Diazirine-containing RNA photocrosslinking probes for the study of siRNA-protein interactions

Satoru Kuboe[a], Mayuko Yoda[c,d], Aya Ogata[a], Yukio Kitade[a,b], Yukihide Tomari*[c,d] and Yoshihito Ueno*[a,b]

[a]Department of Biomolecular Science, Faculty of Engineering, Gifu University; [b]United Graduate School of Drug Discovery and Medical Information Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan; [c]Institute of Molecular and Cellular Biosciences, The University of Tokyo, Tokyo, 113-0032, Japan; [d]Department of Medical Genome Sciences, The University of Tokyo, 113-0032, Japan

Supplementary Data

General remarks

Thin-layer chromatography was carried out on Merck coated plates $60F_{254}$. Silica gel column chromatography was carried out on Wakogel C-300. ^{1}H, ^{13}C, and ^{19}F NMR spectra were obtained with a JEOL JNM AL–400 spectrometer. $CDCl_3$ (CIL) or DMSO-d_6 (CIL) was used as a solvent for obtaining NMR spectra. Chemical shifts (δ) are given in parts per million (ppm) downfield from $(CH_3)_4Si$ (δ 0.00 for ^{1}H NMR in $CDCl_3$), CF_3CO_2H (δ 0.00 for ^{19}F NMR), or a solvent (for ^{13}C NMR and ^{1}H NMR in DMSO-d_6) as an internal reference with coupling constants (J) in Hz. The abbreviations s, d, and q signify singlet, doublet, and quartet, respectively.

3′,5′-Bis(*tert*-butyldimethylsilyloxymethyl)-2,2,2-trifluoromethylacetophenone (5). To a solution of 1,3-bis(*tert*-butyldimethylsilyloxymethyl)-5-iodobenzene (**4**)[1] (3.94 g, 8.00 mmol) in THF (80 mL) at –78 ºC was added dropwise over 30 min *n*-BuLi (1.65 M in hexane, 10.2 ml, 16.8 mmol). The solution was stirred for 15 min then ethyl trifluoroacetate (1.14 ml, 9.58 mmol) was added over 15 min. The resulting mixture was stirred at –78 ºC for 1 h, quenched at –78 ºC using saturated $NaHCO_3$ (50 mL) then extracted three times with EtOAc. The combined organic layer was washed with saturated $NaHCO_3$, brine, dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography (SiO_2, 70% toluene in hexane) to give **5** (2.46 g, 5.32 mmol, 67%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.13 (s, 12H), 0.97 (s, 18H), 4.80 (s, 4H), 7.65 (s, 1H), 7.92 (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ –5.4, 18.3, 25.9, 64.2, 116.7 (q, $^{1}J_{C–F}$ = 311 Hz), 126.0, 129.9, 130.5, 142.8, 180.2 (q, $^{2}J_{C–F}$ = 31 Hz); ^{19}F NMR (372 MHz, $CDCl_3$) δ –87.5. Anal. Calcd for $C_{22}H_{37}F_3O_3Si_2$: C, 57.11; H, 8.06. Found: C, 57.17; H,

7.84.

3′,5′-Bis(*tert*-butyldimethylsilyloxymethyl)-2,2,2-trifluoromethylacetophenone *O*-tosyl-oxime (6). To a solution of **5** (0.47 g, 1.00 mmol) in pyridine (5 mL) and EtOH (5 mL) was added $HONH_2$•HCl (0.10 g, 1.44 mmol). The resulting mixture was srirred at 60 ºC overnight, cooled to room temperature and concentrated. The residual oil was dissolved in $CHCl_3$. The organic layer was washed with H_2O, brine, dried (Na_2SO_4), and concentrated. The residual oil was dissolved in $CHCl_3$ (10 mL) then Et_3N (0.41 g, 4.05 mmol), a catalytic amount of DMAP and *p*-toluenesulfonyl chloride (0.28 g, 1.47 mmol) were added. The final mixture was allowed to react at room temperature overnight. The volatiles were evaporated and the residue was dissolved in $CHCl_3$. The organic layer was washed with saturated $NaHCO_3$, brine, dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography (SiO_2, 17% MeOH in $CHCl_3$) to give **6** (0.54 g, 0.85 mmol, 85 %): ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.10 (s, 12H), 0.94 (s, 18H), 2.46 (s, major stereoisomer, 3/5H), 2.48 (s, minor stereoisomer, 2/5H), 4.81–4.73 (m, 4H), 7.92–7.18 (m, 7H); ^{19}F NMR (372 MHz, $CDCl_3$) δ –87.5. Anal. Calcd for $C_{29}H_{44}F_3NO_5SSi_2$: C, 55.12; H, 7.02; N, 2.22. Found: C, 54.93; H, 7.12; N, 2.23.

