Tunable spontaneous release of a carboxylic acid via a β-eliminative cleavable linker

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General Experimental Procedures

Materials and Reagents

Chemicals were obtained from commercial sources and used without further purification. Oxalyl chloride, 4-(trifluoromethyl)phenyl methyl sulfone, and *N*,*N*′-diisopropylcarbodiimide (DIC) were from Oakwood Chemical (Estill, SC, USA). Sodium azide, trichloroisochyanuric acid, TEMPO, 2.5 M butyl lithium in hexanes, DMAP, piperazine, 3-bromopropionic acid, trifluoroacetic acid (TFA), 5-hexynoic acid, and human serum (from male AB plasma) were from Sigma–Aldrich (St. Louis, MO, USA). Rhodamine B was from Alfa Aesar (Ward Hill, MA, USA). 6-Chloro-1-hexanol, methyl phenyl sulfone, 4-chlorophenyl methyl sulfone, and mouse serum (REF 10410, LOT XB333039) were from Thermo Fisher Scientific (Waltham, MA, USA). H_2O or water indicates "Milli-Q" water prepared with a Milli-Q IQ 7000 Ultrapure Water System from Merck Millipore (Billerica, MA, USA).

Conditions

Procedures were performed at ambient temperature (~22 °C) and pressure (1.0 atm) unless indicated otherwise. An Isotemp Incubator from Thermo Fisher Scientific was used for procedures at 37 °C, and an Isotemp fridge from Thermo Fisher Scientific was used for procedures performed at 4 °C. Syntheses using air- or moisture-sensitive reagents were performed under N_2(g) using oven-dried glassware and conventional Schlenk-line techniques.

Compound Purification

Solvents were removed by "concentration under reduced pressure" using a Büchi R210 rotary evaporator at water aspirator pressure (<30 mbar), maintaining the temperature of the water bath at 40 °C. Residual solvent was removed from compounds at high vacuum (<0.1 mbar) using a mechanical belt-drive oil pump. Lyophilization was achieved with a FreeZone 1.5-Liter Freeze Dry System from Labconco (Kansas City, MO, USA). Flash column chromatography was performed manually using SiliaFlash silica gel obtained from SiliCycle (Québec City, Canada) or with an Isolera One system from Biotage (Uppsala, Sweden) equipped with Biotage Sfar Silica D Duo columns. Reversed-phase flash column chromatography was performed with an Isolera One system from Biotage using Biotage Sfär C18 D Duo columns. H_2O containing 0.1% v/v TFA (solvent A) and CH_3CN containing 0.1% v/v TFA (solvent B) were used as the mobile phase.

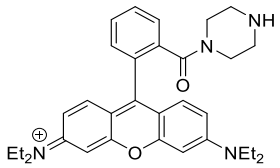
Analytical Instrumentation

NMR spectra were recorded with an Avance Neo 500 MHz or on an Avance-III HD Nanobay 400 MHz spectrometer from Bruker (Billerica, MA, USA). ^{13}C and ^{19}F spectra were proton-decoupled. ^{1}H chemical shifts are reported in parts per million (ppm, δ scale) referenced to the solvent residual signal (D_2O: δ 1.79, $CDCl_3$: δ 7.26). ^{13}C chemical shifts are reported in parts per million (ppm, δ scale) referenced to the solvent carbon resonance ($CDCl_3$: δ 77.16). For ^{13}C spectra in D_2O, samples were referenced by means of absolute referencing to the solvent residual signal in ^{1}H NMR using Ξ-values recommended by IUPAC. ^{19}F NMR measurements were used for confirmation when compounds were obtained as TFA salts, and for these compounds the peaks corresponding to ^{13}C nuclei in trifluoroacetate are not individually reported. High-resolution mass spectra were obtained on an AccuTOF 4G LC-plus spectrometer from JEOL (Peabody, MA, USA) equipped with an ionSense DART source or were acquired on an Accurate Mass 6530 quadrupolar time-of-flight (QTOF) mass spectrometer from Agilent Technologies attached to a 1290 Infinity II liquid chromatography system from Agilent Technologies. Chromatography relied on a PLRP-S column (1000 Å, 50 µm,

