Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Cas9-mediated DNA cleavage guided by enzymatically prepared 4′-thio-modified RNA

Noriko Saito-Tarashima, [a]* Mana Ueno, [a] Akiho Murai, [a] Ayako Matsuo, [a] Noriaki Minakawa [a]*

[a]*Graduate School of Pharmaceutical Science, Tokushima University, Shomachi 1-78-1, Tokushima, 770-8505, Japan.*

Table of Contents

Fig. S1. Preparation of the DNA template for *in vitro* transcription by PCR. The target sequence is shown in blue. 4′-Thio nucleotides are shown in red.

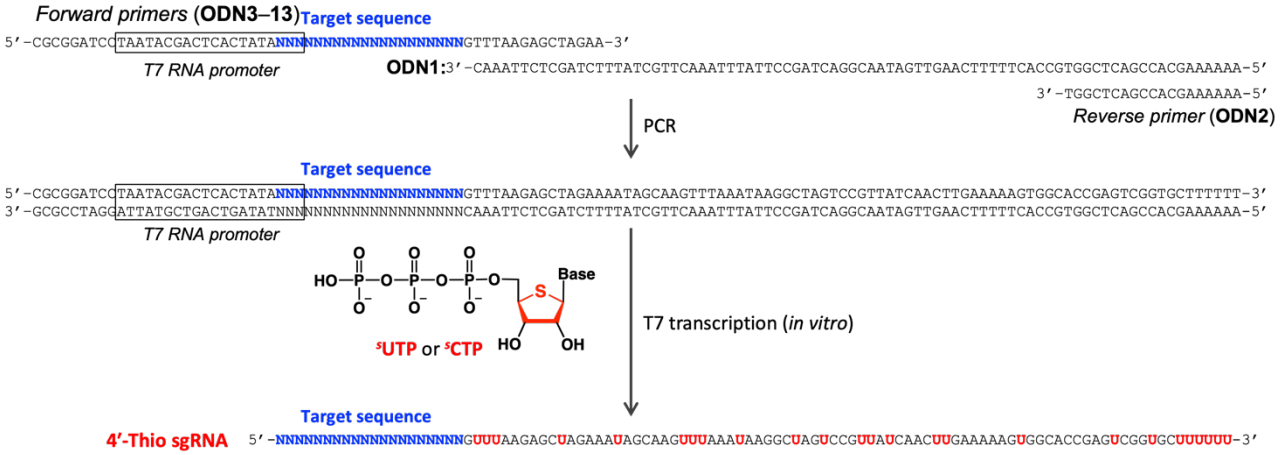

Fig. S2. *In vitro* Cas9-mediated cleavage assay with ^{S}C sgRNA. (a) Native PAGE analysis of *in vitro* Cas9-mediated cleavage reactions. FITC-labeled bands were visualized at 488 nm. (b) Modified scheme, number of modifications in each guide sequences, and cleavage efficiency. For each reaction, cleavage efficiency was estimated from the ratio of cleaved band intensity to total band intensity. Red circles indicate ^{S}C.