3-[3,5-Bis(*tert*-butyldimethylsilyloxymethyl)phenyl]-3-trifluoromethyldiaziridine (7). NH_3 gas was bubbled through a solution of oxime **6** (0.33g, 0.52mmol) in 5% NH_3/THF (30 mL) at –78 ºC. The tube was sealed and the resulting solution was stirred at room temperature for 2 days. After cooling the mixture, the tube was opened and the excess NH_3 was allowed to escape slowly. The mixture was concentrated. The residue was purified by column chromatography (SiO_2, 10–30% EtOAc in hexane) to give **7** (0.15g, 0.31 mmol, 60 %): ^{1}H NMR ($CDCl_3$) δ 0.11 (s, 12H), 0.94 (s, 18H), 2.20 (d, 1H, J = 8 Hz), 2.77 (d, 1H, J = 8 Hz), 4.76 (s, 4H), 7.39 (s, 1H), 7.45 (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ –5.3, 18.4, 25.9, 58.0 (q, $^2J_{C–F}$ = 35 Hz), 64.5, 123.5 (q, $^1J_{C–F}$ = 279 Hz), 124.1, 125.2, 131.5, 142.3; ^{19}F NMR (372 MHz, $CDCl_3$) δ –91.6. Anal. Calcd for $C_{22}H_{39}F_3N_2O_2Si_2$: C, 55.43; H, 8.25; N, 5.88. Found: C, 55.64; H, 8.01; N, 5.77.

3-[3,5-Bis(*tert*-butyldimethylsilyloxymethyl)phenyl]-3-trifluoromethyl-3*H*-diazirine (8). To a solution of diaziridine **7** (95 mg, 0.20 mmol) in MeOH (4 mL) was added Et_3N (70 μL, 0.50 mmol) and I_2 (56 mg, 0.22 mmol). The whole was stirred at room temperature for 30 min. The mixture was partitioned between Et_2O and aqueous $Na_2S_2O_3$. The organic layer was washed with brine, dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography (SiO_2, 10% EtOAc in hexane) to give **8** (70 mg, 0.15 mmol, 74%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.10 (s, 12H), 0.95 (s, 18H), 4.72 (s, 4H), 7.03 (s, 2H), 7.31 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ –5.4, 18.4,

25.9, 28.52 (q, $^2J_{C–F}$ = 40 Hz), 64.4, 122.2 (q, $^1J_{C–F}$ = 276 Hz), 122.4, 124.5, 129.4, 142.4; ^{19}F NMR (372 MHz, $CDCl_3$) δ –81.2. Anal. Calcd for $C_{22}H_{37}F_3N_2O_2Si_2$: C, 55.66; H, 7.86; N, 5.90. Found: C, 55.50; H, 7.69; N, 5.92.

3-[3,5-Bis(hydroxymethyl)phenyl]-3-trifluoromethyl-3*H*-diazirine (9). To a solution of diazirine **8** (91 mg, 0.19 mmol) in THF (3.8 mL) was added TBAF (1 M in THF, 0.4 mL), and the mixture was stirred at room temperature for 2 h. The solvent was evaporated in vacuo, and the resulting residue was purified by column chromatography (SiO_2, 10–50% EtOAc in hexane) to give **9** (40 mg, 0.16 mmol, 85%): ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.20 (s, 2H), 4.52 (s, 4H), 7.06 (s, 2H), 7.33 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 28.1 (q, $^2J_{C–F}$ = 40 Hz), 62.2, 122.0 (q, $^1J_{C–F}$ = 275 Hz), 122.2, 126.0, 127.3, 143.9; ^{19}F NMR (372 MHz, DMSO-d_6) δ –80.3. Anal. Calcd for $C_{10}H_9F_3N_2O_2 \cdot 1/10H_2O$: C, 48.43; H, 3.74; N, 11.30. Found: C, 48.45; H, 3.83; N, 11.11.

3-[3-(4,4′-dimethoxytrityl)oxymethyl-5-hydroxymethylphenyl]-3-trifluoromethyl-3*H*-diazirine (10). A mixture of **9** (0.20 g, 0.81 mmol) and DMTrCl (0.40 g, 1.18 mmol) in pyridine (11 mL) was stirred at room temperature for 5 h. The mixture was partitioned between EtOAc and aqueous $NaHCO_3$ (saturated). The organic layer was washed with brine, dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography (SiO_2, 40% EtOAc in hexane) to give **10** (0.17 g, 0.31 mmol, 38%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.78 (s, 6H), 4.17 (s, 2H), 4.67 (d, 2H, *J* = 4 Hz), 6.80–7.47 (m, 16H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 28.4 (q, $^2J_{C–F}$ = 40 Hz), 55.2, 64.7, 65.0, 86.7, 113.2, 122.1 (q, $^1J_{C–F}$ = 274 Hz), 123.3, 124.1, 126.5, 126.9, 127.9, 128.1, 129.3, 130.0, 136.0, 140.7, 141.7, 144.8, 158.5; ^{19}F NMR (372 MHz, $CDCl_3$) δ –98.0.

