Electronic Supplementary Information for

Chiral figure-eight molecular scaffold for fluorescent probe development

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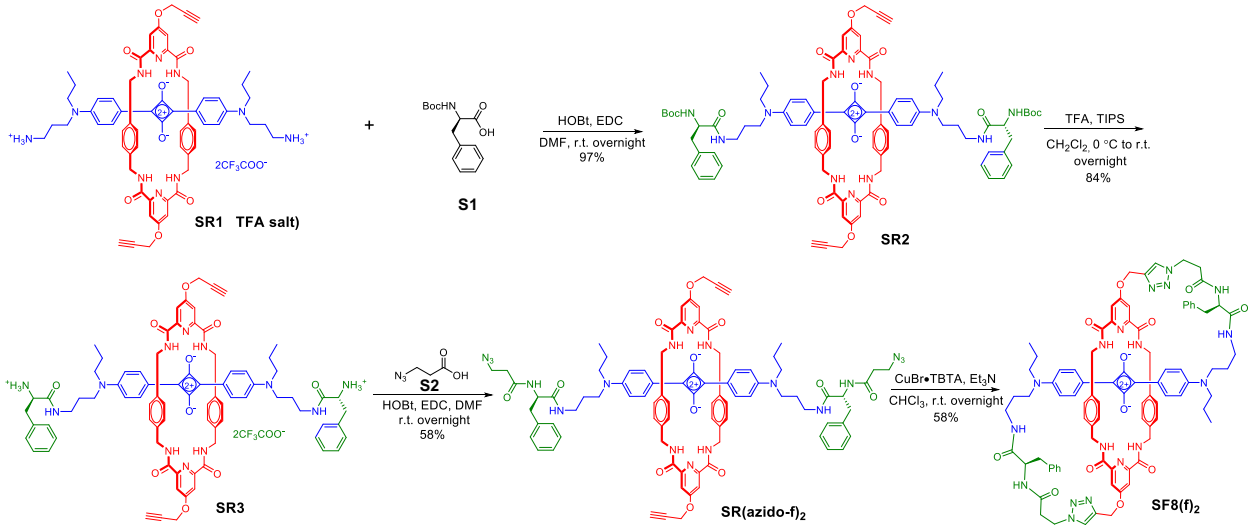
Table of Contents

1. Synthesis and Characterization



Scheme S1. Synthesis of **SF8(f)$_2$**.

Note: Compound **SR1•(TFA salt)**[1] and **S2**[2] were synthesized according to reported procedures.

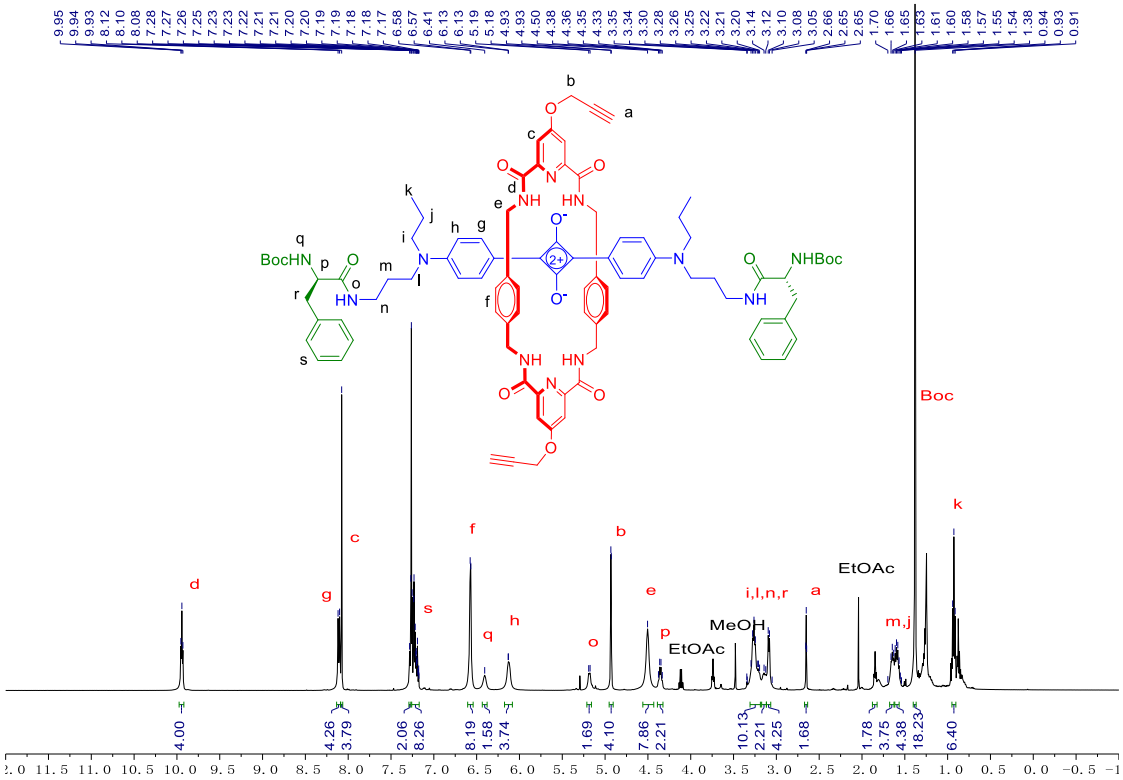
SR2. A mixture of **SR1•(TFA salt)** (15 mg, 0.0138 mmol), **S1** (44 mg, 0.165 mmol), 1-hydroxybenzotriazole hydrate (HOBt) (32 mg, 0.206 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (40 mg, 0.206 mmol) in DMF (0.5mL) was stirred at room temperature overnight. The solvent was removed by rotary evaporation and the resulting residue was purified by silica gel column chromatography (0-5% MeOH in $CHCl_3$) to give product **SR2** as green blue solid (17 mg, 78%). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 9.94 (t, *J* = 5.9 Hz, 4H), 8.11 (d, *J* = 9.0 Hz, 4H), 8.08 (s, 4H), 7.28 (s, 2H), 7.26-7.17 (m, 8H), 6.57 (d, *J* = 3.5 Hz, 8H), 6.41 (s, 2H), 6.17-6.08 (m, 4H), 5.18 (d, *J* = 8.0 Hz, 2H), 4.93 (d, *J* = 2.4 Hz, 4H), 4.50 (s, 8H), 4.35 (q, *J* = 7.4 Hz, 2H), 3.31-3.19 (m, 10H), 3.13 (d, *J* = 10.1 Hz, 2H), 3.09 (d, *J* = 7.2 Hz, 4H), 2.65 (t, *J* = 2.4 Hz, 2H), 1.68-1.63 (m, 4H), 1.59 (q, *J* = 7.6 Hz, 4H), 1.38 (s, 18H), 0.93 (t, *J* = 7.3 Hz, 6H). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 185.29, 184.49, 171.79, 166.65, 163.57, 153.29, 151.83, 136.95, 136.70, 133.77, 129.54, 129.11, 128.86, 127.19, 119.03, 111.90, 80.46, 77.46, 76.96, 68.19, 56.60, 56.40, 53.38, 49.01, 43.61, 38.65, 37.01, 31.81, 29.92, 28.49, 27.67, 25.83, 22.88, 21.09, 14.42, 14.36, 11.49. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{92}H_{103}N_{12}O_{14}^+$ 1599.7711; Found 1599.7709.

SR3. Trifluoroacetic acid (0.2 mL) was added dropwise to a solution of **SR2** (17 mg) in CH_2Cl_2 (2 mL) under ice bath. The reaction was stirred overnight. Solvent was removed and the residue was washed with Et_2O (×3) to give blue powder product **SR3** (14 mg, 96%). ^{1}H NMR (400 MHz, Methanol-d_4) δ 10.12 (t, *J* = 6.0 Hz, 4H), 8.07 (d, *J* = 10.2 Hz, 8H), 7.35-7.24 (m, 10H), 6.56 (s, 8H), 6.24 (d, *J* = 9.0 Hz, 4H), 5.11 (d, *J* = 2.4 Hz, 4H), 4.49 (d, *J* = 5.9 Hz, 8H), 3.97 (t, *J* = 7.5 Hz, 2H), 3.38-3.31 (m, 8H), 3.22 (t, *J* = 2.3 Hz, 2H), 3.20-3.04 (m, 8H), 1.60 (m, 8H), 0.92 (t, *J* = 7.4 Hz, 6H). ^{13}C NMR (101 MHz, Methanol-d_4) δ 185.50, 182.09, 168.90, 166.91, 165.50, 163.52, 153.61, 151.09, 136.49, 134.74, 133.29, 129.11, 128.64, 128.51, 127.32, 118.40, 111.63, 111.57, 77.51, 76.77, 56.19, 54.74, 48.24, 48.03, 42.84, 37.85, 36.63, 26.83, 20.37, 10.10. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{82}H_{87}N_{12}O_{10}^+$ 1399.6663; Found 1399.6643.

