Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Assembly of Long L-RNA by Native RNA Ligation

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S1. Supplementary Text.

Materials and Methods.

General. Oligonucleotides were either purchased from Integrated DNA Technologies (Coralville, IA) or prepared by solid-phase synthesis on an Expedite 8909 DNA/RNA Synthesizer. D-Nucleoside phosphoramidites and solid supports, as well as all synthesis reagents were purchased from Glen Research (Sterling, Va). L-Nucleoside phosphoramidites and solid supports were purchased from ChemGenes (Wilmington, Ma). T4 RNA ligase and RNA length marker were purchased from New England Biolabs (Ipswich, MA). All other reagents were purchased from Sigma Aldrich (St. Louis, MO).

Oligonucleotide synthesis and purification. All L-oligonucleotides were synthesized in house using protocols recommended by the manufacturer. Donor strands were phosphorylated at their 5′ ends using the chemical phosphorylation reagent (Glen Research, Sterling, Va) following procedures recommended by the manufacturer. Terminal labeling of oligonucleotides with cyanine3 (Cy3) was accomplished using the Cy3 phosphoramidite (Glen Research, Sterling, Va) following procedures recommended by the manufacture. Prior to use, oligonucleotides were purified by 20% denaturing polyacrylamide gel electrophoresis (PAGE; 19:1 acrylamide:bisacrylamide). Purified oligonucleotides were excised from the gel and eluted overnight at 23 °C in a buffer consisting of 200 mM NaCl, 10 mM EDTA, and 10 mM Tris (pH 7.6). The solution was then filtered to remove gel fragments, and eluted oligonucleotides were concentrated using an Amicon Ultra Centrifugal Filter (MilliporeSigma, Burlington, MA) having a membrane pore size of 3 kDa. Following concentration, all samples were desalted by ethanol precipitation, and the concentration determined by absorbance at 260 nm on a NanoDrop 2000c (ThermoFisher, Waltham, MA).

Ligation of L-RNA. For small-scale optimization experiments, ligation reaction mixtures contained 2 μM of the acceptor strand, 4 μM of the donor strand, T4 Buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 10%-40% PEG 8000 (v/v %), 10%-30% DMSO (v/v %)), 1 mM ATP, and 10 U/μL T4 RNA Ligase 1, and were incubated at the indicated temperature for 24 hours. Ligation products were resolved by 20% denaturing PAGE (19:1). The gel was imaged by fluorescence emission (Cy3; excitation/emission: 550 nm/570 nm) using a Typhoon FLA9500 Multimode Imager and quantified using ImageQuant TL software (GE Healthcare Life Sciences, Marlborough, MA).

Large-scale ligation reactions were carried out similarly, using T4 Buffer containing 40% PEG 8000 and 10% DMSO. The concentrations of all other reaction components were the same as listed for small-scale reactions. After incubation at 23 °C for 24 hours, the ligated L-oligonucleotide was purified by 20% denaturing PAGE (19:1 acrylamide:bisacrylamide) and processed as describe above for synthetic oligonucleotides.

Hammerhead ribozyme (HRz) cleavage assay. Cleavage reaction mixtures contained 450 nM of either D- or L-HRz RNA, 450 nM of either D- or L-S, 100 mM NaCl, and 50 mM Tris (pH 7) in a final volume of 20 µL. Were indicated, 10% fetal bovine serum (FBS) was also added to the reaction mixture. For the non-ligated control (split), 450 nM of each L-RNA donor (HRz-D) and acceptor (HRz-A) were used instead of L-HRz (Table S1). The cleavage reaction was initiated by the addition of 5 mM $MgCl_2$ (final concentration). The reaction was incubated at 23 °C and aliquots (2 µL) were removed at the indicated time points. Cleavage products were resolved by 10% denaturing PAGE (19:1 acrylamide:bisacrylamide) and gel was imaged by fluorescence emission (SYBR gold; excitation/emission: 495 nm/537 nm) using a Typhoon FLA9500 Multimode Imager and quantified using ImageQuant TL software.

Electrophoretic mobility shift assay (EMSA). Dissociation constants for aptamer–TAR complexes were determined as previously described.[1] Briefly, the indicated concentration of either L-6-4t or L-6-4t_{LIG} was incubated with 1 nM of D-TAR RNA in a reaction mixture containing 50 mM NaCl, 10 mM $MgCl_2$, 25 mM Tris (pH 7.6), and 0.1 mg/mL yeast tRNA. The binding reactions were incubated at 23 °C for 30 min then resolved by 10% native PAGE (29:1, acrylamide:bis-acrylamide), which were run at 30 mA for 1 hour. The gel was imaged by fluorescence emission (Cy5; excitation/emission: 649 nm/670 nm) using Typhoon FLA9500 Multimode Imager and quantified using ImageQuant TL software.

Determination of HRz stability in serum. Either D-HRz or L-RHz (450 nM) were added to a reaction mixture containing 100 mM NaCl, 50 mM Tris (pH 7)) and 10% FBS and were incubated at 23°C. Aliquots (2 µL) were removed at the indicated time point and quenched by the addition of 8 µL formamide loading buffer. Degradation products were resolved by 10% denaturing PAGE (19:1 acrylamide:bisacrylamide) and visualized using SYBR Gold nucleic acid stain (Thermo Fisher, Waltham, MA). The stained gel was imaged using a Typhoon FLA9500 Multimode Imager (SYBR gold; excitation/emission: 495 nm/537 nm).

Analysis of the theophylline biosensor. Either D- or L-HRz_{Theo} (1 μM) was added to a reaction mixture containing 125 mM KCl, 40 mM HEPES (pH 7.4), 200 μM theophylline, and 20 uM DFHBI-1T. The cleavage reaction was initiated by the addition of 10 mM $MgCl_2$ (final concentration) and allowed to incubate for 2 hours at 23 °C. For gel analysis, an aliquot (5 μL) of the cleavage reaction was resolved by 10% denaturing PAGE (19:1 acrylamide:bisacrylamide) and visualized using SYBR Gold nucleic acid stain. The stained gel was imaged using a Typhoon FLA9500 Multimode Imager (SYBR gold; excitation/emission: 495/537 nm). For fluorescence analysis, the concentration of D- or L-HRz_{Theo} in the reaction mixture was reduced to 100 nM. Fluorescence emission was measured on a GloMax Discover multi-well plate reader from Promega Corp. (Madison, WI) with excitation/emission wavelengths at 473 nm/500-550 nm. The background fluorescence from DFHBI-1T alone was subtracted from the signals (a.u.) reported in Figure 4c.

S2. Supplementary Figures.

Figure S1.

