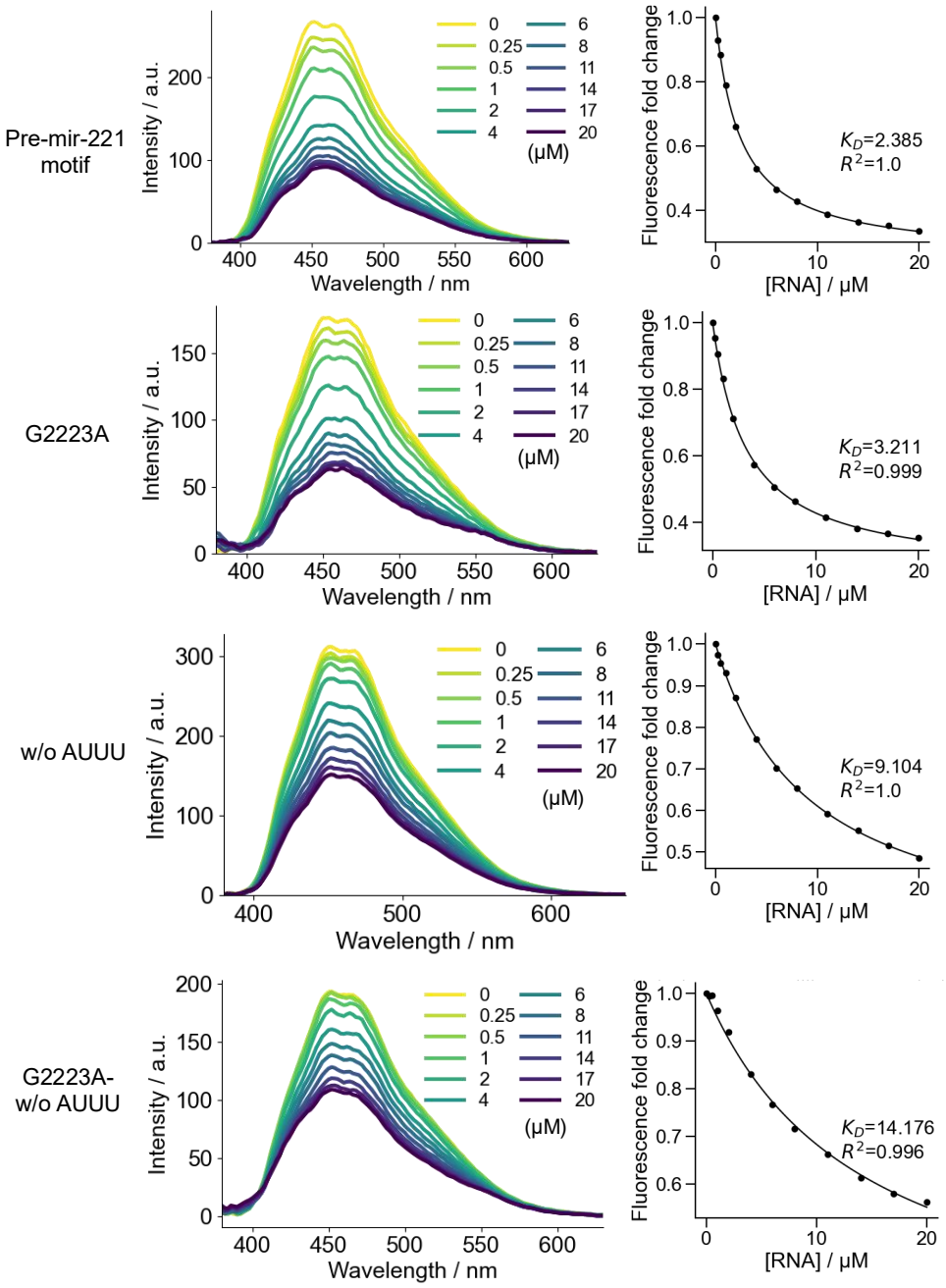
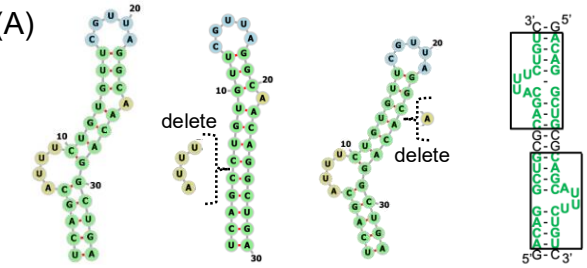


Figure S22. Fluorescence titrations to determine the apparent dissociation constants (K_{Dapp}) of G-clamp-N_3 against pre-mir-221 mutant motifs. Fluorescence titration spectra were measured using G-clamp-N_3 (0.1 μM) upon addition of RNA (0-20 μM) in phosphate buffer (pH 7.0) including 5% DMSO. Conditions: λex: 360 nm, λem: 452 nm. The representative data and K_{Dapp} values are shown.

(A)



Pre-mir-221 motif | w/o AUUU | w/o A | w/o hairpin loop

(B)

K_{Dapp} (μM)	Pre-mir-221 motif	w/o AUUU	w/o A	w/o hairpin loop[1)]
TO-PRO-1	0.44 ± 0.09[2)]	0.30 ± 0.02	0.62 ± 0.05	1.1 ± 0.1
AZ191	34 ± 1	74 ± 8	66 ± 4	105 ± 8

1) Calculated by 2:1 binding model. 2) Previously reported in *Commun Chem.*, **2024**, *7*, 98.

Figure S23. Mutation experiments with TO-PRO-1 and AZ191. (A) Secondary structure of pre-mir-221 and its mutant motifs, predicted by RNAcofold for w/o hairpin loop and RNAfold for the others (-T 37). The nucleotides of w/o harpin loop in green and the black rectangles are extracted nucleotides from pre-mir-221 motif. (B) K_{Dapp} values of TO-PRO-1 and AZ191 in mutation experiments with pre-mir-221 analogs. The data are mean ± standard error from three independent experiments.

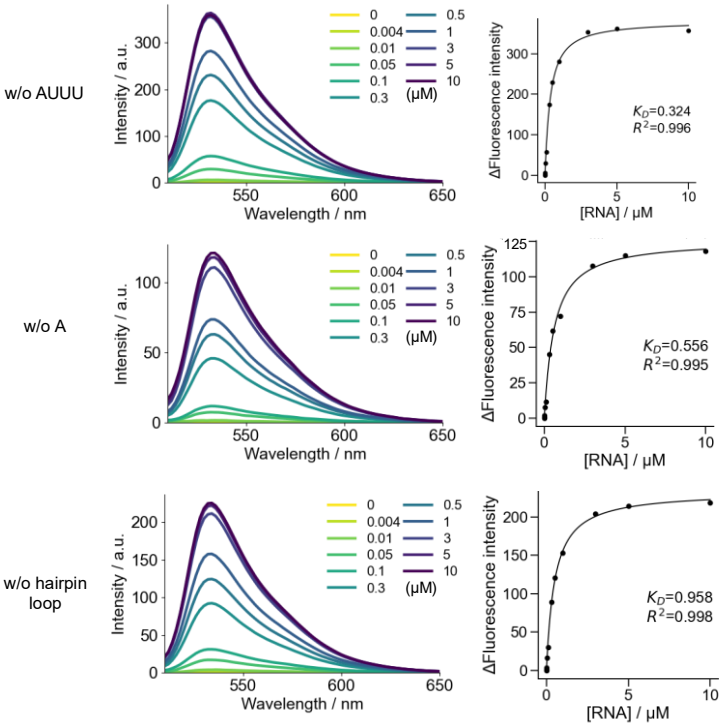


Figure S24. Fluorescence titrations to determine the apparent dissociation constants (K_{Dapp}) of TO-PRO-1 against pre-mir-221 mutant motifs. Fluorescence titration spectra were measured using TO-PRO-1 (0.1 μM) upon addition of RNA (0-10 μM) in phosphate buffer (pH 7.0) including 1% DMSO. Conditions: λex: 501 nm, λem: 530 nm. The representative data and K_{Dapp} values are shown.

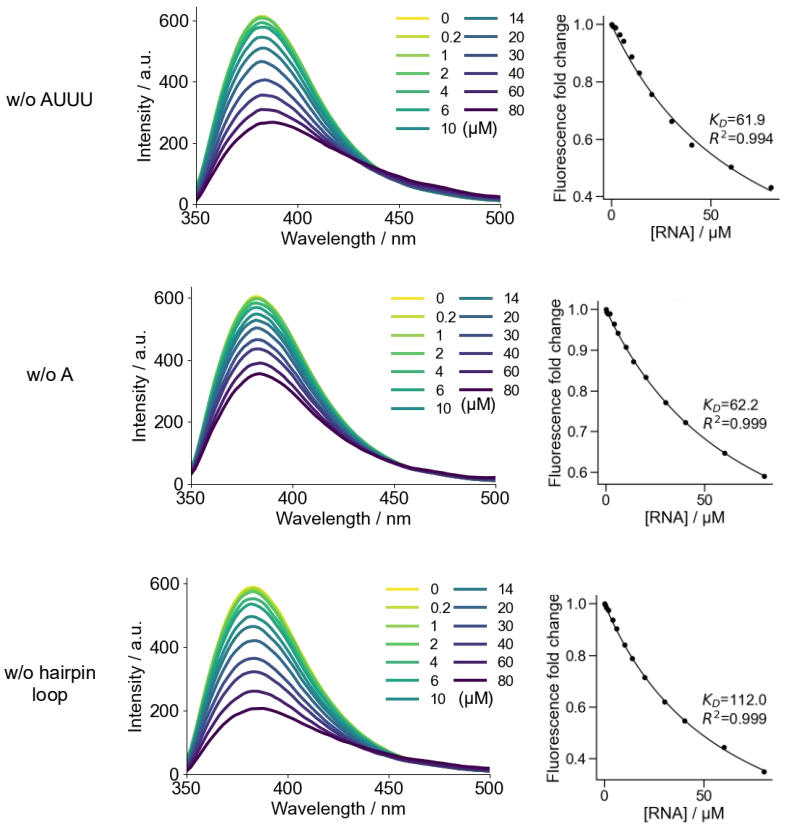
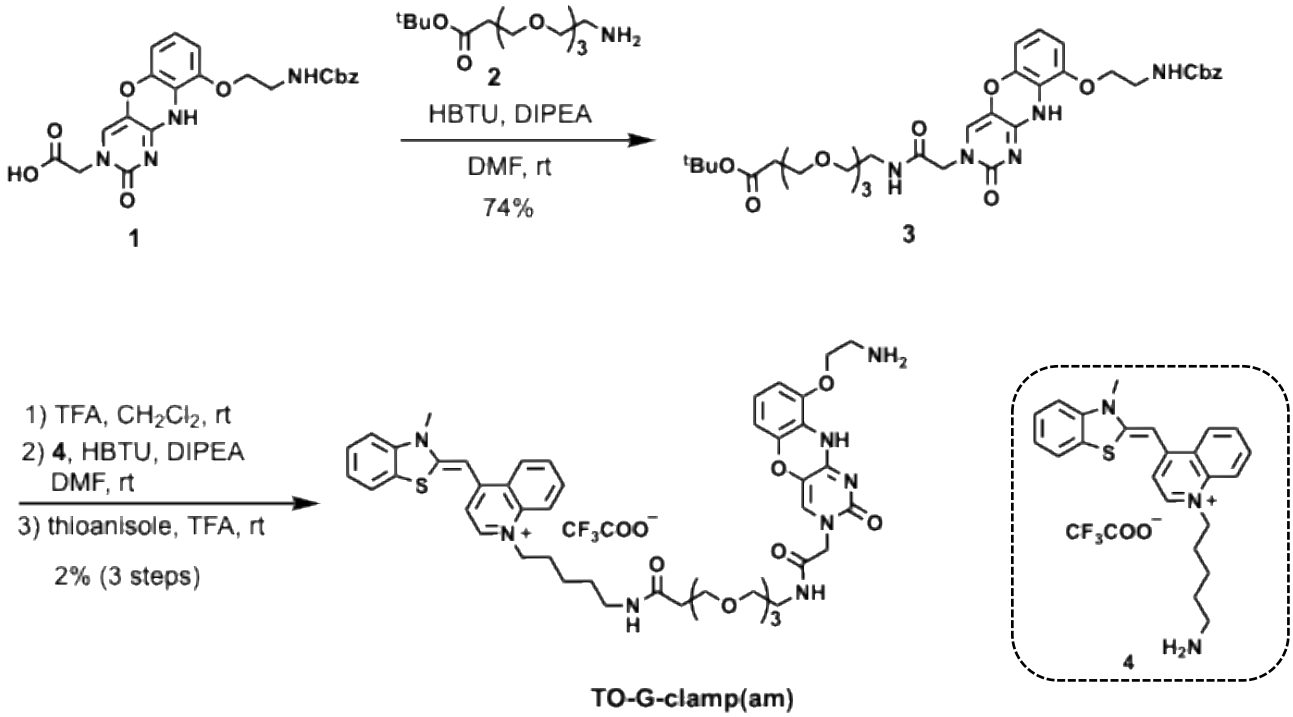


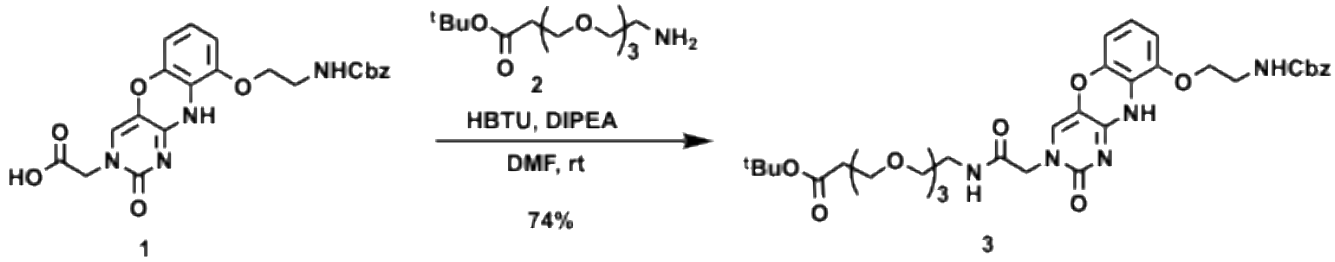
Figure S25. Fluorescence titrations to determine the apparent dissociation constants (K_{Dapp}) of AZ191 against pre-mir-221 mutant motifs. Fluorescence titration spectra were measured using AZ191 (10 μM) upon addition of RNA (0-80 μM) in phosphate buffer (pH 7.0) including 1% DMSO. Conditions: λex: 334 nm, λem: 384 nm. The representative data and K_{Dapp} values are shown.