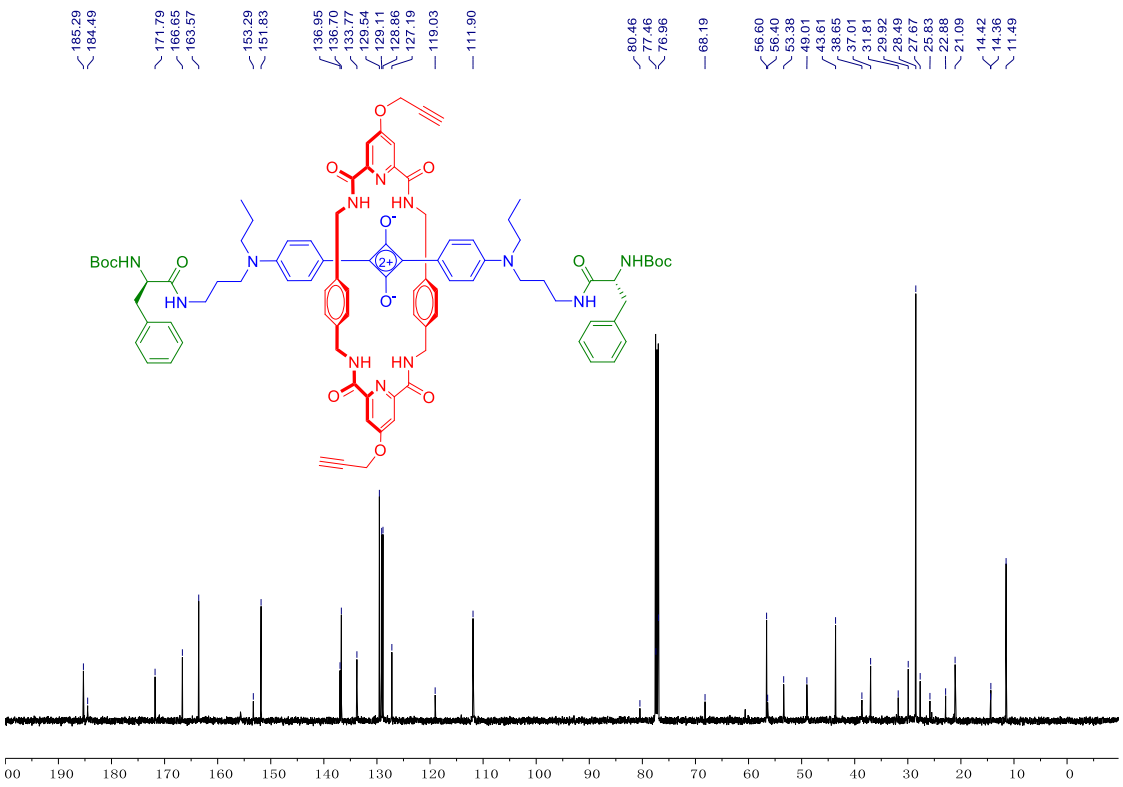
SR(azido-f)$_2$. A mixture of **SR3** (14 mg, 0.01 mmol), **S2** (14 mg, 0.12 mmol), HOBt (23 mg, 0.15 mmol) and EDC (29 mg, 0.15 mmol) in DMF (0.5mL) was stirred at room temperature overnight. The solvent was removed by rotary evaporation and the resulting residue was purified by silica gel column chromatography (0-5% MeOH in $CHCl_3$) to give product **SR(azido-f)$_2$** as green blue solid (9 mg, 58%). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.88 (t, *J* = 5.9 Hz, 4H), 8.05 (d, *J* = 8.7 Hz, 4H), 8.00 (s, 4H), 7.22 (d, *J* = 1.2 Hz, 1H), 7.19-7.07 (m, 9H), 6.51 (s, 8H), 6.39 (s, 2H), 6.06 (s, 4H), 4.88 (d, *J* = 2.4 Hz, 4H), 4.67-4.60 (m, 2H), 4.44 (d, *J* = 5.8 Hz, 8H), 3.60-3.43 (m, 6H), 3.20 (s, 6H), 3.06 (dd, *J* = 7.2, 4.1 Hz, 8H), 2.58 (t, *J* = 2.4 Hz, 2H), 2.39 (t, *J* = 6.2 Hz, 4H), 1.68 (s, 4H), 1.61-1.47 (m, 10H), 0.87 (t, *J* = 7.3 Hz, 6H). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 185.31, 171.04, 170.28, 166.66, 163.61, 151.77, 136.70, 136.68, 133.78, 129.48, 129.12,

128.91, 127.34, 111.96, 77.47, 56.63, 55.04, 47.49, 43.62, 38.33, 37.12, 35.90, 29.93, 11.50. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{88}H_{93}N_{18}O_{12}^+$ 1593.715; Found 1593.7210.

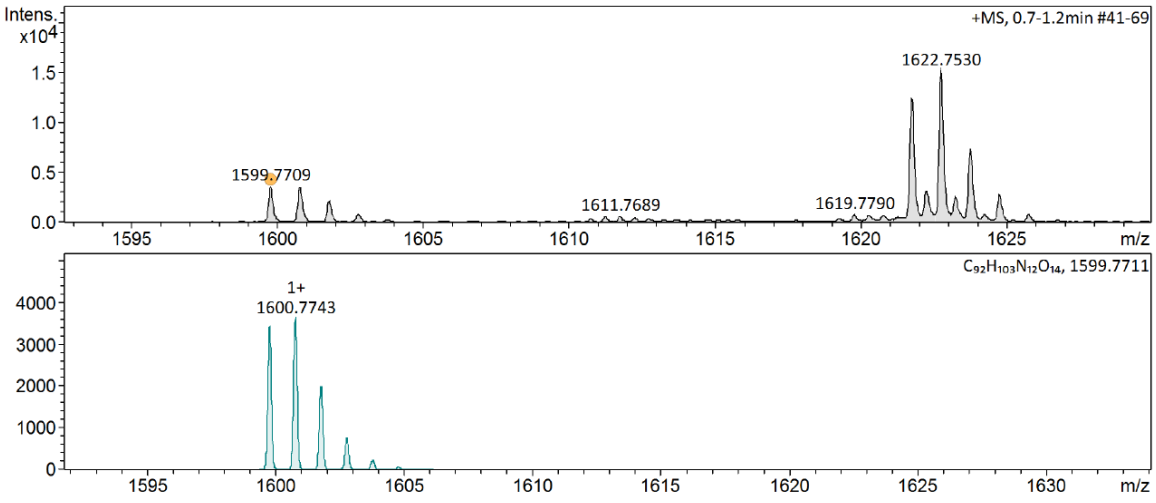
SF8(f)$_2$. Compound **SR(azido-f)$_2$** (8 mg, 0.00489 mmol) was dissolved in $CHCl_3$ (10 mL). CuBr·TBTA (6 mg) and Et_3N (14 μL, 0.0979 mmol) were added to the solution. The reaction mixture was stirred at room temperature overnight. The solvent was removed by rotary evaporation and the resulting residue was purified by silica gel column chromatography (0-10% MeOH in CH_2Cl_2) to give product **SF8(f)$_2$** as green blue solid (4.5 mg, 58%). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 10.37 (t, *J* = 8.3 Hz, 2H), 8.14 (d, *J* = 2.5 Hz, 2H), 8.03 (d, *J* = 2.5 Hz, 3H), 7.82 (s, 2H), 7.78 (s, 2H), 7.32-7.19 (m, 10H), 6.95-6.32 (m, 12H), 5.52-5.42 (m, 4H), 5.37 (s, 2H), 5.11 (m, 4H), 4.87-4.78 (m, 2H), 4.70 (dt, *J* = 13.1, 4.0 Hz, 2H), 4.54 (t, *J* = 7.7 Hz, 2H), 3.85 (dd, *J* = 14.6, 3.7 Hz, 4H), 3.10 (m, 4H), 2.99-2.86 (m, 6H), 2.83-2.69 (m, 4H), 1.65 (m, 4H), 1.53 (d, *J* = 21.6 Hz, 4H), 1.01 (t, *J* = 7.4 Hz, 6H). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 185.21, 184.08, 171.38, 170.68, 167.36, 164.05, 163.97, 153.27, 152.16, 151.77, 136.89, 136.33, 129.29, 128.65, 126.98, 124.21, 118.08, 112.18, 111.27, 62.70, 55.20, 53.17, 47.04, 43.82, 37.22, 37.06, 36.55, 29.81, 27.15, 21.05, 11.32. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{88}H_{93}N_{18}O_{12}^+$ 1593.715; Found 1593.7212.



