Supplementary Information

MicroRNA Detection in Biological Media Using a Split Aptamer Platform

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Chemicals and Materials

Reagents: 2-Amino-4'-dimethylaminoacetophenone hydrochloride was purchased from Combi Blocks. Pyridine, methylene chloride, chloroform, sodium hydroxide, sodium carbonate (all anhydrous), HPLC grade acetonitrile, ACS grade acetone, 4-(fluorosulfonyl)benzoyl chloride, auramine O (AO, 85% dye content), benzoic acid, and bovine serum albumin (BSA), were purchased from Millipore-Sigma. 98% sulfuric acid was purchased from VWR Chemicals BDH. 2-amino-4'-dimethylaminoacetophenone was purchased from TCI America. Dulbecco's Modified Eagle Medium (DMEM) with phenol red was purchased from Corning. 10% (v/v) fetal bovine serum (FBS) was purchased from Hyclone Laboratories Inc. Dapoxyl sulfonate (DS) and dapoxyl sulfonyl fluoride (DSF) were synthesized according to published procedures[1] with modification (detailed below).

Buffers and Solvents: Tris(hydroxymethyl)aminomethane (Tris) was purchased from Millipore-Sigma. Water was deionized and filtered to a resistivity of 18.2 ΩM with a Milli-Q® Plus water purification system (Millipore, Massachusetts). Buffers were prepared freshly in Milli-Q® water and their pH was adjusted using HCl or NaOH. Fluorescence assay buffer (FAB): 20 mM Tris, 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, pH 7.6.

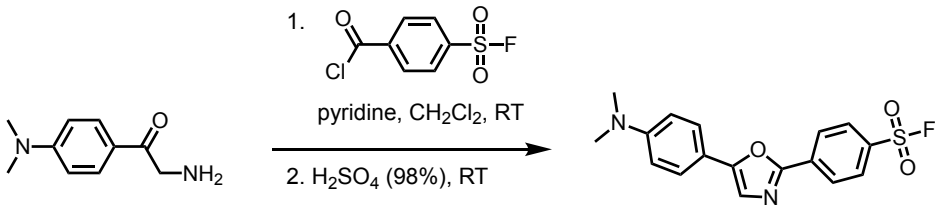
Biological Media: CM: Cell media, HS: Human serum, and HP: Human plasma.
CM was obtained from HeLa cell (CCL-2, ATCC) cultured in DMEM that was supplemented with 10% FBS. HS was purchased from Millipore-Sigma as heat inactivated, from male AB clotted whole blood. HP was purchased from Equitech-Bio, Inc. as human unfiltered sodium heparin plasma.

Oligonucleotides: DNA and RNA sequences used in this work were obtained as dry thin film from Millipore-Sigma. All of the oligonucleotides were cartridge purified by the manufacturer. Stock solutions of oligonucleotides were prepared with Milli-Q® Plus water and stored at -80 ºC.

General Synthetic Methods

For the chemical synthesis of organic compounds, all reactions were performed under a dry nitrogen atmosphere unless otherwise stated. All glassware was oven-dried before use. Purification of the synthesized compounds was performed using a Büchi Reveleris® flash chromatography system equipped with a FlashPure EcoFlex C-18 (50 µm sphere) column. Nuclear Magnetic Resonance (NMR) spectroscopic analyses were carried out on either a Bruker Avance Neo 500 MHz or Varian VNMRS 500 MHz spectrometer. ^{1}H NMR spectra were acquired at 500 MHz, ^{13}C NMR spectra were acquired at 126 MHz, and ^{19}F NMR spectrum was acquired at 471 MHz. Chemical shifts (δ) for ^{1}H NMR spectra were referenced to (C***H***$_3$)$_4$Si at δ = 0.00 ppm and to C***H***D$_2$S(O)CD$_3$ at δ = 2.50 ppm. ^{13}C NMR spectra were referenced to ***C***D$_3$S(O)***C***D$_3$ at δ = 39.52 ppm. The following abbreviations are used to describe NMR resonances: s (singlet) and d (doublet). Coupling constants (*J*) are reported in Hz. Liquid chromatography followed by high-resolution mass spectroscopy (LC-HRMS) analysis in the ESI mode was carried out on a Waters Acquity-Xevo G2-XS QTof.

Preparation Procedure and Characterization Data for DSF and DS



dapoxyl sulfonyl fluoride (DSF)

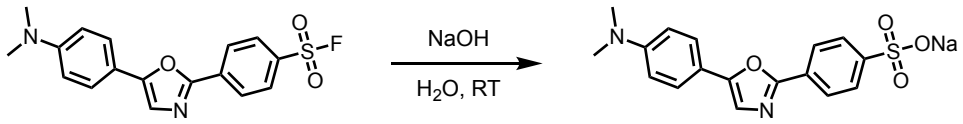
Synthesis of 4-(5-(4-(dimethylamino)phenyl)oxazol-2-yl)benzenesulfonyl fluoride (dapoxyl sulfonyl fluoride, DSF): In a 100 mL round bottomed flask, 2-amino-4'-dimethylaminoacetophenone (247 mg, 1.15 mmol, 1.15 equiv) and 4-(fluorosulfonyl)benzoyl chloride (1.00 mmol, 1.00 eq, 247 mg) were dissolved in 50 mL methylene chloride and anhydrous pyridine (3.00 mmol, 3.00 eq, 241 µL) was added dropwise. The orange heterogenous mixture was stirred overnight at room temperature (RT) and the solvent was evaporated under reduced pressure. The resulting orange powder was dissolved in 10 mL H_2SO_4 (98%) and stirred for two hours at RT. The clear orange solution was poured into a flask containing ice cubes and mixed, which was then neutralized with saturated Na_2CO_3 solution and then centrifuged. The crude product was collected by decantation and purified by silica gel column chromatography (CH_2Cl_2/MeOH step gradient). Concentration of the product fractions afforded DSF as a yellow solid (210 mg, 0.60 mmol, 60% yield, ≥95 % purity by NMR).

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.29 (d, *J* = 8.4 Hz, 2H), 8.09 (d, *J* = 8.4 Hz, 2H), 7.61 (d, *J* = 8.6 Hz, 2H), 7.34 (s, 1H), 6.77 (d, *J* = 8.6, 2H), 3.04 (s, 6H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 157.3, 154.1, 150.9, 134.1, 133.0, 132.8, 129.0, 126.5, 125.9, 121.6, 115.0, 112.1, 40.2.

^{19}F NMR (471 MHz, $CDCl_3$) δ 66.3.

HRMS (ESI) m/z: $[M + H]^+$ Calculated for $C_{17}H_{16}FN_2O_3S^+$, 347.0860; found 347.0864.



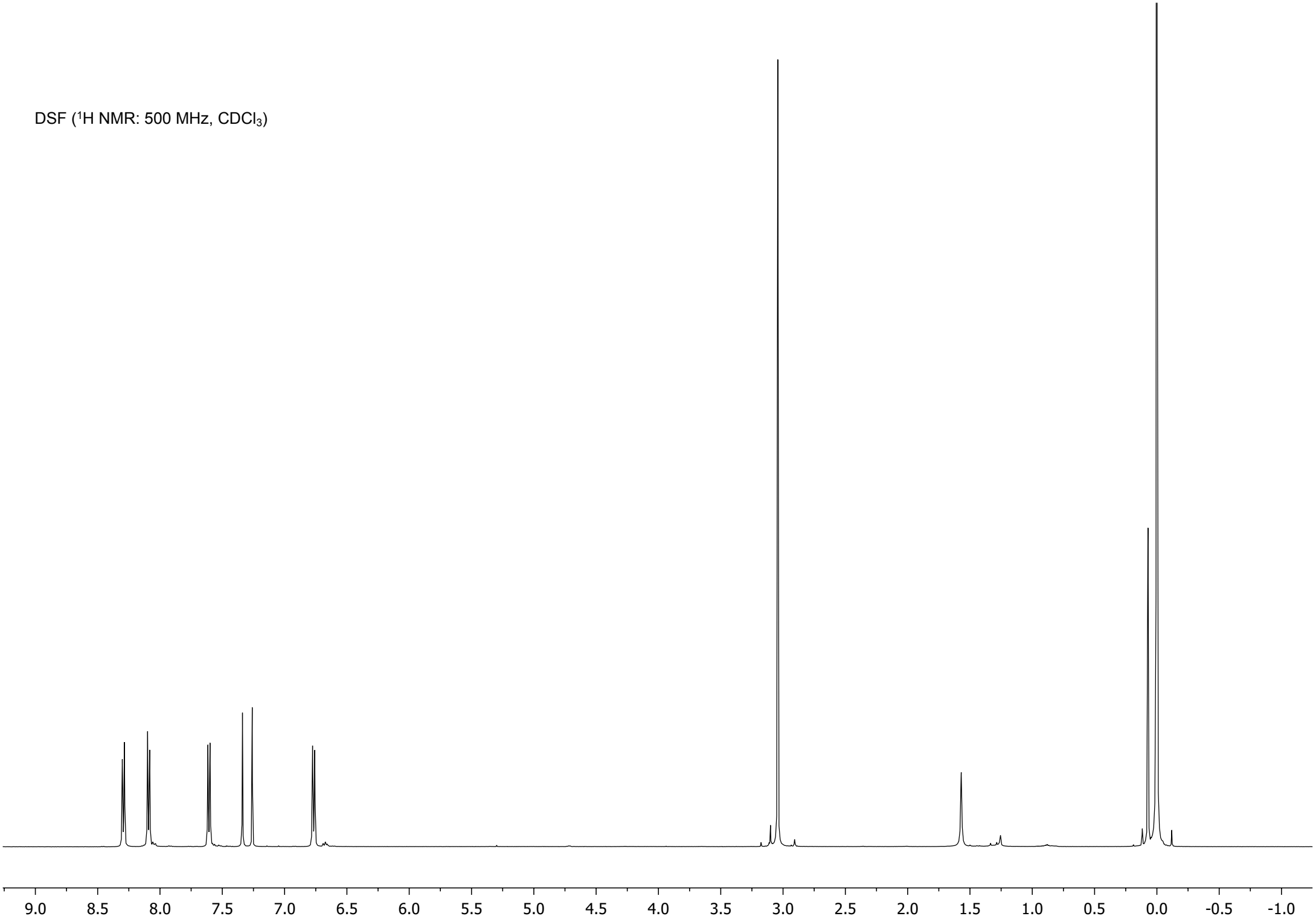
dapoxyl sulfonyl fluoride sodium dapoxyl sulfonate (DS)

Synthesis of sodium 4-(5-(4-(dimethylamino)phenyl)oxazol-2-yl)benzenesulfonate (sodium dapoxyl sulfonate, DS): In a 25-mL round-bottom flask, DSF (15.0 mg, 0.0433 mmol) was suspended with 10% aqueous NaOH (5 mL) and refluxed (110 ºC) overnight while stirring. The mixture was then cooled down to RT and neutralized with 1 M HCl. The resulting yellow solid was chilled in an ice bath and recovered by decantation, followed by centrifugation. The isolated DS precipitate was dissolved in Milli-Q® water. The solution was filtered and lyophilized. The crude product was collected by filtration and purified by reversed-phase column chromatography (water / acetonitrile, step gradient from 0 to 100% acetonitrile). Fractions containing the product were lyophilized to afford DS as pale yellow powder (10.5 mg, 0.0287 mmol, 66% yield, ≥95 % purity by NMR).