Synthesis of TO-G-clamp(am)



Scheme S1 Synthesis of TO-G-clamp(am) via amide coupling

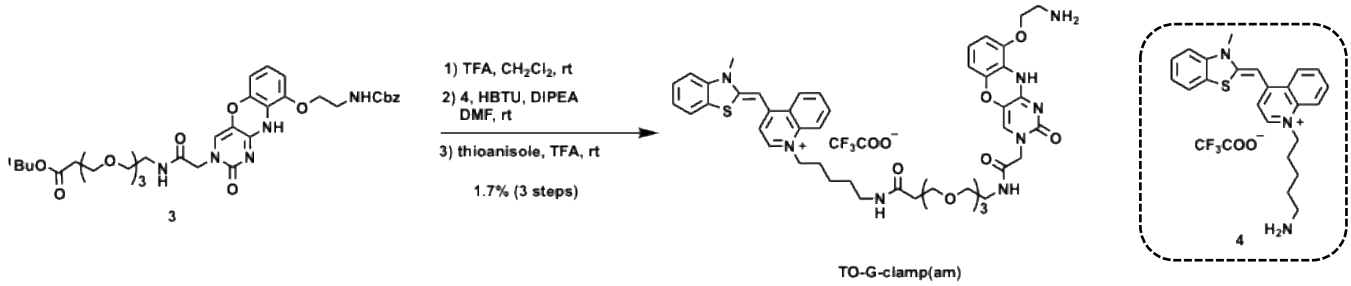
Synthesis of compound 3



To a solution of compound **1** (51.8 mg, 0.114mmol), prepared following the procedure described in the previous report,[12] in DMF (1.0 mL), DIPEA (22.3 mg, 0.173 mmol) and HBTU (62.8 mg, 0.166 mmol) were added at 0°C and stirred. The mixture was added to a solution of Amino-PEG3-acid *tert*-Butyl Ester **2** (48.3 mg, 0.174 mmol) in DMF (1.7 mL), and the solution was warmed to room temperature and stirred for 16 h. After the reaction, the mixture was diluted with AcOEt (40 mL), washed with sat. NH_4Cl aq. (5 mL×2), sat. $NaHCO_3$ aq. (5 mL×2), and brine (5 mL), and dried over Na_2SO_4. The solution was filtered and evaporated under reduced pressure. Crude (0.116 g) was purified by column chromatography ($CHCl_3$/MeOH = 40/1 to 30/1 to 20/1) to afford compound **3** as a yellow solid (60.4 mg, 74% yield). ^{1}H NMR (600 MHz, MeOD) δ1.43 (9H, s), 2.46 (2H, t, *J* = 6.3 Hz), 3.40 (2H, t, *J* = 5.4 Hz), 3.54 (4H, m), 3.60 (8H, m), 3.68 (2H, t, *J* = 6.3 Hz), 4.03 (2H, t, *J* = 5.1 Hz), 4.38 (2H, s), 5.10 (2H, s), 6.38 (1H, d, *J* = 8.4 Hz), 6.56 (2H, d, *J* = 8.4), 6.78 (1H, t, *J* = 8.4 Hz), 7.21 (1H, s), 7.29 (5H, m). ^{13}C NMR (600 MHz, MeOD) δ27.0, 35.8, 39.2, 39.8, 51.3, 66.2, 66.5, 68.1, 69.1, 69.9, 70.0, 70.1, 70.2, 80.4, 106.9, 107.9, 115.5, 123.5, 127.4, 127.6, 128.0, 128.1, 137.0, 142.8, 146.5, 154.8, 156.2, 157.7, 168.3, 171.4. ESI-HRMS (*m/z*): $[M+H]^+$ calcd

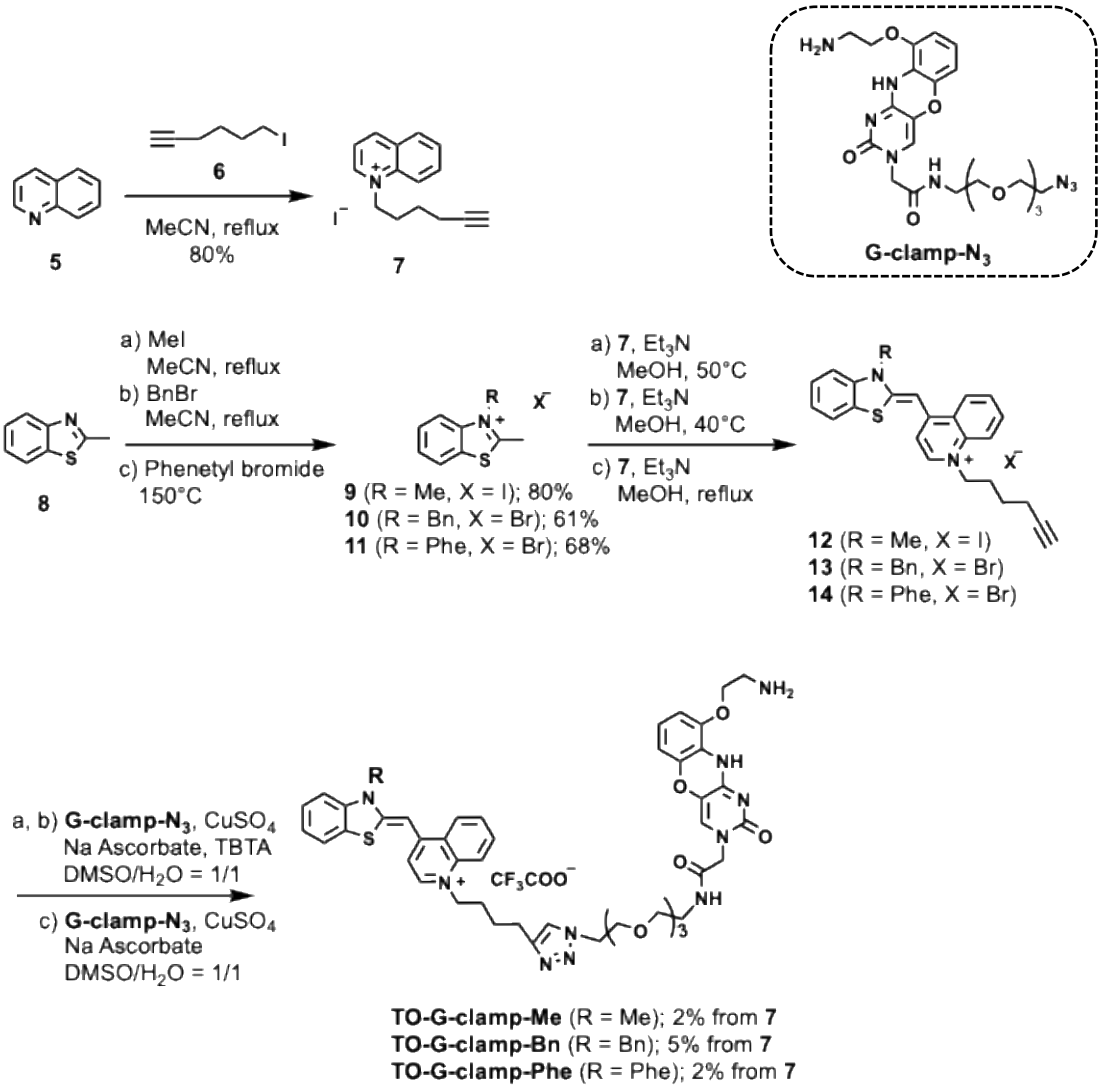
for $C_{35}H_{46}N_5O_{11}^+$, 712.3188, found 712.3187.

Synthesis of TO-G-clamp(am)



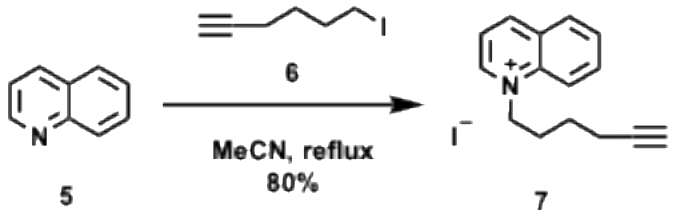
To a solution of compound **3** (18.9 mg, 26.6 μmol) in CH_2Cl_2 (50 μL), TFA (0.30 mL) was added and stirred at room temperature for 7 h. The mixture was evaporated under reduced pressure to obtain the residue. The residue was dissolved in DMF (0.20 mL), and DIPEA (33.4 mg, 0.258 mmol) and HBTU (15.2 mg, 40.1 μmol) were added to the mixture at 0°C, followed by stirring. To the solution of the mixture, a solution of compound **4** (37.9 μmol), prepared following the procedure described in the previous report,[8] in DMF (0.40 mL) was added and stirred at room temperature for 20 h. The mixture was evaporated under reduced pressure to obtain a red oil residue. TFA (0.35 mL) and thioanisole (82.7 mg, 0.666 mmol) were added to the residue and stirred at room temperature for 24 h. Then, the mixture was evaporated under reduced pressure to obtain a residue. The residue was filtrated with DMSO (1.0 mL) and purified by reverse phased HPLC to afford **TO-G-clamp(am)** as a red solid (1.7% yield in 3 steps). The concentration of **TO-G-clamp(am)** was determined by quantitative ^{1}H-NMR using maleic acid as an internal standard. HPLC conditions; A: 0.1% TFA in distilled water, B: 0.1% TFA in MeCN; B: 0%→60% (～20 min)→100% (～25 min)→100%(～30 min). Flow rate = 4 mL/min; Temp. = 35°C; UV = 254 nm, C-18 column (Nacalai Tesque: COSMOSIL 5C_{18}-AR-II, 10×250 mm). ^{1}H NMR (600 MHz, MeOD) δ 1.48 (2H, quintet, *J* = 7.6 Hz), 1.59 (2H, quintet, *J* = 7.0 Hz), 2.00 (2H, quintet, *J* = 7.4 Hz), 2.42 (2H, t, *J* = 6.0 Hz), 3.22, (2H, t, *J* = 7.2 Hz), 3.39 (2H, t, *J* = 5.4 Hz), 3.54 (2H, t, *J* = 5.7 Hz), 3.60 (10H, m), 3.71 (2H, t, *J* = 6.0 Hz), 3.98 (3H, s), 4.21 (2H, t, *J* = 4.8 Hz), 4.34 (2H, s), 4.59 (2H, t, *J* = 7.5 Hz), 6.41 (1H, d, *J* = 8.4 Hz), 6.60 (1H, d, *J* = 8.4 Hz), 6.82 (1H, t, *J* = 8.4 Hz), 7.16 (1H, m), 7.19 (1H, s), 7.42 (1H, t, *J* = 7.5 Hz), 7.47 (1H, d, *J* = 7.2 Hz), 7.61 (1H, t, *J* = 7.8 Hz), 7.66 (1H, d, *J* = 7.8 Hz), 7.75 (1H, t, *J* = 7.8 Hz), 7.90 (1H, d, *J* = 7.8 Hz), 7.99 (1H, t, *J* = 7.8 Hz), 8.08 (1H, d, *J* = 8.4 Hz), 8.43 (1H, d, *J* = 7.2 Hz), 8.65 (1H, d, *J* = 8.4 Hz). ^{13}C NMR (150 MHz, MeOD) δ 15.8, 15.9, 16.0, 23.3, 28.4, 28.5, 32.7, 36.3, 38.4, 38.8, 39.2, 51.1, 54.6, 55.9, 56.1, 56.2, 56.4, 66.9, 69.0, 69.9, 69.9, 70.0, 70.2, 108.4, 108.7, 112.3, 115.9, 117.6, 117.9, 122.3, 123.7, 124.4, 124.5, 124.8, 125.2, 126.8, 127.7, 128.1, 133.2, 137.5, 140.6, 142.9, 143.6, 149.5, 160.7, 168.1, 172.6. ESI-HRMS (*m/z*): $[M\text{-}CF_3COO]^+$ calcd for $C_{46}H_{55}N_8O_8^+$, 879.385, found 879.3835.

Synthesis of TO-G-clamp-R derivatives (R = Me, Bn, and Phe)



Scheme S2 Synthesis of TO-G-clamp-R derivatives via copper-catalyzed click reactions. Reaction conditions a, b, and c are for synthesis of TO-G-clamp-Me, -Bn, and -Phe, respectively.

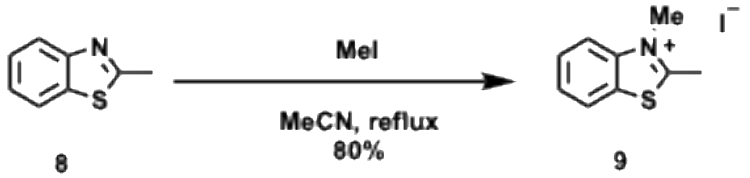
Synthesis of compound 7 for TO-G-clamp-R derivatives



To a solution of quinoline **5** (0.28 mL, 2.36 mmol) in MeCN (7.2 mL), 6-iodo-1-hexyne **6** (1.0 g, 4.81 mmol) was added and stirred at reflux for 23 h. Then, the mixture was cooled to room temperature, and the solvent was removed by vacuo. To the crude mixture, EtOAc (10 mL) was added, and the solid was corrected by filtration. The solid was washed with EtOAc, dried in vacuo to afford compound **7** as an ocher powder (637 mg, 80% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ9.56 (d, J = 8.4 Hz, 1H), 9.30 (d, J = 8.4 Hz, 1H), 8.65 (d, J = 9.2 Hz, 1H), 8.51 (d, J = 8.0 Hz, 1H), 8.30 (t, J = 7.2 Hz, 1H), 8.21 (t, J = 7.2 Hz, 1H), 8.05 (t, J = 7.2 Hz, 1H), 5.09 (t, J = 7.6 Hz, 2H),

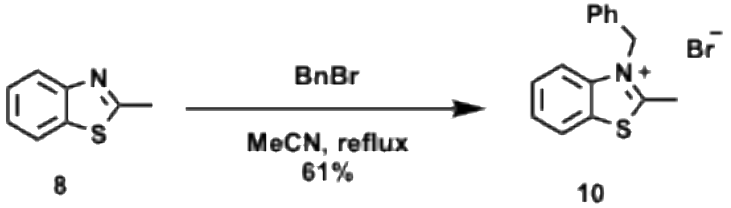
2.83 (t, *J* = 2.8 Hz, 1H), 2.25 (dt, *J* = 2.8, 7.2 Hz, 2H), 2.10-2.02 (m, 2H), 1.62-1.55 (m, 2H). ESI-HRMS(*m/z*): $[M-I]^+$ calcd for $C_{15}H_{16}N^+$, 210.1277, found 210.1287.

Synthesis of compound 9 for TO-G-clamp-Me



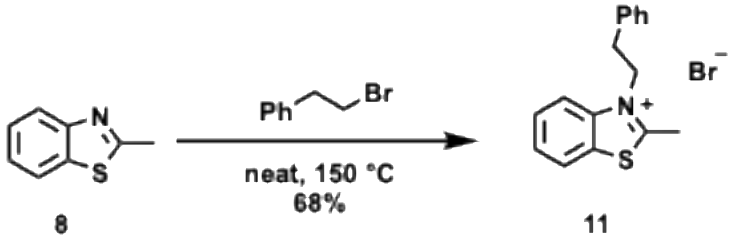
To a solution of compound **8** (499 mg, 3.34 mmol) in MeCN (12 mL), iodomethane (0.42 mL, 6.75 mmol) was added and stirred at reflux. After 19 h, additional iodomethane (0.42 mL, 6.75 mmol) was added to the mixture and stirred at reflux for further 3 h. The reaction mixture was cooled to room temperature, and the solid was filtered, washed with EtOAc (30 mL), and dried in vacuo to afford compound **9** as a green-white powder (774 mg, 80% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.44 (d, *J* = 8.0 Hz, 1H), 8.30 (d, *J* = 8.4 Hz, 1H), 7.90 (t, *J* = 8.0 Hz, 1H), 7.81 (t, *J* = 7.6 Hz, 1H), 4.20 (s, 3H), 3.17 (s, 3H). ESI-HRMS(*m/z*): $[M-I]^+$ calcd for $C_9H_{10}NS^+$, 164.0528, found 164.0538.

Synthesis of compound 10 for TO-G-clamp-Bn



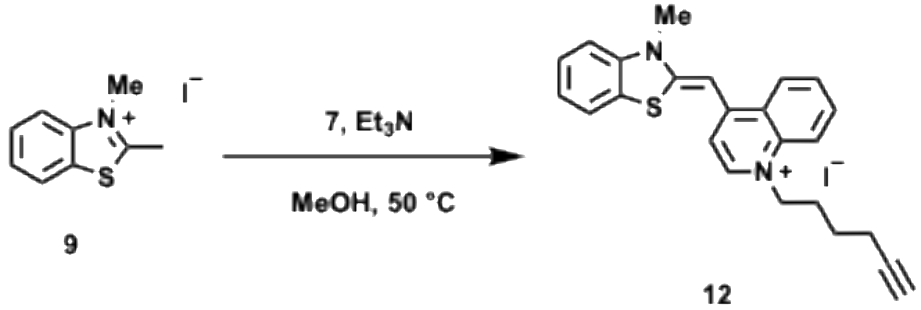
To a solution of compound **8** (509 mg, 3.34 mmol) in MeCN (12 mL), benzyl bromide (0.60 mL, 5.05 mmol) was added and stirred at reflux for 47 h. The reaction mixture was cooled to room temperature, and the solid was filtered, washed with EtOAc (30 mL), and dried in vacuo to afford compound **10** as a green-white powder (665 mg, 61% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.50 (d, *J* = 7.6 Hz, 1H), 8.20 (d, *J* = 8.0 Hz, 1H), 7.87-7.78 (m, 2H), 7.43-7.32 (m, 5H), 6.09 (s, 2H), 3.27 (s, 3H). ESI-HRMS(*m/z*): $[M-Br]^+$ calcd for $C_{15}H_{14}NS^+$, 240.0841, found 240.0852.