^{1}H NMR (500 MHz, $CDCl_3$) of **SR2**

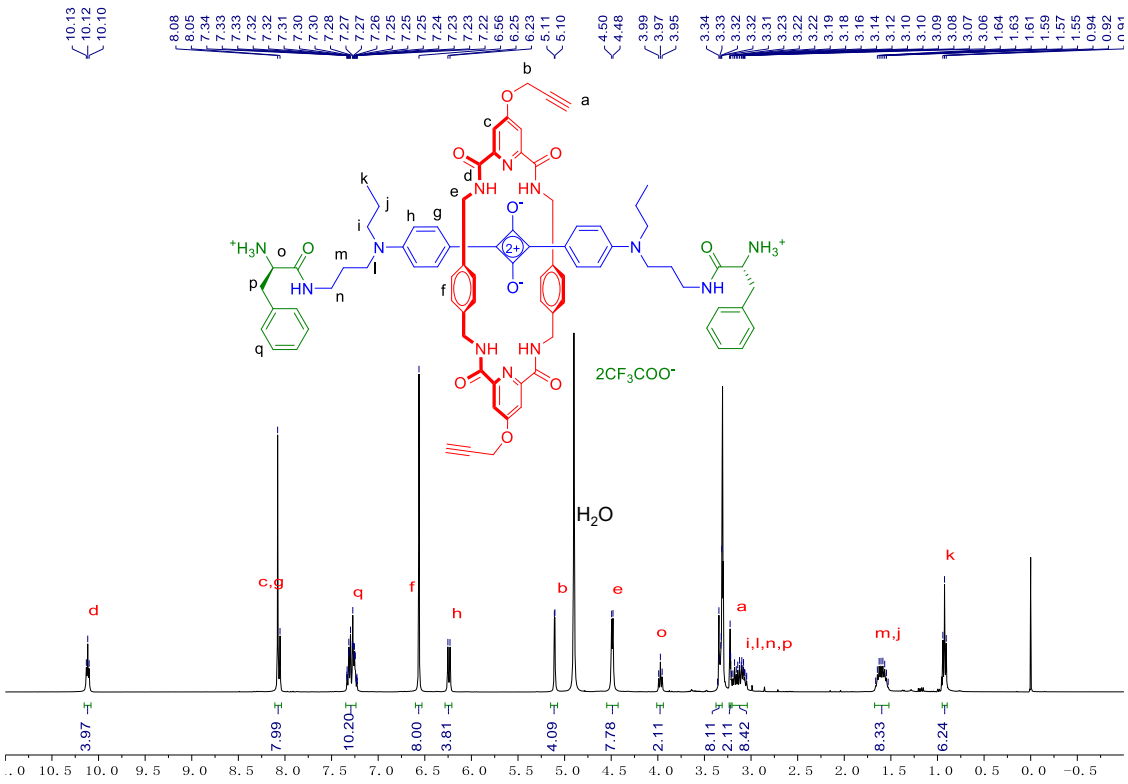


^{13}C NMR (126 MHz, $CDCl_3$) of **SR2**

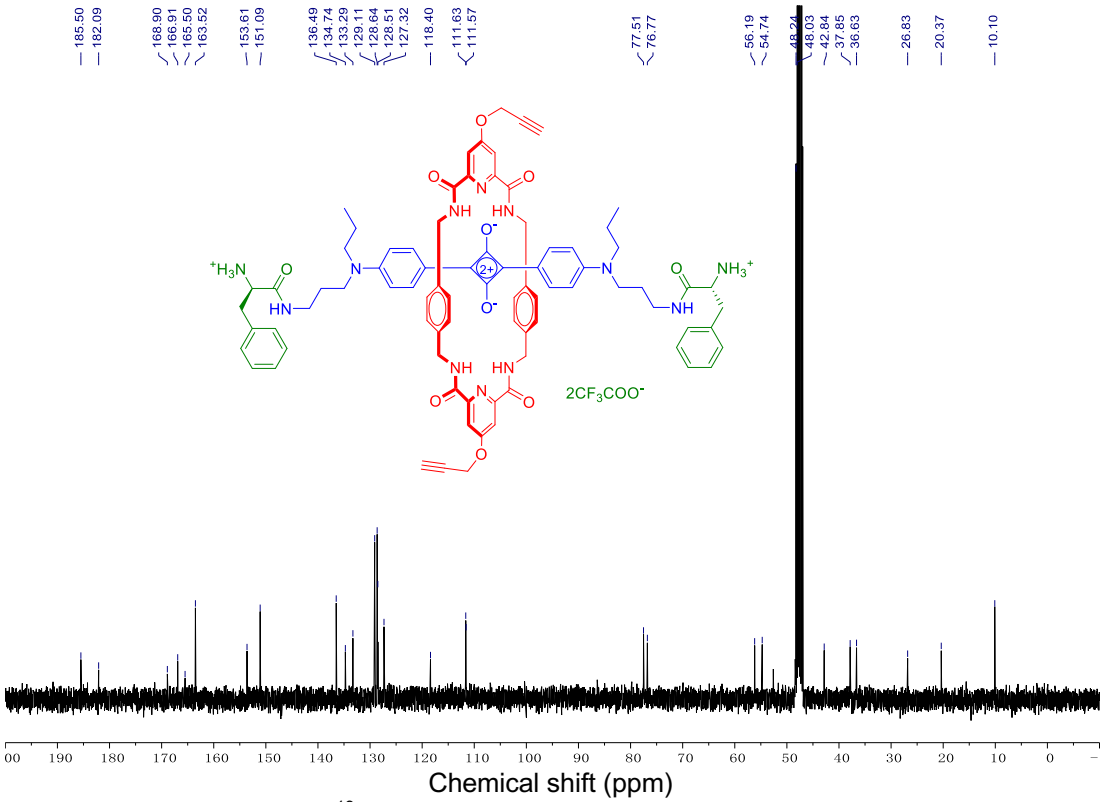


Meas. m/z	#	Ion Formula	m/z	err [ppm]	Mean err [ppm]	rdb	N-Rule	e¯ Conf
1599.770912	1	C92H103N12O14	1599.771122	0.1	-1.7	47.5	ok	even

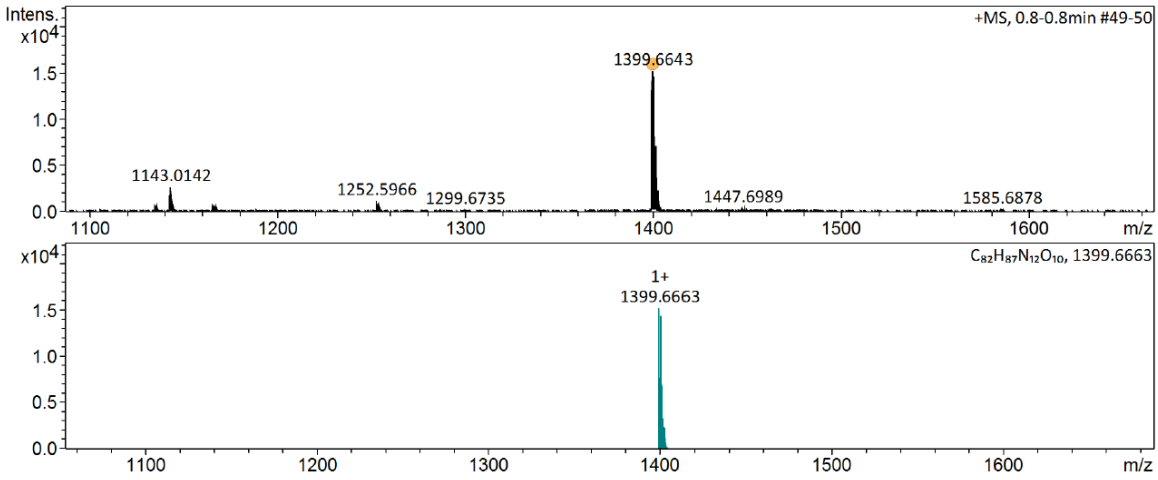
HRMS-ESI of **SR2**



^{1}H NMR (400 MHz, CD_3OD) of **SR3**

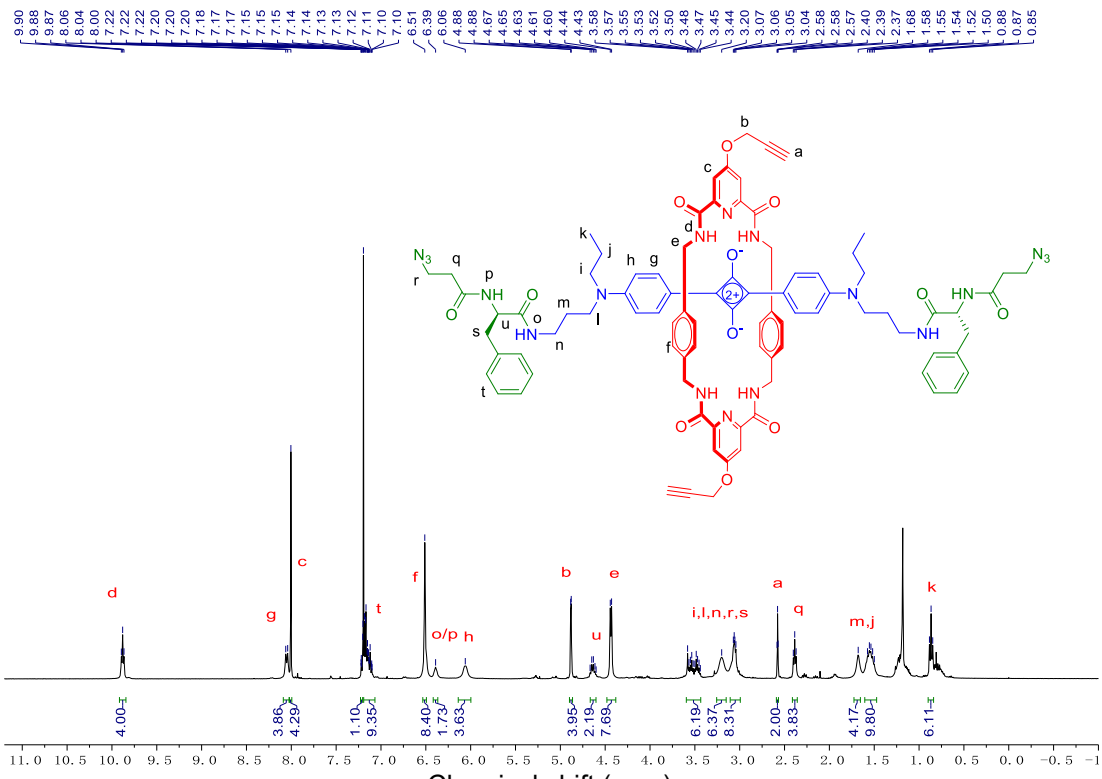


^{13}C NMR (101 MHz, CD_3OD) of **SR3**

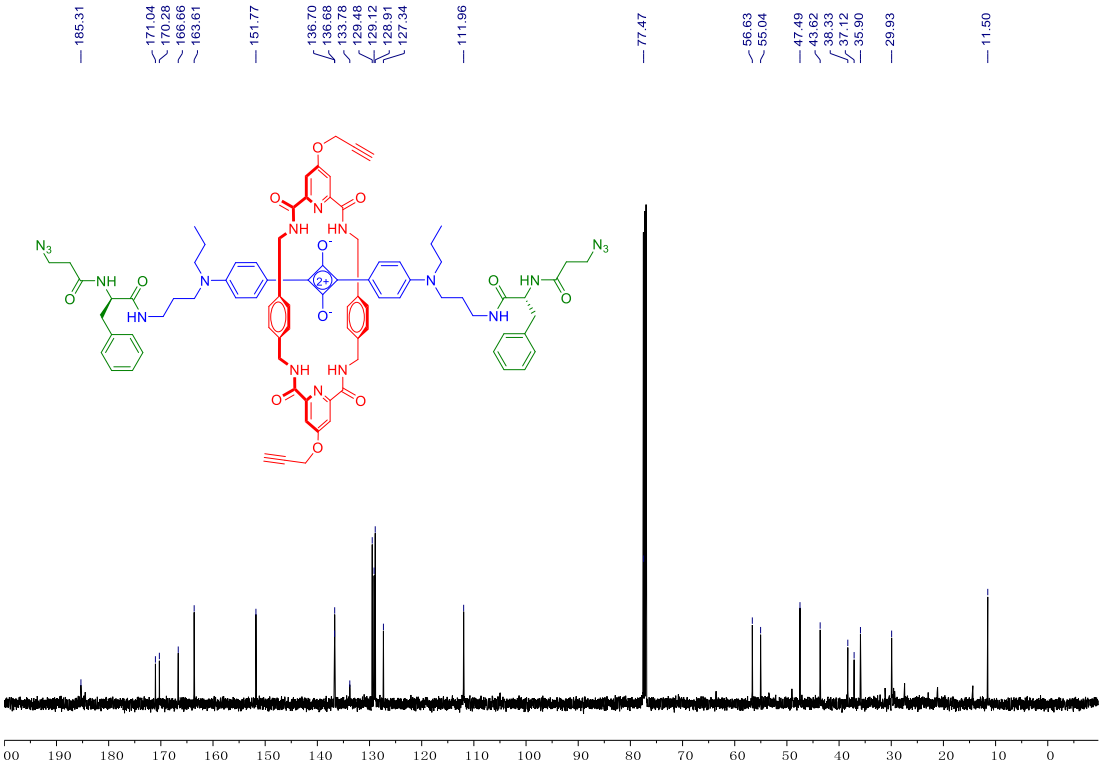


Meas. m/z	#	Ion Formula	m/z	err [ppm]	Mean err [ppm]	rdb	N-Rule	e⁻ Conf
1399.664300	1	C82H87N12O10	1399.666263	1.4	2.5	45.5	ok	even