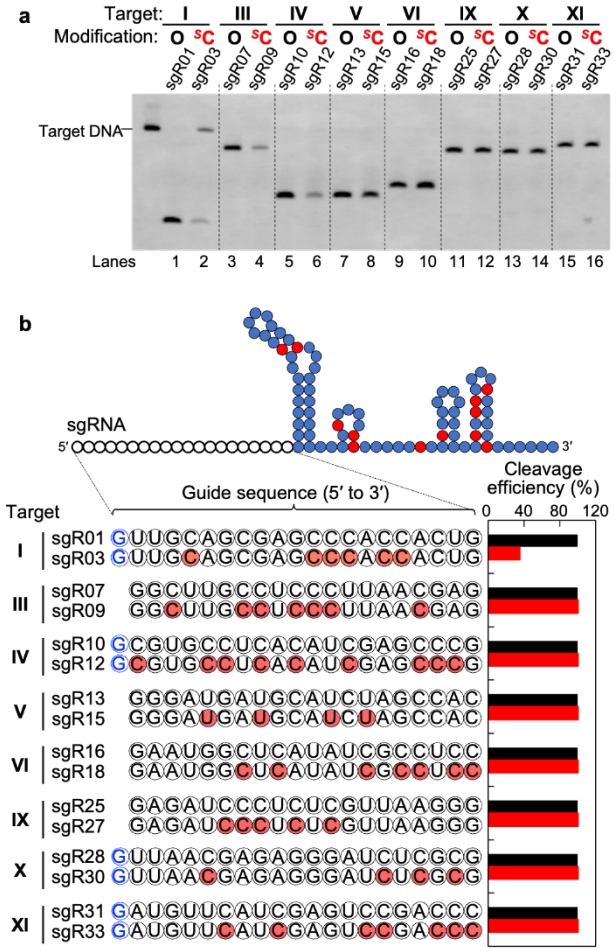

Fig. S3. Thermal stability of target DNA–sgRNA duplexes. Prior to the thermal denaturation study, a solution containing sgRNA and complementary DNA sequence (0.5 μM) in a buffer comprising 20 mM cacodylate (pH 7.0), 150 mM KCl, and 1 mM $MgCl_2$ (total volume 100 μL) was heated at 90 °C for 3 minutes, and then cooled gradually to room temperature. Samples were then heated at 0.5 °C/min.

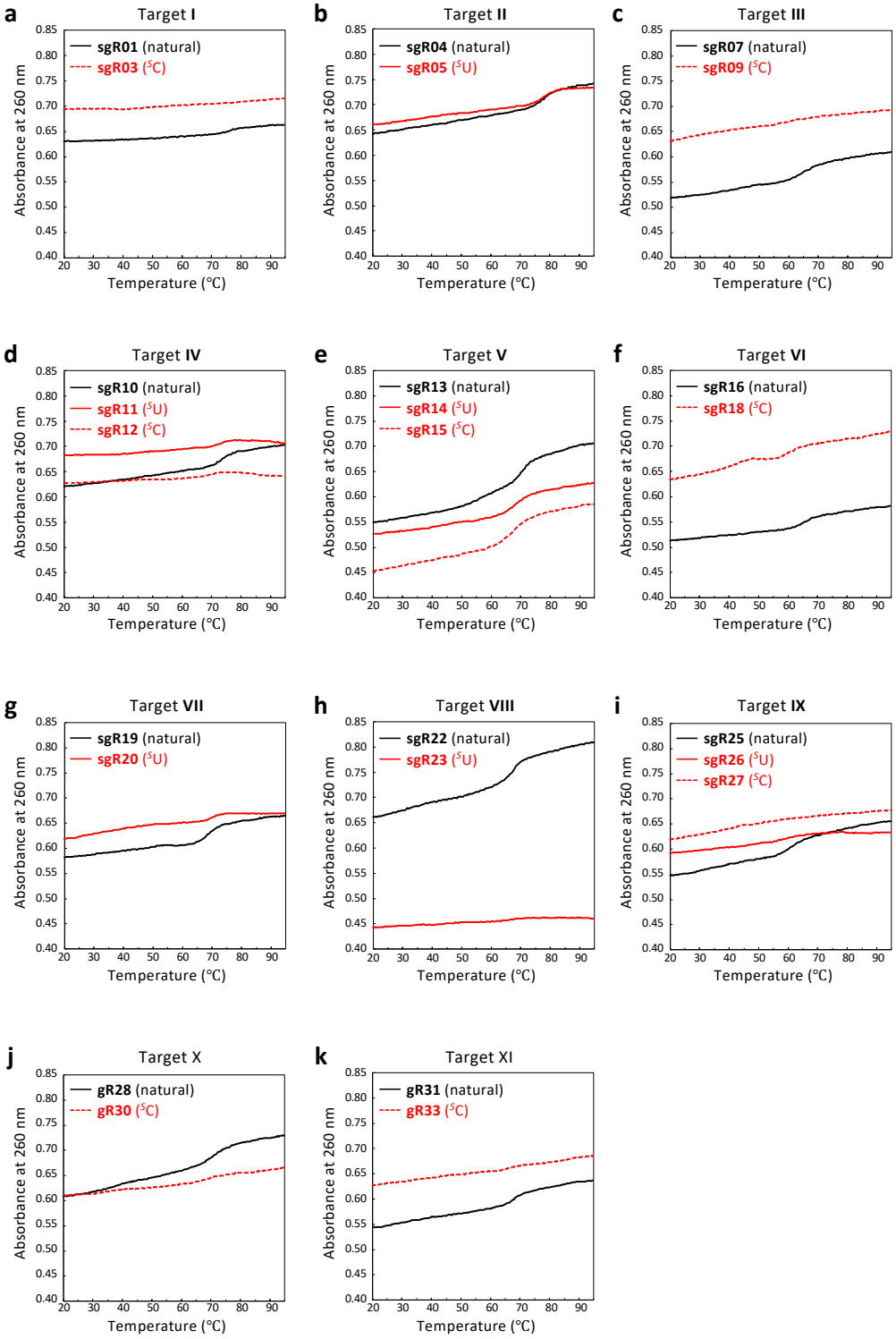

Table S1. HRMS analysis of transcribed sgRNAs.