Synthesis of compound 11 for TO-G-clamp-Phe



To compound **8** (500 mg, 3.35 mmol), 2-bromoethylbenzene (0.68 mL, 5.03 mmol) was added and stirred at 150°C for 19 h. The reaction mixture was cooled to room temperature and triturated with EtOAc (2 mL). The solid was filtered, washed with EtOAc (30 mL), and dried in vacuo to afford compound **11** as a brown-white powder (763 mg, 68% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.50 (d, *J* = 8.4 Hz, 1H), 8.33 (d, *J* = 8.4 Hz, 1H), 7.87 (t, *J* = 7.2 Hz, 1H), 7.80 (t, *J* = 7.2 Hz, 1H), 7.29-7.18 (m, 5H), 4.99 (t, *J* = 7.2 Hz, 2H), 3.22 (t, *J* = 7.2 Hz, 2H), 2.92 (s, 3H). ESI-HRMS(*m/z*): $[M-Br]^+$ calcd for $C_{16}H_{16}NS^+$, 254.0998, found 254.1009.

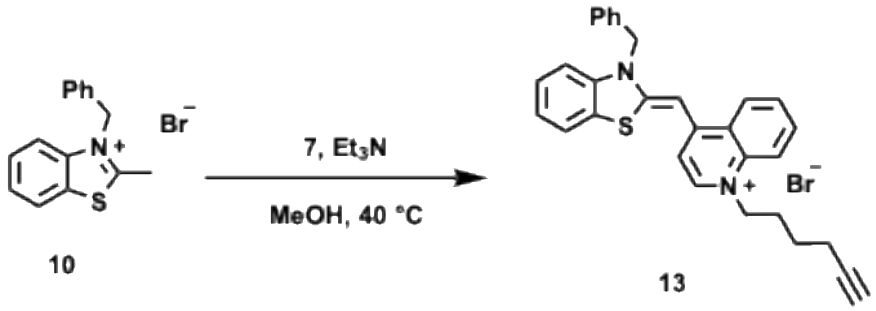
Synthesis of compound 12 for TO-G-clamp-Me



9 12

To a solution of compound **9** (104 mg, 0.356 mmol) and compound **7** (100 mg, 0.297 mmol) in MeOH (10.8 mL), Et_3N (0.41 mL, 2.97 mmol) was added and stirred at 50°C. The solution turned to be red. After 23 h, the solvent was removed by vacuo. The crude was roughly purified by column chromatography (amino silica gel, $CHCl_3$/MeOH) to afford a dark-red solid containing compound **12** (15.3 mg). The crude was implemented to the next reaction without further purification. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.97 (d, *J* = 7.2 Hz, 1H), 8.66 (d, *J* = 8.0 Hz, 1H), 7.74-7.62 (m, 4H), 7.46 (t, *J* = 8.0 Hz, 1H), 7.32 (t, *J* = 8.0 Hz, 3H), 6.71 (s, 1H), 4.61 (t, *J* = 7.6 Hz, 2H), 3.99 (s, 3H), 3.11 (s, 1H), 2.32-2.29 (m, 2H), 2.08-2.06 (m, 2H), 2.01-1.97 (m, 2H). ESI-HRMS(*m/z*): $[M-I]^+$ calcd for $C_{24}H_{23}N_2S^+$, 371.1576, found : 371.1539.

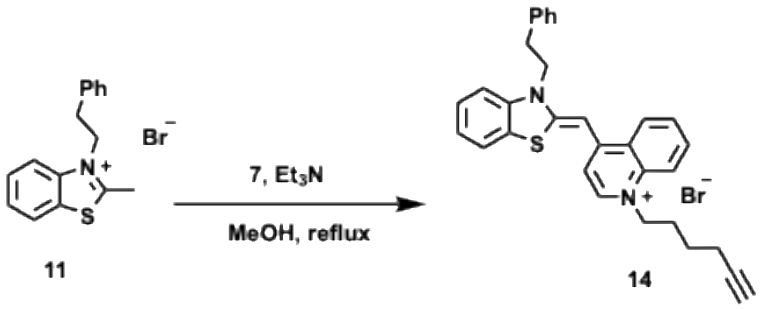
Synthesis of compound 13 for TO-G-clamp-Bn



10 13

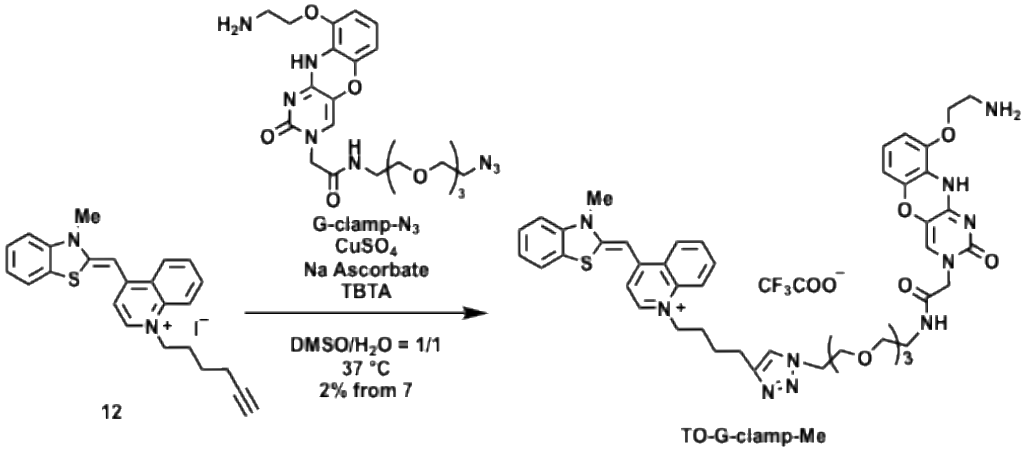
To a solution of compound **10** (115 mg, 0.358 mmol) and compound **7** (101 mg, 0.298 mmol) in MeOH (19 mL), Et_3N (0.42 mL, 3.01 mmol) was added and stirred at 40°C. The solution turned to be red. After 22 h, to the reaction mixture, additional compound **10** (76.1 mg, 0.238 mmol) was added and stirred at 40°C for further 24 h. The solvent was removed by vacuo. The residue was washed and filtered with water (20 mL) and Et_2O (20 mL). To the resulting solid, DCM was added and filtered to remove insoluble materials. The filtrate was concentrated by vacuo and then roughly purified by column chromatography (amino silica gel, $CHCl_3$/MeOH) to afford a dark-red solid containing compound **13** (13.8 mg). The crude was implemented to the next reaction without further purification. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.21 (d, *J* = 7.2 Hz, 1H), 8.13 (d, *J* = 8.4 Hz, 1H), 7.77 (d, *J* = 8.0 Hz, 3H), 7.57 (t, *J* = 14.8 Hz, 1H), 7.51 (d, *J* = 7.2 Hz, 1H), 7.44 (t, *J* = 6.0 Hz, 1H), 7.40-7.37 (m, 2H), 7.34-7.31 (m, 5H), 6.64 (s, 1H), 4.71 (t, *J* = 7.6 Hz, 2H), 2.29 (dt, *J* = 2.4, 6.8 Hz, 2H), 2.06 (t, *J* = 7.6 Hz, 2H), 1.94 (t, *J* = 2.8 Hz, 1H), 1.75-1.68 (m, 4H). ESI-HRMS(*m/z*): $[M-Br]^+$ calcd for $C_{30}H_{27}N_2S^+$, 447.1889, found 447.1885.

Synthesis of compound 14 for TO-G-clamp-Phe



To a solution of compound **11** (120 mg, 0.358 mmol) and compound **7** (100 mg, 0.297 mmol) in MeOH (19 mL), Et_3N (0.42 mL, 3.01 mmol) was added and stirred at room temperature for 26 h, but the reaction did not proceed well. Then the reaction mixture was stirred at reflux for 17 h. The solution turned to be red. The solvent was removed by vacuo. The residue was washed and filtered with water (20 mL) and Et_2O (20 mL). To the resulting solid, DCM was added and filtered to remove insoluble materials. The filtrate was concentrated by vacuo to afford a dark-red solid (132 mg). Then 40.2 mg of this crude was roughly purified by column chromatography (amino silica gel, $CHCl_3$/MeOH) to afford a dark-red solid containing compound **14** (3.2 mg). The crude was implemented to the next reaction without further purification. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.04 (d, *J* = 7.2 Hz, 1H), 8.16 (d, *J* = 8.4 Hz, 1H), 7.80 (t, *J* = 6.8 Hz, 1H), 7.72 (t, *J* = 7.2 Hz, 2H), 7.65 (t, *J* = 7.2 Hz, 1H), 7.44 (d, *J* = 7.2 Hz, 1H), 7.38 (d, *J* = 7.2 Hz, 1H), 7.30-7.12 (m, 7H), 6.44 (s, 1H), 4.68 (m, 4H), 3.25 (t, *J* = 6.8 Hz, 4H), 2.31 (m, 2H), 2.08 (m, 2H), 1.97 (t, *J* = 2.8 Hz, 1H), 1.73 (m, 2H). ESI-HRMS(*m/z*): $[M-Br]^+$ calcd for $C_{31}H_{29}N_2S^+$, 461.2046, found 461.2039.

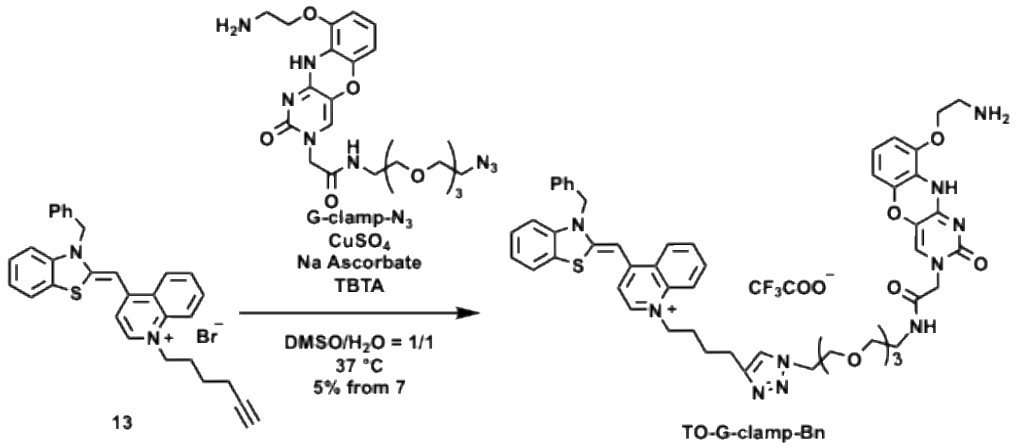
Synthesis of TO-G-clamp-Me



To a solution of compound **G-clamp-N_3** (3.11 mg, 6.0 μmol), prepared following the procedure described in the previous report,[8] in DMSO (100 μL), compound **12** (2.99 mg, 6.0 μmol), TBTA (1.3 mg, 2.4 μmol), a solution of sodium ascorbate (3.0 mg, 15 μmol) in water (50 μL), and a solution of copper(II) sulfate pentahydrate (0.6 mg, 2.4 μmol) in water (50 μL) were added. The mixture was stirred at 37°C for 22 h, then diluted with DMSO (300 μL), and filtered to remove insoluble materials. Purification by reverse phased HPLC afforded **TO-G-clamp-Me** as a dark-red solid (0.869 μmol, 2% yield in 2 steps from compound **7**). The concentration of **TO-G-clamp-Me** was determined by quantitative ^{1}H-NMR using maleic acid as an internal standard. HPLC conditions; A: 0.1% TFA in distilled water, B: 0.1% TFA in MeCN; B: 0%→60% (～20 min)→100% (～25 min)→100%(～30 min). Flow rate = 4 mL/min; Temp. = 35°C; UV = 254 nm, C-18 column (Nacalai Tesque: COSMOSIL 5C_{18}-AR-II, 10×250 mm). ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.78 (d, *J* = 9.0 Hz, 1H), 8.62 (d, *J* = 7.2 Hz, 1H), 8.19 (t, *J* = 5.4 Hz, 1H), 8.14 (d, *J* = 9.0 Hz, 1H),

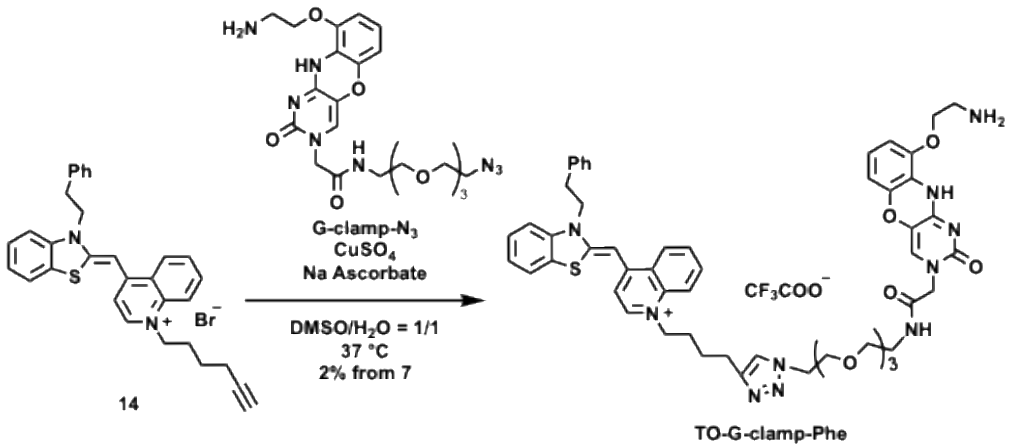
8.04 (d, *J* = 7.2 Hz, 1H), 7.98 (t, *J* = 7.8 Hz, 3H), 7.79 (t, *J* = 9.0 Hz, 1H), 7.75 (t, *J* =7.2 Hz, 1H), 7.61 (t, *J* = 7.8 Hz, 1H), 7.42 (t, *J* = 7.2 Hz, 2H), 7.37 (d, *J* = 7.2 Hz, 1H), 6.91 (s, 1H), 6.80 (t, *J* =7.8 Hz, 1H), 6.64 (d, *J*= 7.2 Hz, 1H), 6.45 (d, *J* =7.8 Hz, 1H), 4.63(t, *J* = 7.2 Hz, 2H), 4.45 (d, *J* = 5.4 Hz, 3H), 4.24 (s, 2H), 4.13 (t, *J* = 4.8 Hz, 2H), 4.01 (s, 3H), 3.77 (t, *J* = 5.4 Hz, 3H), 3.22 (t, *J* = 6.0 Hz, 2H), 2.68 (t, *J* = 7.8 Hz, 2H), 1.91 (m, 2H), 1.69 (t, *J* = 7.2 Hz, 2H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 167.2, 160.1, 148.6, 146.1, 144.4, 140.5, 137.0, 133.3, 128.2, 126.8, 125.8, 124.5, 124.3, 123.9, 122.9, 122.3, 122.3, 118.1, 113.0, 107.9, 88.1, 69.7, 69.6, 69.6, 69.0, 68.8, 53.9, 50.7, 49.2, 33.8, 28.4, 26.0, 24.5. ESI-HRMS(*m/z*): $[M+H-CF_3COO]^{2+}$ calcd for $C_{46}H_{54}N_{10}O_7S^{2+}$ 445.1944, found 445.1967.