Solid Support Synthesis. A mixture of **10** (0.17 g, 0.31 mmol), succinic anhydride (93 mg, 0.93 mmol), and DMAP (2.4 mg, 20 μmol) in pyridine (3 mL) was stirred at room temperature. After 2 days, the solution was partitioned between $CHCl_3$ and H_2O, and the organic layer was washed with H_2O and brine. The separated organic phase was dried (Na_2SO_4) and concentrated to give a succinate. Aminopropyl controlled pore glass (0.65 g, 78 μmol) was added to a solution of the succinate and EDCI (60 mg, 0.31 mmol) in DMF (8 mL), and the mixture was kept for 3 days at room temperature. After the resin was washed with pyridine, a capping solution (15 mL, 0.1 M DMAP in pyridine:Ac_2O = 9:1, v/v) was added and the whole mixture was kept for 12 h at room temperature. The resin was washed with MeOH and acetone, and dried *in vacuo*. Amount of loaded compound **10** to solid support was 28 μmol/g from calculation of released dimethoxytrityl cation by a solution of 70% $HClO_4$:EtOH (3:2, v/v).

RNA Synthesis. Synthesis was carried out with a DNA/RNA synthesizer by phosphoramidite method. Deprotection of bases and phosphates was performed in

concentrated NH_4OH:EtOH (3:1, v/v) at room temperature for 12 h. 2′-TBDMS groups were removed by TBAF (Aldrich) in THF at room temperature for 12 h. The reaction was quenched with 0.1 M TEAA buffer (pH 7.0) and desalted on a Sep-Pak C18 cartridge. Deprotected ONs were purified by 20% PAGE containing 7 M urea to give the highly purified ON**5** (14), ON**9** (11). The yields are indicated in parentheses as OD units at 260 nm starting from 1.0 μmol scale.

MALDI-TOF/MS Analysis of RNAs. Spectra were obtained with a SHIMAZU/KRATOS time-of-flight mass spectrometer equipped with a nitrogen laser (337 nm, 3-ns pulse). A solution of 3-hydroxypicolinic acid (3-HPA) and diammonium hydrogen citrate in H_2O was used as the matrix. ON**5**: *m/z* = 6708.2 ($[M–H]^-$, calculated 6707.9; $C_{203}H_{243}F_3N_{84}O_{136}P_{20}$ (MW = 6708.9). ON**9**: *m/z* = 6790.0 ($[M–H]^-$, calculated 6792.9; $C_{203}H_{242}F_3N_{81}O_{144}P_{20}$ (MW = 6793.9).

Dual-Luciferase Assay. HeLa cells were grown at 37 °C in a humidified atmosphere of 5% CO_2 in air in Minimum Essential Medium (MEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS). Twenty-four hours before transfection, HeLa cells (4×10^4/mL) were transferred to 96-well plates (100 μL per well). They were transfected, using TransFast (Promega), according to instructions for transfection of adherent cell lines. Cells in each well were transfected with a solution (35 μL) of 20 ng of psiCHECK-2 vector (Promega), the indicated amounts of siRNAs, and 0.3 μg of TransFast in Opti-MEM I Reduced-Serum Medium (Invitrogen), and incubated at 37 °C. Transfection without siRNA was used as a control. After 1 hour, MEM (100 μL) containing 10% FBS and antibiotics was added to each well, and the whole was further incubated at 37 °C. After 24 h, cell extracts were prepared in Passive Lysis Buffer (Promega). Activities of firefly and *Renilla* luciferases in cell lysates were determined with a dual-luciferase assay system (Promega) according to a manufacturer's protocol. The results were confirmed by at least three independent transfection experiments with two cultures each and are expressed as the average from four experiments as mean ± SD.

In vitro RISC assembly and photocrosslinking. *Drosophila* embryo lysate, lysis buffer, and 40x reaction mix were prepared as described before.[2] RISC assembly was typically performed in 50 μL reaction, containing 25 μL of embyo lysate, 15 μL of 40× reaction mix and 5 μL of 100 nM ^{32}P radiolabeled siRNA duplexes at 25 ºC. 10-μL aliquots were then taken at indicated time points, irradiated with 302 nm UV-B or 365 nm UV-A for 5 min (~1 cm under 6W UV-B or UV-A bulb), and subjected to SDS-PAGE.

References for supplementary data

1. Y. Ueno, A. Kawamura, K. Takasu, S. Komatsuzaki, T. Kato, S. Kuboe, Y. Kitamura and Y. Kitade, *Org. Biomol. Chem.*, 2009, **7**, 2761–2769.
2. B. Haley, G. Tang and P. D. Zamore, *Methods*, 2003, **30**, 330–336.