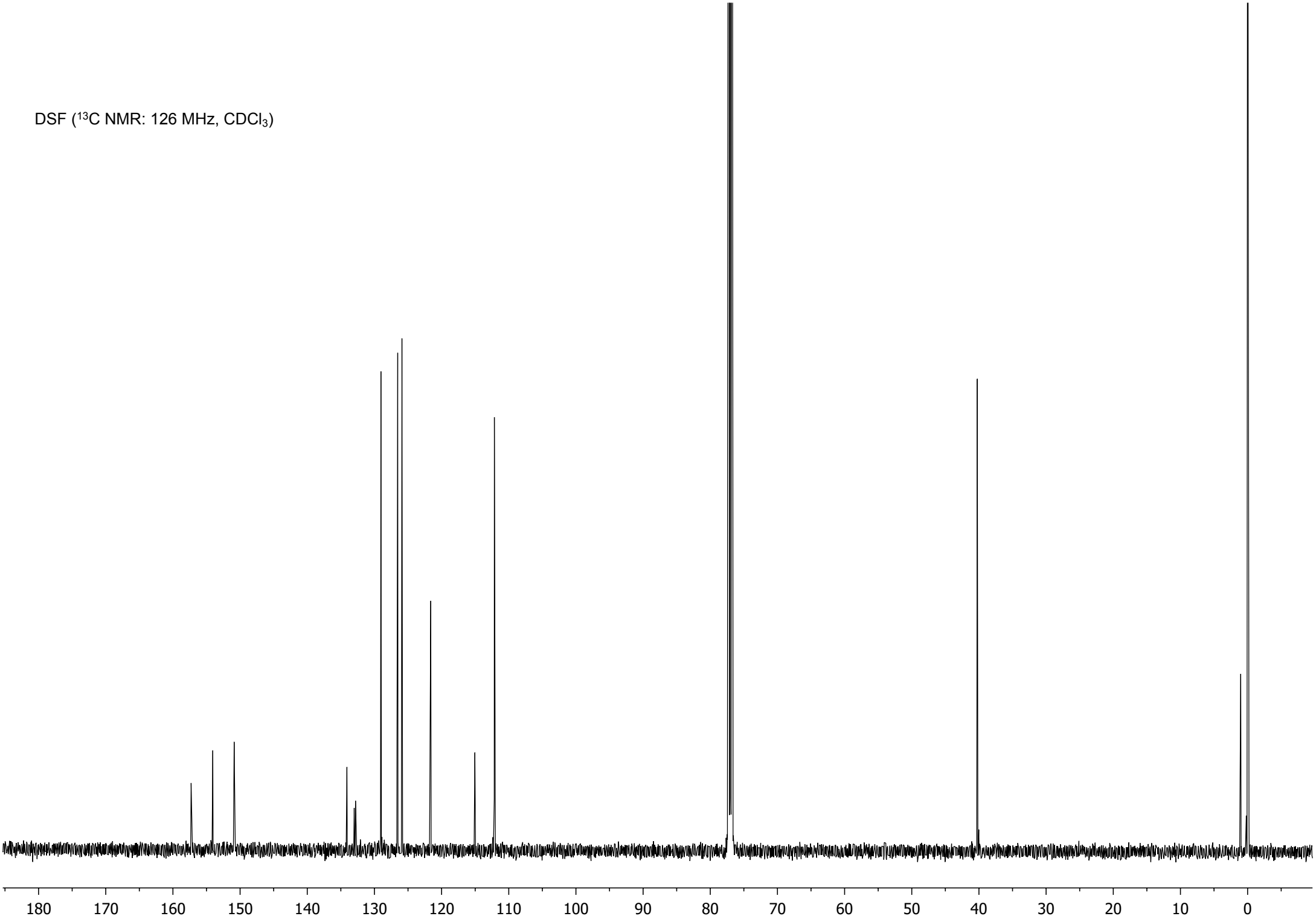
^{1}H NMR (500 MHz, d_6-DMSO) δ 7.99 (d, *J* = 8.4 Hz 2H), 7.73 (d, *J* = 8.5 Hz, 2H), 7.65 (d, *J* = 8.9 Hz, 2H), 7.55 (s, 1H), 6.81 (d, *J* = 8.9, Hz, 2H), and 2.98 (s, 6H).

^{13}C NMR (126 MHz, d_6-DMSO) δ 158.6, 151.9, 150.4, 149.7, 126.9, 126.3, 125.3, 125.0, 121.0, 115.0, 112.2, and 39.8.

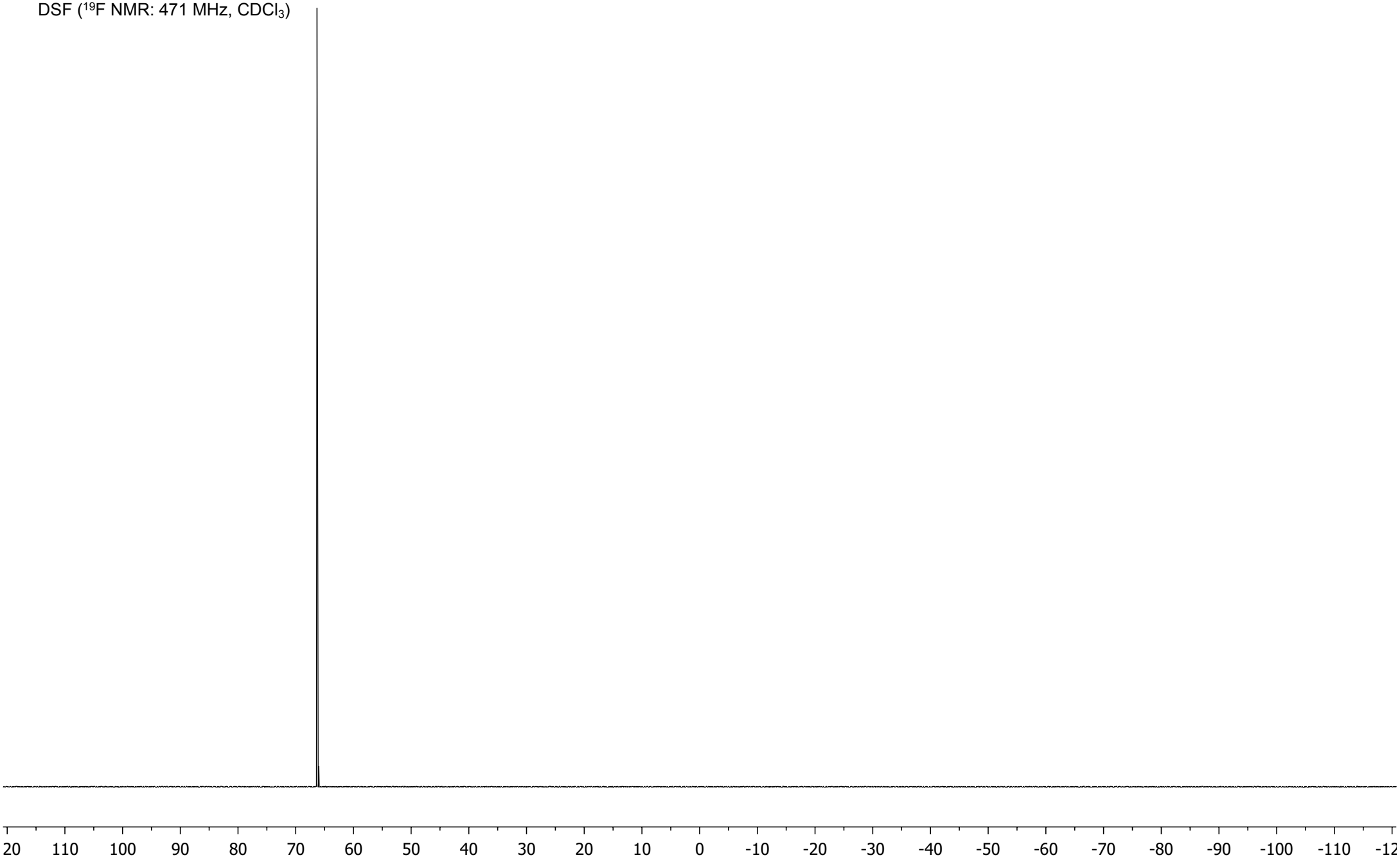
HRMS (ESI) m/z: $[M + H]^+$ Calculated for $C_{17}H_{17}N_2O_4S^+$ 345.0904; found 345.0841.