Synthesis of TO-G-clamp-Bn



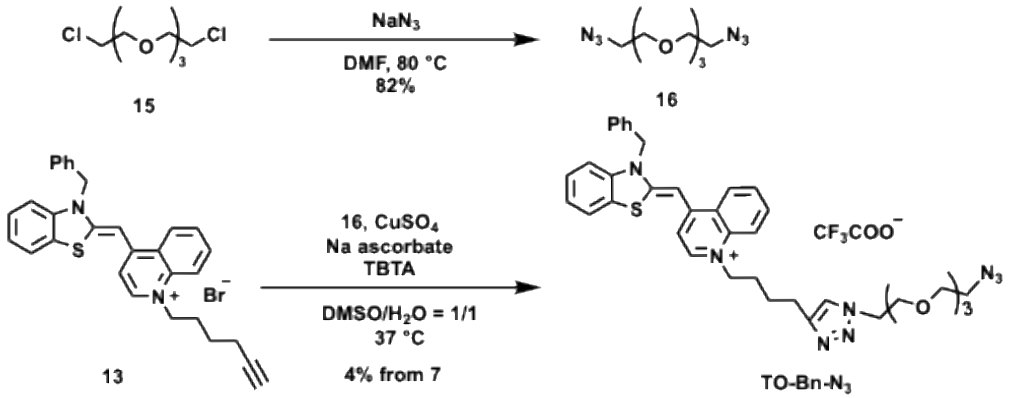
To a solution of compound **G-clamp-N$_3$** (4.7 mg, 9.0 μmol), prepared following the procedure described in the previous report,[8] in DMSO (150 μL), compound **13** (4.8 mg, 9.0 μmol), TBTA (1.9 mg, 3.6 μmol), a solution of sodium ascorbate (4.5 mg, 22.5 μmol) in water (75 μL), and a solution of copper(II) sulfate pentahydrate (0.9 mg, 3.6 μmol) in water (75 μL) were added. The mixture was stirred at 37°C for 24 h, then diluted with DMSO (300 μL), and filtered to remove insoluble materials. Purification by reverse phased HPLC afforded **TO-G-clamp-Bn** as a dark-red solid (1.78 μmol, 5% yield in 2 steps from compound **7**). The concentration of **TO-G-clamp-Bn** was determined by quantitative ^{1}H-NMR using maleic acid as an internal standard. HPLC conditions; A: 0.1% TFA in distilled water, B: 0.1% TFA in MeCN; B: 0%→60% (～20 min)→100% (～25 min)→100%(～30 min). Flow rate = 4 mL/min; Temp. = 35°C; UV = 254 nm, C-18 column (Nacalai Tesque: COSMOSIL 5C$_{18}$-AR-II, 10×250 mm). ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.68 (d, *J* = 7.2 Hz, 1H), 8.53 (d, *J* = 8.4 Hz, 1H), 8.16, (t, *J* = 9.0 Hz, 1H), 8.09 (d, *J* = 7.8 Hz, 1H), 8.08 (d, *J* = 8.4 Hz, 1H), 7.96 (t, *J* = 7.2 Hz, 3H), 7.79 (t, *J* = 8.4 Hz, 2H), 7.72 (t, *J* = 7.2 Hz, 1H), 7.59 (t, *J* = 7.8 Hz, 1H), 7.44 (t, *J* =6.6 Hz, 3H), 7.37 (t, *J* = 7.2 Hz, 2H), 7.33 (d, *J* = 7.2 Hz, 2H), 7.30 (t, *J* = 7.2 Hz, 1H), 6.98 (s, 1H), 6.80 (t, *J* = 8.4 Hz, 1H), 6.66 (d, *J*= 7.8 Hz, 1H), 6.46 (d, *J* =7.8 Hz, 1H), 5.95 (s, 2H), 4.65 (t, *J* = 7.2 Hz, 2H), 4.46 (t, *J* = 5.4 Hz, 2H), 4.26 (s, 2H), 4.14 (t, *J* = 4.8 Hz, 2H), 3.78 (t, *J* = 5.4 Hz, 2H), 2.67 (t, *J* = 7.2 Hz, 2H), 1.90 (t, *J* = 7.2 Hz, 2H), 1.69 (t, *J* = 7.2 Hz, 2H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 167.2, 159.6, 148.8, 146.1, 144.6, 140.4, 137.0, 135.2, 133.4, 129.0, 127.9, 126.7, 125.4, 124.7, 124.3, 123.8, 123.1, 122.3, 118.3, 113.0, 108.5, 88.2, 69.7, 69.6, 69.6, 69.5, 69.0, 68.8, 54.1, 49.2, 48.7, 42.1, 28.4, 25.9, 24.4. ESI-HRMS(*m/z*): $[M+H-CF_3COO]^{2+}$ calcd for $C_{52}H_{58}N_{10}O_7S^{2+}$ 483.2100, found 483.2114

Synthesis of TO-G-clamp-Phe



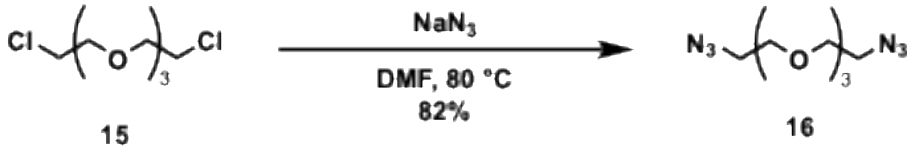
To a solution of compound **G-clamp-N_3** (9.3 mg, 18 μmol), prepared following the procedure described in the previous report,[8] in DMSO (300 μL), compound **14** (9.6 mg, 18 μmol), a solution of sodium ascorbate (9.0 mg, 45 μmol) in water (150 μL), and a solution of copper(II) sulfate pentahydrate (1.8 mg, 7.2 μmol) in water (150 μL) were added. The mixture was stirred at 37°C for 20 h, then diluted with DMSO (300 μL), and filtered to remove insoluble materials. Purification by reverse phased HPLC afforded **TO-G-clamp-Phe** as a dark-red solid (2.16 μmol, 2% yield in 2 steps from compound **7**). The concentration of **TO-G-clamp-Phe** was determined by quantitative ^{1}H-NMR using maleic acid as an internal standard. HPLC conditions; A: 0.1% TFA in distilled water, B: 0.1% TFA in MeCN; B: 0%→60% (～20 min)→100% (～25 min)→100%(～30 min). Flow rate = 4 mL/min; Temp. = 35°C; UV = 254 nm, C-18 column (Nacalai Tesque: COSMOSIL 5C_{18}-AR-II, 10×250 mm). ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.58 (d, *J* = 7.2 Hz, 1H), 8.50 (d, *J* = 8.4 Hz, 1H), 8.19 (t, *J* = 6.0 Hz, 1H), 8.12, (d, *J* = 8.4 Hz, 1H), 7.98 (t, *J* = 7.8 Hz, 2H), 7.81 (s, 1H), 7.76 (t, *J* = 7.8 Hz, 1H), 7.67 (d, *J* = 8.4 Hz, 1H), 7.54 (t, *J* = 7.8 Hz, 1H), 7.39 (t, *J* = 7.8 Hz, 1H), 7.34 (d, *J* =7.2 Hz, 1H), 7.23 (t, *J* = 7.2 Hz, 2H), 7.18 (t, *J* = 7.2 Hz, 2H), 7.09 (t, *J* = 7.2 Hz, 1H), 6.81 (t, *J* = 8.4 Hz, 1H), 6.73 (s, 1H), 6.64 (d, *J* = 9.0 Hz, 1H), 6.46 (d, *J* =7.8 Hz, 1H), 4.88 (t, *J* = 7.2 Hz, 2H), 4.61 (t, *J* = 7.2 Hz, 2H), 4.45 (t, *J* = 5.4 Hz, 2H), 4.25 (s, 2H), 3.77 (t, *J* = 5.4 Hz, 2H), 3.21 (t, *J* = 6.0 Hz, 2H), 3.15 (t, *J* = 7.2 Hz, 2H), 2.68 (t, *J* = 7.2 Hz, 2H), 1.90 (t, *J* = 7.2 Hz, 2H), 1.69 (t, *J* = 7.2 Hz, 2H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 167.3, 159.7, 148.6, 146.2, 144.3, 139.9, 137.6, 137.1, 133.4, 129.2, 128.5, 128.3, 126.8, 125.9, 124.7, 124.2, 123.8, 122.9, 122.4, 118.2, 113.2, 108.0, 88.3, 69.7, 69.7, 69.6, 69.6, 69.0, 68.8, 54.0, 49.3, 47.1, 32.9, 28.5, 26.0, 24.5. ESI-HRMS(*m/z*): $[M+H-CF_3COO]^{2+}$ calcd for $C_{53}H_{60}N_{10}O_7S^{2+}$ 490.2178, found 490.2197.

Synthesis of TO-Me, -Bn, and -Phe-N_3



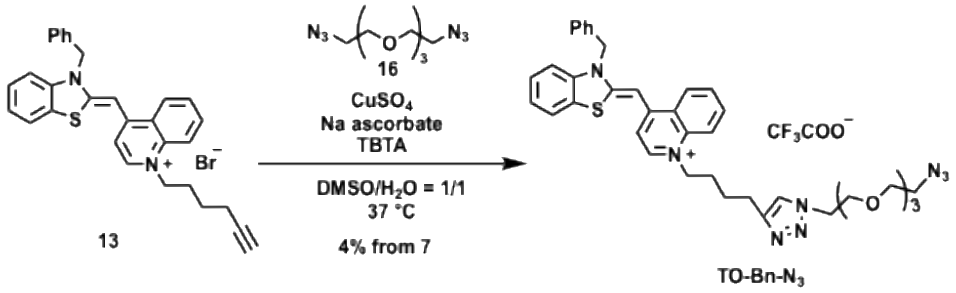
Scheme S3

Synthesis of compound 16



To a solution of diethylene glycol bis(2-chloroethyl) ether **15** (952 mg, 4 mmol) in DMF (10 mL), sodium azide (1.04 g, 16 mmol) was added and stirred at 80°C for 18 h. The reaction mixture was diluted with water (10 mL), extracted with Et_2O (20 mL x3), and washed with water (20 mL x3). The combined organic layer was dried over Na_2SO_4, filtered, and concentrated by vacuo to afford compound **16** as a yellow oil (798 mg, 82% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ3.39 (4H, t, *J* = 4.8 Hz), 3.68 (m, 12H). ESI-HRMS(*m/z*): $[M+H]^+$ calcd for $C_8H_{17}N_6O_3^+$ 245.1357, found 245.1362.

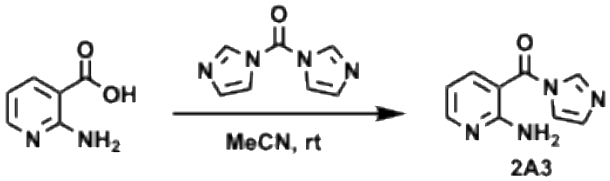
Synthesis of TO-Bn-N_3



To a solution of compound **16** (24.4 mg, 100 μmol) in DMSO (167 μL), compound **13** (5.3 mg, <10 μmol), water (167 μL), sodium ascorbate (5.0 mg, 25 μmol), TBTA (2.1 mg, 4 μmol), and copper(II) sulfate pentahydrate (1.0 mg, 4 μmol) were added. The mixture was stirred at 37°C for 22 h, then diluted with DMSO (300 μL), and filtrated. The filtrate was washed with diethyl ether (300 μL) 3 times. Combined DMSO phase was purified by reverse phased HPLC to afford **TO-Bn-N_3** as a dark-red solid (5.0 μmol, 4% yield in 2 steps from compound **7**). The concentration of **TO-Bn-N_3** was determined by quantitative ^{1}H-NMR using maleic acid as an internal standard. HPLC conditions;

A: 0.1% TFA in distilled water, B: 0.1% TFA in MeCN; B: 0%→60% (～20 min)→100% (～25 min)→100%(～30 min). Flow rate = 4 mL/min; Temp. = 35°C; UV = 254 nm, C-18 column (Nacalai Tesque: COSMOSIL 5C_{18}-AR-II, 10×250 mm). ^{1}H NMR (600 MHz, DMSO-d_6) δ1.69 (m, 2H), 1.90 (m, 2H), 2.68 (t, 2H, *J* = 7.2 Hz), 3.35 (t, 2H, *J* = 4.8 Hz), 3.56 (m, 10H), 3.61 (t, 2H, *J* = 4.8 Hz), 3.77 (t, 2H, *J* = 5.4 Hz), 4.45 (t, 2H, *J* = 5.4 Hz), 4.66 (t, 2H, *J* = 7.8 Hz), 6.99 (s, 1H), 7.30 (t, 1H, *J* = 7.2 Hz), 7.34 (d, 2H, *J* = 7.2 Hz), 7.39 (t, 2H, *J* = 7.2 Hz), 7.44 (m, 2H), 7.60 (t, 1H, *J* = 8.4 Hz), 7.73 (m, 1H), 7.79 (d, 2H, *J* = 6.0 Hz), 7.98 (m, 1H), 8.09 (d, 1H, *J* = 8.4 Hz), 8.16 (d, 1H, *J* = 8.4 Hz), 8.55 (d, 1H, *J* = 7.8 Hz), 8.69 (d, 1H, *J* = 7.2 Hz). ^{13}C NMR (151 MHz, DMSO-d_6) δ 24.4, 25.9, 28.4, 48.7, 49.2, 50.0, 54.1, 68.8, 69.2, 69.3, 69.5, 69.7, 69.7, 69.7, 69.9, 88.3, 108.5, 113.1, 118.3, 122.3, 123.1, 123.8, 124.4, 124.7, 125.5, 126.7, 127.0, 127.9, 128.4, 129.1, 133.4, 135.2, 137.0, 140.4, 144.6, 146.1, 148.8, 159.6. ESI-HRMS(*m/z*): $[M\text{-}CF_3COO]^+$ calcd for $C_{38}H_{43}N_8O_3S^+$ 691.3173, found 691.3184.