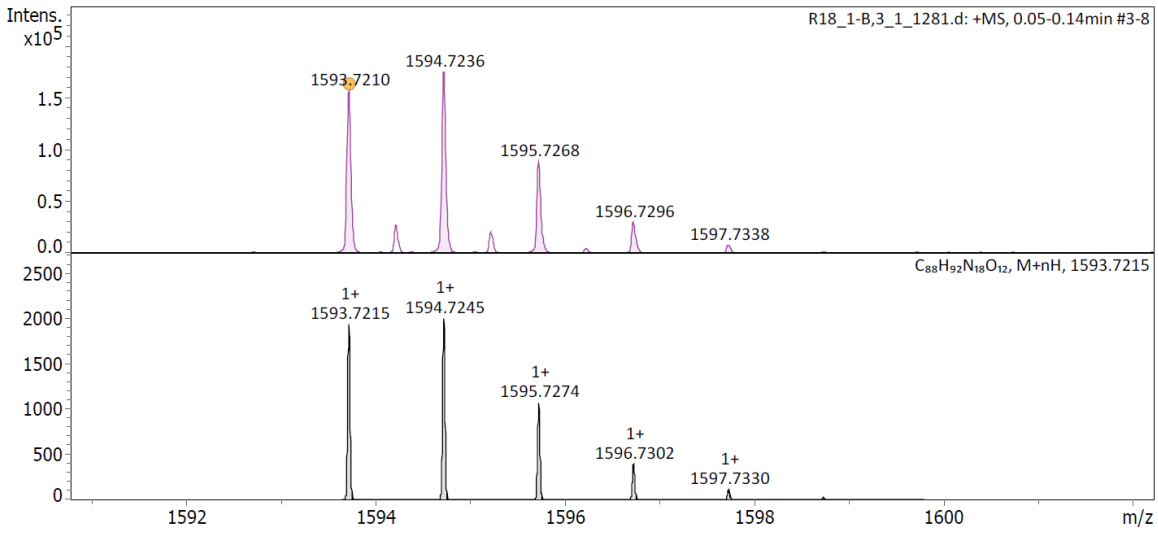
HRMS-ESI of **SR3**



^{1}H NMR (400 MHz, $CDCl_3$) of **SR(azido-f)$_2$**

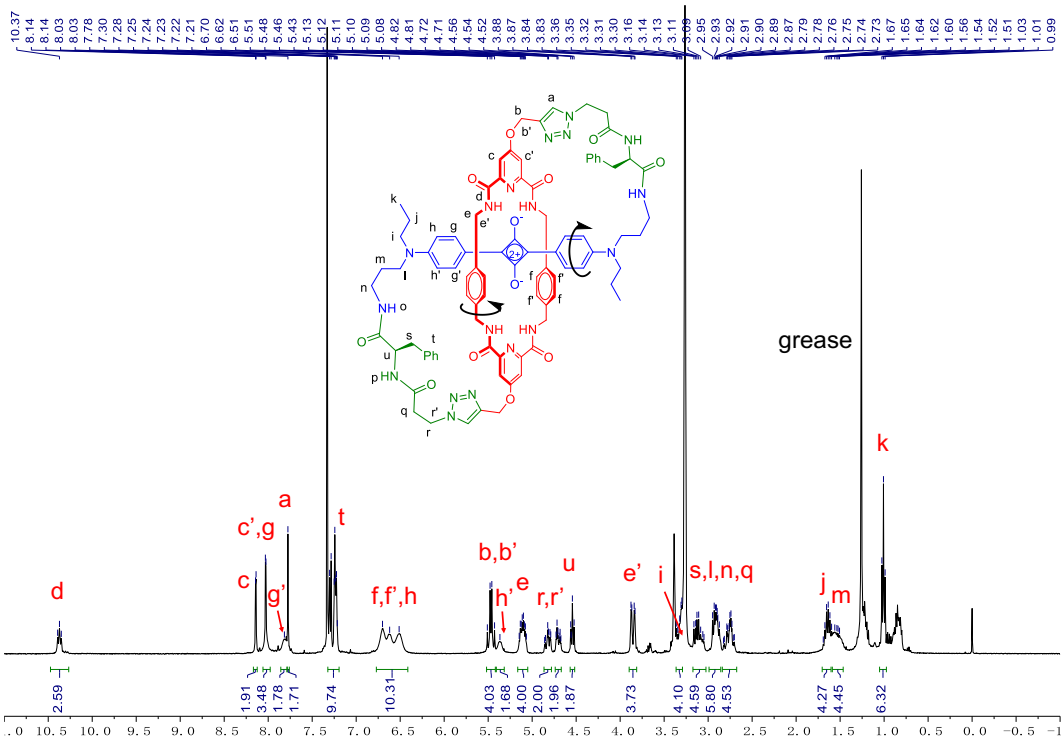


^{13}C NMR (126 MHz, $CDCl_3$) of **SR(azido-f)$_2$**

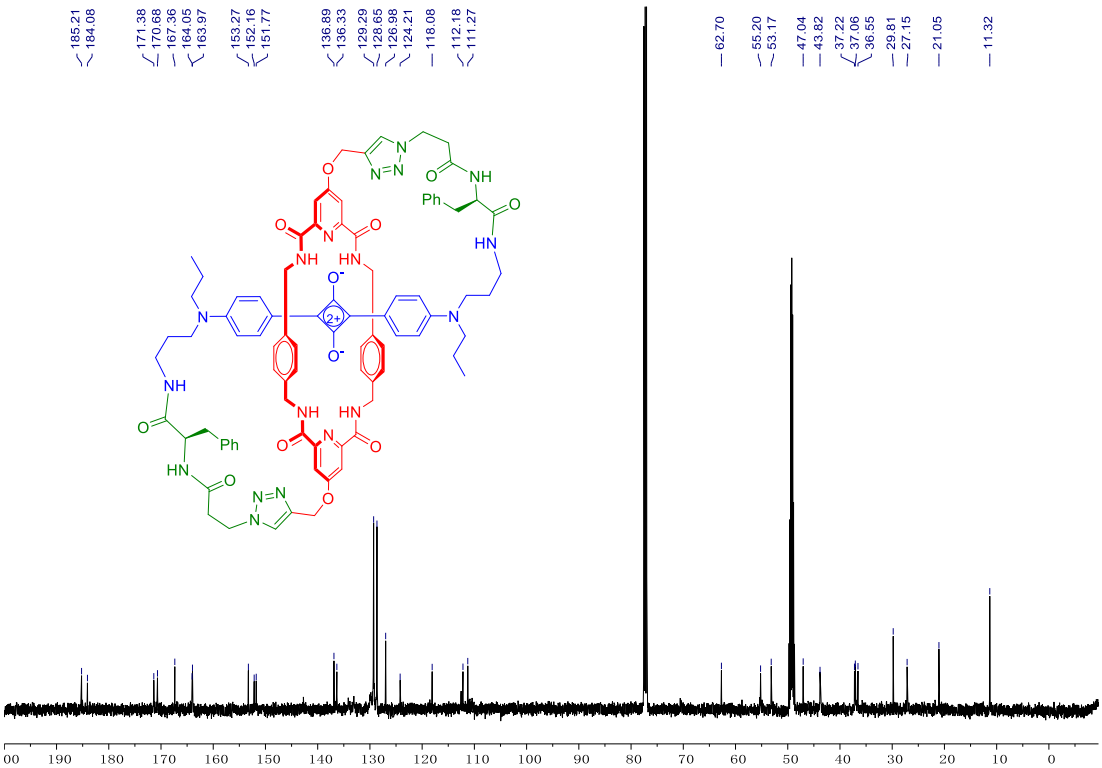


Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma	# mSigma	Score	rdb	e$^-$ Conf	N-Rule
1593.7210	1	C88H93N18O12	1593.7215	0.3	33.0	1	100.00	52.0	even	ok