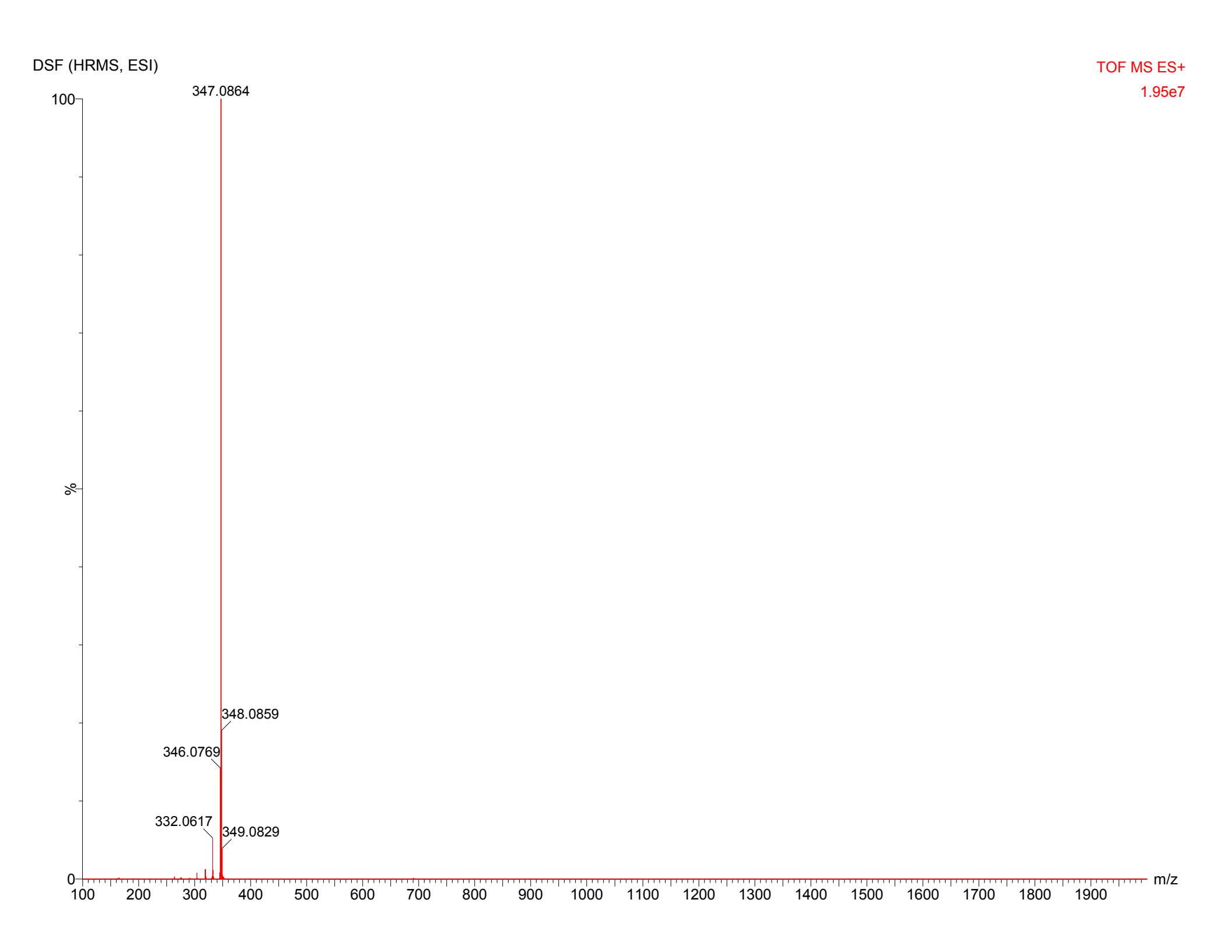

DSF (^{1}H NMR: 500 MHz, $CDCl_3$)
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0

DSF (^{13}C NMR: 126 MHz, $CDCl_3$)

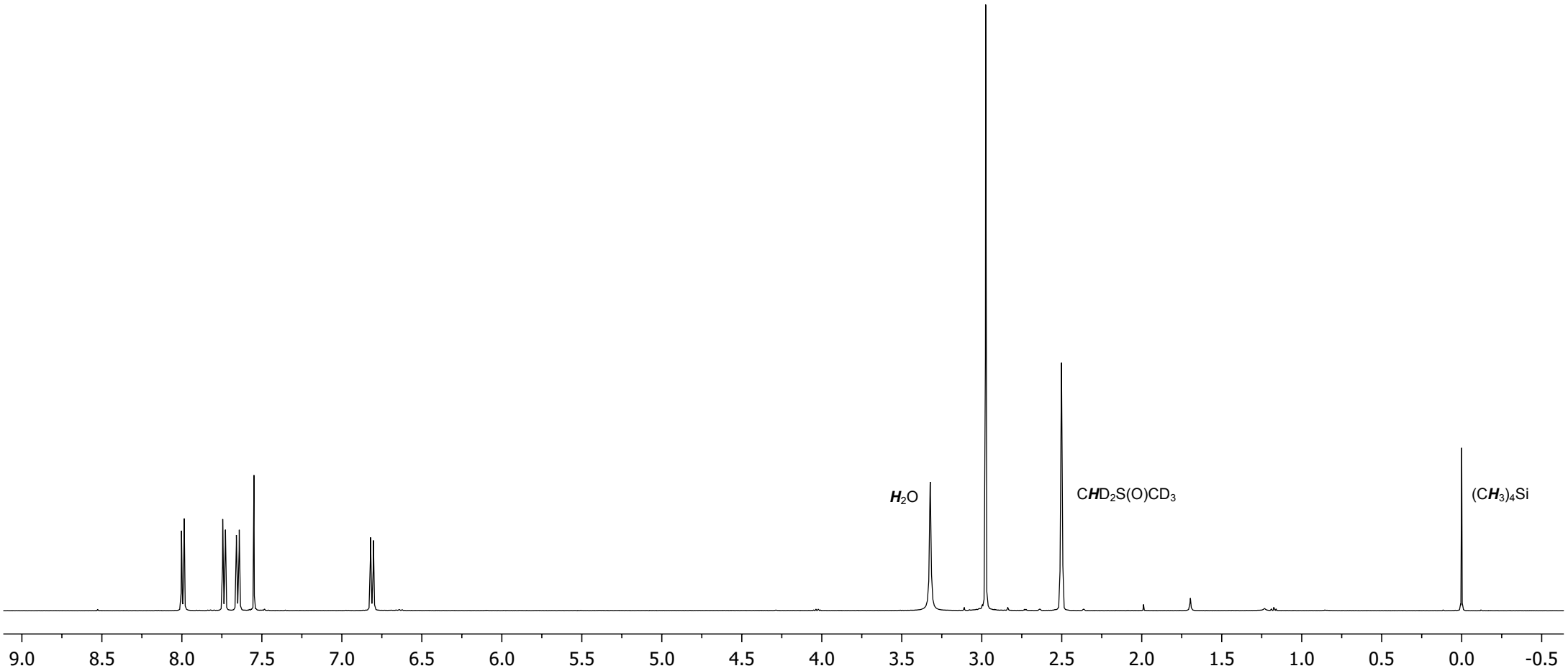


DSF (^{19}F NMR: 471 MHz, $CDCl_3$)