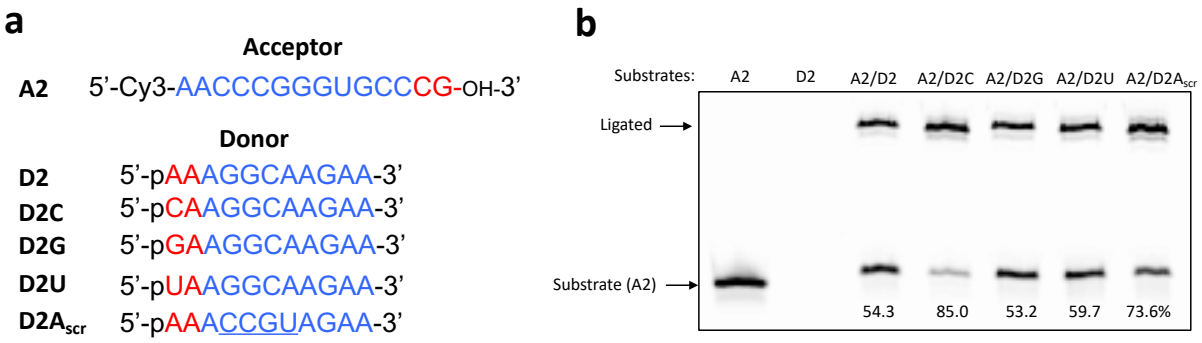

Figure S1. (a) Sequences of the acceptor (A2) and various donor substrates tested for ligation efficiency. Blue text: L-RNA; red text: D-RNA. The underlined bases in $D2A_{scr}$ were scrambled relative to D2 to prevent formation of a hairpin with A2. (b) Representative 20% PAGE gel showing the ligation of A2 (acceptor) and various donor sequences shown in (a). Reaction mixtures contained 2 μM of indicated RNA substrates, T4 Buffer substituted with 40% PEG 8000, and 25 U of T4 RNA ligase 1 and were incubated at 23 °C for 24 hours. The gel was visualized using the Cy3 on acceptor A2. Ligation yield are indicated at the bottom of the gel image.

Figure S2

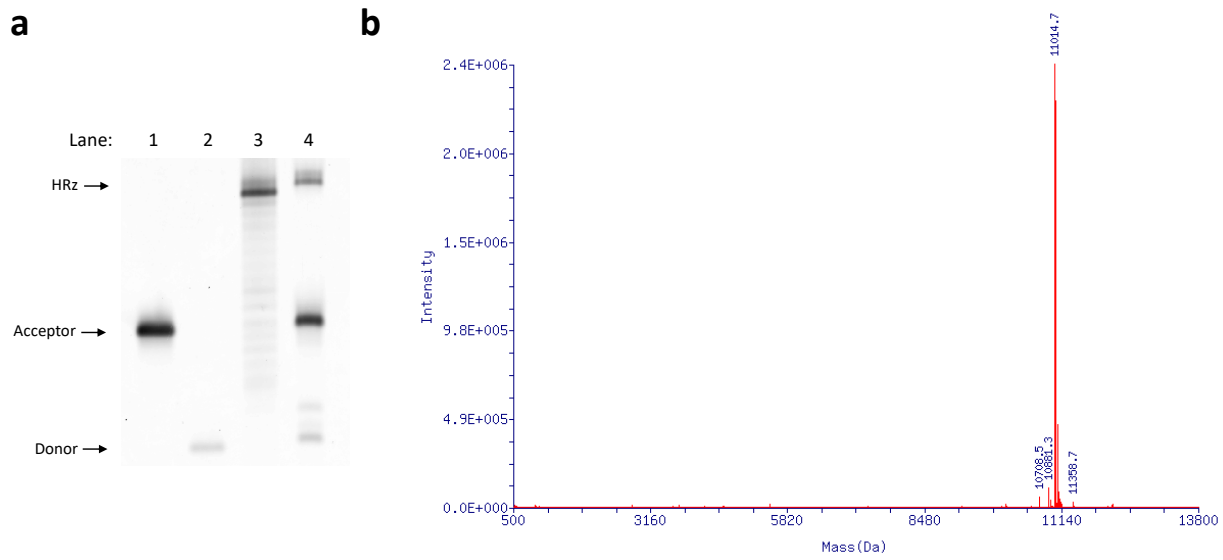

Figure S2. (a) Representative 20% PAGE gel showing the ligation of HRz-A (acceptor) and HRz-D (donor) (Table S1) to generate the full-length ribozyme L-HRz. The gel was visualized using SYBR Gold nucleic acid stain. Lane 1: HRz-A; lane 2: HRz-D; lane 3: D-HRz prepared by *in vitro* transcription; lane 4: ligation reaction to prepare L-HRz. (b) Mass analysis of L-HRz. Expected mass: 11013.7 Da; Observed mass: 11014.7 Da.

Figure S3

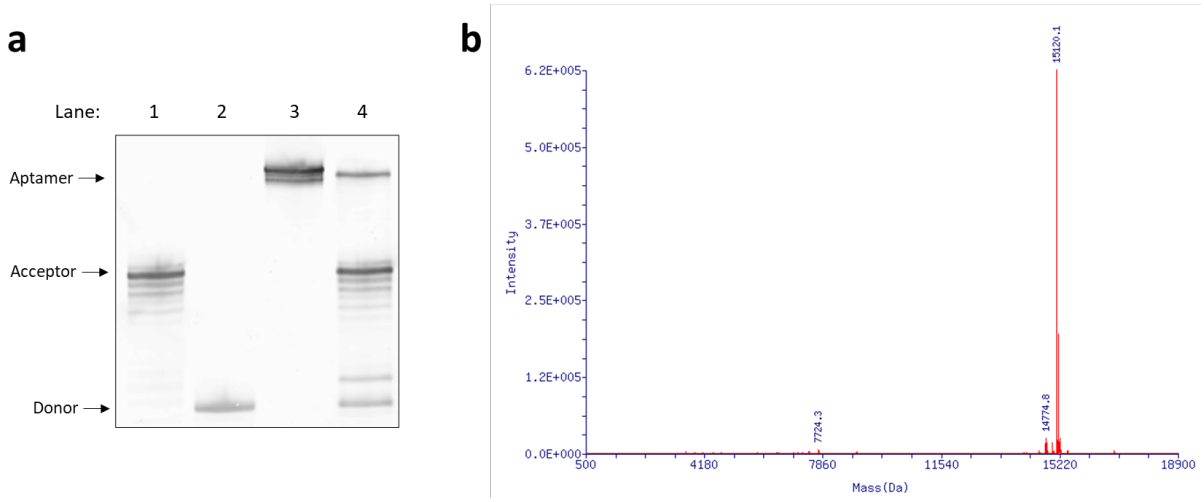

Figure S3. Representative 20% PAGE gel showing the ligation of 6-4t-A (acceptor) and 6-4t-D (donor) (Table S1) to generate the full-length aptamer L-6-4t$_{LIG}$. The gel was visualized using SYBR Gold nucleic acid stain. Lane 1: 6-4t-A; lane 2: 6-4t-D; lane 3: L-6-4t prepared by solid-phase synthesis; lane 4: ligation reaction to prepare L-6-4t$_{LIG}$. (b) Mass analysis of L-6-4t$_{LIG}$. Expected mass: 15119.3 Da; Observed mass: 15120.1 Da.