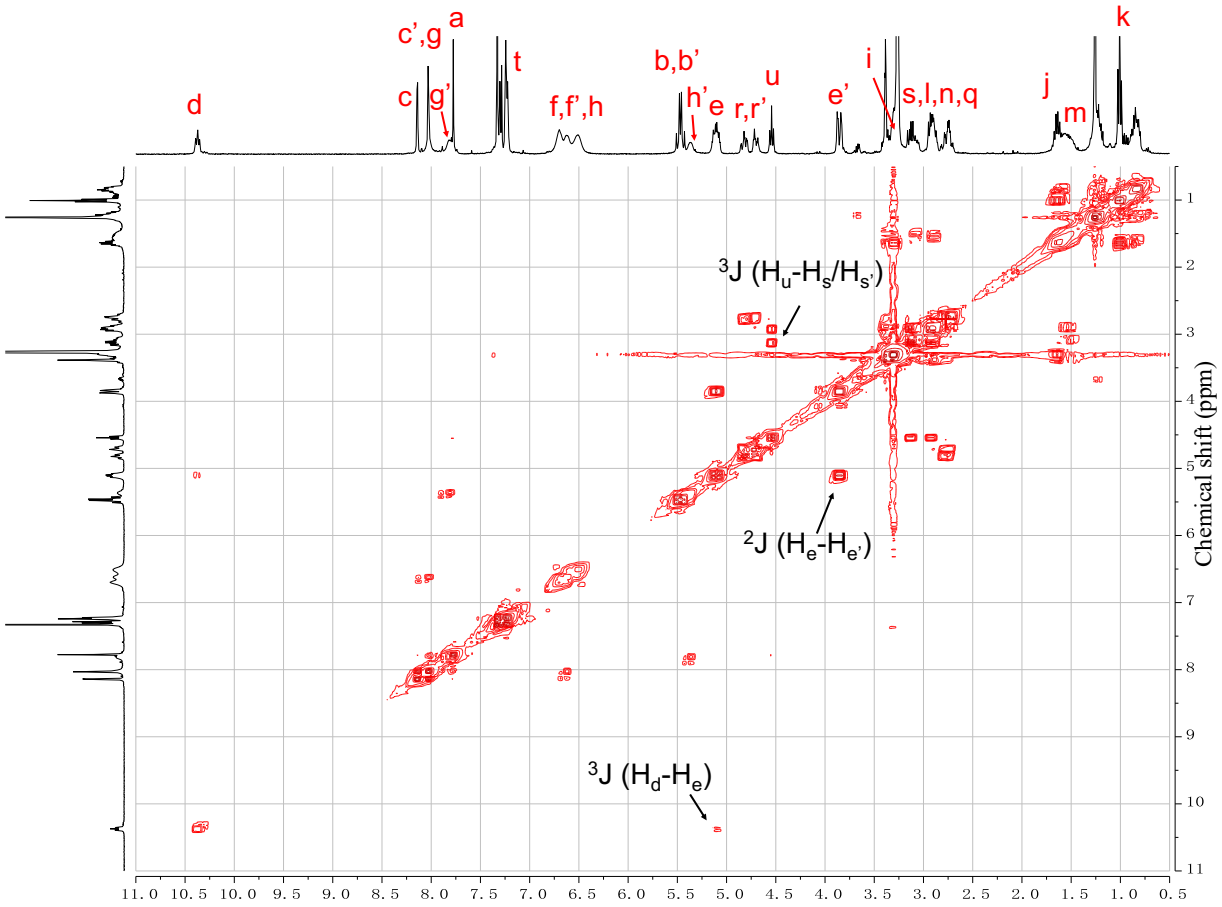
HRMS-ESI of **SR(azido-f)$_2$**



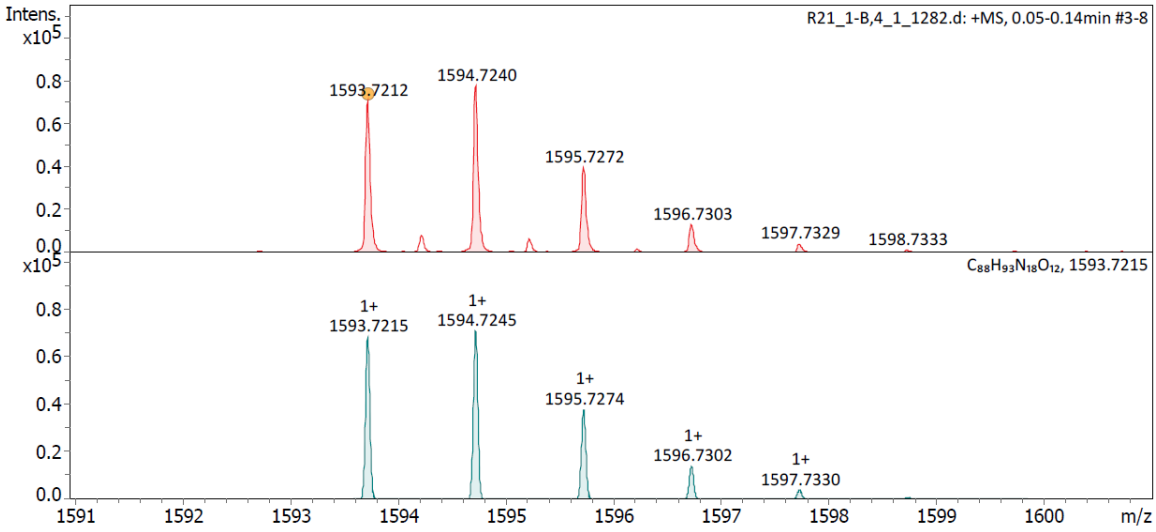
^{1}H NMR (400 MHz, 10% CD_3OD in $CDCl_3$) of **SF8(f)$_2$**



^{13}C NMR (126 MHz, 10% CD_3OD in $CDCl_3$) of **SF8(f)$_2$**

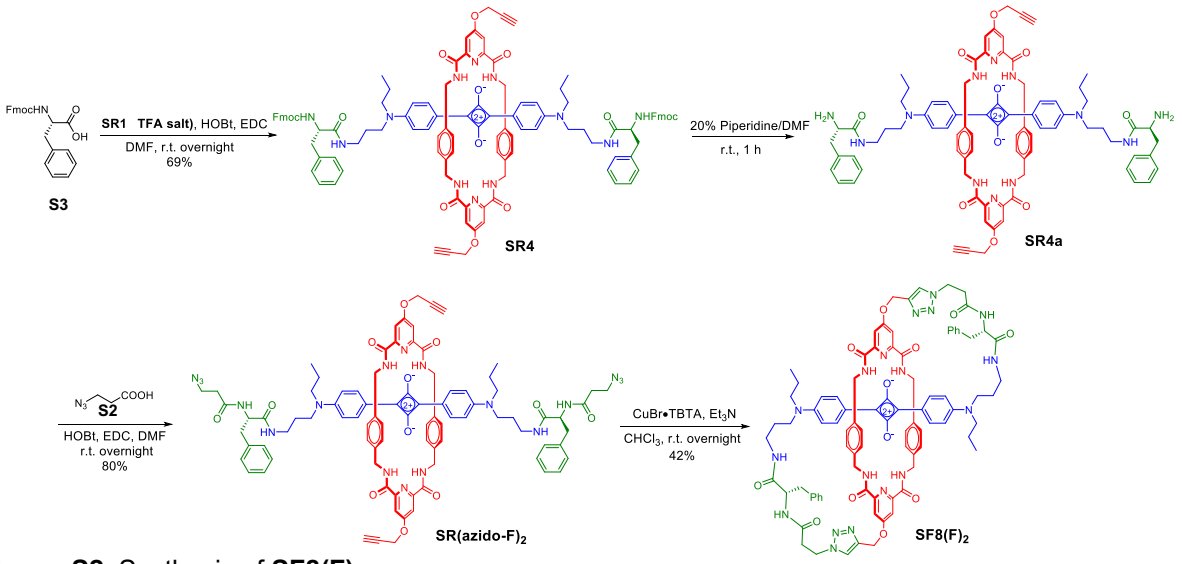


^{1}H-^{1}H COSY NMR (400 MHz, 10% CD_3OD in $CDCl_3$) of **SF8(f)$_2$**



Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma	# mSigma	Score	rdb	e¯ Conf	N-Rule
1593.7212	1	C88H93N18O12	1593.7215	0.2	28.2	1	100.00	52.0	even	ok

HRMS-ESI of **SF8(f)$_2$**

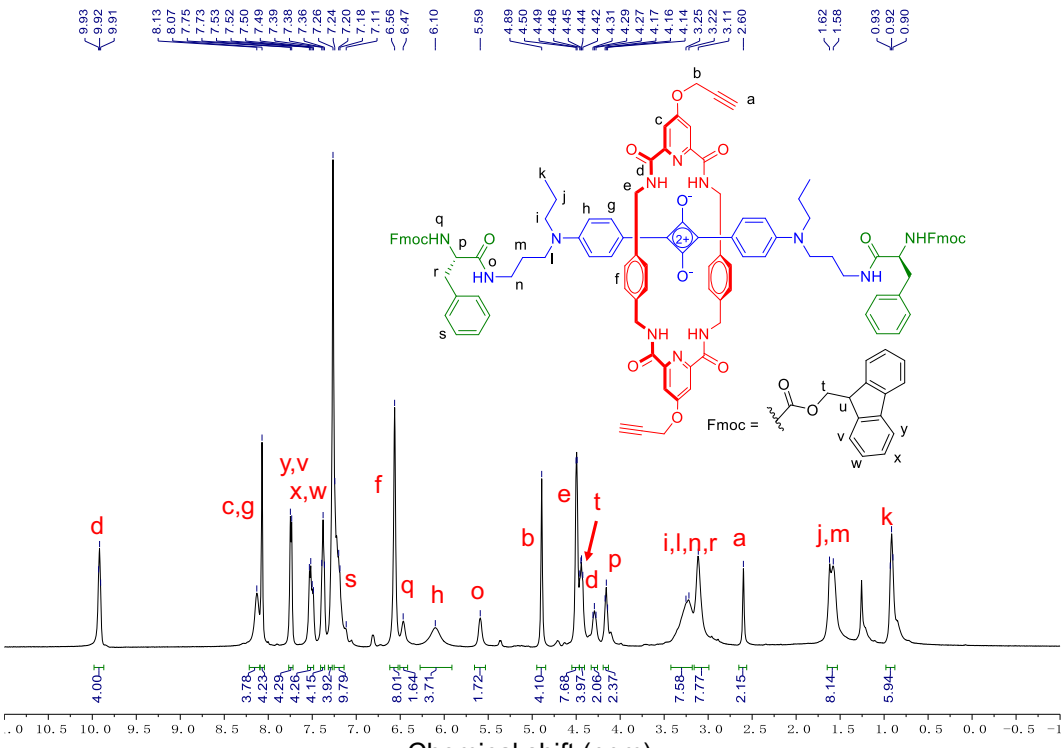


Scheme S2. Synthesis of **SF8(F)$_2$**.

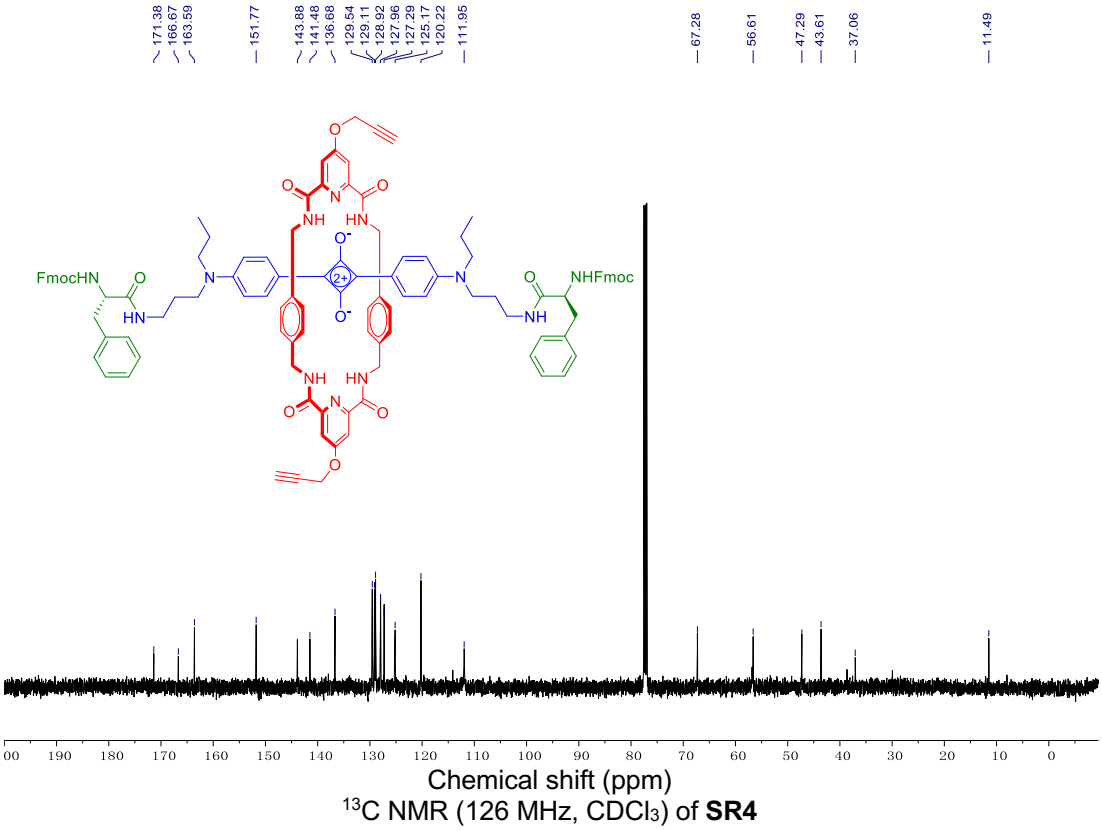
SR4. A mixture of **SR1●(TFA salt)** (15 mg, 0.013 mmol), **S3** (61 mg, 0.156 mmol), HOBt (30 mg, 0.195 mmol) and EDC (38 mg, 0.195 mmol) in DMF (0.5 mL) was stirred at room temperature overnight. The solvent was removed by rotary evaporation and the resulting residue was purified by silica gel column chromatography (0-5% MeOH in $CHCl_3$) to give product **SR4** as green blue solid (17 mg, 78%).^{1}H NMR (500 MHz, Chloroform-*d*) δ 9.92 (t, *J* = 5.8 Hz, 4H), 8.13 (s, 5H), 8.07 (s, 5H), 7.74 (d, *J* = 7.5 Hz, 5H), 7.51 (dd, *J* = 15.6, 7.4 Hz, 6H), 7.38 (t, *J* = 7.6 Hz, 6H), 7.21 (d, *J* = 29.6 Hz, 20H), 6.56 (s, 10H), 6.10 (s, 4H), 5.59 (s, 2H), 4.89 (s, 5H), 4.46 (dd, *J* = 31.7, 6.5 Hz, 13H), 4.29 (t, *J* = 8.7 Hz, 2H), 4.16 (t, *J* = 7.0 Hz, 3H), 3.18 (d, *J* = 70.1 Hz, 19H), 2.60 (s, 2H), 1.60 (d, *J* = 21.5 Hz, 11H), 0.91 (d, *J* = 7.7 Hz, 7H). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 171.38, 166.67, 163.59, 151.77, 143.88, 141.48, 136.68, 129.54, 129.11, 128.92, 127.96, 127.29, 125.17, 120.22, 111.95, 67.28, 56.61, 47.29, 43.61, 37.06, 11.49. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{112}H_{107}N_{12}O_{14}^+$ 1843.8024; Found 1843.8046.