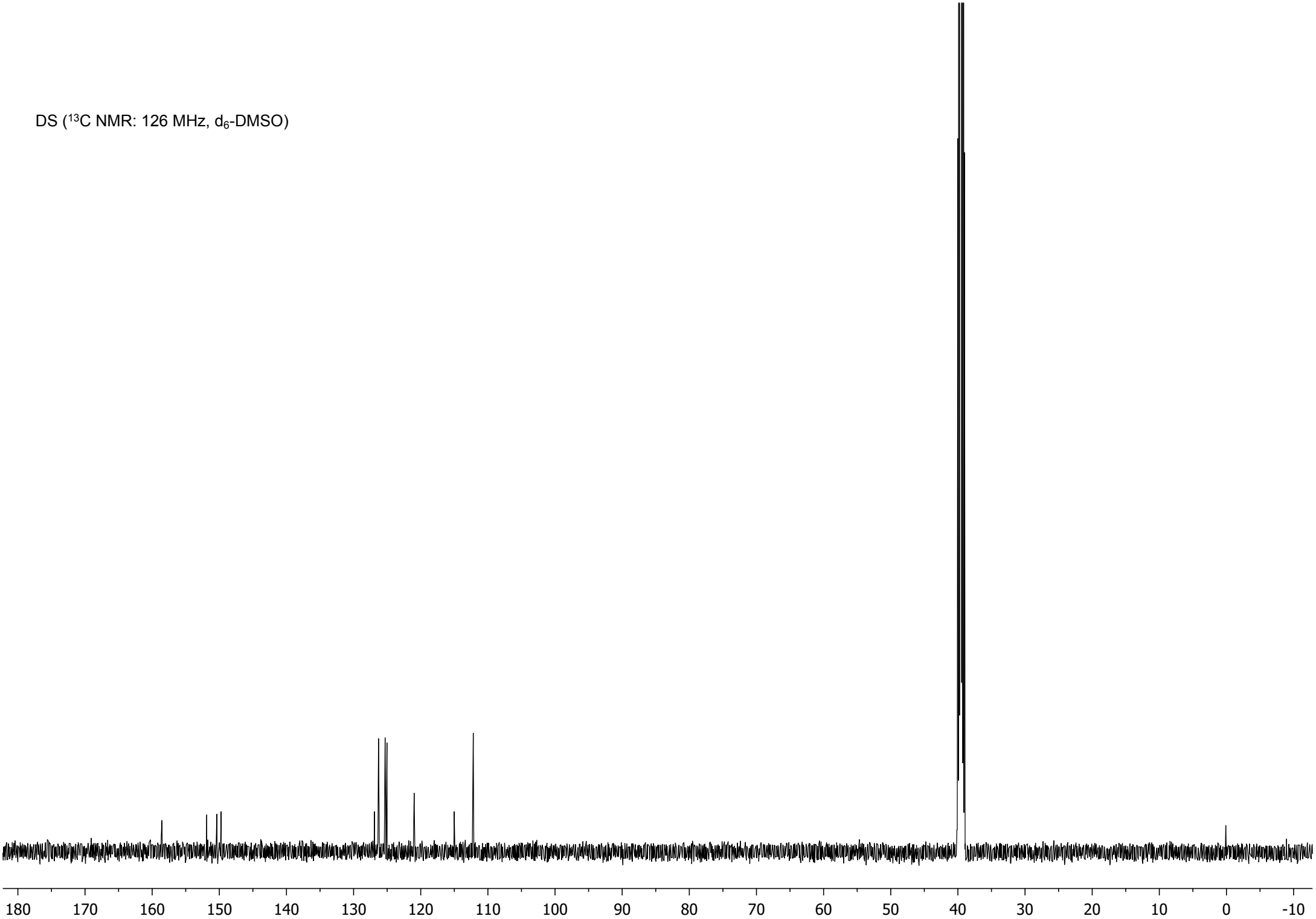


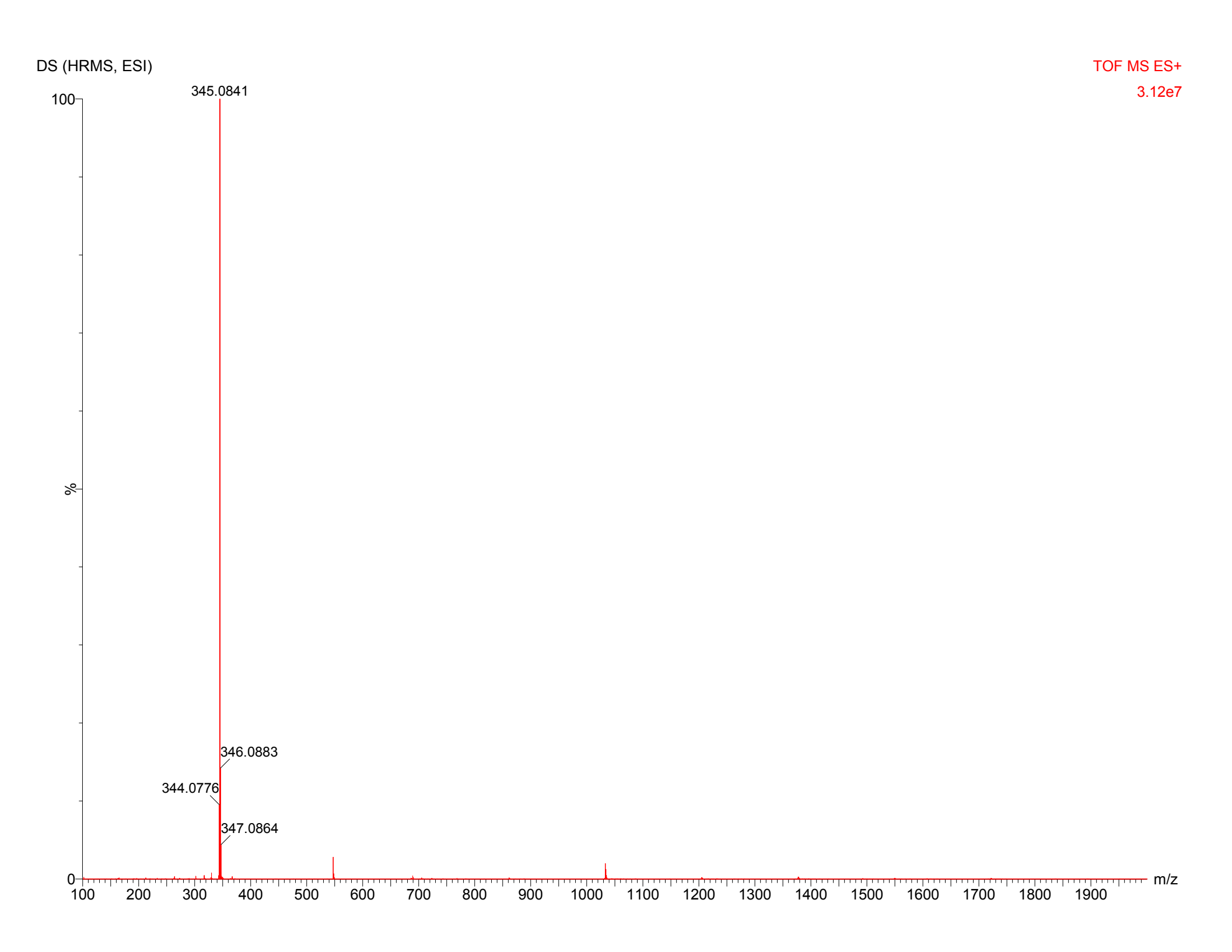

DSF (HRMS, ESI)
TOF MS ES+
1.95e7
347.0864
348.0859
346.0769
332.0617
349.0829
100
%
0
m/z
100
200
300
400
500
600
700
800
900
1000
1100
1200
1300
1400
1500
1600
1700
1800
1900

DS (^{1}H NMR: 500 MHz, d_6-DMSO)

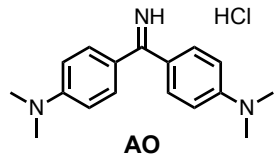


DS (^{13}C NMR: 126 MHz, d_6-DMSO)




DS (HRMS, ESI)
TOF MS ES+
3.12e7
345.0841
346.0883
344.0776
347.0864
100
%
0
100
200
300
400
500
600
700
800
900
1000
1100
1200
1300
1400
1500
1600
1700
1800
1900
m/z

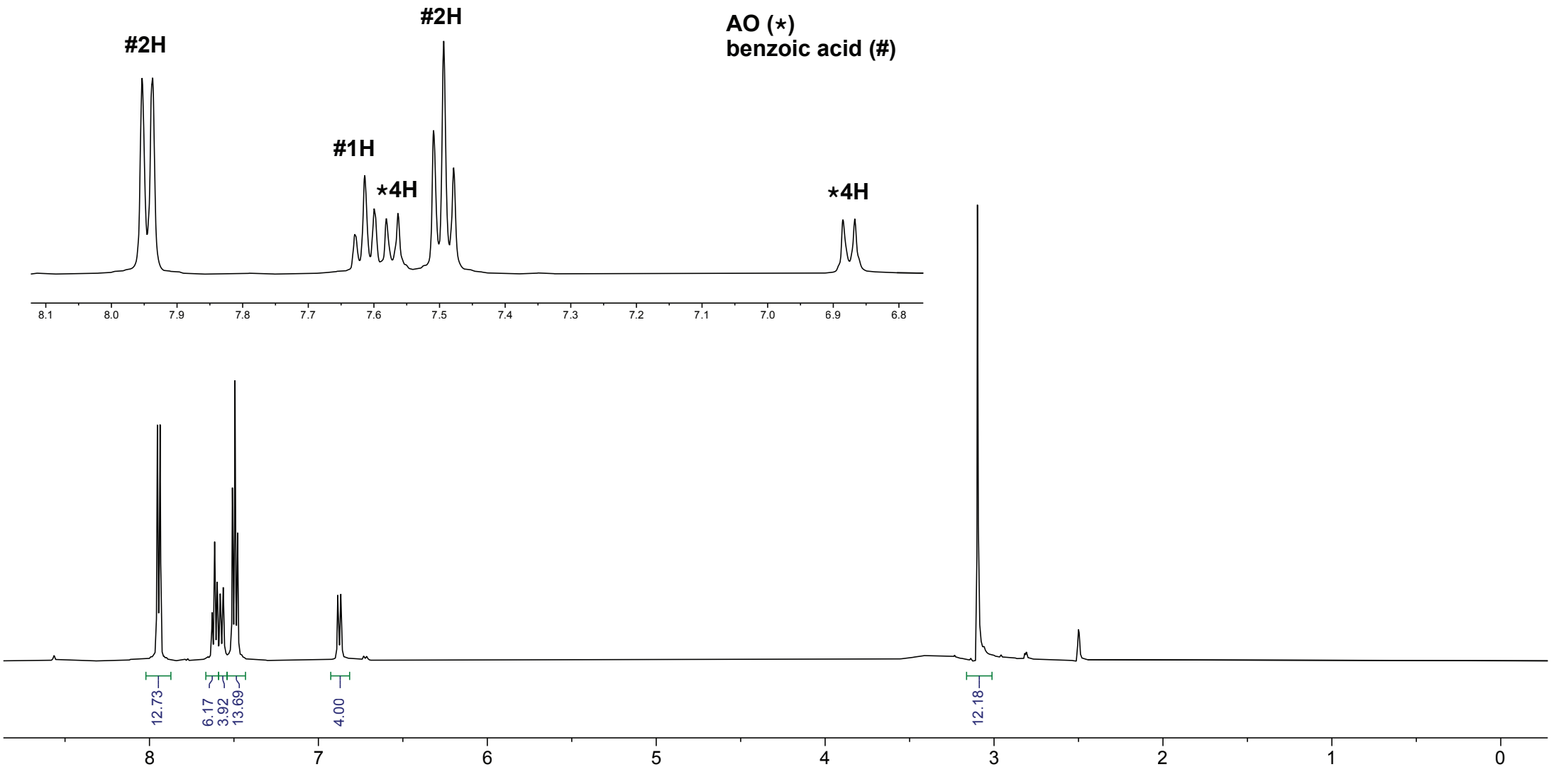
Preparation and ^{1}H NMR Quantification of AO