Figure S4

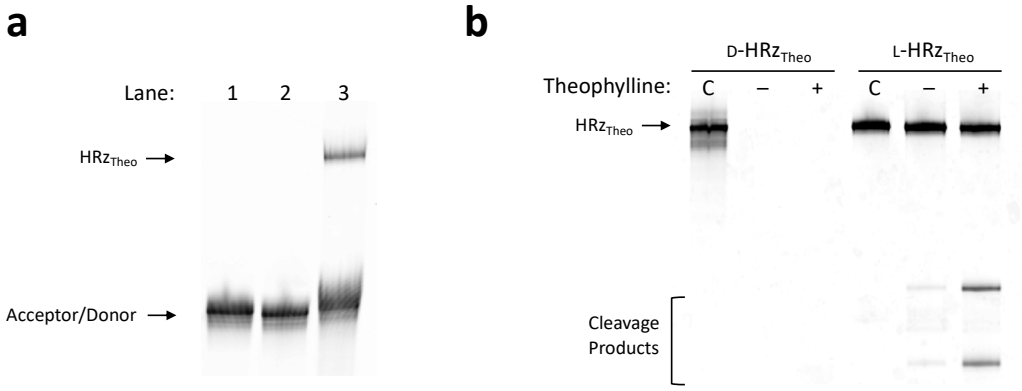

Figure S4. (a) Representative 10% PAGE gel showing the ligation of HRz_{Theo}-A (acceptor) and HRz_{Theo}-D (donor) (Table S1) to generate the full-length biosensor L-HRz_{Theo}. The gel was visualized using SYBR Gold nucleic acid stain. Lane 1: HRz_{Theo}-A; lane 2: HRz_{Theo}-D; lane 3: ligation reaction to generate L-HRz_{Theo}. (b) Stability of D- and L-HRz_{Theo} biosensors in 10% FBS. Either D- or L-HRz_{Theo} were added into a reaction mixture containing 125 mM KCl, 10 mM $MgCl_2$, 40 mM HEPES buffer (pH 7.4), 200 µM theophylline, 20 µM DFHBI-1T, and 10% FBS and incubated for 2 hours. Reaction products were resolved by 10% denaturing PAGE and visualized by SYBR Gold nucleic acid stain. C = Non-treated control for each D- or L-HRz_{Theo} sensor.

Figure S5

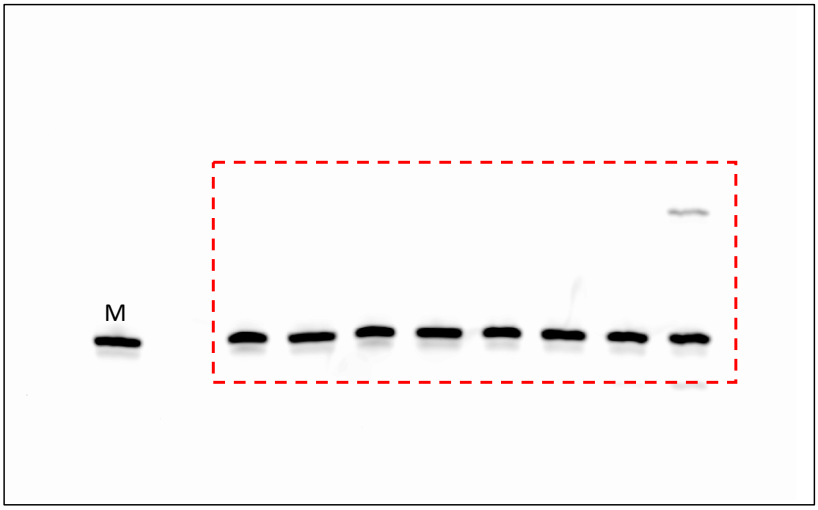

Figure S5. Uncropped gel images for Figure 1c. Red frame indicates the cropped region shown in Figure 1c. M = Marker (acceptor A1).

Figure S6

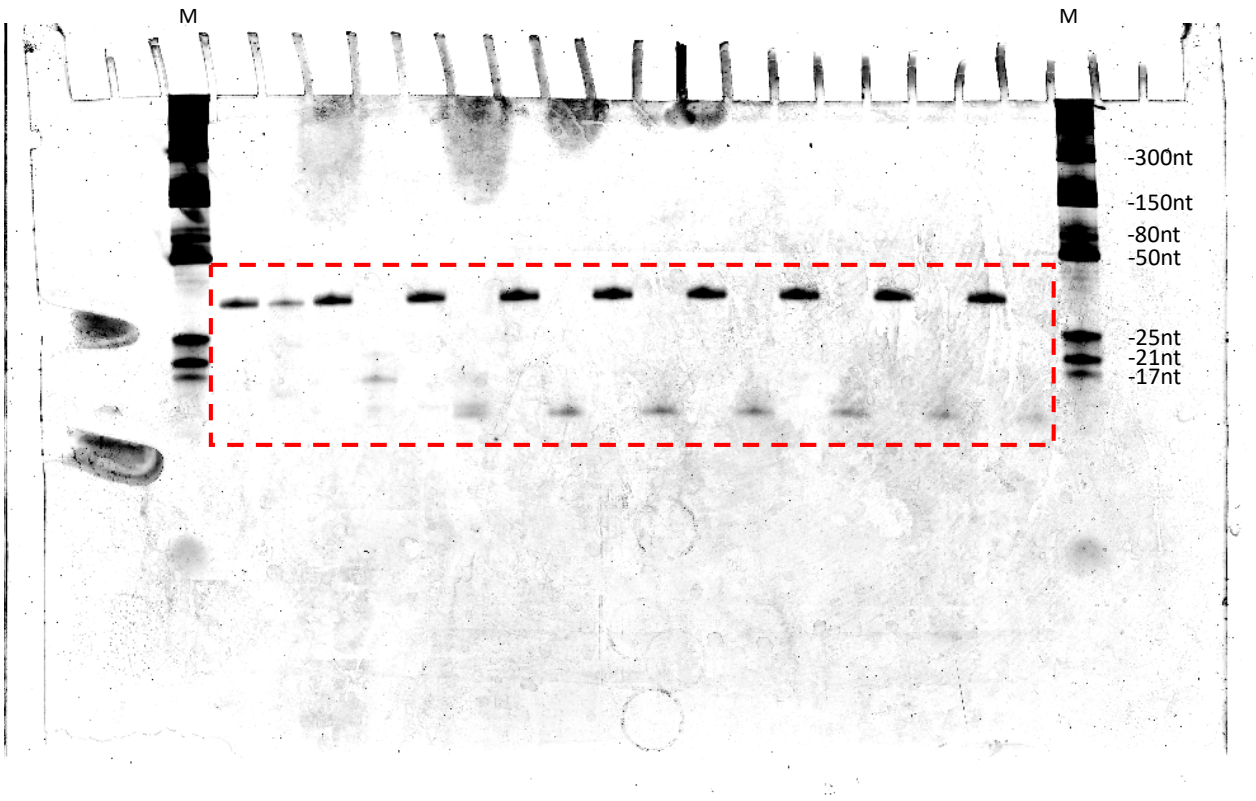

Figure S6. Uncropped gel images for Figure 2c. Red frame indicates the cropped region shown in Figure 2c. M = Size marker.