Synthesis of 2A3

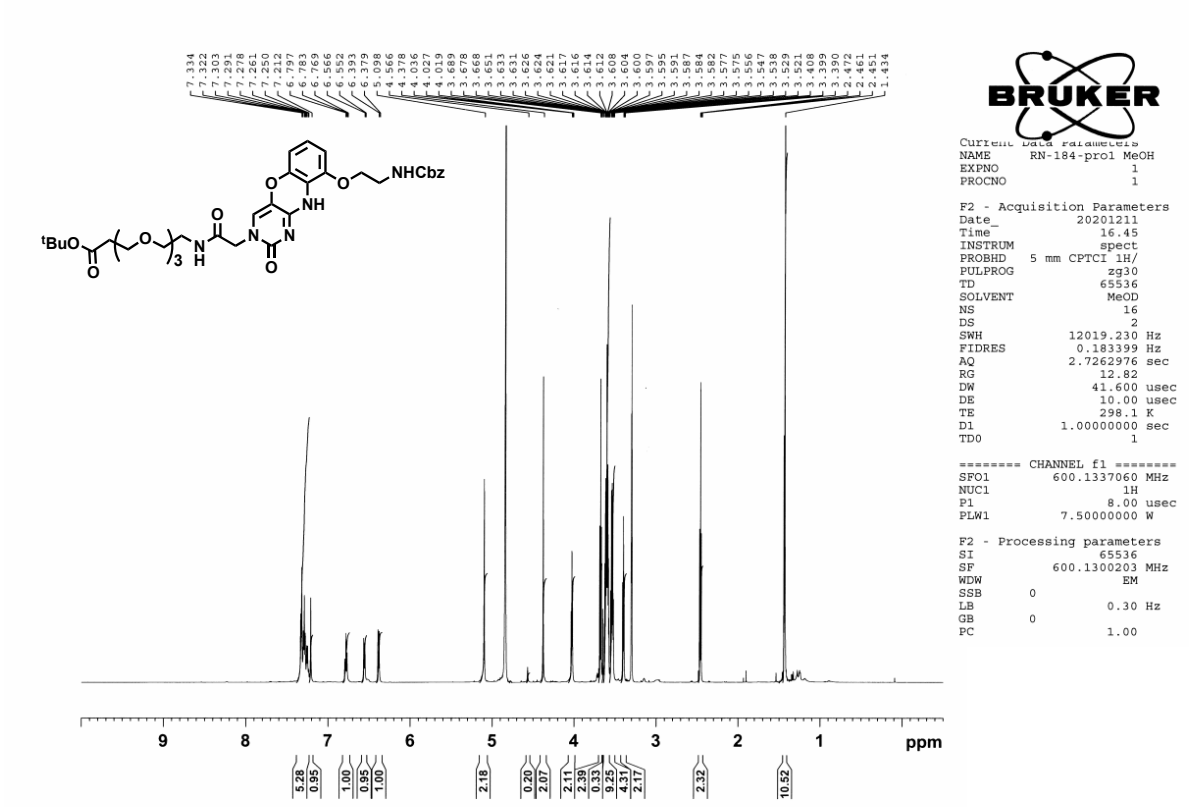


2A3

2A3 was synthesized by following the protocol[13] with minor modifications. To a solution of 2-aminopyridine-3-carboxylic acid (1.00 g, 7.21 mmol) in MeCN (3.6 mL), a solution of 1,1'-carbonyldiimidazole (1.17 g, 7.19 mmol) in MeCN (3.6 mL) was added over 5 min. Then the mixture was stirred at room temperature for 21 h. The mixture was filtrated with CH_2Cl_2 (3 mL) followed by addition of another CH_2Cl_2 (10 mL) and sat. $NaHCO_3$ (15 mL). After separation of an aqueous layer and organic layer, the aqueous layer was extracted with CH_2Cl_2 (10 mL×2) and dried over Na_2SO_4. The residue was evaporated under reduced pressure to obtain crude (0.50 g). The crude was added hexane (45 mL) and CH_2Cl_2 (45 mL) followed by wash with H_2O (10 mL) and brine (10 mL×2). The organic layer was dried over Na_2SO_4 and evaporated under reduced pressure to obtain desired product (0.155 g, 11% yield) as a white solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 6.69 (1H, dd, J = 5.0, 7.8 Hz), 7.03 (2H, br s), 7.13 (1H, dd, J = 1.0, 1.5 Hz), 7.62 (1H, t, J = 1.5 Hz), 7.76 (1H, dd, J = 2.0, 8.0 Hz), 8.15 (1H, t, J = 1.0 Hz), 8.27 (1H, dd, J = 2.0, 4.5 Hz). ESI-HRMS (*m/z*): $[M+H]^+$ calcd for $C_9H_9N_4O^+$, 189.0771, found 189.0761.

NMR Data

Compound **3**, ^{1}H NMR (600 MHz, MeOD)

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EXPNO                 1
PROCNO                1

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INSTRUM          spect
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PULPROG          zg30
TD              65536
SOLVENT          MeOD
NS                 16
DS                  2
SWH          12019.230 Hz
FIDRES        0.183399 Hz
AQ            2.7262976 sec
RG               12.82
DW              41.600 usec
DE              10.00 usec
TE              298.1 K
D1           1.00000000 sec
TD0                 1

======== CHANNEL f1 ========
SFO1        600.1337060 MHz
NUC1              1H
P1               8.00 usec
PLW1        7.50000000 W

F2 - Processing parameters
SI              65536
SF         600.1300203 MHz
WDW               EM
SSB       0
LB             0.30 Hz
GB        0
PC              1.00
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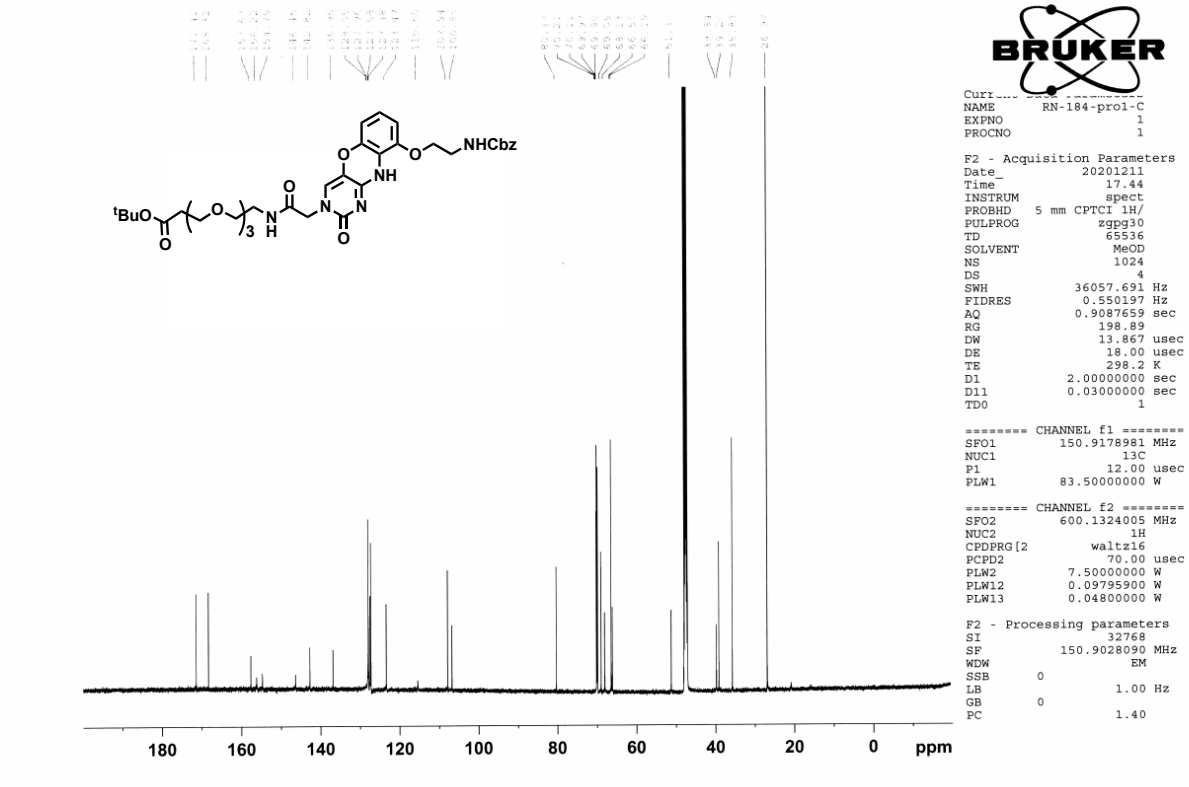
Compound **3**, ^{13}C NMR (151 MHz, MeOD)

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Current Data Parameters
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EXPNO                 1
PROCNO                1

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Time             17.44
INSTRUM          spect
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PULPROG        zgpg30
TD              65536
SOLVENT          MeOD
NS               1024
DS                  4
SWH          36057.691 Hz
FIDRES        0.550197 Hz
AQ            0.9087659 sec
RG              198.89
DW              13.867 usec
DE              18.00 usec
TE              298.2 K
D1           2.00000000 sec
D11          0.03000000 sec
TD0                 1

======== CHANNEL f1 ========
SFO1        150.9178981 MHz
NUC1              13C
P1              12.00 usec
PLW1        83.50000000 W

======== CHANNEL f2 ========
SFO2        600.1324005 MHz
NUC2              1H
CPDPRG[2        waltz16
PCPD2           70.00 usec
PLW2         7.50000000 W
PLW12        0.09795900 W
PLW13        0.04800000 W

F2 - Processing parameters
SI              32768
SF         150.9028090 MHz
WDW               EM
SSB       0
LB             1.00 Hz
GB        0
PC              1.40
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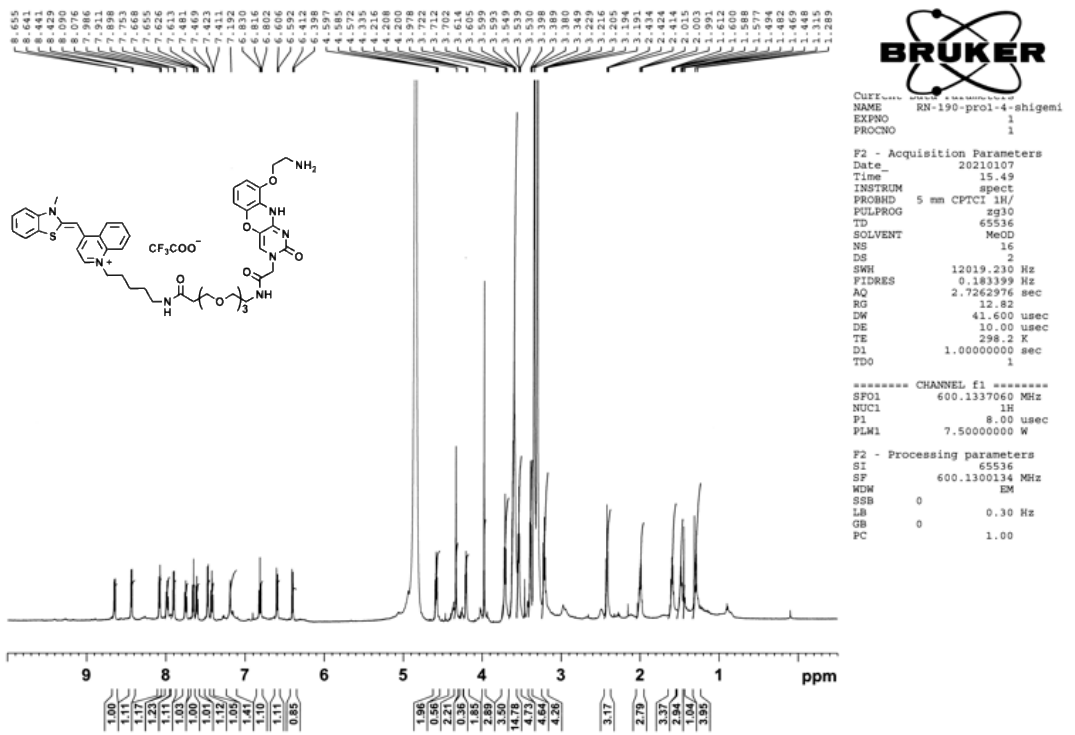
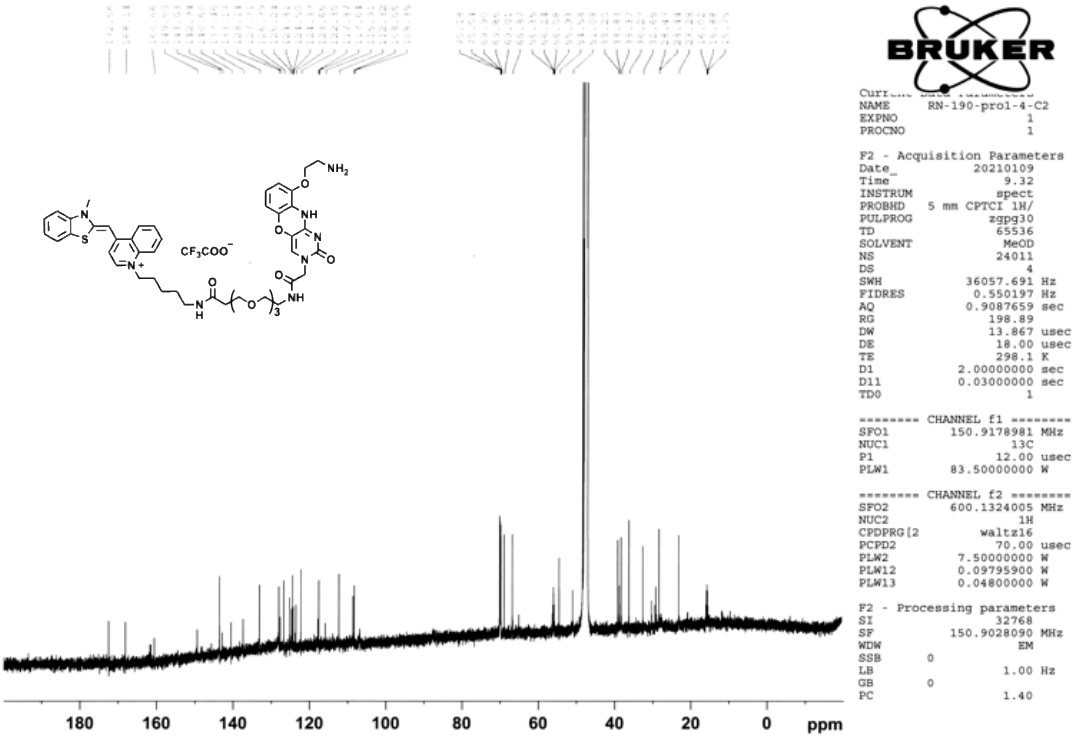
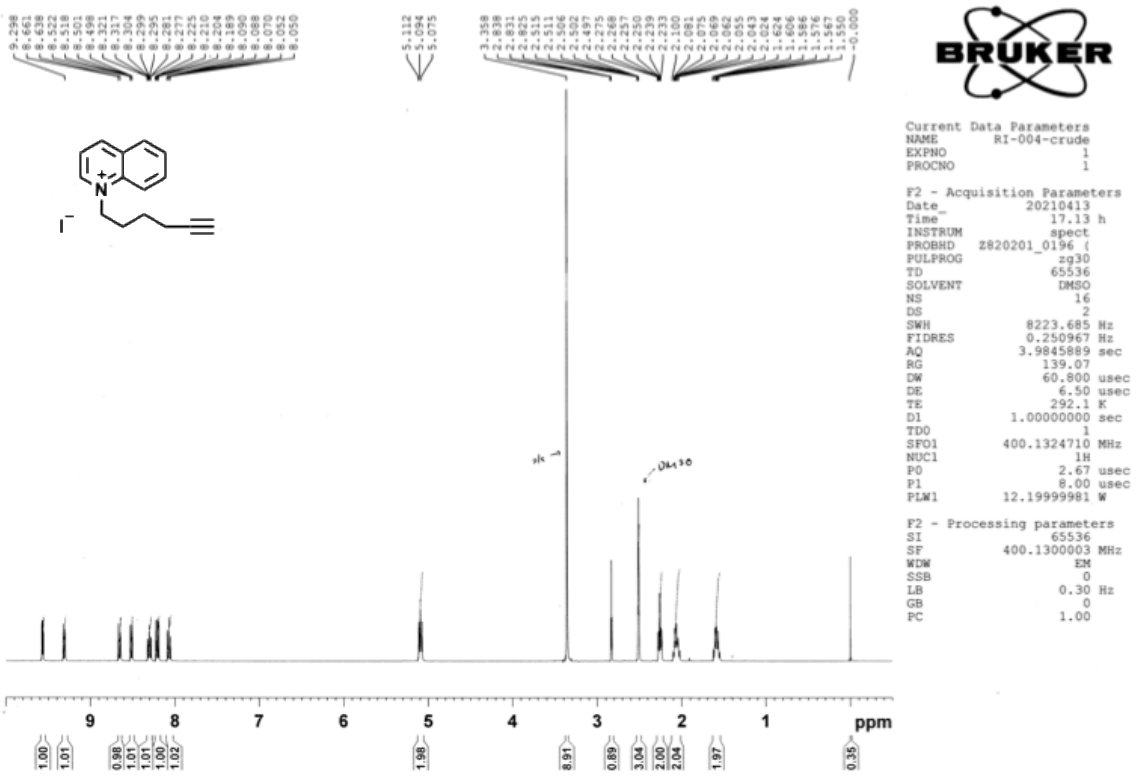
TO-G-clamp(am), ^{1}H NMR (600 MHz, MeOD)