SR(azido-F)$_2$. Compound **SR4** (17 mg, 0.009 mmol) was dissolved in 20% piperidine/DMF solution. The reaction was stirred at room temperature for 1 hour. Then the solvent was removed by rotary evaporation to produce the intermediate **SR4a**. A mixture of **S2** (13 mg, 0.108 mmol), HOBt (21 mg, 0.135 mmol), and EDC (26 mg, 0.135 mmol) in DMF (0.5 mL) was stirred at room temperature overnight. The solvent was removed by rotary evaporation and the resulting residue was purified by silica gel column chromatography (0-5% MeOH in $CHCl_3$) to give product **SR(azido-F)$_2$** as green blue solid (11.5 mg, 80%). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 9.95 (t, *J* = 5.9 Hz, 4H), 8.12 (d, *J* = 8.7 Hz, 4H), 8.07 (s, 4H), 7.28 (s, 3H), 7.25-7.17 (m, 8H), 6.58 (s, 9H), 6.52 (d, *J* = 8.0 Hz, 2H), 6.42 (t, *J* = 6.8 Hz, 2H), 6.13 (d, *J* = 8.7 Hz, 4H), 4.95 (s, 4H), 4.70 (q, *J* = 7.5 Hz, 2H), 4.50 (d, *J* = 5.7 Hz, 8H), 3.72 (q, *J* = 7.1 Hz, 1H), 3.62 (m, 2H), 3.54 (m, 6H). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 185.30, 170.99, 170.25, 166.67, 163.61, 151.79, 136.69, 133.79, 129.47, 129.12, 128.91, 127.34, 111.95, 56.62, 55.04, 53.45, 48.99, 47.49, 43.63, 38.32, 37.13, 35.92, 29.93, 27.48, 21.14, 11.50. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{88}H_{93}N_{18}O_{12}^+$ 1593.7215; Found 1593.7221.

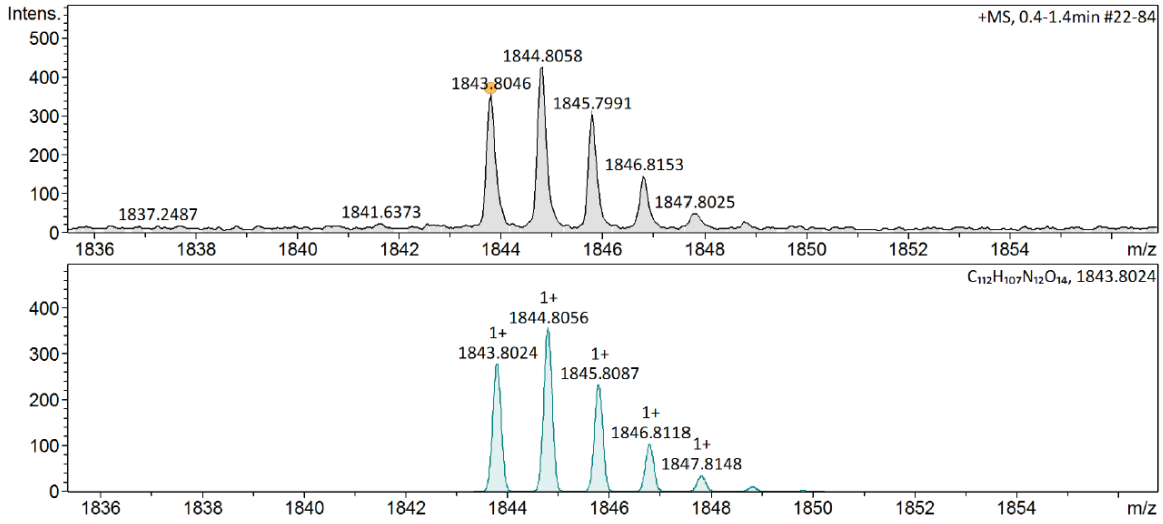
SF8(F)$_2$. Compound **SR(azido-F)$_2$** (11 mg, 0.00703 mmol) was dissolved in $CHCl_3$ (14 mL). CuBr·TBTA (6 mg) and Et_3N (29 μL, 0.211 mmol) were added to the solution. The reaction mixture was stirred at room temperature overnight. The solvent was removed by rotary evaporation and the resulting residue was purified by silica gel column chromatography (0-10% MeOH in CH_2Cl_2) to give product **SF8(F)$_2$** as green blue solid (4.7 mg, 42%).^{1}H NMR (500 MHz, Chloroform-*d*) δ 10.31 (d, *J* = 9.8 Hz, 4H), 8.15 (s, 2H), 8.03 (d, *J* = 19.6 Hz, 4H), 7.77 (d, *J* = 32.8 Hz, 4H), 7.30 (d, *J* = 7.1 Hz, 4H), 7.23 (d, *J* = 7.8 Hz, 5H), 6.71 (s, 6H), 6.50 (s, 5H), 6.05 (s, 2H), 5.49 (d, *J* = 16.9 Hz, 4H), 5.34 (s, 2H), 5.14 (dt, *J* = 15.3, 9.0 Hz, 4H), 4.88 (s, 2H), 4.68 – 4.60 (m, 8H), 3.83 (d, *J* = 14.4 Hz, 4H), 3.33 (s, 4H), 3.13 (dd, *J* = 28.8, 8.5 Hz, 6H), 2.98 (d, *J* = 44.9 Hz, 4H), 2.83 – 2.73 (m, 4H), 1.00 (s, 6H). HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{88}H_{93}N_{18}O_{12}^+$ 1593.7215; Found 1593.7217.