Figure S7

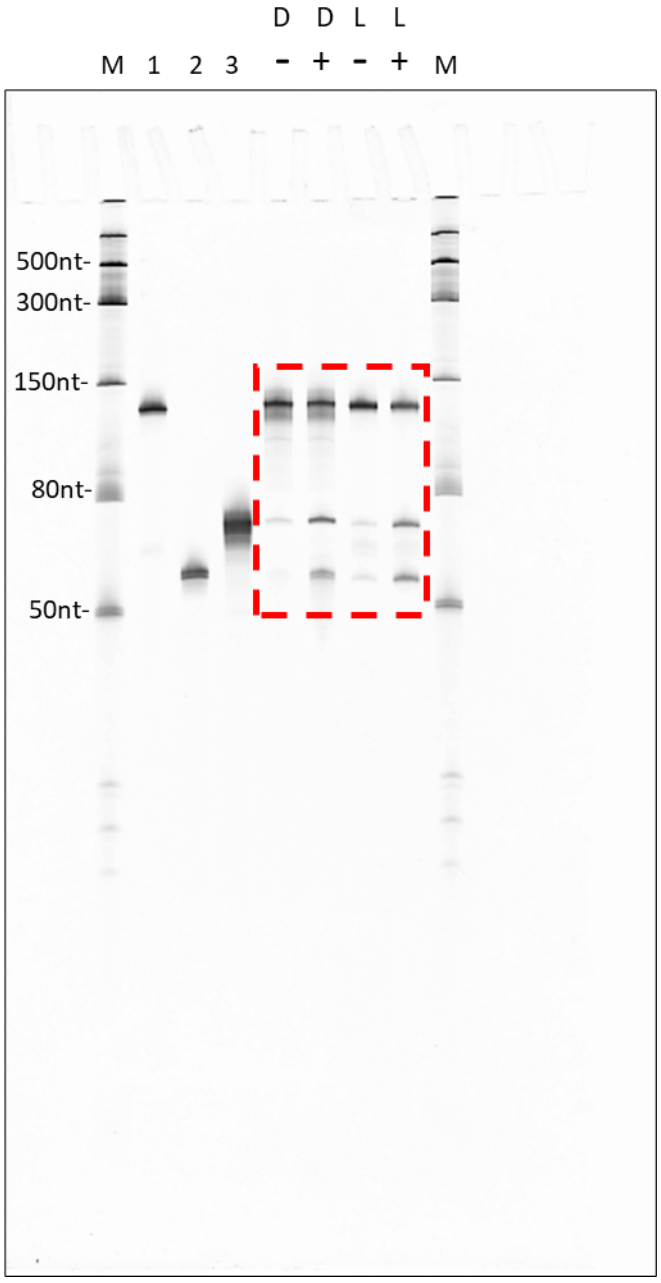

Figure S7. Uncropped gel images for Figure 4b. Red frame indicates the cropped region shown in Figure 4b. M = Size marker. Lane 1: HRz_{theo}, lane 2: *in vitro* transcribed broccoli aptamer, lane 3: cleaved HRz_{theo}.

Figure S8

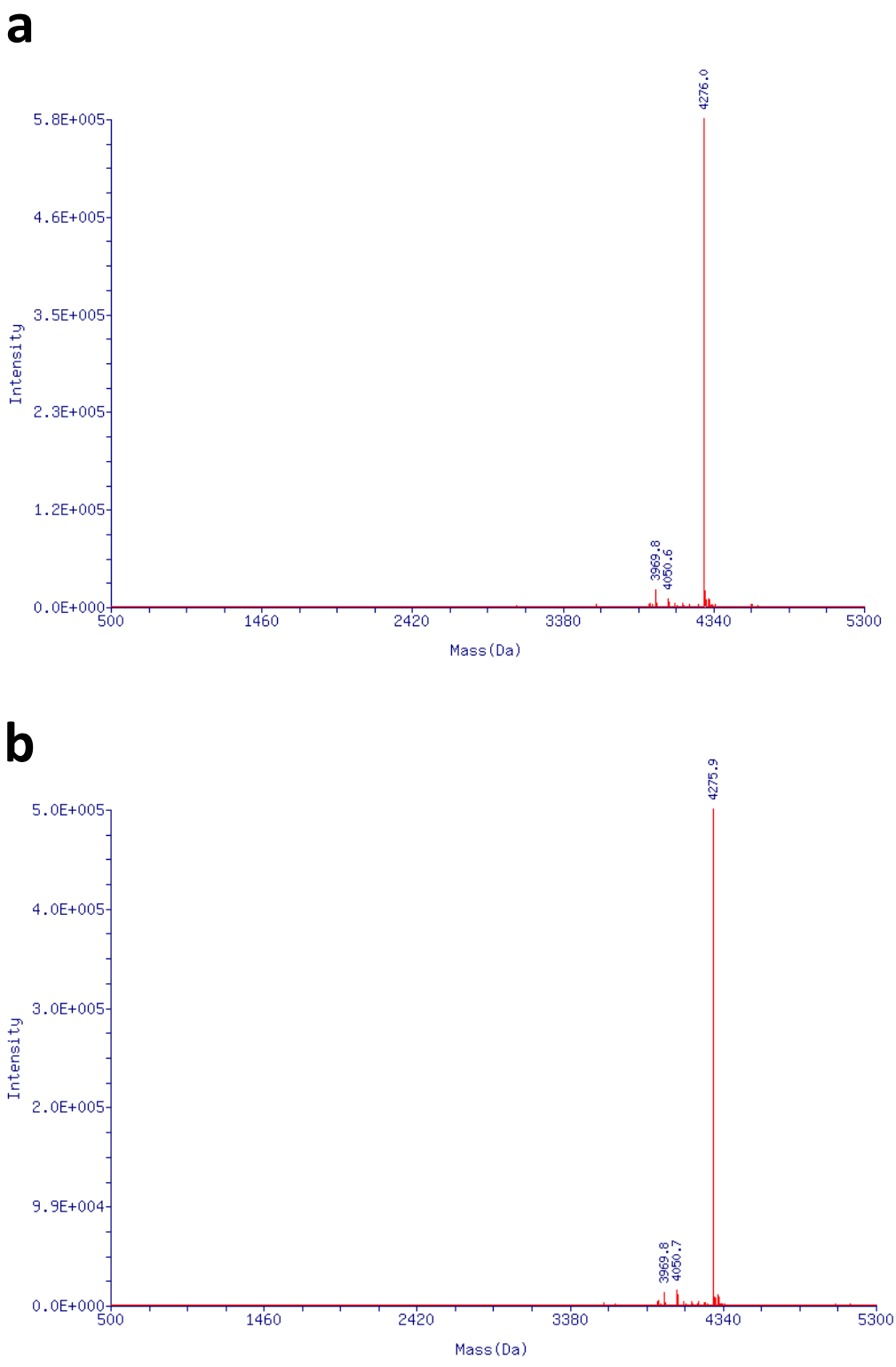

Figure S8. (a) Mass analysis of A1. Expected mass: 4276.9 Da; Observed mass: 4276.0 Da. (b) Mass analysis of A2. Expected mass: 4276.9 Da; Observed mass: 4275.9 Da.