Target	sgRNA	Modification	Length of polyU tail[a]	Molecular formula	Calcd.	Anal.	MS error (Da)
I	sgR01	Natural	7	$C_{991}H_{1220}N_{393}O_{725}P_{102}$	33426.7860	33425.87216	0.9138
	sgR03	^{S}C	6	$C_{982}H_{1209}N_{391}O_{697}P_{102}S_{20}$	33441.9320	33441.62794	0.3041
II	sgR04	Natural	6	$C_{992}H_{1219}N_{394}O_{728}P_{103}$	33499.7936	33502.88111	–3.0875
	sgR05	^{S}U	7	$C_{992}H_{1219}N_{394}O_{698}P_{103}S_{30}$	33981.7616	33980.69410	1.0675
III	sgR07	Natural	6	$C_{971}H_{1195}N_{382}O_{713}P_{101}$	32753.3595	32746.80581	6.5537
	sgR09	^{S}C	6	$C_{971}H_{1195}N_{382}O_{694}P_{101}S_{19}$	33058.6059	33054.21607	4.3898
IV	sgR10	Natural	7	$C_{990}H_{1220}N_{391}O_{726}P_{103}$	33402.7613	33394.34774	8.4136
	sgR11	^{S}U	7	$C_{990}H_{1220}N_{391}O_{697}P_{103}S_{29}$	33868.6637	33864.98739	3.6763
	sgR12	^{S}C	7	$C_{990}H_{1220}N_{391}O_{705}P_{103}S_{21}$	33740.1389	33739.17047	0.9684
V	sgR13	Natural	7	$C_{983}H_{1207}N_{391}O_{718}P_{102}$	33146.6142	33138.47216	8.1420
	sgR14	^{S}U	7	$C_{983}H_{1207}N_{391}O_{688}P_{102}S_{30}$	33628.5822	33632.42417	–3.8420
	sgR15	^{S}C	7	$C_{983}H_{1207}N391O_{701}P102S_{17}$	33419.7294	33416.07364	3.6558
VI	sgR16	Natural	7	$C_{980}H_{1206}N_{384}O_{720}P_{102}$	33043.5261	33038.63016	4.8959
	sgR18	^{S}C	7	$C_{980}H_{1206}N_{384}O_{701}P_{102}S_{19}$	33348.7725	33350.39057	–1.6181
VII	sgR19	Natural	7	$C_{992}H_{1218}N_{393}O_{728}P_{103}$	33484.7790	33477.06701	7.7120
	sgR20	^{S}U	7	$C_{992}H1_{218}N_{393}O_{697}P_{103}S_{31}$	33982.8126	33982.84846	–0.0359
VIII	sgR22	Natural	7	$C_{983}H_{1208}N_{392}O_{716}P_{102}$	33129.6300	33127.18121	2.4488
	sgR23	^{S}U	7	$C_{983}H_{1208}N_{392}O_{687}P_{102}S_{29}$	33595.5324	33594.99630	0.5361
IX	sgR25	Natural	7	$C_{982}H_{1206}N_{388}O_{720}P_{102}$	33123.5743	33115.93081	7.6435
	sgR26	^{S}U	6	$C_{973}H_{1195}N_{386}O_{682}P_{101}S_{30}$	33299.3763	33298.97875	0.3976
	sgR27	^{S}C	6	$C_{973}H_{1195}N_{386}O_{695}P_{101}S_{17}$	33090.5235	33090.55469	–0.0312
X	sgR28	Natural	8	$C_{1003}H_{1230}N_{400}O_{733}P_{104}$	33838.0096	33839.93729	–1.9277
	sgR30	^{S}C	8	$C_{1004}H_{1230}N_{400}O_{717}P_{104}S_{16}$	34107.0699	34107.95262	–0.8827
XI	sgR31	Natural	8	$C_{990}H_{1218}N_{389}O_{727}P_{103}$	33388.7314	33379.86521	8.8661
	sgR33	^{S}C	8	$C_{990}H_{1218}N_{389}O_{708}P_{103}S_{19}$	33693.9778	33693.10720	0.8706

[a]The length of polyU tail in the mainly identified sgRNAs.