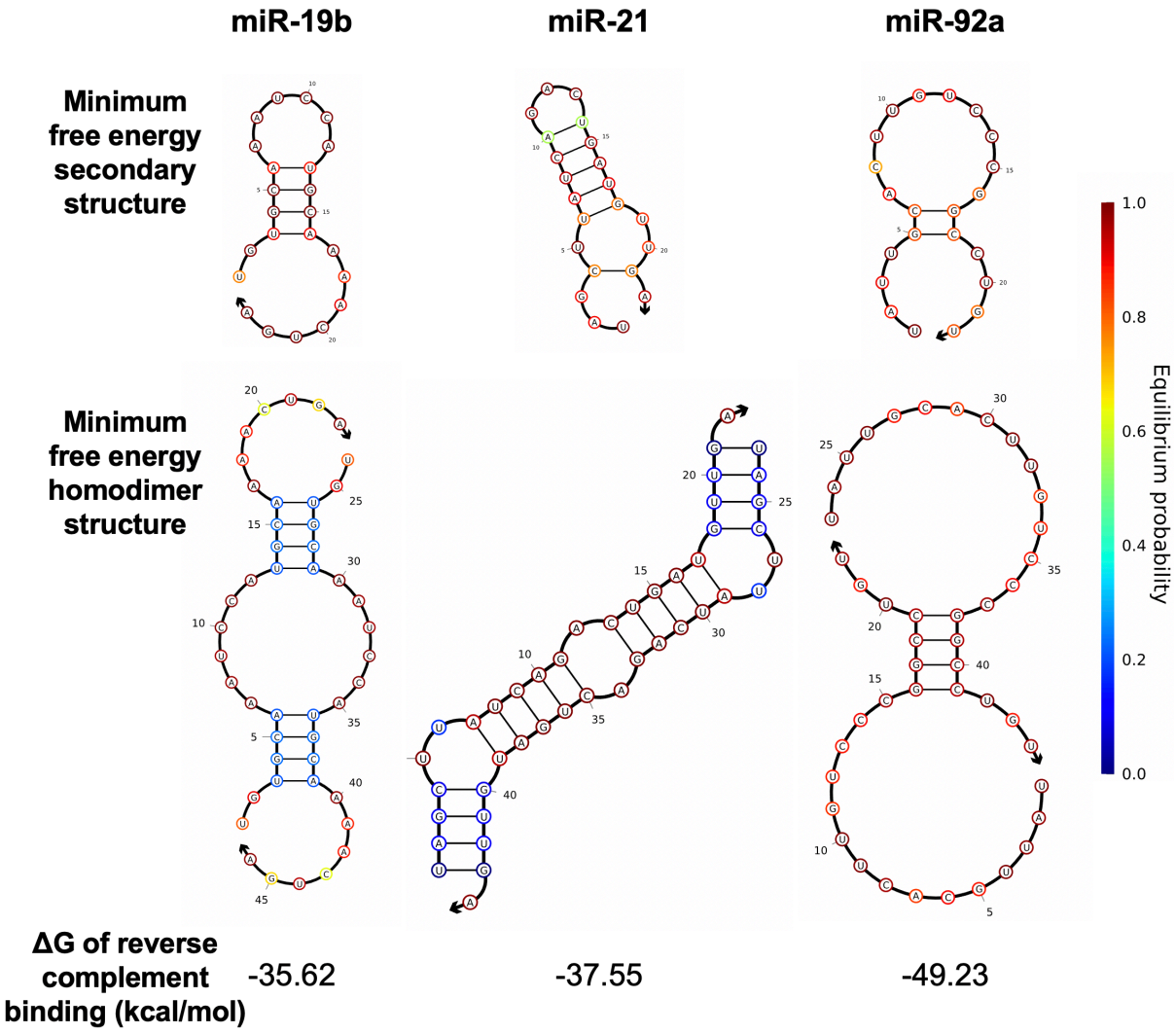
AO

To a 1.5 mL Eppendorf tube was placed AO (126 mg, 85% dye content per manufacturer) and ACS grade acetone (200 µL). The suspension was vortexed for 5 min and centrifuged to settle the solid. The supernatant was removed, and the solid was sequentially washed with acetone (200 µL) twice and diethyl ether (200 µL). The isolated solid was left under reduced pressure to remove residual organic volatiles, which provided a final amount of 83 mg of AO. For the purity assessment, 3.0 mg of AO and 7.6 mg (62.3 mmol) of benzoic acid (≥99% per manufacturer) as an internal standard were dissolved in 350 µL of d_6-DMSO. The purity of AO was determined to be ≥98% by ^{1}H NMR spectroscopy (see below). A 5 mM stock solution of AO was then made by dissolving 10 mg in 6.6 mL of Milli-Q® water. Depending on the fluorescence assay, this stock solution was diluted with Milli-Q® water to prepare a secondary stock solution.

AO (^{1}H NMR: 500 MHz, d_6-DMSO)

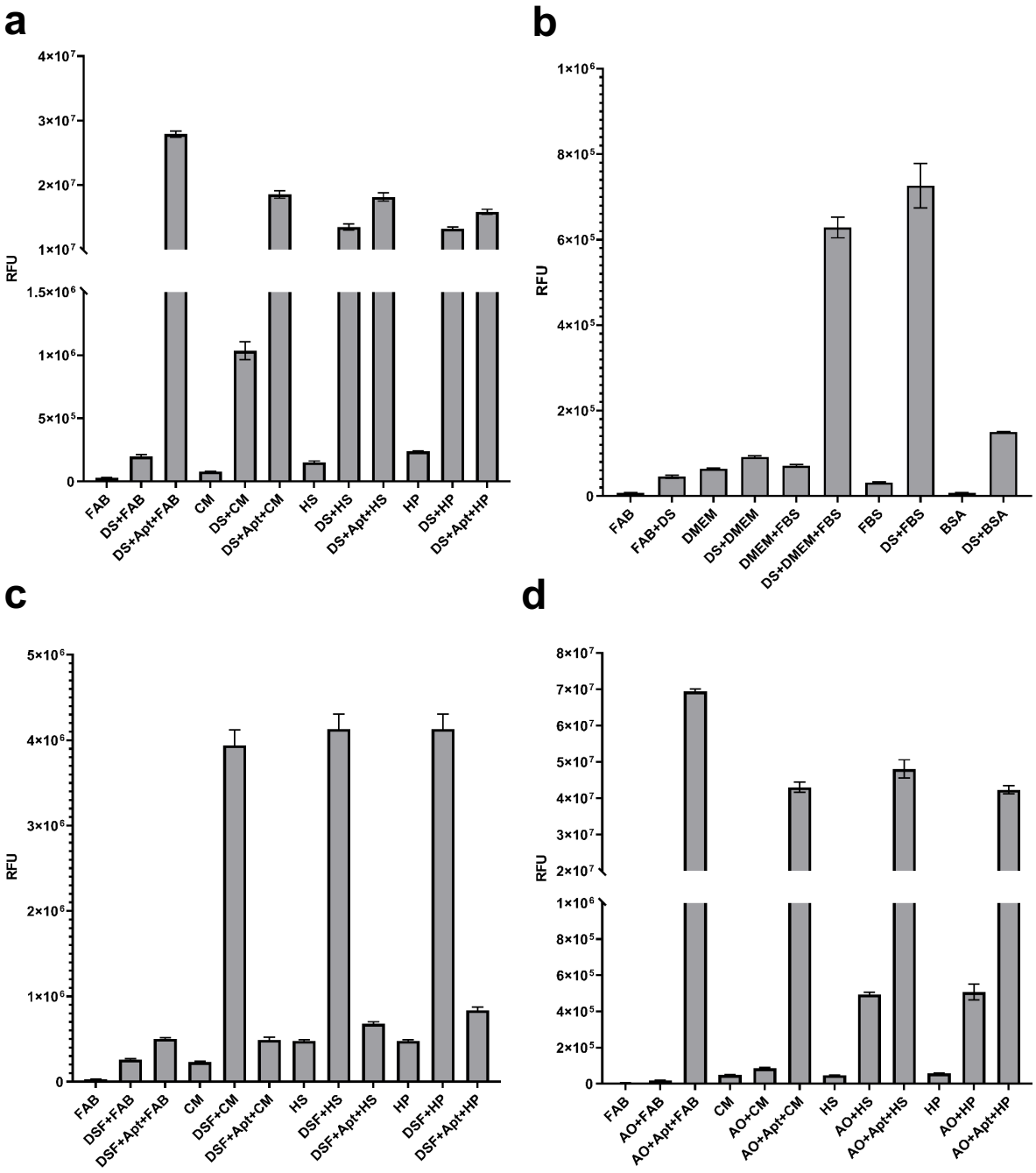


MiRNA Secondary Structures and Hybridization Energies



Supplementary Fig. 1. Comparison of secondary structures and hybridization energies of miRNAs used in this study. MiR-19b exhibits the strongest secondary structure. miR-21 forms a homodimer with high equilibrium probability. miR-19 has the highest binding energy to a complementary sequence. Visualization was performed with NUPACK[2]. ΔG calculations were performed with Vienna RNA Websuite[3].

Comparative Analysis of Fluorogenicity of DSF, DS, and AO in Buffer and Biological Media



Supplementary Fig. 2. Comparison of fluorescence intensity of (**a**-**b**) DS, (**c**) DSF, and (**d**) AO in fluorescence assay buffer (FAB) and biological media (10%). 10 μM fluorophore, 2 μM DAP-10 (5′-CAATTACGGGGGAGGGTGTGTGGTCTTGCTTGGTTCGTATTG), and 10% (v/v) media were used. Fluorescence intensities were measured at the following λ_{ex} / λ_{em}: DS: 391 / 497 nm; DSF: 390 / 505 nm; AO: 475 / 540 nm. Error bars denote the standard deviation of three replicates. Final concentrations of DMEM: 10%, FBS: 1% v/v, BSA: 0.025 mg/mL.

MiRNAs and MiRNA Mutants

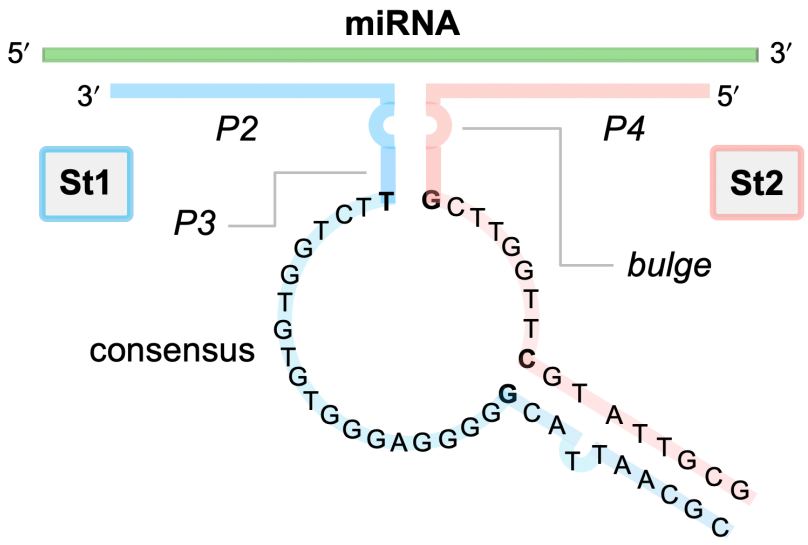
The complete list of oncogenic miRNAs and mutant miRNAs used in this study is provided in Supplementary Table 1. Mutants include single nucleotide variation compared to the native miRNA and are classified as *NXM*, where *N* designates the native nucleotide, *X* is the nucleotide number in the sequence, and *M* designates the nucleotide variant.

Supplementary Table 1. Sequences of miRNAs and miRNA mutants.

miRNA	Mutation	Sequence (5′ to 3′)
miR-19b	None	UGUGCAAAUCCAUGCAAAACUGA
	G2A	U**A**UGCAAAUCCAUGCAAAACUGA
	U3G	UG**G**GCAAAUCCAUGCAAAACUGA
	G4A	UGU**A**CAAAUCCAUGCAAAACUGA
	C5G	UGUG**G**AAAUCCAUGCAAAACUGA
	A6U	UGUGC**U**AAUCCAUGCAAAACUGA
	A16U	UGUGCA**A**AUCCAUGCUAAACUGA
	A17U	UGUGCAAAUCCAUGCA**U**AACUGA
	A18U	UGUGCAAAUCCAUGCAA**U**ACUGA
	A19U	UGUGCAAAUCCAUGCAAA**U**CUGA
	C20G	UGUGCAAAUCCAUGCAAAA**G**UGA
	U21G	UGUGCAAAUCCAUGCAAAAC**G**GA
miR-21	None	UAGCUUAUCAGACUGAUGUUGA
	A2U	U**U**GCUUAUCAGACUGAUGUUGA
	G3A	UA**A**CUUAUCAGACUGAUGUUGA
	C4G	UAG**G**UUAUCAGACUGAUGUUGA
	U5G	UAGC**G**UAUCAGACUGAUGUUGA
	U6G	UAGCU**G**AUCAGACUGAUGUUGA
	A16U	UAGCUUAUCAGACUG**U**UGUUGA
	U17G	UAGCUUAUCAGACUGA**G**GUUGA
	G18A	UAGCUUAUCAGACUGAU**A**UUGA
	U19G	UAGCUUAUCAGACUGAUG**G**UGA
	U20G	UAGCUUAUCAGACUGAUGU**G**GA
	G21A	UAGCUUAUCAGACUGAUGUU**A**A
miR-92a	None	UAUUGCACUUGUCCCGGCCUGU
	A2U	U**U**UUGCACUUGUCCCGGCCUGU
	U3G	UA**G**UGCACUUGUCCCGGCCUGU
	U4G	UAU**G**GCACUUGUCCCGGCCUGU
	G5A	UAUU**A**CACUUGUCCCGGCCUGU

	C6G	UAUUG**G**ACUUGUCCCGGCCUGU
	G16A	UAUUGCACUUGUCCC**A**GCCUGU
	G17A	UAUUGCACUUGUCCCG**A**CCUGU
	C18G	UAUUGCACUUGUCCCGG**G**CUGU
	C19G	UAUUGCACUUGUCCCGGC**G**UGU
	U20G	UAUUGCACUUGUCCCGGCC**G**GU
	G21A	UAUUGCACUUGUCCCGGCCU**A**U

Split Aptamer Design



Supplementary Fig. 3. Design of **St1:St2:miRNA**.