Figure S9

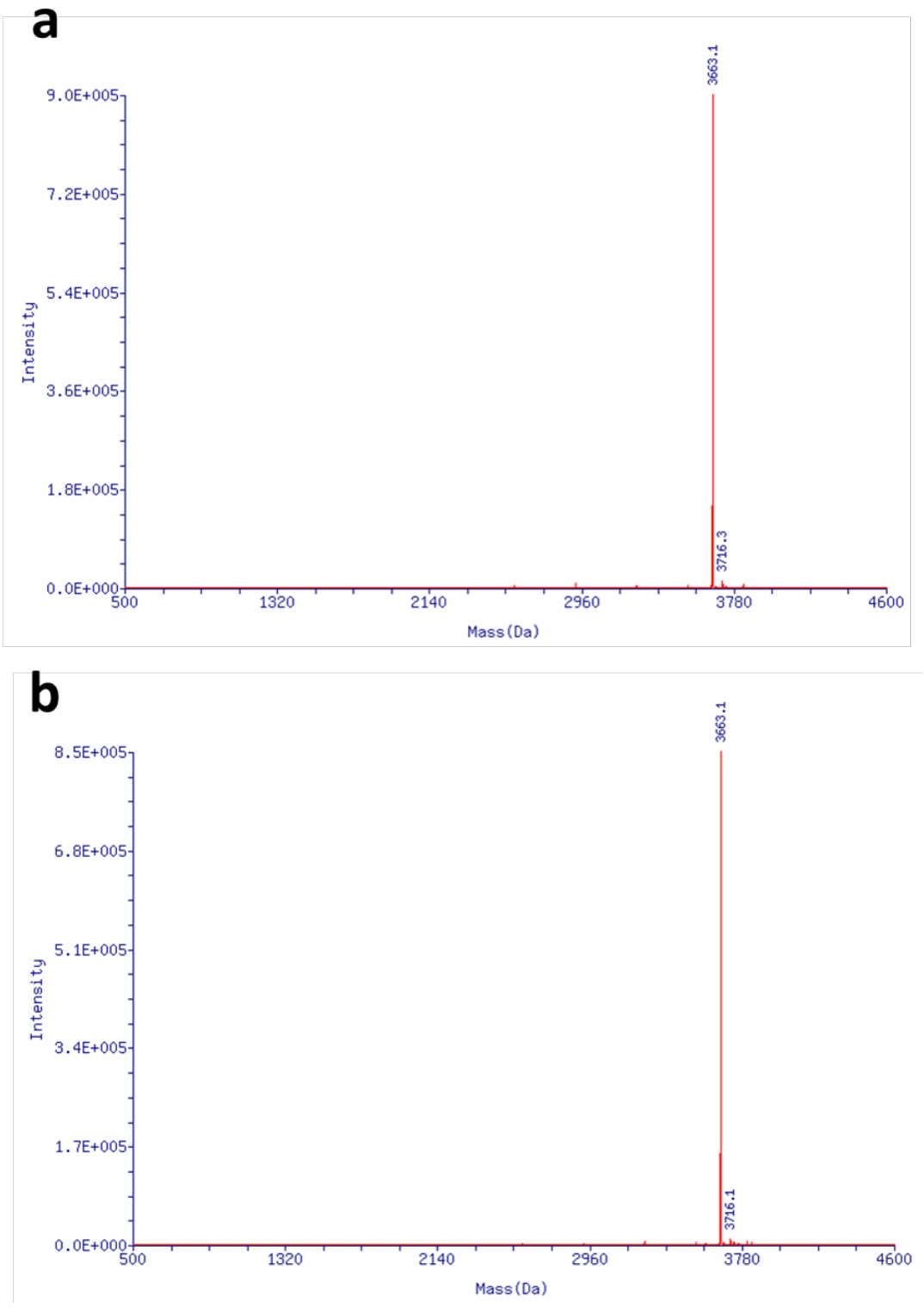

Figure S9. (a) Mass analysis of D1. Expected mass: 3663.3 Da; Observed mass: 3663.1 Da. (b) Mass analysis of D2. Expected mass: 3663.3 Da; Observed mass: 3663.1 Da.