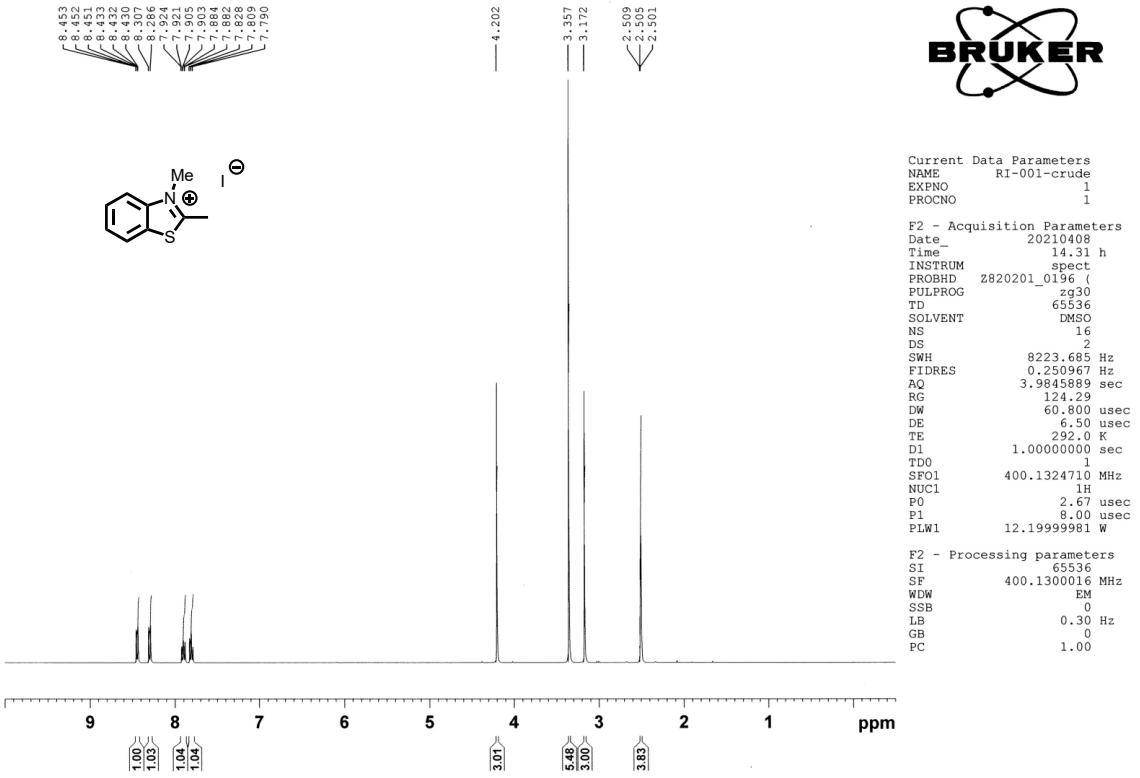
TO-G-clamp(am), ^{13}C NMR (151 MHz, MeOD)



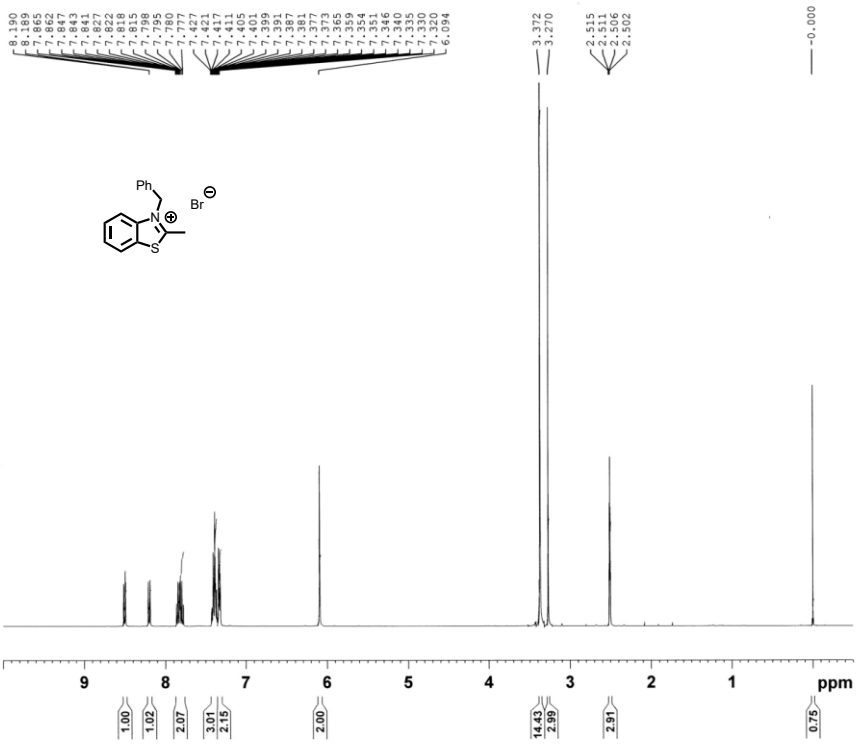
Compound **7**, ^{1}H NMR (400 MHz, DMSO-d_6)



Compound **9**, ^{1}H NMR (400 MHz, DMSO-d_6)



Compound **10**, ^{1}H NMR (400 MHz, DMSO-d_6)



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EXPNO                   1
PROCNO                  1

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INSTRUM              spect
PROBHD    Z820201_0196 (
PULPROG               zg30
TD                   65536
SOLVENT               DMSO
NS                      16
DS                       2
SWH               8223.685 Hz
FIDRES           0.250967 Hz
AQ               3.9845889 sec
RG                  113.74
DW                  60.800 usec
DE                    6.50 usec
TE                  291.8 K
D1              1.00000000 sec
TD0                     1
SFO1           400.1324710 MHz
NUC1                   1H
P0                    2.67 usec
P1                    8.00 usec
PLW1           12.19999981 W

F2 - Processing parameters
SI                   65536
SF              400.1300007 MHz
WDW                     EM
SSB                      0
LB                    0.30 Hz
GB                       0
PC                    1.00
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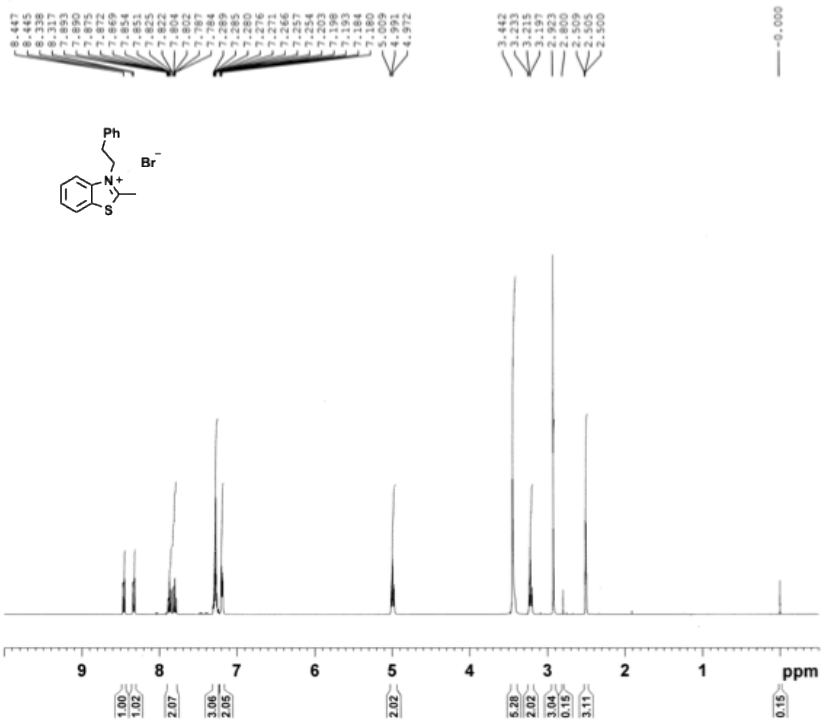
Compound **11**, ^{1}H NMR (400 MHz, DMSO-d_6)



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Current Data Parameters
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EXPNO                   1
PROCNO                  1

F2 - Acquisition Parameters
Date_             20210414
Time                16.19 h
INSTRUM              spect
PROBHD    Z820201_0196 (
PULPROG               zg30
TD                   65536
SOLVENT               DMSO
NS                      16
DS                       2
SWH               8223.685 Hz
FIDRES           0.250967 Hz
AQ               3.9845889 sec
RG                  161.99
DW                  60.800 usec
DE                    6.50 usec
TE                  292.7 K
D1              1.00000000 sec
TD0                     1
SFO1           400.1324710 MHz
NUC1                   1H
P0                    2.67 usec
P1                    8.00 usec
PLW1           12.19999981 W

F2 - Processing parameters
SI                   65536
SF              400.1300012 MHz
WDW                     EM
SSB                      0
LB                    0.30 Hz
GB                       0
PC                    1.00
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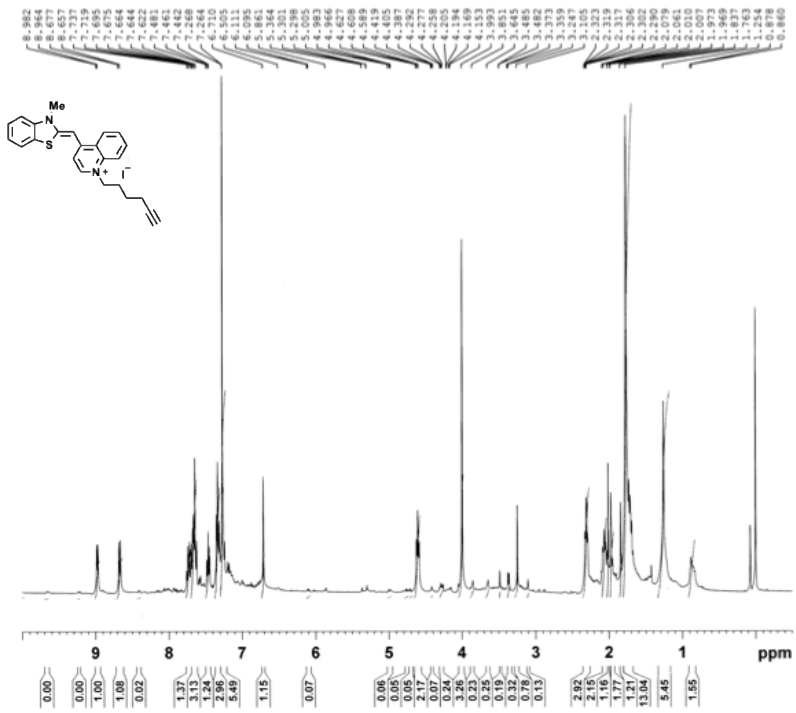
Compound **12**, ^{1}H NMR (400 MHz, $CDCl_3$)

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Current Data Parameters
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EXPNO                      1
PROCNO                     1

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Date_              20210924
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INSTRUM                spect
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PULPROG                zg30
TD                    65536
SOLVENT               CDCl3
NS                       16
DS                        2
SWH                8223.685 Hz
FIDRES             0.250967 Hz
AQ                 3.9845889 sec
RG                    139.07
DW                    60.800 usec
DE                     6.50 usec
TE                   297.8 K
D1                 1.00000000 sec
TD0                        1
SFO1             400.1324710 MHz
NUC1                      1H
P0                     2.67 usec
P1                     8.00 usec
PLW1            12.19999981 W

F2 - Processing parameters
SI                    65536
SF               400.1300069 MHz
WDW                       EM
SSB                        0
LB                      0.30 Hz
GB                         0
PC                      1.00
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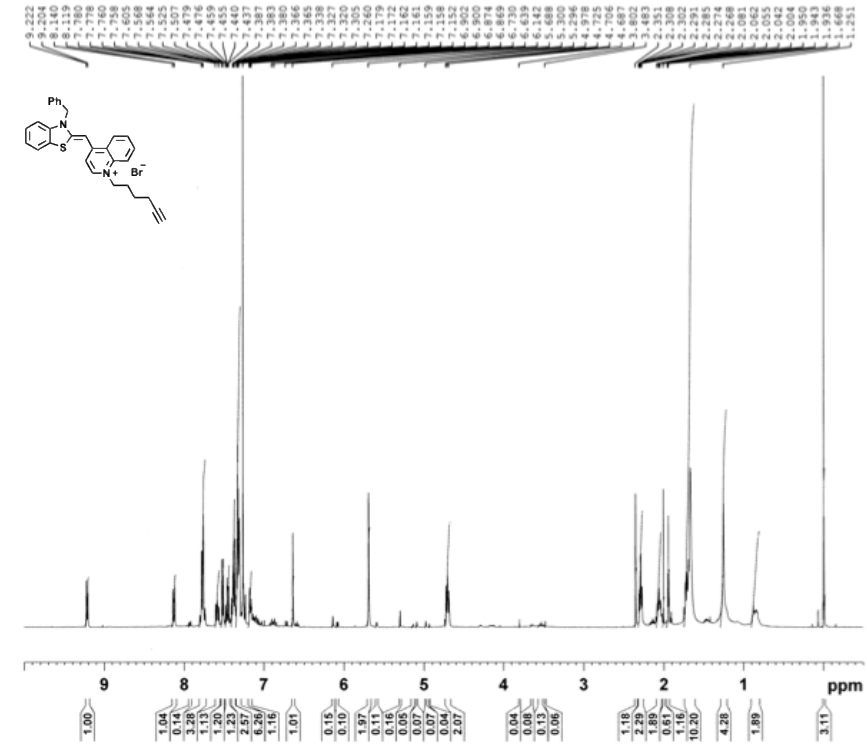
Compound **13**, ^{1}H NMR (400 MHz, $CDCl_3$)

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Current Data Parameters
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EXPNO                      1
PROCNO                     1

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Date_              20210820
Time                 18.49 h
INSTRUM                spect
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PULPROG                zg30
TD                    65536
SOLVENT               CDCl3
NS                       16
DS                        2
SWH                8223.685 Hz
FIDRES             0.250967 Hz
AQ                 3.9845889 sec
RG                    161.99
DW                    60.800 usec
DE                     6.50 usec
TE                   296.9 K
D1                 1.00000000 sec
TD0                        1
SFO1             400.1324710 MHz
NUC1                      1H
P0                     2.67 usec
P1                     8.00 usec
PLW1            12.19999981 W