Supplementary Table 2. St1:St2 Designations.

Letter Designation	P2 (nts)	P4 (nts)	35G
a	9	10	+
b	9	9	+
c	9	9	-
d	8	9	-
e	8	10	-

Numeric Designation	bulge (nts)	P3 (nts)
1	2	3
2	1	3
3	0	3
4	2	2
5	1	2
6	2	1
7	1	1
8	0	0

Supplementary Table 3. DNA aptamer precursor strands **St1** and **St2**.

ID	Aptamer DNA Precursor	
	Sequence (5′ to 3′)	
	St1	St2
Targeting miR-19b		
a1	CGCAATTACGGGGGAGGGTGTGTGGTCTTGTCTTGGATTTGCA	AGTTTTGCATTTGACGCTTGGTTCGTATTGCG
a2	CGCAATTACGGGGGAGGGTGTGTGGTCTTGTCTGGATTTGCA	AGTTTTGCATTGACGCTTGGTTCGTATTGCG
a3	CGCAATTACGGGGGAGGGTGTGTGGTCTTGTCGGATTTGCA	AGTTTTGCATGACGCTTGGTTCGTATTGCG
a4	CGCAATTACGGGGGAGGGTGTGTGGTCTTGTTTGGATTTGCA	AGTTTTGCATTTACGCTTGGTTCGTATTGCG
a5	CGCAATTACGGGGGAGGGTGTGTGGTCTTGTTGGATTTGCA	AGTTTTGCATTACGCTTGGTTCGTATTGCG
a6	CGCAATTACGGGGGAGGGTGTGTGGTCTTGTTGGATTTGCA	AGTTTTGCATTTCGCTTGGTTCGTATTGCG
a7	CGCAATTACGGGGGAGGGTGTGTGGTCTTGTGGATTTGCA	AGTTTTGCATTCGCTTGGTTCGTATTGCG
a8	CGCAATTACGGGGGAGGGTGTGTGGTCTTGGATTTGCA	AGTTTTGCATGCTTGGTTCGTATTGCG
b1	CGCAATTACGGGGGAGGGTGTGTGGTCTTGTCTTGGATTTGCA	GTTTTGCATTTGACGCTTGGTTCGTATTGCG
b2	CGCAATTACGGGGGAGGGTGTGTGGTCTTGTCTGGATTTGCA	GTTTTGCATTTGACGCTTGGTTCGTATTGCG
b3	CGCAATTACGGGGGAGGGTGTGTGGTCTTGTCGGATTTGCA	GTTTTGCATTGACGCTTGGTTCGTATTGCG
b4	CGCAATTACGGGGGAGGGTGTGTGGTCTTGTTTGGATTTGCA	GTTTTGCATTTACGCTTGGTTCGTATTGCG
b5	CGCAATTACGGGGGAGGGTGTGTGGTCTTGTTGGATTTGCA	GTTTTGCATTACGCTTGGTTCGTATTGCG
b6	CGCAATTACGGGGGAGGGTGTGTGGTCTTGTTGGATTTGCA	GTTTTGCATTTCGCTTGGTTCGTATTGCG
b7	CGCAATTACGGGGGAGGGTGTGTGGTCTTGTGGATTTGCA	GTTTTGCATTCGCTTGGTTCGTATTGCG
b8	CGCAATTACGGGGGAGGGTGTGTGGTCTTGGATTTGCA	GTTTTGCATGCTTGGTTCGTATTGCG
c1	CGCAATTACGGGGGAGGGTGTGTGGTCTTGTCTTGATTTGCAC	GTTTTGCATTTGACGCTTGGTTCGTATTGCG
c2	CGCAATTACGGGGGAGGGTGTGTGGTCTTGTCTGATTTGCAC	GTTTTGCATTTGACGCTTGGTTCGTATTGCG
c3	CGCAATTACGGGGGAGGGTGTGTGGTCTTGTCGATTTGCAC	GTTTTGCATTGACGCTTGGTTCGTATTGCG
c4	CGCAATTACGGGGGAGGGTGTGTGGTCTTGTTTGATTTGCAC	GTTTTGCATTTACGCTTGGTTCGTATTGCG
c5	CGCAATTACGGGGGAGGGTGTGTGGTCTTGTTGATTTGCAC	GTTTTGCATTACGCTTGGTTCGTATTGCG
c6	CGCAATTACGGGGGAGGGTGTGTGGTCTTGTTGATTTGCAC	GTTTTGCATTTCGCTTGGTTCGTATTGCG
c7	CGCAATTACGGGGGAGGGTGTGTGGTCTTGTGATTTGCAC	GTTTTGCATTCGCTTGGTTCGTATTGCG
c8	CGCAATTACGGGGGAGGGTGTGTGGTCTTGATTTGCAC	GTTTTGCATGCTTGGTTCGTATTGCG
d1	CGCAATTACGGGGGAGGGTGTGTGGTCTTGTCTTGATTTGCA	GTTTTGCATTTGACGCTTGGTTCGTATTGCG
d2	CGCAATTACGGGGGAGGGTGTGTGGTCTTGTCTGATTTGCA	GTTTTGCATTTGACGCTTGGTTCGTATTGCG
d3	CGCAATTACGGGGGAGGGTGTGTGGTCTTGTCGATTTGCA	GTTTTGCATTGACGCTTGGTTCGTATTGCG
d4	CGCAATTACGGGGGAGGGTGTGTGGTCTTGTTTGATTTGCA	GTTTTGCATTTACGCTTGGTTCGTATTGCG
d5	CGCAATTACGGGGGAGGGTGTGTGGTCTTGTTGATTTGCA	GTTTTGCATTACGCTTGGTTCGTATTGCG
d6	CGCAATTACGGGGGAGGGTGTGTGGTCTTGTTGATTTGCA	GTTTTGCATTTCGCTTGGTTCGTATTGCG
d7	CGCAATTACGGGGGAGGGTGTGTGGTCTTGTGATTTGCA	GTTTTGCATTCGCTTGGTTCGTATTGCG
d8	CGCAATTACGGGGGAGGGTGTGTGGTCTTGATTTGCA	GTTTTGCATGCTTGGTTCGTATTGCG
e1	CGCAATTACGGGGGAGGGTGTGTGGTCTTGTCTTGATTTGCA	AGTTTTGCATTTGACGCTTGGTTCGTATTGCG
e2	CGCAATTACGGGGGAGGGTGTGTGGTCTTGTCTGATTTGCA	AGTTTTGCATTGACGCTTGGTTCGTATTGCG
e3	CGCAATTACGGGGGAGGGTGTGTGGTCTTGTCGATTTGCA	AGTTTTGCATGACGCTTGGTTCGTATTGCG
e4	CGCAATTACGGGGGAGGGTGTGTGGTCTTGTTTGATTTGCA	AGTTTTGCATTTACGCTTGGTTCGTATTGCG
e5	CGCAATTACGGGGGAGGGTGTGTGGTCTTGTTGATTTGCA	AGTTTTGCATTACGCTTGGTTCGTATTGCG

e6	CGCAATTACGGGGGAGGGTGTGTGGTCTTGTTGATTTGCA	AGTTTTGCATTTCGCTTGGTTCGTATTGCG
e7	CGCAATTACGGGGGAGGGTGTGTGGTCTTGTGATTTGCA	AGTTTTGCATTCGCTTGGTTCGTATTGCG
e8	CGCAATTACGGGGGAGGGTGTGTGGTCTTGATTTGCA	AGTTTTGCATGCTTGGTTCGTATTGCG
Targeting miR-21		
a1	CGCAATTACGGGGGAGGGTGTGTGGTCTTGTCTTCTGATAAGC	CAACATCAGTTTGACGCTTGGTTCGTATTGCG
a2	CGCAATTACGGGGGAGGGTGTGTGGTCTTGTCTCTGATAAGC	CAACATCAGTTGACGCTTGGTTCGTATTGCG
a6	CGCAATTACGGGGGAGGGTGTGTGGTCTTGTTCTGATAAGC	CAACATCAGTTTCGCTTGGTTCGTATTGCG
a7	CGCAATTACGGGGGAGGGTGTGTGGTCTTGTCTGATAAGC	CAACATCAGTTCGCTTGGTTCGTATTGCG
a8	CGCAATTACGGGGGAGGGTGTGTGGTCTTCTGATAAGC	CAACATCAGTGCTTGGTTCGTATTGCG
b1	CGCAATTACGGGGGAGGGTGTGTGGTCTTGTCTTCTGATAAGC	AACATCAGTTTGACGCTTGGTTCGTATTGCG
b7	CGCAATTACGGGGGAGGGTGTGTGGTCTTGTCTGATAAGC	AACATCAGTTCGCTTGGTTCGTATTGCG
b8	CGCAATTACGGGGGAGGGTGTGTGGTCTTCTGATAAGC	AACATCAGTGCTTGGTTCGTATTGCG
c1	CGCAATTACGGGGGAGGGTGTGTGGTCTTGTCTTTGATAAGCT	AACATCAGTTTGACGCTTGGTTCGTATTGCG
c2	CGCAATTACGGGGGAGGGTGTGTGGTCTTGTCTTGATAAGCT	AACATCAGTTGACGCTTGGTTCGTATTGCG
c3	CGCAATTACGGGGGAGGGTGTGTGGTCTTGTCTGATAAGCT	AACATCAGTGACGCTTGGTTCGTATTGCG
c4	CGCAATTACGGGGGAGGGTGTGTGGTCTTGTTTTGATAAGCT	AACATCAGTTTACGCTTGGTTCGTATTGCG
Targeting miR-92a		
a1	CGCAATTACGGGGGAGGGTGTGTGGTCTTGTCTTCAAGTGCAA	CAGGCCGGGATTGACGCTTGGTTCGTATTGCG
a2	CGCAATTACGGGGGAGGGTGTGTGGTCTTGTCTCAAGTGCAA	CAGGCCGGGATGACGCTTGGTTCGTATTGCG
a6	CGCAATTACGGGGGAGGGTGTGTGGTCTTGTTCAAGTGCAA	CAGGCCGGGATTCGCTTGGTTCGTATTGCG
a7	CGCAATTACGGGGGAGGGTGTGTGGTCTTGTCAAGTGCAA	CAGGCCGGGATCGCTTGGTTCGTATTGCG
a8	CGCAATTACGGGGGAGGGTGTGTGGTCTTCAAGTGCAA	CAGGCCGGGAGCTTGGTTCGTATTGCG
b1	CGCAATTACGGGGGAGGGTGTGTGGTCTTGTCTTCAAGTGCAA	AGGCCGGGATTGACGCTTGGTTCGTATTGCG
b7	CGCAATTACGGGGGAGGGTGTGTGGTCTTGTCAAGTGCAA	AGGCCGGGATCGCTTGGTTCGTATTGCG
b8	CGCAATTACGGGGGAGGGTGTGTGGTCTTCAAGTGCAA	AGGCCGGGAGCTTGGTTCGTATTGCG
c1	CGCAATTACGGGGGAGGGTGTGTGGTCTTGTCTTAAGTGCAAT	AGGCCGGGATTGACGCTTGGTTCGTATTGCG
c2	CGCAATTACGGGGGAGGGTGTGTGGTCTTGTCTAAGTGCAAT	AGGCCGGGATGACGCTTGGTTCGTATTGCG
c3	CGCAATTACGGGGGAGGGTGTGTGGTCTTGTCAAGTGCAAT	AGGCCGGGAGACGCTTGGTTCGTATTGCG
c4	CGCAATTACGGGGGAGGGTGTGTGGTCTTGTTTAAGTGCAAT	AGGCCGGGATTACGCTTGGTTCGTATTGCG