Figure S10

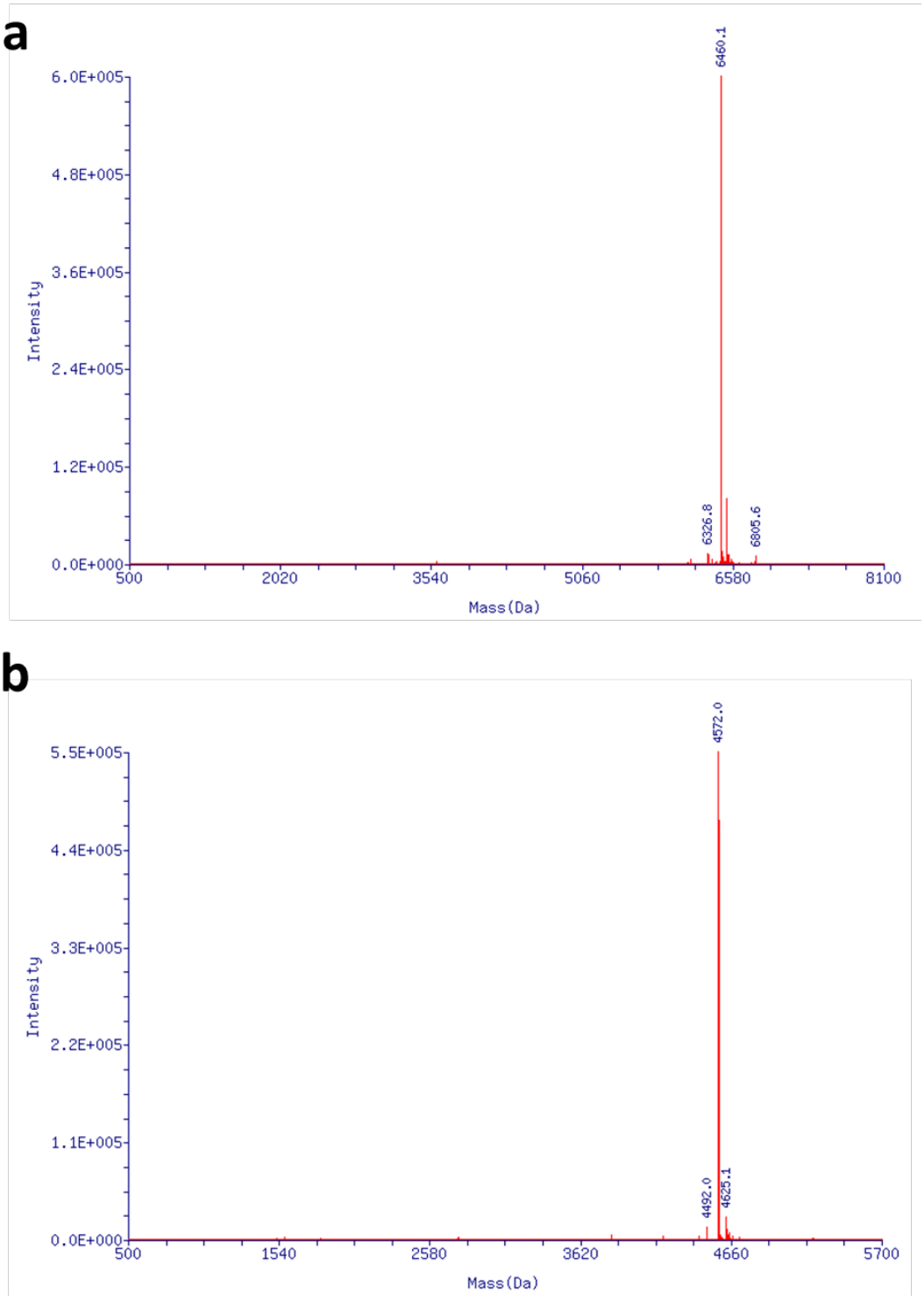

Figure S10. (a) Mass analysis of HRz-A. Expected mass: 6460.0 Da; Observed mass: 6460.1 Da. (b) Mass analysis of HRz-D. Expected mass: 4571.8 Da; Observed mass: 4572.0 Da.

Figure S11

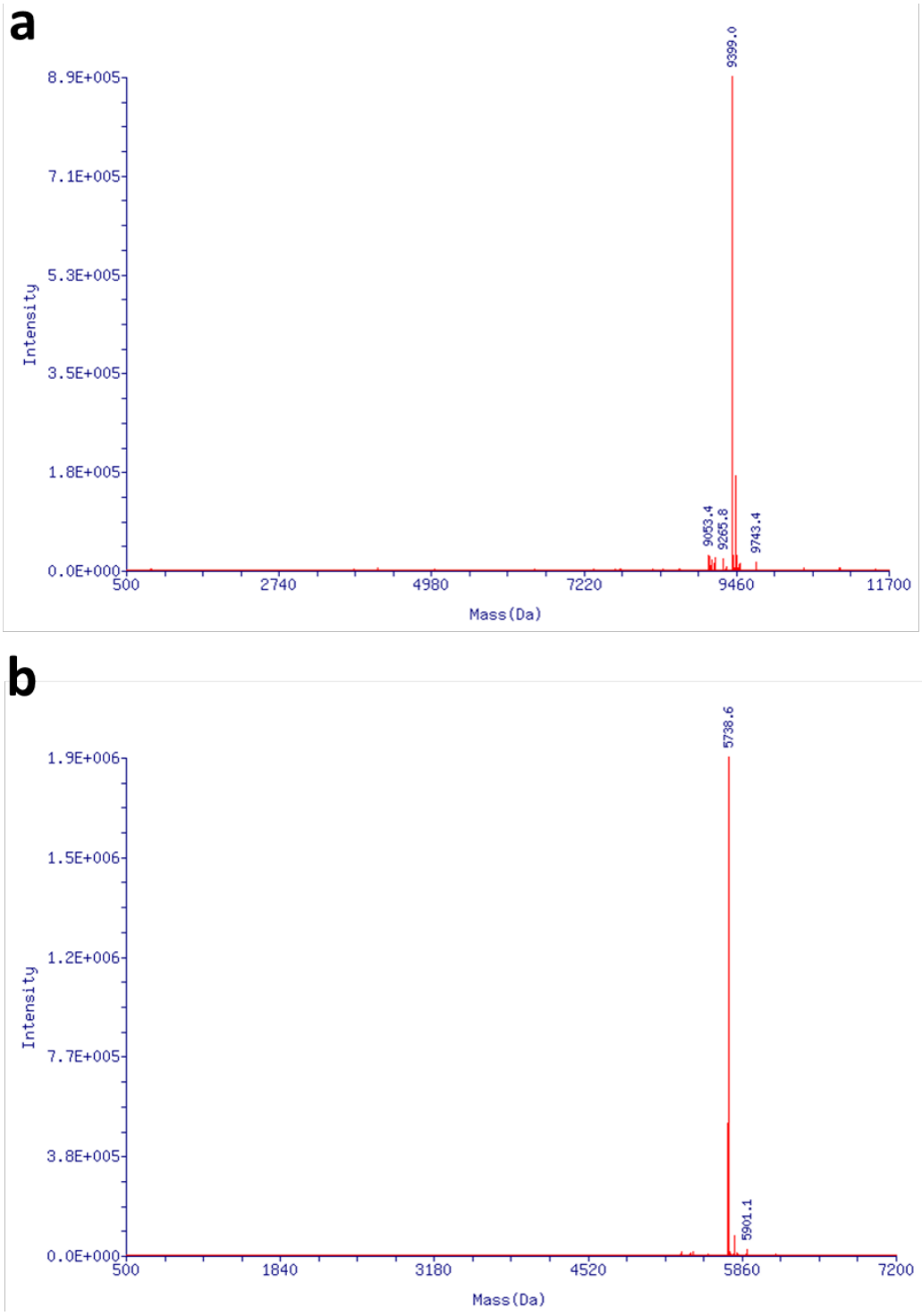

Figure S11. (a) Mass analysis of 6-4t-A. Expected mass: 9398.8 Da; Observed mass: 9399.0 Da. (b) Mass analysis of 6-4t-D. Expected mass: 5738.5 Da; Observed mass: 5738.6 Da.