F2 - Processing parameters
SI                    65536
SF               400.1300097 MHz
WDW                       EM
SSB                        0
LB                      0.30 Hz
GB                         0
PC                      1.00
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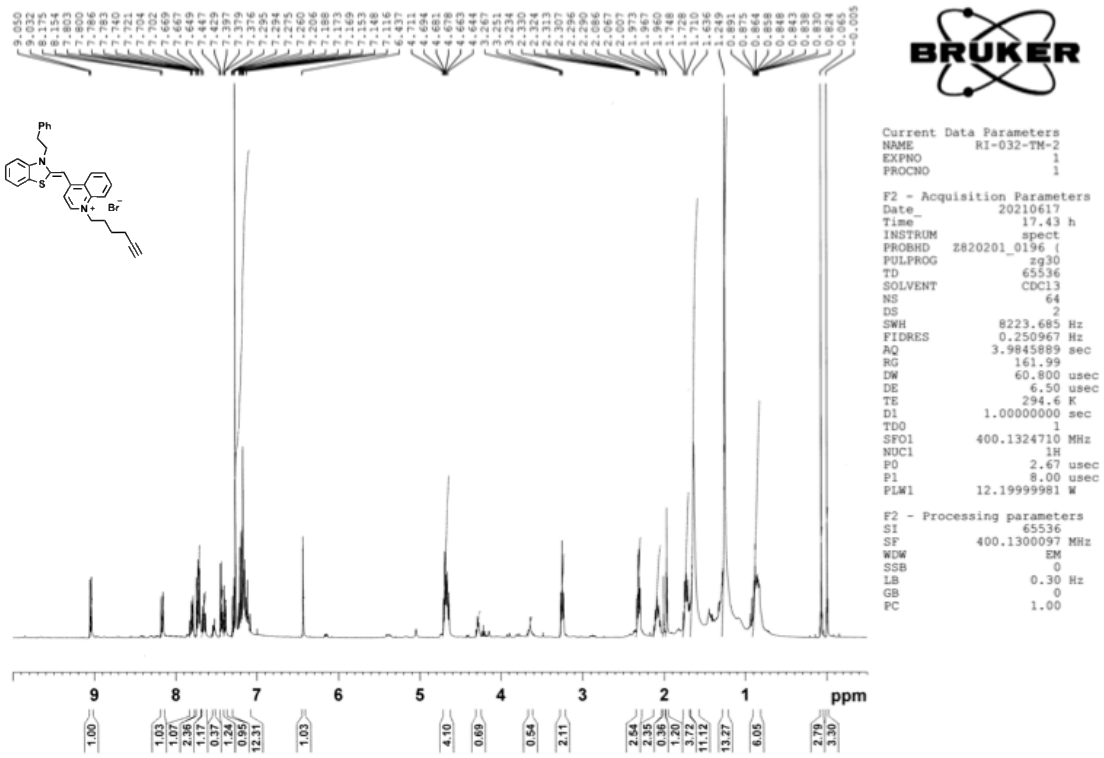
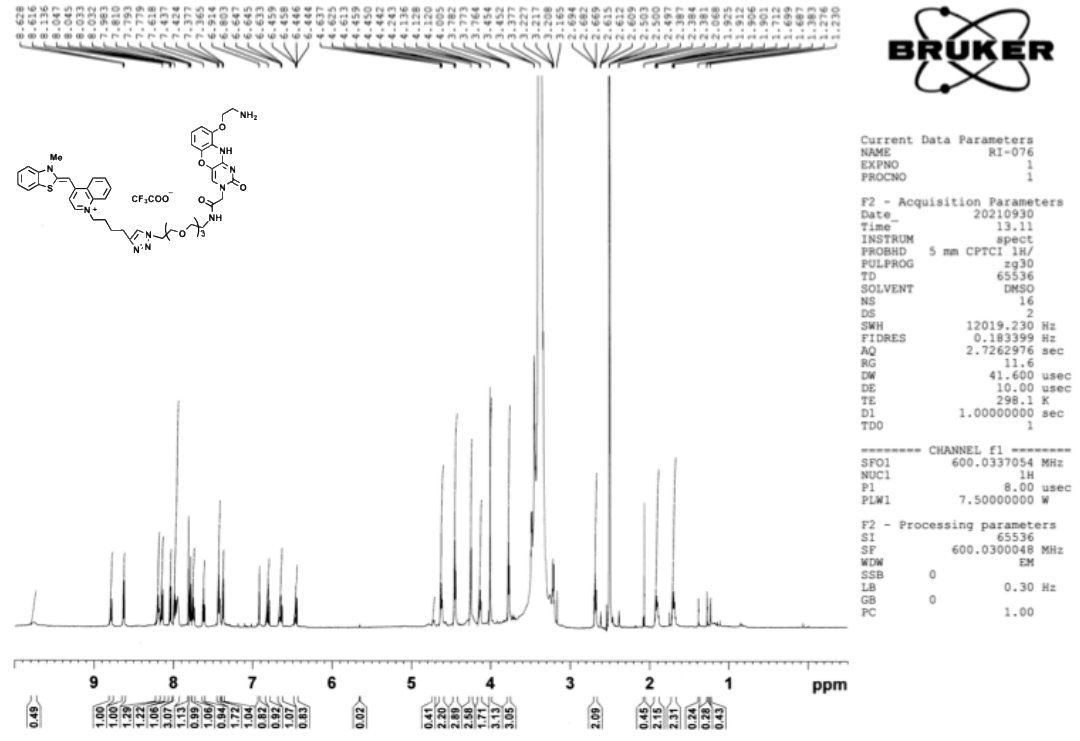
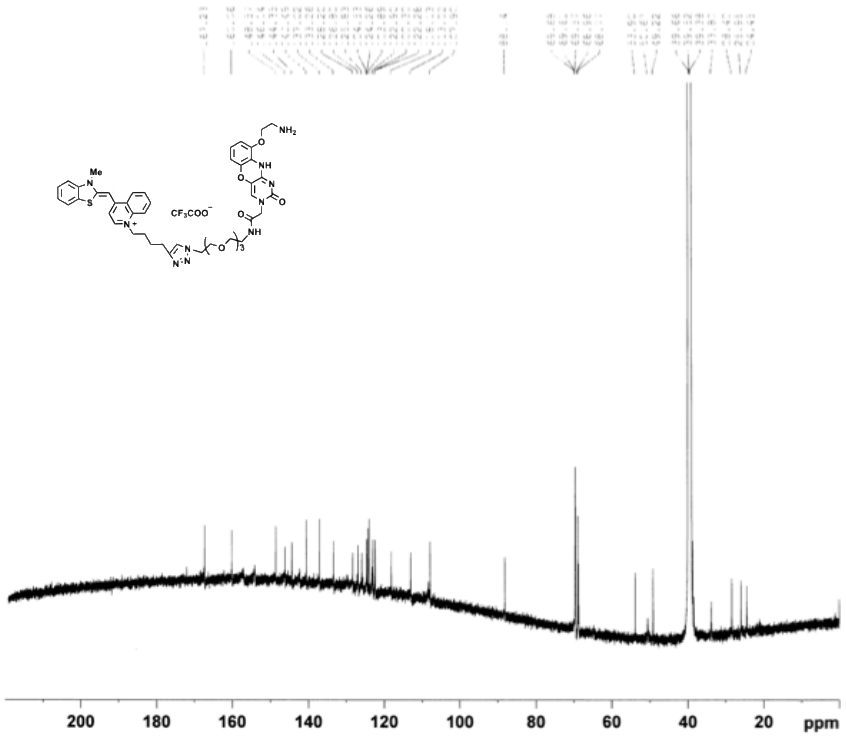
Compound **14**, ^{1}H NMR (400 MHz, $CDCl_3$)



TO-G-clamp-Me, ^{1}H NMR (600 MHz, DMSO-d_6)



TO-G-clamp-Me, ^{13}C NMR (151 MHz, DMSO-d_6)

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Current Data Parameters
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PROCNO                   1

F2 - Acquisition Parameters
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Time                   8.50
INSTRUM               spect
PROBHD   5 mm CPTCI 1H/
PULPROG              zgpg30
TD                    65536
SOLVENT                DMSO
NS                     16384
DS                         4
SWH                36057.691 Hz
FIDRES              0.550197 Hz
AQ                 0.9087659 sec
RG                    198.89
DW                    13.867 usec
DE                     18.00 usec
TE                     298.2 K
D1                2.00000000 sec
D11               0.03000000 sec
TD0                        1

======== CHANNEL f1 ========
SFO1          150.8927501 MHz
NUC1                    13C
P1                    12.00 usec
PLW1          92.00000000 W

======== CHANNEL f2 ========
SFO2          600.0324001 MHz
NUC2                    1H
CPDPRG[2           waltz16
PCPD2                 70.00 usec
PLW2           7.50000000 W
PLW12          0.09795900 W
PLW13          0.04800000 W

F2 - Processing parameters
SI                     32768
SF            150.8777349 MHz
WDW                       EM
SSB       0
LB                     1.00 Hz
GB        0
PC                     1.40
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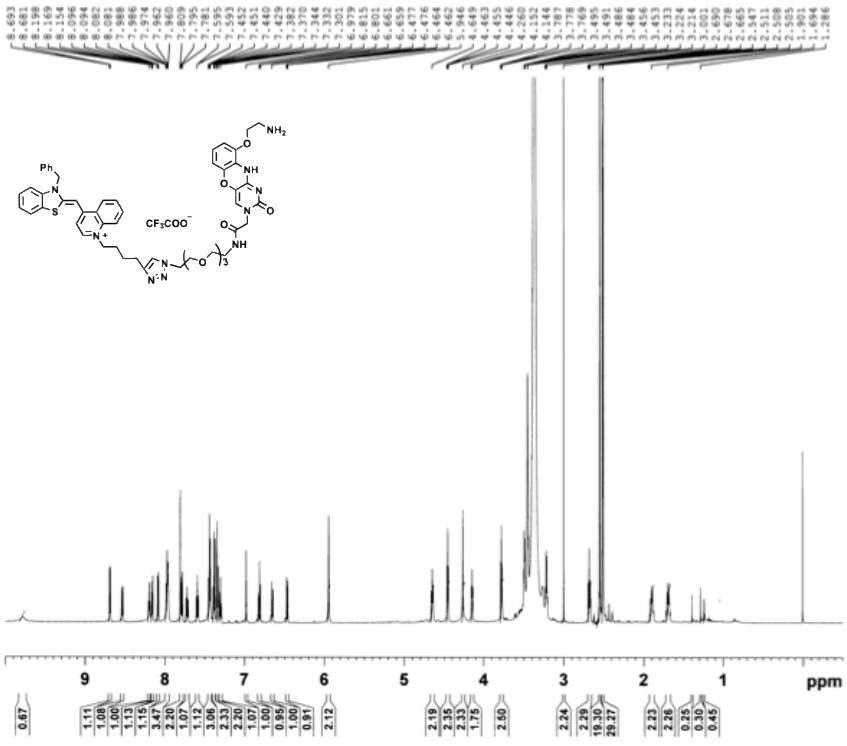
TO-G-clamp-Bn, ^{1}H NMR (600 MHz, DMSO-d_6)

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Current Data Parameters
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EXPNO                      1
PROCNO                     1

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Time                  14.16
INSTRUM               spect
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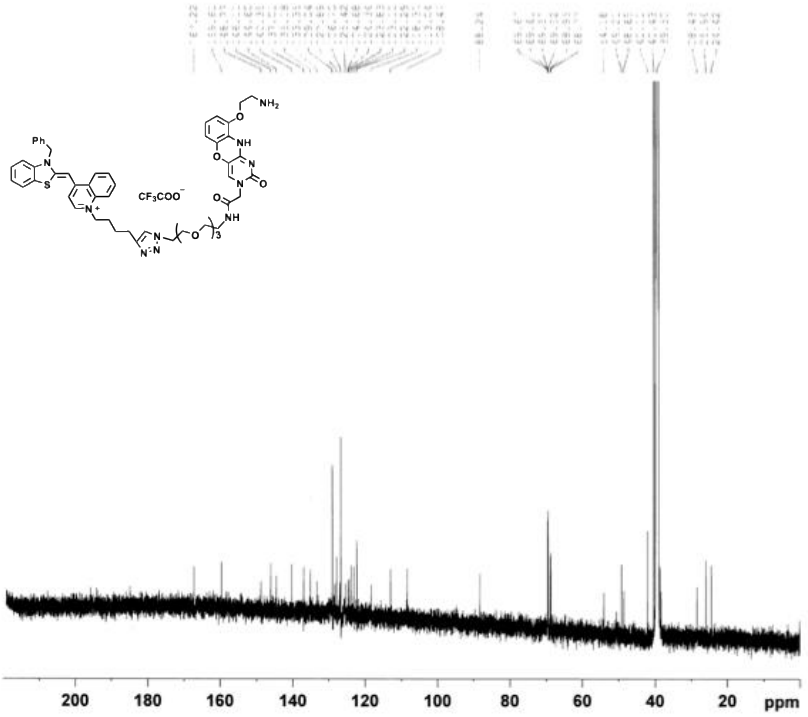
PULPROG                zg30
TD                    65536
SOLVENT                DMSO
NS                        16
DS                         2
SWH                12019.230 Hz
FIDRES              0.183399 Hz
AQ                 2.7262976 sec
RG                     12.82
DW                    41.600 usec
DE                     10.00 usec
TE                     298.1 K
D1                1.00000000 sec
TD0                        1

======== CHANNEL f1 ========
SFO1          600.0337054 MHz
NUC1                    1H
P1                     8.00 usec
PLW1           7.50000000 W

F2 - Processing parameters
SI                     65536
SF            600.0300000 MHz
WDW                       EM
SSB       0
LB                     0.30 Hz
GB        0
PC                     1.00
```

TO-G-clamp-Bn, ^{13}C NMR (151 MHz, DMSO-d_6)




```
Current Data Parameters
NAME        RI-065-carbon
EXPNO                   1
PROCNO                  1

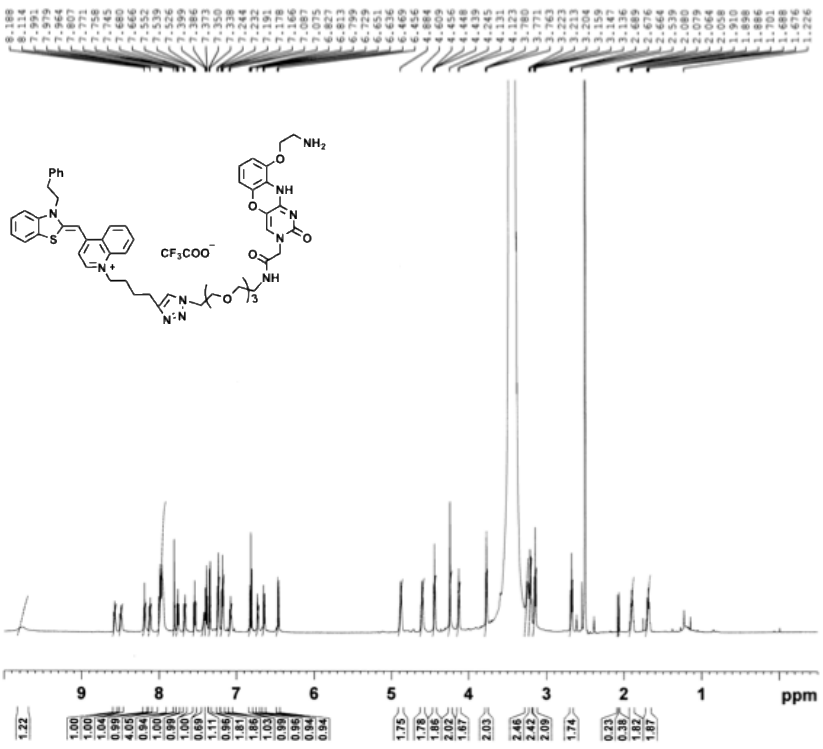
F2 - Acquisition Parameters
Date_            20210828
Time                15.20
INSTRUM             spect
PROBHD   5 mm CPTCI 1H/
PULPROG          zgpg30
TD                65536
SOLVENT             DMSO
NS                  1024
DS                     4
SWH           36057.691 Hz
FIDRES         0.550197 Hz
AQ             0.9087659 sec
RG                198.89
DW                13.867 usec
DE                18.00 usec
TE                298.1 K
D1            2.00000000 sec
D11           0.03000000 sec
TD0                    1

======== CHANNEL f1 ========
SFO1         150.8927501 MHz
NUC1                  13C
P1                  12.00 usec
PLW1         92.00000000 W

======== CHANNEL f2 ========
SFO2         600.0324001 MHz
NUC2                   1H
CPDPRG[2          waltz16
PCPD2               70.00 usec
PLW2          7.50000000 W
PLW12         0.09795900 W
PLW13         0.04800000 W

F2 - Processing parameters
SI                 32768
SF           150.8777344 MHz
WDW                   EM
SSB       0
LB                 1.00 Hz
GB        0
PC                 1.40
```

TO-G-clamp-Phe, ^{1}H NMR (600 MHz, DMSO-d_6)



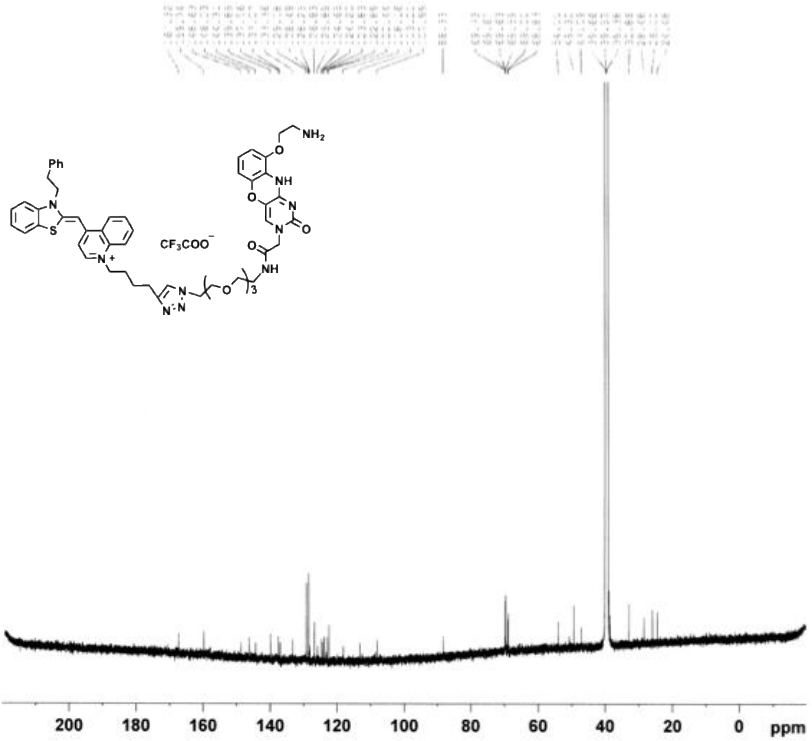