Table S2. Sequences of oligodeoxynucleotides (ODNs) used in this study.

ODNs		Sequence (5′ to 3′)
1	template for PCR of *in vitro* transcription template	d(aaaaaagcaccgactcggtgccacttttcaagttgataacggactagccttatttaaacttgctatttctagctcttaaac)
2	reverse primer for PCR of *in vitro* transcription template	d(aaaaaagcaccgactcggt)
3	forward primer for PCR of target I	d(cgcggatcctaatacgactcactatagttgcagcgagcccaccactggtttaagagctagaa)
4	forward primer for PCR of target II	d(cgcggatcctaatacgactcactatagccacgactgggggcttgtcgtttaagagctagaa)
5	forward primer for PCR of target III	d(cgcggatcctaatacgactcactataggcttgcctcccttaacgaggtttaagagctagaa)
6	forward primer for PCR of target IV	d(cgcggatcctaatacgactcactatagcgtgcctcacatcgagcccggtttaagagctagaa)
7	forward primer for PCR of target V	d(cgcggatcctaatacgactcactatagggatgatgcatctagccacgtttaagagctagaa)
8	forward primer for PCR of target VI	d(cgcggatcctaatacgactcactatagaatggctcatatcgcctccgtttaagagctagaa)
9	forward primer for PCR of target VII	d(cgcggatcctaatacgactcactatagcgtccatgctgagagtgtcggtttaagagctagaa)
10	forward primer for PCR of target VIII	d(cgcggatcctaatacgactcactatagacactctcagcatggacgagtttaagagctagaa)
11	forward primer for PCR of target IX	d(cgcggatcctaatacgactcactatagagatccctctcgttaaggggtttaagagctagaa)
12	forward primer for PCR of target X	d(cgcggatcctaatacgactcactatagttaacgagagggatctcgcggtttaagagctagaa)
13	forward primer for PCR of target XI	d(cgcggatcctaatacgactcactatagatgttcatcgagtccgacccgtttaagagctagaa)
14	forward primer for PCR of target V [α]	d(cgcggatcctaatacgactca)
15	forward primer for PCR of target DNA	FITC-d(aagcttgattcttctgacacaacagtc)
16	reverse primer for PCR of target DNA	Cy3-d(ctcacacaaaaaaccaacacacagatg)
17	DNA of target I for T_m measurement	d(cagtggtgggctcgctgcaa)
18	DNA of target II for T_m measurement	d(gacaagccccccagtcgtgg)
19	DNA of target III for T_m measurement	d(ctcgttaagggaggcaagcc)
20	DNA of target IV for T_m measurement	d(cgggctcgatgtgaggcacg)
21	DNA of target V for T_m measurement	d(gtggctagatgcatcatccc)
22	DNA of target VI for T_m measurement	d(ggaggcgatatgagccattc)
23	DNA of target VII for T_m measurement	d(cgacactctcagcatggacg)
24	DNA of target VIII for T_m measurement	d(tcgtccatgctgagagtgtc)
25	DNA of target IX for T_m measurement	d(cccttaacgagagggatctc)
26	DNA of target X for T_m measurement	d(cgcgagatccctctcgttaa)
27	DNA of target XI for T_m measurement	d(gggtcggactcgatgaacat)

[*] The *in vitro* transcription template for target **V** was prepared by PCR using ODN14 and ODN2 after PCR using ODN7 and ODN2.

Experimental procedures

General

4′-Thiouridine 5′-triphosphates (^{S}UTPs) and 4′-thiocytidine 5′-triphosphates (^{S}CTPs) were prepared according to our previous reports.[21] Natural dNTPs and rNTPs were purchased from GE Healthcare Japan (Tokyo, Japan). Oligodeoxynucleotides (ODNs) were chemically synthesized by FASMAC (Kanagawa, Japan).

PCR amplification of the DNA template for in vitro transcription

The reaction mixture contained 1.6 nM template (ODN1), 0.2 mM primers (forward primer, ODN3–ODN13; reverse primer, ODN2), and 1× PrimeSTAR Max Premix (Takara Bio, Shiga, Japan) in a volume of 50 μL. The PCR cycling conditions were 33 cycles of denaturation at 98 °C for 10 seconds, and annealing and extension at 68 °C for 10 seconds. The reaction products were analyzed by 2% agarose gel electrophoresis and purified by a High Pure PCR Product Purification Kit (Roche, Basel, Switzerland) and ethanol precipitation.