Figure S12

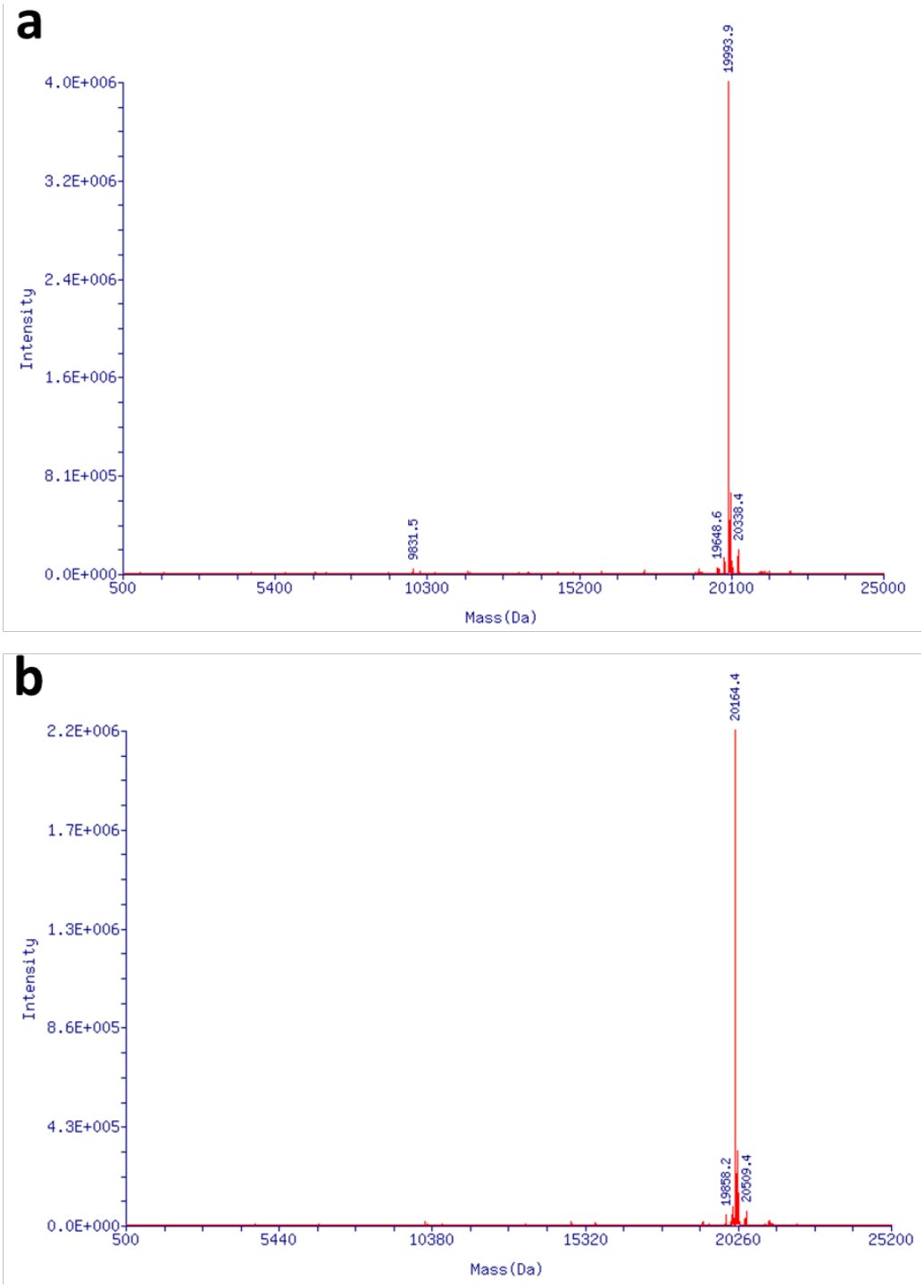

Figure S12. (a) Mass analysis of HRz_{Theo}-A. Expected mass: 19991.0 Da; Observed mass: 19993.9 Da. (b) Mass analysis of HRz_{Theo}-D. Expected mass: 20162.0 Da; Observed mass: 20164.4 Da.

Figure S13

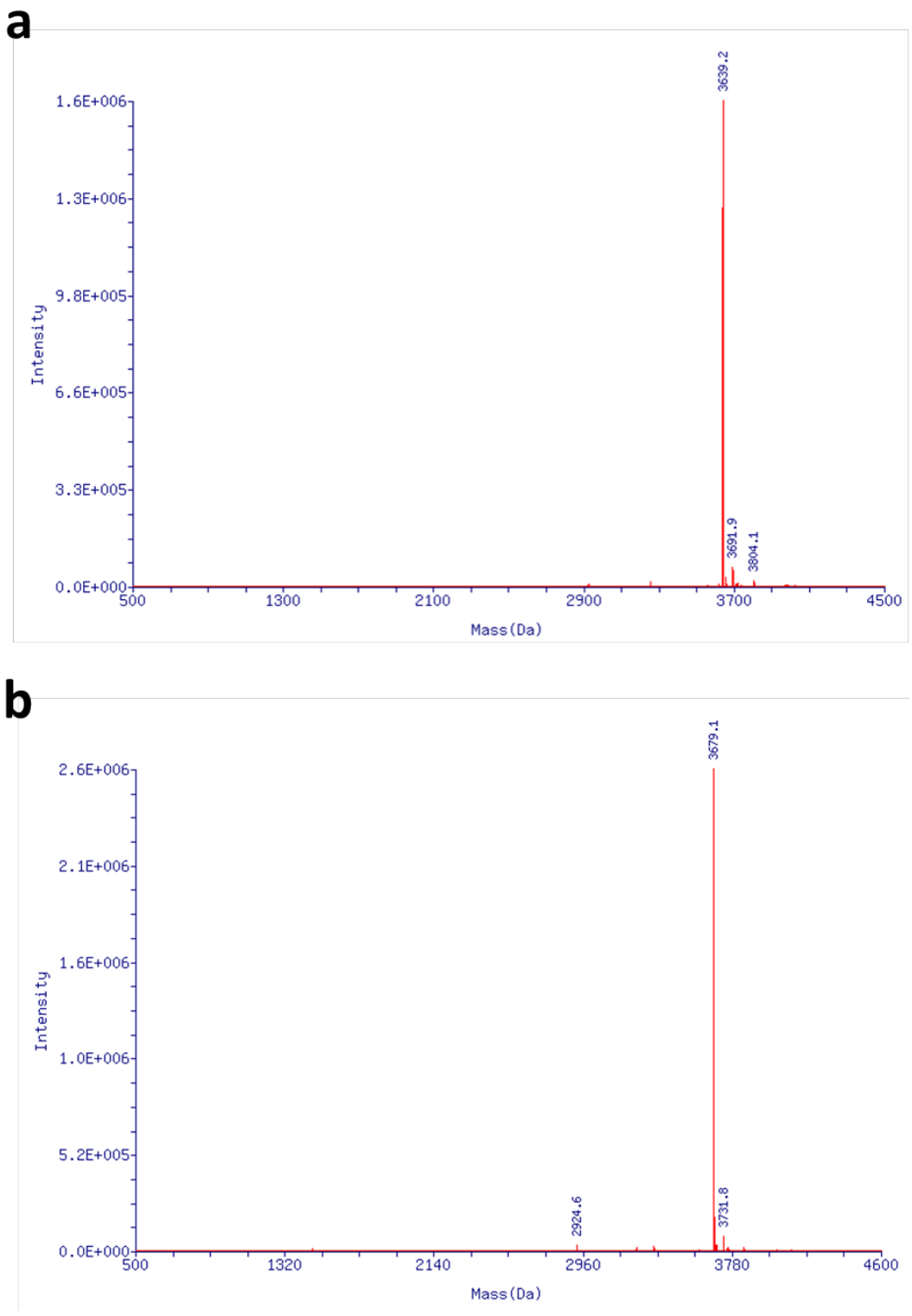

Figure S13. (a) Mass analysis of D2C. Expected mass: 3639.2 Da; Observed mass: 3639.2 Da. (b) Mass analysis of D2G. Expected mass: 3679.3 Da; Observed mass: 3679.1 Da.