```
Current Data Parameters
NAME             RI-041-re
EXPNO                    1
PROCNO                   1

F2 - Acquisition Parameters
Date_             20210713
Time                 12.49
INSTRUM              spect
PROBHD   5 mm CPTCI 1H/
PULPROG              zg30
TD                  65536
SOLVENT               DMSO
NS                      64
DS                       2
SWH            12019.230 Hz
FIDRES          0.183399 Hz
AQ             2.7262976 sec
RG                    11.6
DW                 41.600 usec
DE                 10.00 usec
TE                 298.2 K
D1             1.00000000 sec
TD0                      1

======== CHANNEL f1 ========
SFO1          600.1337060 MHz
NUC1                    1H
P1                   8.00 usec
PLW1           7.50000000 W

F2 - Processing parameters
SI                   65536
SF            600.1300074 MHz
WDW                     EM
SSB       0
LB                  0.30 Hz
GB        0
PC                  1.00
```

TO-G-clamp-Phe, ^{13}C NMR (151 MHz, DMSO-d_6)




```
Current Data Parameters
NAME          RI-041-carbon
EXPNO                     1
PROCNO                    1

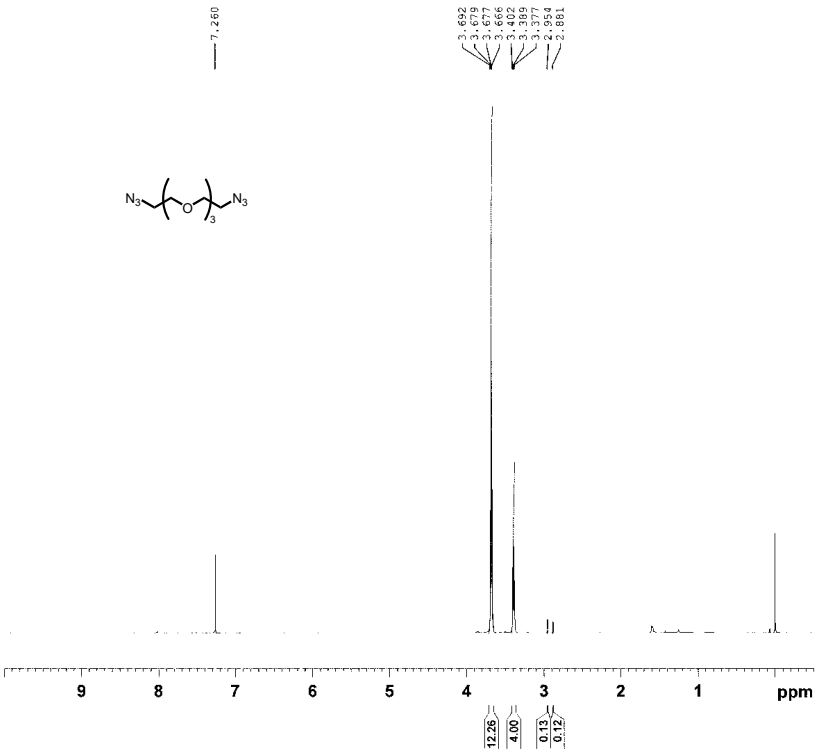
F2 - Acquisition Parameters
Date_              20210713
Time                  12.38
INSTRUM               spect
PROBHD   5 mm CPTCI 1H/
PULPROG           zgpg30
TD                 65536
SOLVENT             DMSO
NS                  4096
DS                     4
SWH            36057.691 Hz
FIDRES         0.550197 Hz
AQ             0.9087659 sec
RG                198.89
DW                13.867 usec
DE                18.00 usec
TE                298.1 K
D1            2.00000000 sec
D11           0.03000000 sec
TD0                    1

======== CHANNEL f1 ========
SFO1          150.9178981 MHz
NUC1                  13C
P1                  12.00 usec
PLW1          83.50000000 W

======== CHANNEL f2 ========
SFO2          600.1324005 MHz
NUC2                  1H
CPDPRG[2          waltz16
PCPD2               70.00 usec
PLW2          7.50000000 W
PLW12         0.09795900 W
PLW13         0.04800000 W

F2 - Processing parameters
SI                 32768
SF            150.9028693 MHz
WDW                   EM
SSB       0
LB                 1.00 Hz
GB        0
PC                 1.40
```

Compound **16**, ^{1}H NMR (400 MHz, $CDCl_3$)

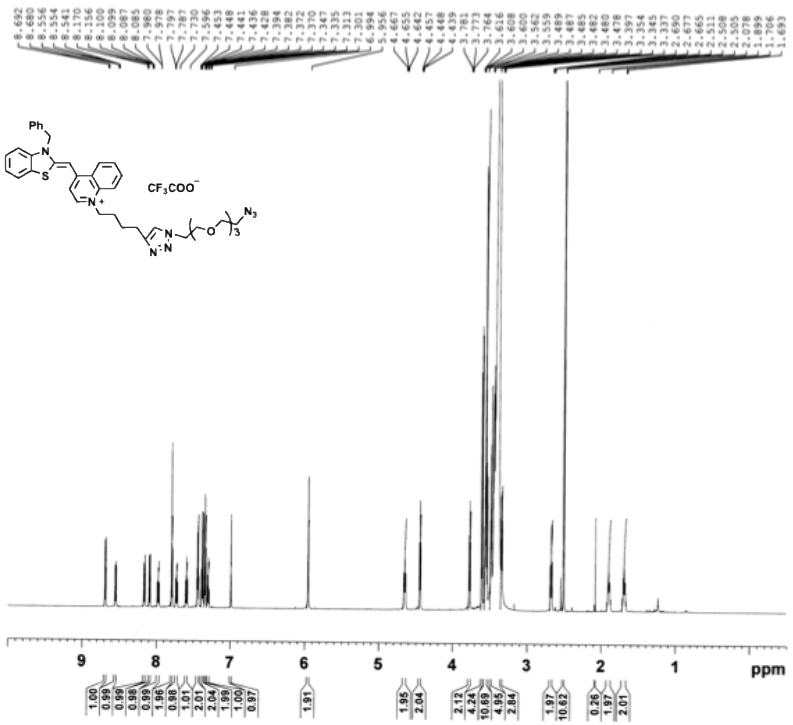



```
Current Data Parameters
NAME                  RI-111
EXPNO                      1
PROCNO                     1

F2 - Acquisition Parameters
Date_              20211111
Time                 14.30 h
INSTRUM               spect
PROBHD   Z820201_0196 (
PULPROG                zg30
TD                    65536
SOLVENT               CDCl3
NS                       16
DS                        2
SWH               8223.685 Hz
FIDRES            0.250967 Hz
AQ                3.9845889 sec
RG                   139.07
DW                   60.800 usec
DE                    6.50 usec
TE                   296.9 K
D1               1.00000000 sec
TD0                       1
SFO1            400.1324710 MHz
NUC1                      1H
P0                    2.67 usec
P1                    8.00 usec
PLW1            12.19999981 W

F2 - Processing parameters
SI                    65536
SF              400.1300095 MHz
WDW                      EM
SSB                       0
LB                    0.30 Hz
GB                        0
PC                    1.00
```

TO-Bn-N$_3$, ^{1}H NMR (600 MHz, DMSO-d_6)



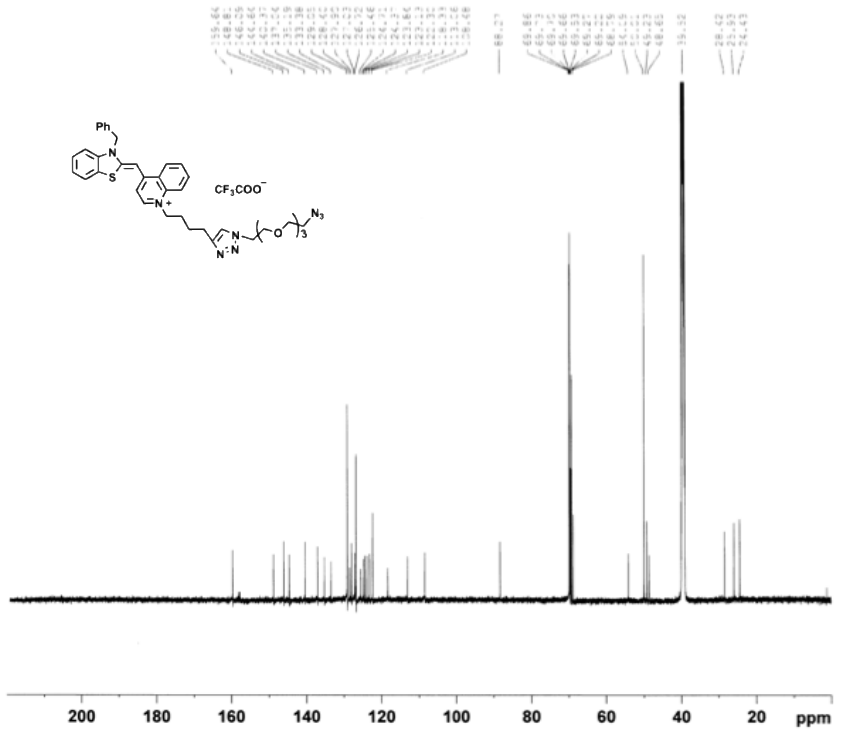

```
Current Data Parameters
NAME                  RI-112
EXPNO                      1
PROCNO                     1

F2 - Acquisition Parameters
Date_              20211122
Time                  15.51
INSTRUM               spect
PROBHD   5 mm CPTCI 1H/
PULPROG               zg30
TD                   65536
SOLVENT               DMSO
NS                       16
DS                        2
SWH               12019.230 Hz
FIDRES             0.183399 Hz
AQ                 2.7262976 sec
RG                     11.6
DW                   41.600 usec
DE                   10.00 usec
TE                   298.2 K
D1                1.00000000 sec
TD0                        1

======== CHANNEL f1 ========
SFO1             600.0337054 MHz
NUC1                      1H
P1                     8.00 usec
PLW1              7.50000000 W

F2 - Processing parameters
SI                       65536
SF               600.0300000 MHz
WDW                         EM
SSB          0
LB                     0.30 Hz
GB           0
PC                     1.00
```

TO-Bn-N$_3$, ^{13}C NMR (151 MHz, DMSO-d_6)




```
Current Data Parameters
NAME          RI-112-carbon
EXPNO                      1
PROCNO                     1

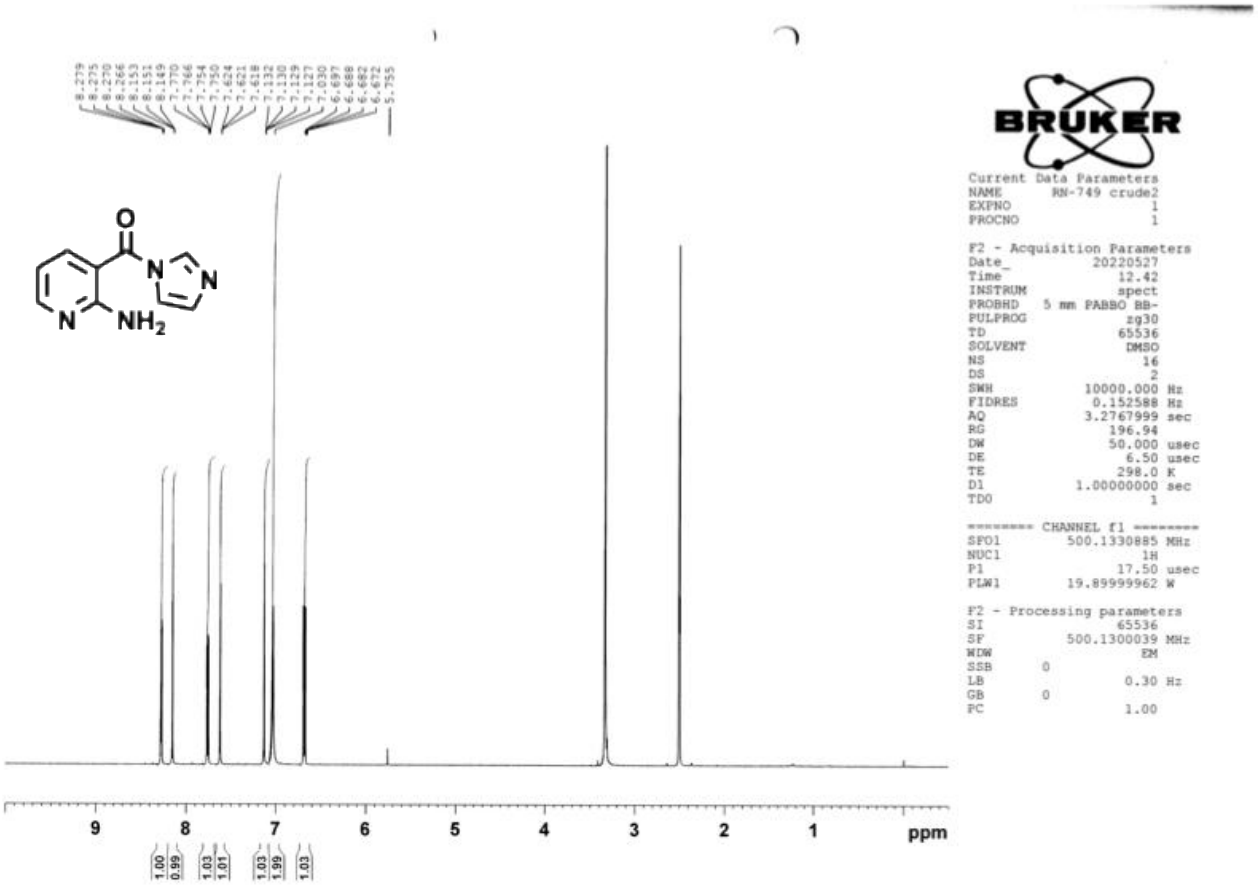
F2 - Acquisition Parameters
Date_              20211122
Time                  17.41
INSTRUM               spect
PROBHD   5 mm CPTCI 1H/
PULPROG           zgpg30
TD                   65536
SOLVENT               DMSO
NS                     2048
DS                        4
SWH               36057.691 Hz
FIDRES             0.550197 Hz
AQ                 0.9087659 sec
RG                    198.89
DW                   13.867 usec
DE                   18.00 usec
TE                   298.2 K
D1                2.00000000 sec
D11               0.03000000 sec
TD0                        1

======== CHANNEL f1 ========
SFO1             150.8927501 MHz
NUC1                      13C
P1                    12.00 usec
PLW1             92.00000000 W

======== CHANNEL f2 ========
SFO2             600.0324001 MHz
NUC2                      1H
CPDPRG[2              waltz16
PCPD2                 70.00 usec
PLW2              7.50000000 W
PLW12             0.09795900 W
PLW13             0.04800000 W

F2 - Processing parameters
SI                       32768
SF               150.8777323 MHz
WDW                         EM
SSB          0
LB                     1.00 Hz
GB           0
PC                     1.40
```

2A3, ^{1}H NMR (500 MHz, DMSO-d_6)



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