MiRNA Detection

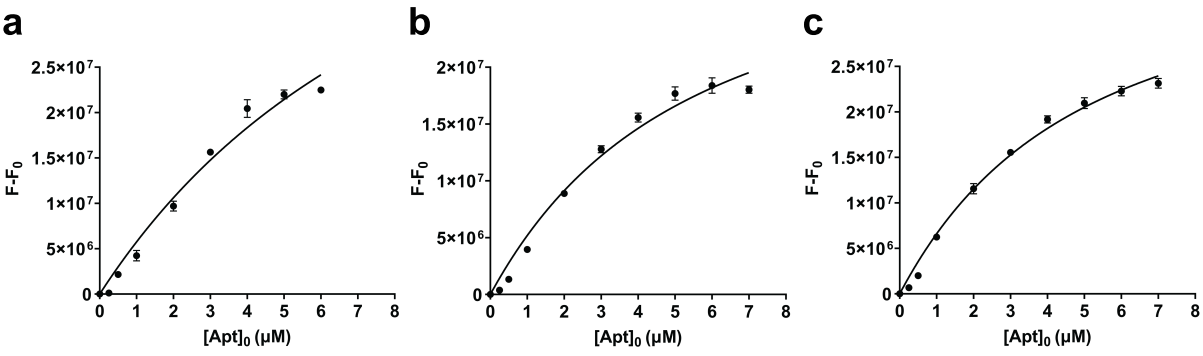
DNA strands St1 and St2 (100 μM stock solutions) were mixed and diluted with FAB (volumes vary depending on the assay). The mixtures were incubated at 80 ºC for 5 minutes and then cooled to RT. The resulting solution was aliquoted into 96 well plates (Greiner Bio-one). To these wells were sequentially added stock solutions of AO (25 μM) and miRNA (20 μM). The final concentrations were the following: 2 μM St1, 2 μM St2, 10 μM AO, and 2 μM miRNA. Fluorescence intensity of the samples were measured at 475 nm excitation over one hour on a microplate reader (SpectraMax iD3, Molecular Devices, LLC). For the samples containing biological media, undiluted CM, HS, or HP was first incubated with murine RNase inhibitor at RT for 5 minutes. The resulting solution was then added into 96 well plates. The pre-incubated St1 and St2, followed by AO and miRNA solutions were added into these wells. The final concentrations were the following: 10% or 30% (v/v) CM, HS, or HP, 2 μM St1, 2 μM St2, 10 μM AO, 2 μM miRNA, and 1 unit/μL murine RNase inhibitor. Of note, fluorescence intensities were observed to typically decrease by ~30% in FAB upon addition of murine RNase inhibitor.

Dissociation Constant Determination

The dissociation constant of AO binding to the LigBR, K_d, was calculated in the presence and absence of the target miRNA using fluorescence assays. To calculate the K_d with miRNA, $K_{d,1}$, a mixture of St1, St2, and miRNA was incubated at 80 ºC for 5 minutes and then cooled to RT to form the aptamer. Next, the aptamer was diluted to concentrations ranging from 0.25 to 7 μM. To these samples was added AO (1 μM final concentration) at RT and fluorescence intensities were continuously recorded by the plate reader. The intensities were used to calculate $K_{d,1}$ by fitting by nonlinear regression analysis to the equation below, which was derived from 1-to-1 binding.

$$[Bound\ AO]_1 = \frac{F_1 - F_0}{F_{max}}[AO]_T = \frac{[Apt]_T + [AO]_T + K_{d,1}}{2} - \sqrt{\left(\frac{[Apt]_T + [AO]_T + K_{d,1}}{2}\right)^2 - [Apt]_T[AO]_T} \quad [1]$$

In this equation, F_1 represents the observed fluorescence intensity, F_0 is the fluorescence intensity of 1 μM AO, F_{max} is the theoretical maximum fluorescence intensity based on a defined AO concentration, and $[Apt]_T$ and $[AO]_T$ are the total concentrations of the aptamer and AO, respectively. $[Bound\ AO]_1$ is the concentration of bound state of AO in the presence of miRNA.



Supplementary Fig. 4. Determination of $K_{d,1}$ for complexes of AO and **(a)** c2-19b:miR-19b, **(b)** a2-21:miR-21, and **(c)** b7-92a:miR-92a. Error bars denote the standard deviation of three replicates.

Fitting by non-linear regression could not be used to determine $K_{d,2}$, which is the dissociation constant of AO binding to LigBR in the absence of the miRNA, because generation of adequate signal for curve fitting would require orders of magnitude higher St1 and St2 concentrations. To estimate the $K_{d,2}$, we used the F_1/F_2 obtained in the miRNA detection section. We first made 2 assumptions: 1) the fluorescence output of LigBR-bound AO is the same both in the presence and absence of the miRNA, and 2) the ratio of fluorescence intensity in the presence of miRNA to in the absence of miRNA is close to the ratio of bound AO in the presence of miRNA to in the absence of miRNA.

$$\frac{[Bound\ AO]_1}{[Bound\ AO]_2} \approx \frac{F_1}{F_2} \qquad [2]$$

In this equation, $[\text{Bound AO}]_2$ is the concentration of bound state of AO in the absence miRNA. Substituting equation [2] into equation [1], $K_{d,2}$ can be solved using the previously determined $K_{d,1}$.

$$[Bound\ AO]_1 \times \frac{F_2}{F_1} = \frac{[Apt]_T + [AO]_T + K_{d,2}}{2} - \sqrt{\left(\frac{[Apt]_T + [AO]_T + K_{d,2}}{2}\right)^2 - [Apt]_T[AO]_T}$$

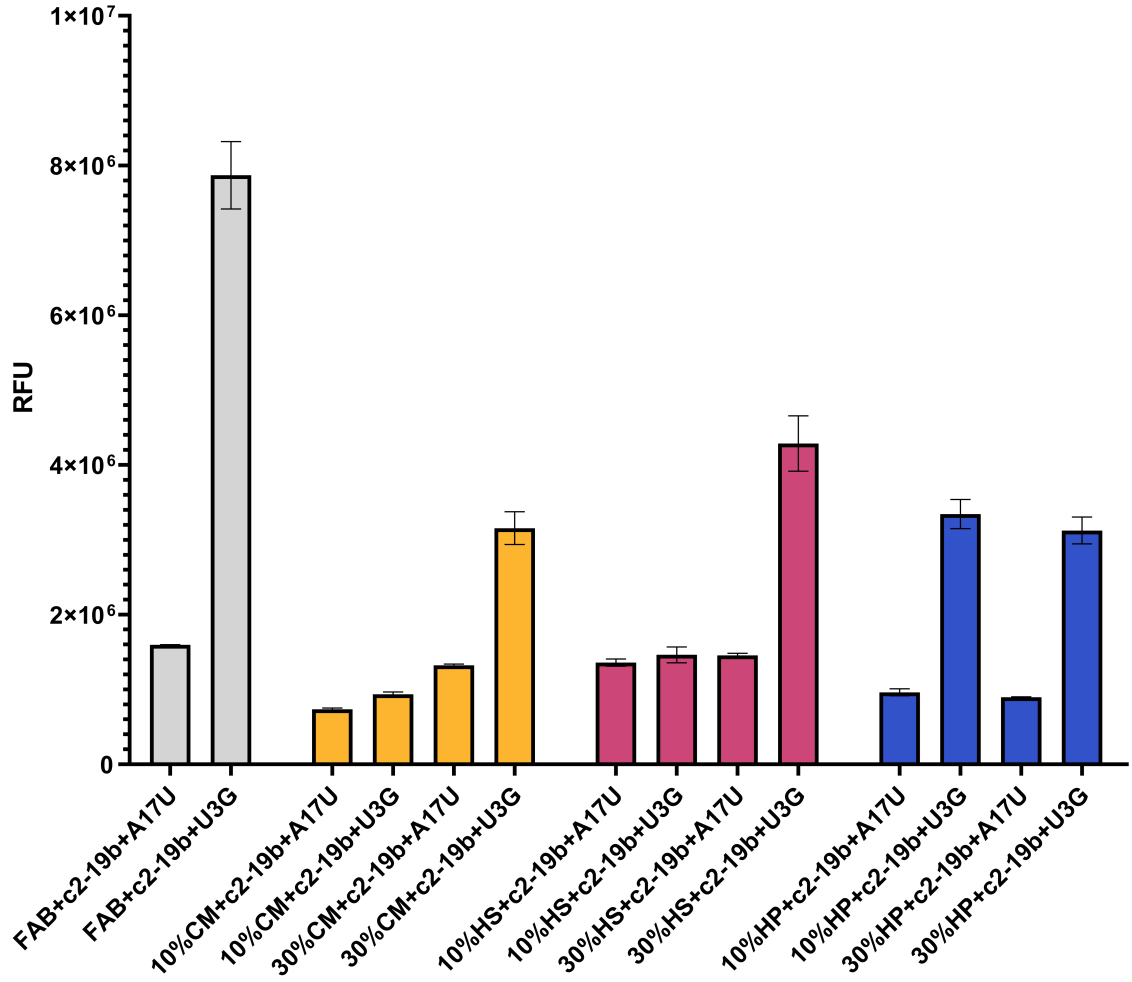
Supplementary Table 4. Summary of data fitting.