T7 transcription with ^{S}UTP or ^{S}CTP

Each DNA template (50 ng) was incubated with T7 RNA polymerase (5 units/μL, Takara Bio) in 40 mM Tris-HCl (pH 8.0), 8 mM $MgCl_2$, 2 mM spermidine, RNase Inhibitor (1 unit/μL, Takara Bio), and rNTPs (each 2 mM) containing r^{S}UTP or r^{S}CTP at 42 °C for 2 hours. The DNA template was then removed by treatment with DNase I (0.5 units/μL, Takara Bio) at 37 °C for 30 minutes. The reaction mixture was analyzed by 5% denaturing PAGE (7 M urea) with SYBR Green II (Takara Bio) staining. The reaction solution was mixed with 2 × gel loading solution [urea, 1 mM EDTA (pH 8.0), 20% (w/v) sucrose, 0.1% SDS, 0.05% (w/x) bromophenol blue, 0.05% (w/x) xylene cyanol, 0.09 M tris, and 0.09 M borate], and heated to 90 °C for 3 minutes before being loaded on the gel. The RNA transcripts were purified by using a Guide-it^{TM} IVT RNA Clean-up Kit (Takara Bio). The transcribed sgRNAs were characterized by HRMS analysis using Bioaccord (Waters, Massachusetts, USA), and the mathematical deconvolution of the raw data was performed by BayesSpray using UNIFI softoware (Waters).

Preparation of target DNA for in vitro genome cleavage assay.

Plasmid DNA containing the *Renilla* luciferase target gene (psiCHECK^{TM}-1 Vector, Promega, Wisconsine, USA) was digested with *Hind*III and *Bam*HI (Takara Bio) and purified by phenol–chloroform extraction,

followed by ethanol precipitation. The resulting product was amplified by PCR in a reaction mixture containing 60 ng of template, 1 μM primers labeled with FITC or Cy3 at the 5′-end (ODN15 and ODN16), and 1× PrimeSTAR Max DNA polymerase (Takara Bio) in a volume of 100 μL. The cycling conditions were 33 cycles of denaturation at 98 °C for 10 seconds, and annealing and extension at 68 °C for 10 seconds. The reaction products were analyzed by 1% agarose gel electrophoresis and purified by phenol–chloroform extraction and ethanol precipitation.

In vitro DNA cleavage assay.

To form a Cas9 RNP complex, Cas9 (0.34 μM, NIPPON GENE, Tokyo, Japan) and sgRNA (0.36 μM) were incubated at room temperature for 5 minutes. Target DNA (360 ng) was combined with the Cas9 RNP complex in 1 × H buffer [50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, 100 mM NaCl], and incubated at 37 °C for 1 hour in a volume of 20 μL. The mixture was heated at 95 °C for 5 minutes, and then purified by High Pure PCR Product Purification Kit (Roche), followed by ethanol precipitation. The precipitate mixed with 5 × gel loading solution [50 mM EDTA, 0.25% (w/v) SDS, 15% (w/v) Ficoll 400, 0.1% (w/v) bromophenol blue) and analyzed by 3% native PAGE. The cleaved bands were visualized by a Molecular Imager FXpro system (BioRad, Hercules, CA, USA), and band intensities were measured by using Quantity One Software (BioRad).

Thermal denaturation monitored by UV absorbance.

Thermally induced transitions were monitored at 260 nm on a Shimadzu UV-1800 UV-Visible spectrophotometer (Kyoto, Japan). Samples were heated at a ramp rate of 0.5 °C/min while monitoring UV absorbance at 260 nm every 0.5 °C. Before thermal denaturation, the samples were prepared as follows:

(i) For the Cas9–sgRNA RNP: a solution containing Cas9 either alone or complexed to sgRNA (0.5 μM each) in 20 mM cacodylate (pH 7.0), 150 mM KCl, and 1 mM $MgCl_2$ (total 100 μL) was incubated at room temperature for 10 minutes.

(ii) Duplex formation: a solution containing sgRNA and the cDNA sequence (0.5 μM each, ODN17－ODN27) in 20 mM cacodylate (pH 7.0), 150 mM KCl, and 1 mM $MgCl_2$ (total 100 μL) was heated at 90 °C for 3 minutes, and then cooled gradually to room temperature.