2.1 mm × 50 mm) from Agilent with 95%/5% v/v H_2O/CH_3CN containing 0.1% v/v formic acid and 95%/5% v/v CH_3CN/H_2O containing 0.1% v/v formic acid as the mobile phase. LC–MS was performed with a 6125B mass spectrometer from Agilent Technologies using electrospray ionization (ESI) attached to a 1260 Infinity LC from Agilent Technologies featuring a Poroshell 120 SB C18 column (2.7 µm, 2.1 mm × 50 mm) from Agilent Technologies. Generally, a gradient of CH_3CN (2–95% v/v) containing formic acid (0.1% v/v) in H_2O containing formic acid (0.1% v/v) over 10 min was used. UPLC analyses were performed with an Agilent 1290 Infinity II LC System equipped with a Kinetex 2.6 µm C18 100 Å, LC Column 100 × 2.1 mm. The solvents used were Milli-Q water and CH_3CN containing 0.1% and 0.08% v/v TFA, respectively. The following CH_3CN gradient was used for all measurements on the UPLC device: 0–20% in 1 min, 20–100% in 14 min). Absorbances were measured on a DeNovix DS-11 spectrophotometer.

Synthetic Procedures

Rhodamine B Piperazine Amide (**1**)



The following procedure was adapted from the literature.[1] Rhodamine B (3.0 g, 6.8 mmol) was dissolved in 75 mL of dry CH_2Cl_2, and the resulting solution was cooled to 0 °C. Oxalyl chloride (2.6 mL, 30 mmol, 4.5 equiv) was added slowly to the stirred solution, followed by one drop of dry dimethyl formaldehyde. The resulting solution was allowed to warm to ambient temperature. After completion of the reaction, monitored by gas evolution, the solvent was removed under reduced pressure. The residue was dissolved in dry CH_2Cl_2 (50 mL), and the resulting solution was concentrated again to remove excess oxalyl chloride, yielding rhodamine B chloride as a purple solid that was immediately used without further purification. Rhodamine B chloride was dissolved in 100 mL of dry CH_2Cl_2, and the resulting solution was added slowly to a vigorously stirred solution of piperazine (2.3 g, 27 mmol, 4.0 equiv) in 50 mL dry CH_2Cl_2 previously cooled to 0 °C. The solution was then allowed to warm to ambient temperature and was stirred for 2 h. The solvent was removed under reduced pressure, the crude product was dissolved in 20% v/v MeOH in CH_2Cl_2 and purified by column chromatography (silica, 20% v/v MeOH in CH_2Cl_2) to yield rhodamine B piperazine amide (**1**) as a dark purple solid (2.7 g, 5.3 mmol, 78%).

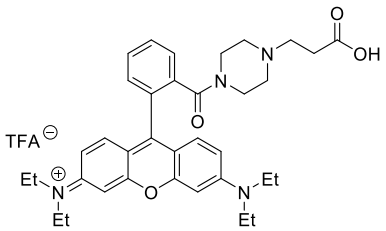
TLC: R_f = 0.3 (20% MeOH/CH_2Cl_2).

^{1}H NMR (500 MHz, D_2O, δ): 7.89 (td, J = 7.6, 1.3 Hz, 1H), 7.83 (td, J = 7.7, 1.3 Hz, 1H), 7.65 (ddd, J = 15.1, 7.6, 1.2 Hz, 2H), 7.14 (d, J = 9.5 Hz, 2H), 6.91 (dd, J = 9.6, 2.3 Hz, 2H), 6.78 (d, J = 2.4 Hz, 2H), 3.66–3.51 (m, 8H), 3.42 (s, 4H), 2.86 (s, 4H), 2.75 (s, 4H), 1.92 (s, 1H), 1.24 (t, J = 7.1 Hz, 12H).

^{13}C{^{1}H} NMR (126 MHz, D_2O, δ): 169.8, 157.2, 155.5, 153.4, 142.8, 133.7, 130.9, 130.6, 130.3, 130.1, 127.8, 111.1, 112.5, 96.3, 45.9, 43.4, 43.0, 12.0.

HRMS (ESI–TOF): m/z calcd for $C_{32}H_{39}N_4O_2$ $[M]^+$, 511.3073; found, 511.3068.