^{1}H NMR (500 MHz, $CDCl_3$) of **SR4**

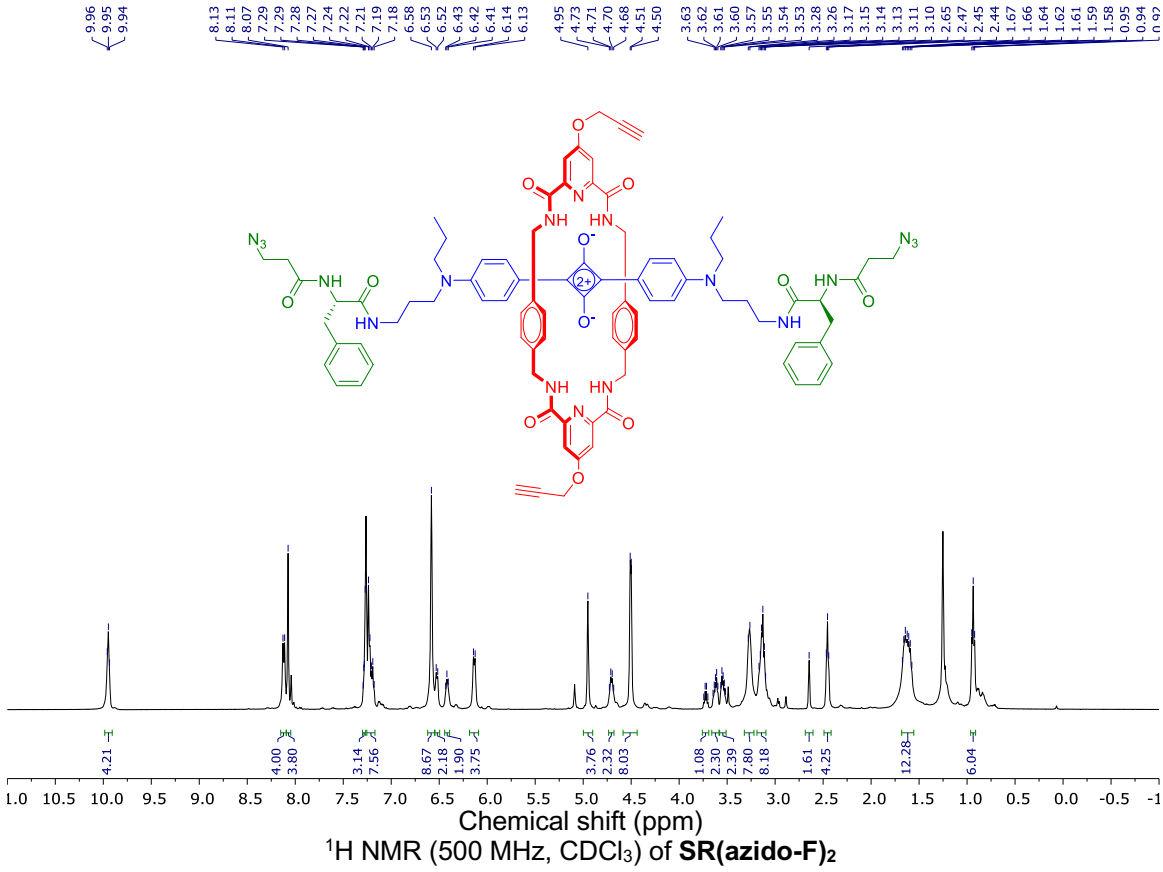


^{13}C NMR (126 MHz, $CDCl_3$) of **SR4**

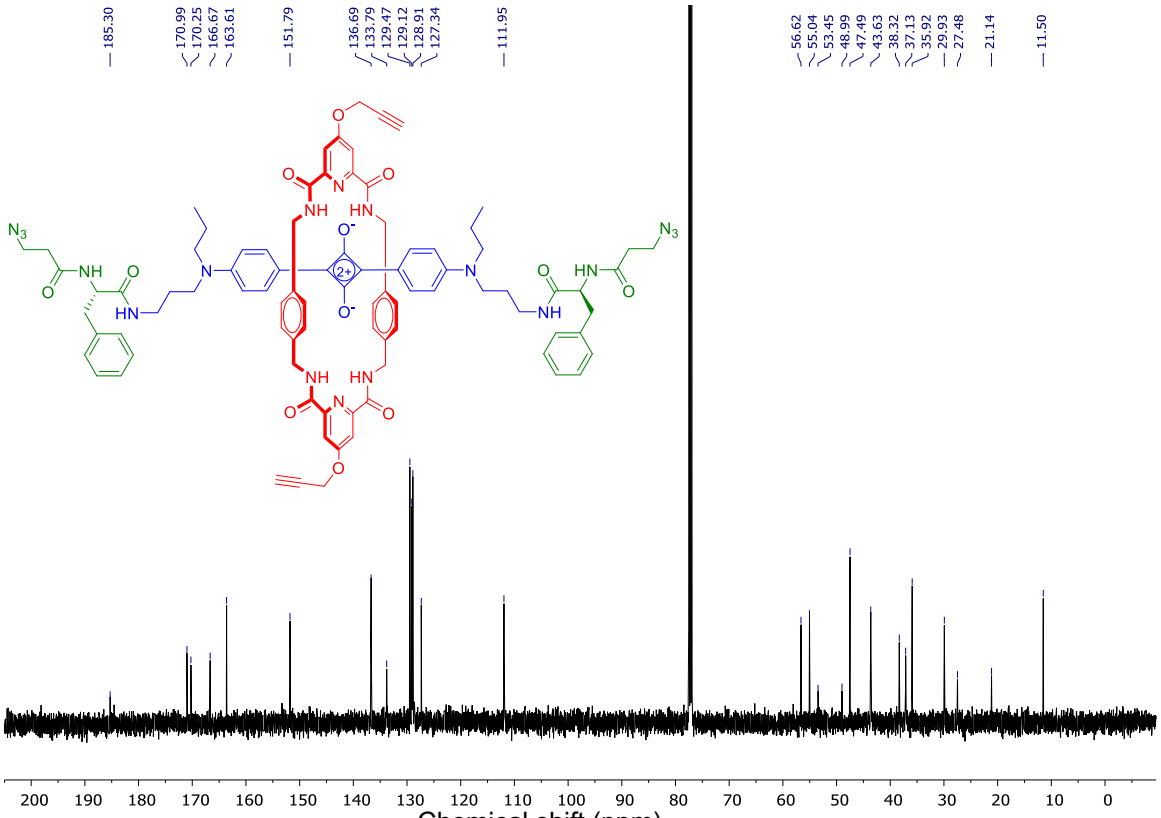


Meas. m/z	#	Ion Formula	m/z	err [ppm]	Mean err [ppm]	rdb	N-Rule	e⁻ Conf
1843.804557	1	C112H107N12O14	1843.802423	-1.2	705.0	65.5	ok	even

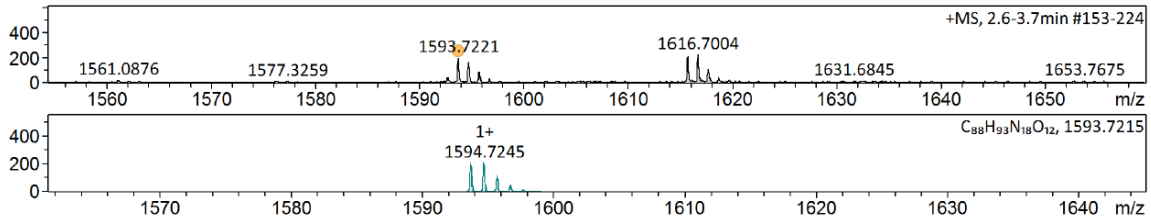
HRMS-ESI of **SR4**



^{1}H NMR (500 MHz, $CDCl_3$) of **SR(azido-F)$_2$**

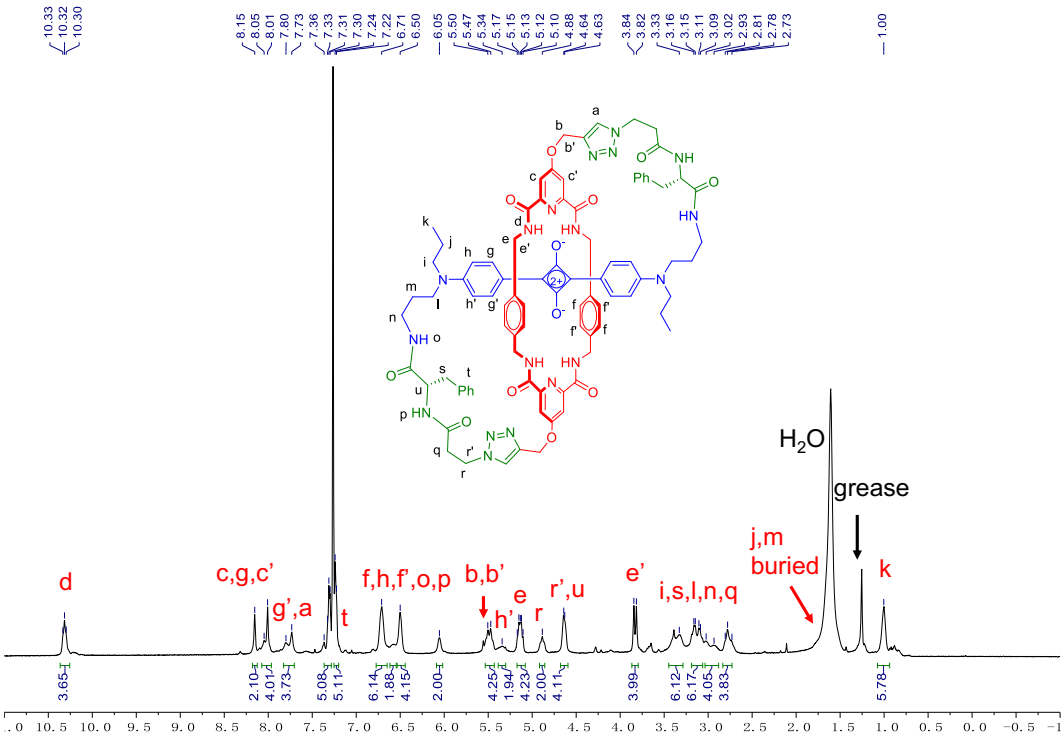


^{13}C NMR (126 MHz, $CDCl_3$) of **SR(azido-F)$_2$**

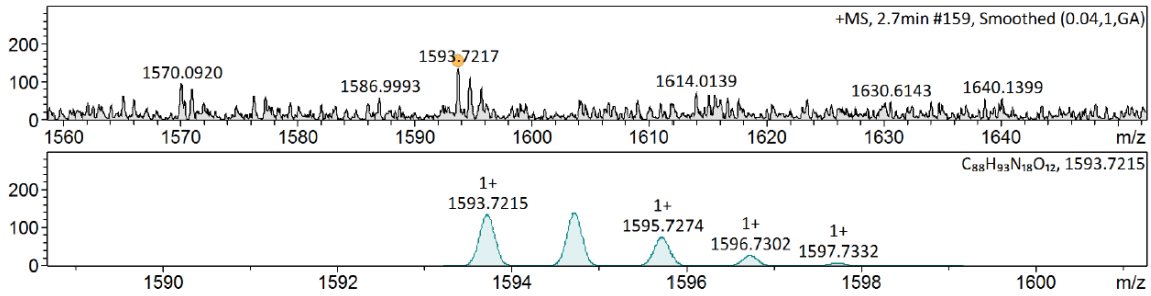


Meas. m/z	#	Ion Formula	m/z	err [ppm]	Mean err [ppm]	rdb	N-Rule	e¯ Conf
1593.722070	1	C88H93N18O12	1593.721487	-0.4	304.9	51.5	ok	even

HRMS-ESI of **SR(azido-F)$_2$**



^{1}H NMR (500 MHz, $CDCl_3$) of **SF8(F)$_2$**



Meas. m/z	#	Ion Formula	m/z	err [ppm]	Mean err [ppm]	rdb	N-Rule	e¯ Conf
1593.721742	1	C88H93N18O12	1593.721487	-0.2	1157.1	51.5	ok	even