complex	$K_{d,1}$ (μM)	R^2	St1:St2	F/F_0	$K_{d,2}$ (μM)*
c2-19b:miR-19b	9.1 ± 2.8	0.9876	c2-19b	9.35	180
a2-21:miR-21	4.6 ± 0.9	0.9804	a2-21	9.84	140
b7-92a:miR-92a	4.2 ± 0.5	0.9912	b7-92a	21.36	310

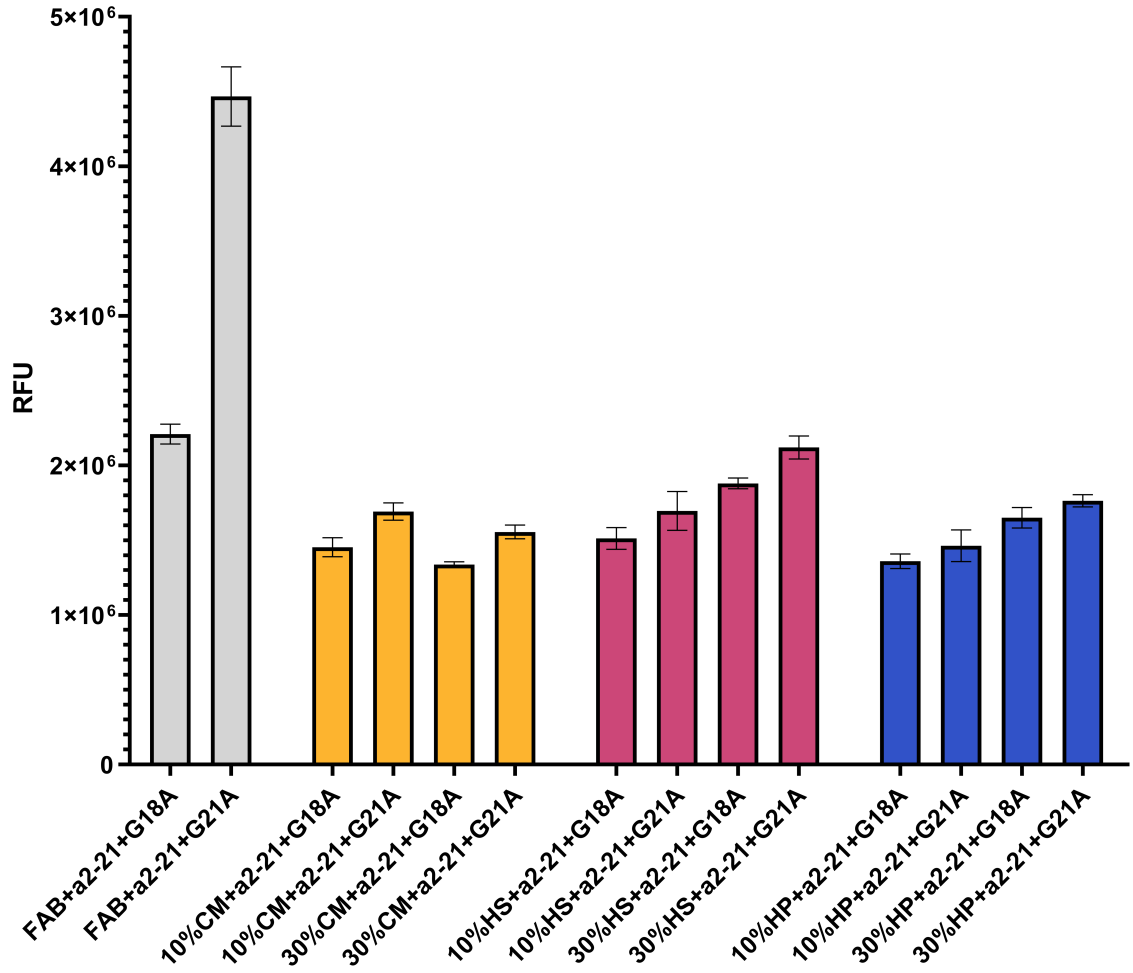
* Estimated for AO binding to St1:St2 by employing F/F_0 and measured K_d.

Sequence Specificity in Biological Media

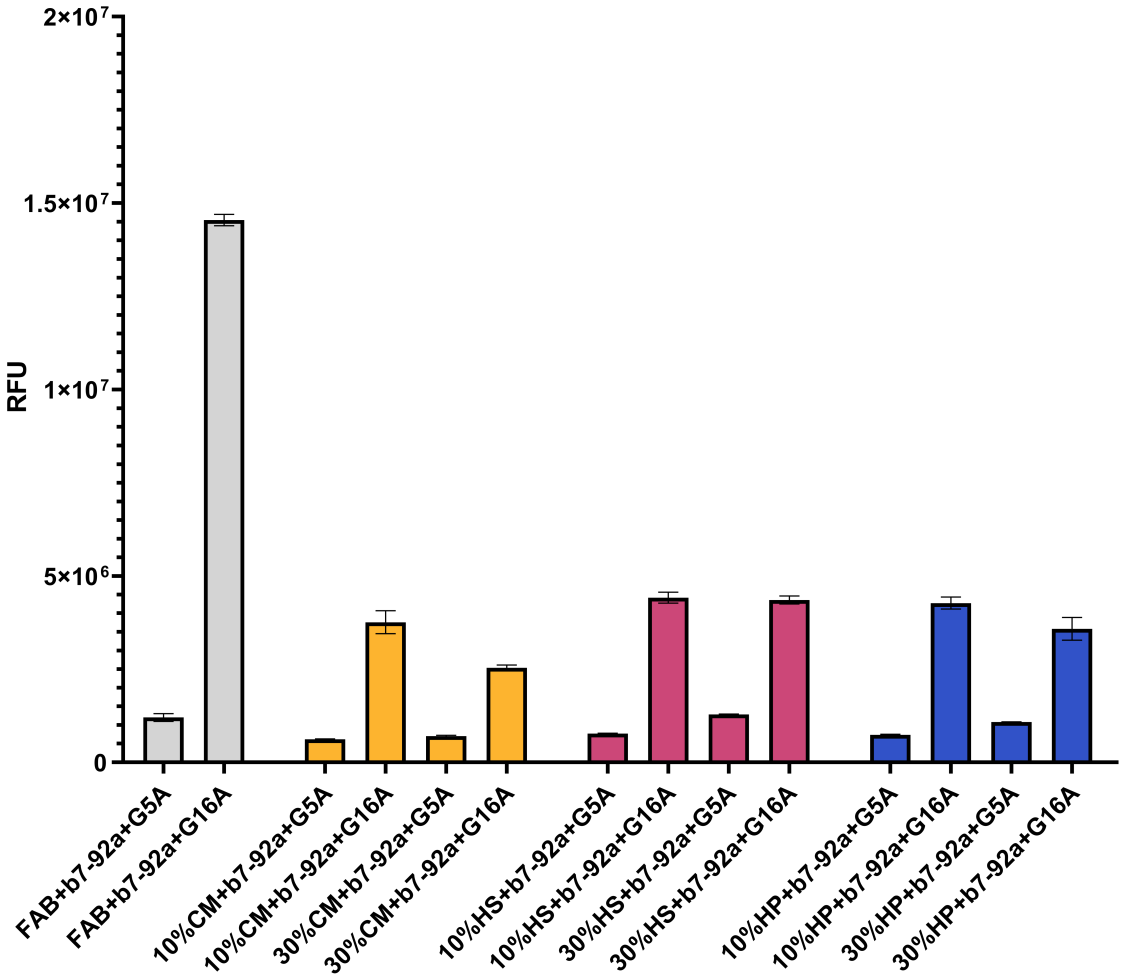
Sequence specificities of the selected St1:St2 sets were assessed in the presence of 2 μM single nucleotide mutants that led to the lowest and highest differentiation factor, D_f, in FAB. Larger D_f indicates better specificity. These mutants were then tested in biological media and the results are presented in bar graphs below. Samples prepared with biological media contained murine RNase inhibitor as described above.



Supplementary Fig. 5a. Selectivity of c2-19b against miR-19b mutants A17U and U3G.



Supplementary Fig. 5b. Selectivity of a2-21 against miR-21 mutants G18A and G21A.



Supplementary Fig. 5c. Selectivity of b7-92a against miR-92a mutants G5A and G16A.

Supplementary Table 5. Summary of D_f in FAB and biological media.

miRNA	mutants	FAB	10% CM	30% CM	10% HS	30% HS	10% HP	30% HP
miR-19b	A17U	9.8	7.1	10.3	8.2	14.6	13.7	17.9
	U3G	2.0	2.9	9.5	2.8	4.2	3.9	14.1
miR-21	G18A	10.2	7.3	12.4	7.4	12.0	6.6	12.3
	G21A	5.1	6.3	11.1	6.6	11.1	6.2	10.6
miR-92a	G5A	23.8	18.3	18.4	13.0	19.0	11.2	24.8
	G16A	2.0	14.3	13.3	10.7	14.9	9.7	15.0

Limit of Detection (LoD)

LoDs were determined by measuring the fluorescence intensities of samples of AO with St1:St2:miRNA prepared in pure buffer or 10% or 30% (v/v) biological media. Samples contained different concentrations of the target miRNA (0–100 nM for assays in FAB or 0–20 nM for assays in biological media), 100 nM St1, 100 nM St2, and 2 μM AO. Samples were prepared and the fluorescence intensities were measured after 1 hour of incubation at RT. Three replicates of experiments were performed and one-tailed Student's *t*-test was used to assess the statistical difference between the intensity at each concentration of miRNA and that at 0 nM miRNA.

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