Figure S14

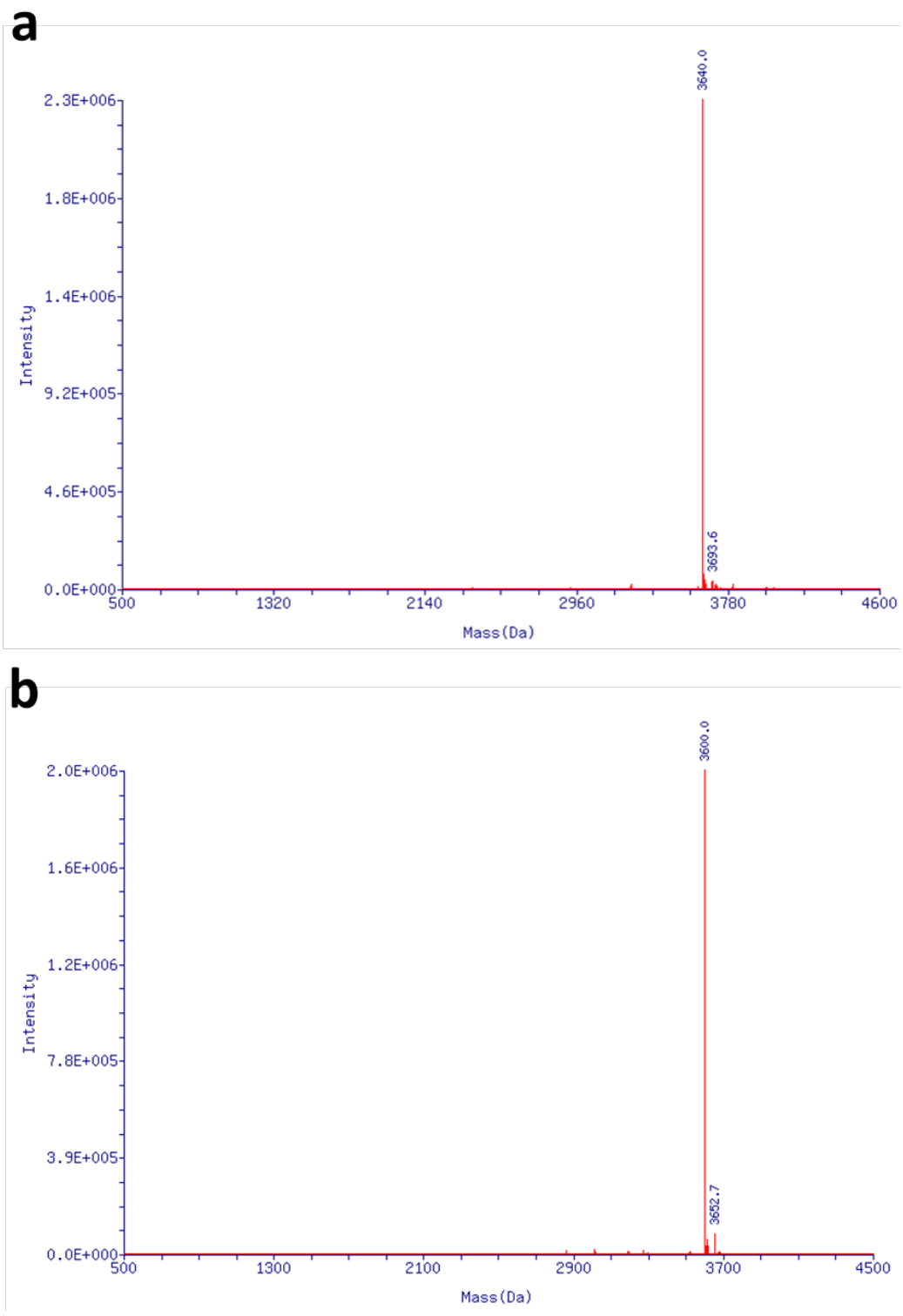

Figure S14. (a) Mass analysis of D2U. Expected mass: 3640.2 Da; Observed mass: 3640.0 Da. (b) Mass analysis of $D2A_{scr}$. Expected mass: 3600.2 Da; Observed mass: 3600.0 Da.

S3. Supplementary Tables.

Table S1. Names and sequences of oligonucleotides used in this work. L-RNA (blue) and D-RNA (red) are indicated by color. /Cy3/ = cyanine3 dye; /Cy5/ = cyanine5 dye.

Sequence Name	Sequence Identity 5′→3′
A1	/Cy3/AACCCGGGUGCCCG
A2	/Cy3/AACCCGGGUGCCCG
D1	pAAAGGCAAGAA
D2	pAAAGGCAAGAA
D2C	pCAAGGCAAGAA
D2G	pGAAGGCAAGAA
D2U	pUAAGGCAAGAA
$D2A_{scr}$	pAAACCGUAGAA
HRz-A	GGCGACCCUGAUGAGGCCGA
HRz-D	pAAGGCCGAAACCGU
L-HRz	GGCGACCCUGAUGAGGCCGAAAGGCCGAAACCGU
D-HRz	GGCGACCCUGAUGAGGCCGAAAGGCCGAAACCGU
L-S	/Cy3/ACGGUCGGUCGCC
D-S	/Cy3/ACGGUCGGUCGCC
6-4t-A	GGACUAGGGCGCGAGCAAACCCGUGCCGA
6-4t-D	pAAGGCAAGAAACAGUCC
$L\text{-}6\text{-}4t_{LIG}$	GGACUAGGGCGCGAGCAAACCCGUGCCGAAAGGCAAGAAACAG UCC
L-6-4t	GGACUAGGGCGCGAGCAAACCCGUGCCGAAAGGCAAGAAACAG UCC
D-TAR	/Cy5/GCCAGAUUUGAGCCUGGGAGCUCUCUGGC
HRz_{Theo}-A	GUCUCCGCCUGAUGAGCCUGGAUACCAGCCGAAAGGCCCUUGG CAGUUAGACGAAACGGUGA
HRz_{Theo}-D	pAAGCCGUAGCGGAGACGGUCGGGUCCAGAUAUUCGUAUCUGU CGAGUAGAGUGUGGGCUCCG
$L\text{-}HRz_{Theo}$	GUCUCCGCCUGAUGAGCCUGGAUACCAGCCGAAAGGCCCUUGG CAGUUAGACGAAACGGUGAAAGCCGUAGCGGAGACGGUCGGGU CCAGAUAUUCGUAUCUGUCGAGUAGAGUGUGGGCUCCG
$D\text{-}HRz_{Theo}$	GUCUCCGCCUGAUGAGCCUGGAUACCAGCCGAAAGGCCCUUGG CAGUUAGACGAAACGGUGAAAGCCGUAGCGGAGACGGUCGGGU CCAGAUAUUCGUAUCUGUCGAGUAGAGUGUGGGCUCCG

Table S2. Raw yields (before purification) and isolated yields of ligation reactions.

	Raw yield	Isolated yield (4 nmole scale)
L-HRz	33.4%	225pmol (5.6%)
L-6-4t_{LIG}	37.2%	292pmol (7.3%)
L-HRz$_{Theo}$	41.4%	287pmol (7.2%)

S4. References.

1. Sczepanski, J. T.; Joyce, G. F., Binding of a structured D-RNA molecule by an L-RNA aptamer. *J Am Chem Soc* **2013,** *135* (36), 13290-3.