HRMS-ESI of **SF8(F)$_2$**

2. Variable Temperature ^{1}H NMR Studies of SF8(f)$_2$

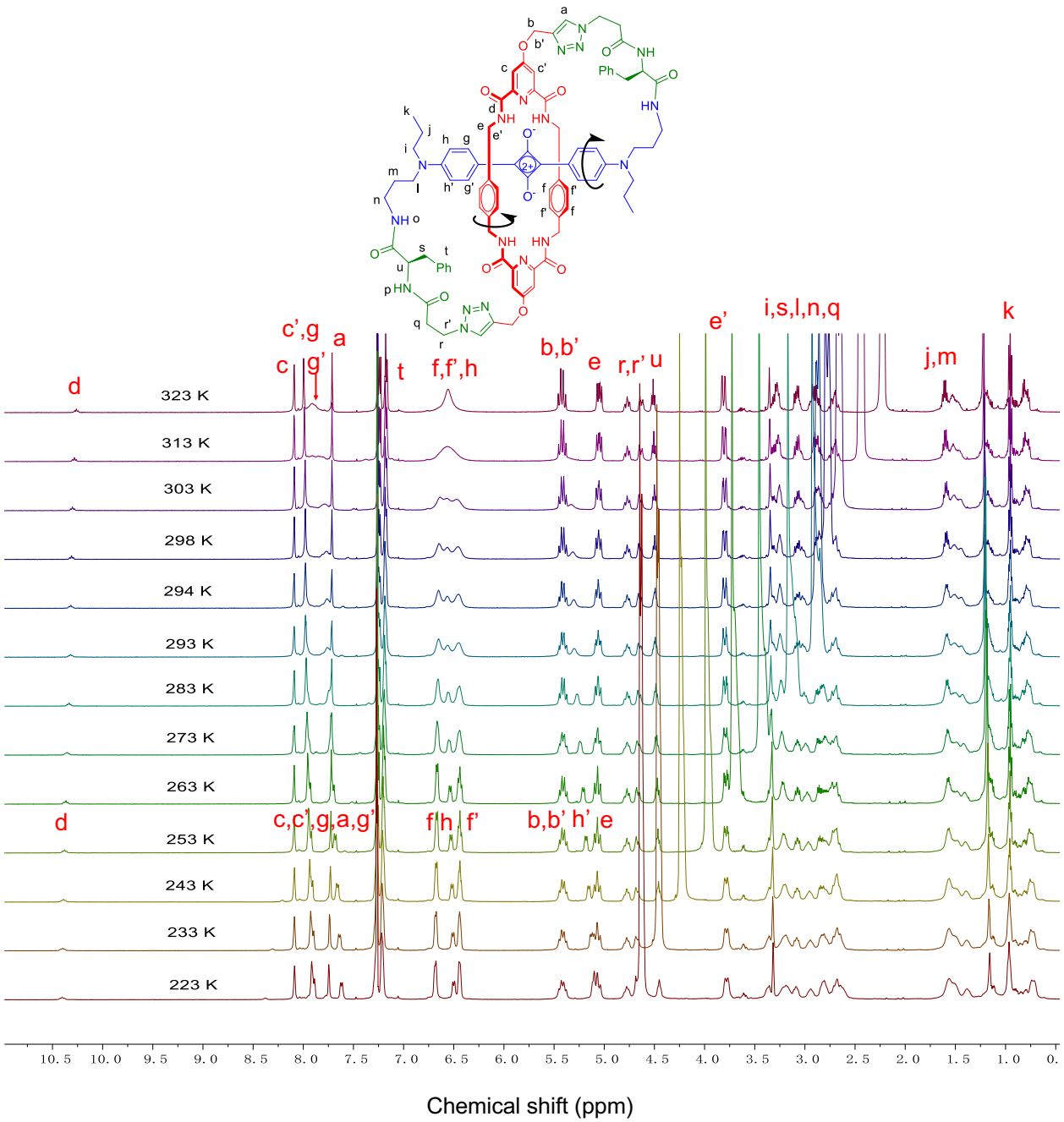


Figure S1. Variable temperature ^{1}H NMR (500 MHz, 10% CD_3OD in $CDCl_3$) of **SF8(f)$_2$**. (*Due to the proton exchange with solvent CD_3OD, proton d is weak)

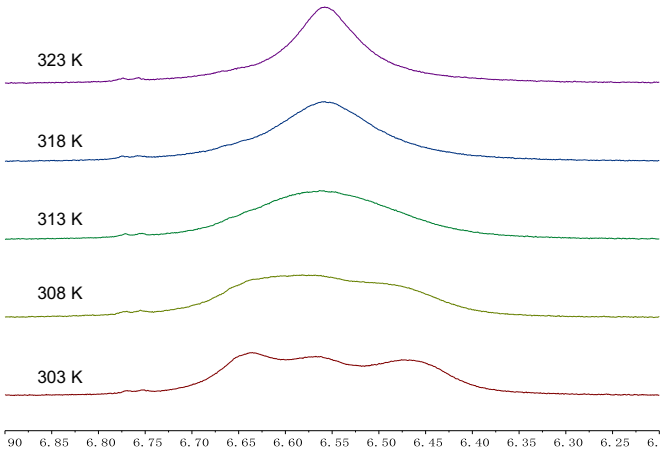


Figure S2. Expanded coalescence region for proton f from Figure S1. (coalescence temperature is 313 K)

Table S1. Summary of variable temperature ^{1}H NMR data for compound **SF8(f)$_2$**.

Compound	T_c (K)	$\Delta\nu$ (Hz)	k (s^{-1})	$\Delta G^{\neq}$ (kJ/mol)
SF8(f)$_2$	313	86	191.0	63.2

The rate of two-site exchange (k) at the coalescence temperature was determined using:

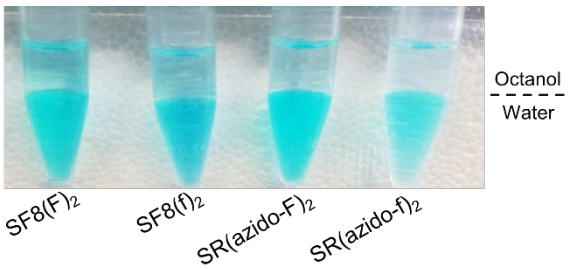
$$k = \frac{\pi}{\sqrt{2}}\Delta\nu_0$$

Where $\Delta\nu_0$ is the chemical shift difference of the two protons (in Hz). To determine the activation energy (in kJ/mol), the Eyring equation was simplified into the following form, where *Tc* is the coalescence temperature (in K):

$$\Delta G^{\neq} = 19.12\, Tc\, [9.97 + \log_{10}(\frac{Tc}{\Delta\nu_0})]$$

3. Log *P* and Stability Measurements

A



B

Probe	$SF8(F)_2$	$SF8(f)_2$	$SR(azido\text{-}F)_2$	$SR(azido\text{-}f)_2$
log *P* (25℃)	-0.5 ± 0.2	-0.5 ± 0.1	-0.6 ± 0.1	-0.5 ± 0.1

Figure S3. Determination of log *P* with octanol−water partitioning at 25 ℃. (A) Photographs of each probe (10 μM) partitioned between octanol and water. (B) Calculated log *P* values for each probe.

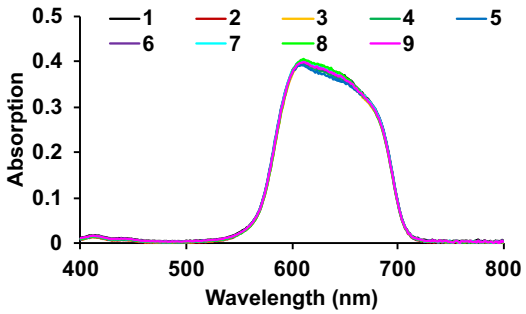


Figure S4. Absorption spectra showing maxima band of **$SF8(f)_2$** (5 μM) in water (1), or after addition of one molar equivalent of Arg (2), Ser (3), Cys (4), Trp (5), DTT (6), GSH (7), Ascorbic acid (8), H_2O_2 (9). The absorption band is broad due to self-aggregation of the probe in water. The additives have no effect on the absorption spectra indicating no chemical or non-covalent interaction with the encapsulated squaraine dye within **$SF8(f)_2$**. T = 25 °C.

4. Cell Toxicity and Fluorescence Microscopy

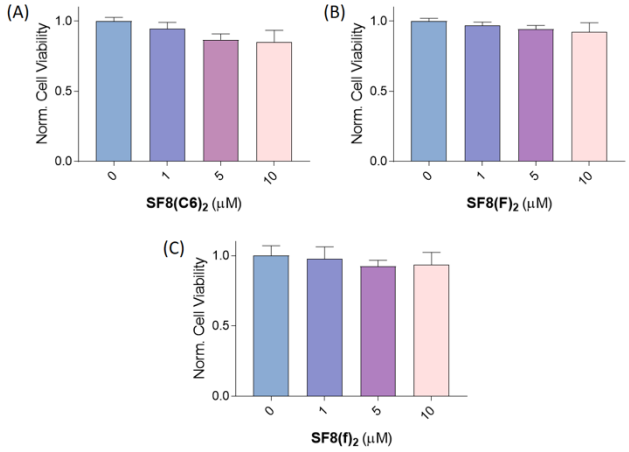


Figure S5. MTT viability assays for HT-1080 cells after 6 hour incubations with SF8 probes.

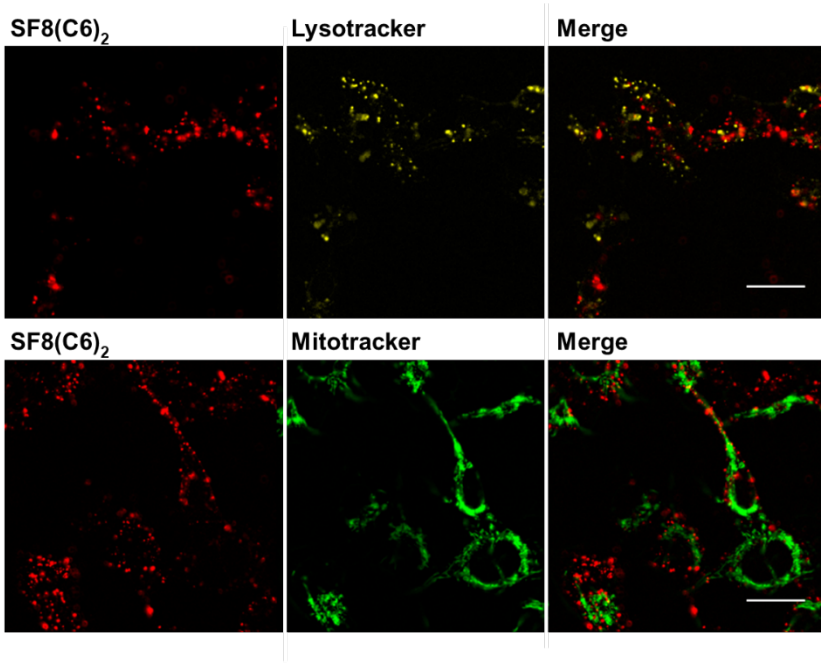


Figure S6. (top) Localization of **SF(C6)$_2$** within cell lysosomes. HT-1080 cells were incubated with 1 μM of **SF(C6)$_2$** for 30 min and co-stained with 100 nM LysoTracker Red DND-99 for 15 min. Representative epifluorescence cell micrographs depict colocalization. Red fluorescence shows SF8 probes; yellow fluorescence shows LysoTracker Red DND-99; orange fluorescence shows colocalization. Scale bar = 30 μm. (bottom) Localization of **SF(C6)$_2$** within cell mitochondria. HT-1080 cells were incubated with 1 μM of **SF(C6)$_2$** for 30 min and co-stained with 100 nM MitoTracker Green FM for 15 min. Representative epifluorescence cell micrographs depict qualitative colocalization. Red fluorescence shows SF8 probes; green fluorescence shows MitoTracker Green FM; yellow fluorescence shows colocalization. Scale bar = 30 μm.

5. References

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2 Y. C. Su, Y. L. Lo, C. C. Hwang, L. F. Wang, M. H. Wu, E. C. Wang, Y. M. Wang and T. P. Wang, *Org. Biomol. Chem.*, 2014, **12**, 6624–6633.