TFA·rhodamine B 4-(3-Carboxypropyl) Piperazine Amide (**2**)



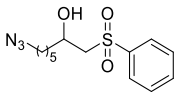
Rhodamine B piperazine amide (**1**) (500 mg, 0.98 mmol) was dissolved in 10 mL of dimethyl formamide, and sodium carbonate (387 mg, 3.7 mmol, 3.7 equiv) was added to the resulting solution with stirring. 3-Bromopropionic acid (419 mg, 2.7 mmol, 2.8 equiv) was added, and the mixture was stirred for 24 h. The solvent was removed under reduced pressure, and the crude product was dissolved in 95% v/v CH_3CN in H_2O containing 0.1% v/v TFA. The obtained crude solution was purified by reversed-phase column chromatography (C18, 5–100% v/v CH_3CN in H_2O containing 0.1% TFA). Fractions containing the product were combined and lyophilized, yielding the TFA salt of rhodamine B 4-(3-carboxypropyl) piperazine amide (**2**) as a dark purple solid (364 mg, 0.52 mmol, 53%).

^{1}H NMR (500 MHz, D_2O, δ): 7.73–7.64 (m, 3H), 7.31 (dd, J = 7.8, 1.4 Hz, 1H), 7.18 (d, J = 9.6 Hz, 2H), 6.93 (dd, J = 9.6, 2.4 Hz, 2H), 6.75 (d, J = 2.5 Hz, 2H), 3.78–3.50 (m, 9H), 3.36 (t, J = 6.6 Hz, 2H), 3.10 (s, 4H), 2.79 (t, J = 6.6 Hz, 2H), 1.31 (t, J = 7.1 Hz, 12H).

^{13}C{^{1}H} NMR (126 MHz, D_2O, δ): 172.0, 167.6, 157.8, 155.9, 155.7, 131.2, 131.9, 130.8, 130.7, 130.5, 130.3, 128.2, 111.4, 113.8, 96.5, 52.3, 51.4, 50.8, 46.2, 41.4, 29.1, 12.6.

HRMS (Q–TOF): *m*/*z* calcd for $C_{35}H_{43}N_4O_4$ $[M]^+$, 583.3284; found, 583.3279.

1-(Phenylsulfonyl)-7-azido-2-heptanol (**3A**)



The following procedure was adapted from the literature.[2] In an oven-dried round-bottom flask under N_2(g), phenyl methyl sulfone (300 mg, 1.9 mmol) was dissolved in anhydrous THF (6.5 mL), and the resulting solution was cooled to −78 °C. 2.5 M *n*-BuLi in hexanes (0.77 mL, 1.9 mmol, 1 equiv) was slowly added to the solution. After the addition, the solution was allowed to warm slowly to 0 °C using an ice bath. The mixture was then cooled again to −78 °C, and 6-azidohexanal (**5**) (298 mg, 2.1 mmol, 1.1 equiv) was added. After stirring the solution for 15 min, the cooling bath was removed, and the mixture was allowed to warm. When the mixture became clear, 2 mL of saturated aqueous NH_4Cl was added, and the mixture was allowed to continue warming to ambient temperature. The mixture was diluted with ethyl acetate and washed successively with H_2O (10 mL) and brine (10 mL) and then dried over Na_2SO_4(s) and filtered. The solvent was removed by concentration under reduced pressure. The resulting oil was purified by column chromatography (silica, 0–50% v/v EtOAc in hexanes) to yield 1-(phenylsulfonyl-7-azido-2-heptanol) (**3A**) as a pale-yellow oil (203 mg, 0.69 mmol, 36%).

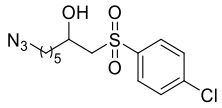
TLC: R_f = 0.4 (30% v/v EtOAc in hexanes).

^{1}H NMR (500 MHz, $CDCl_3$, δ): 7.94 (dd, J = 7.4, 1.4 Hz, 2H), 7.70 (dd, J = 7.4, 1.2 Hz, 1H), 7.61 (dd, J = 8.4, 7.1 Hz, 2H), 1.23–1.09 (m, 1H), 3.36 (d, J = 2.5 Hz, 1H), 3.27–3.14 (m, 4H), 1.62–1.56 (m, 2H), 1.50–1.42 (m, 2H), 1.39–1.32 (m, 2H)

^{13}C{^{1}H} NMR (126 MHz, $CDCl_3$, δ): 139.4, 131.3, 129.7, 128.1, 65.9, 62.4, 51.4, 36.4, 28.8, 26.6, 21.7.

HRMS (ESI–TOF): *m*/*z* calcd for $C_{13}H_{20}N_3O_3S$ $[M + H]^+$, 298.1220; found, 298.1234.

1-(4-Chlorophenylsulfonyl)-7-azido-2-heptanol (**3B**)



The following procedure was adapted from the literature.[2] In an oven-dried round-bottom flask under N_2(g), 4-chlorophenyl methyl sulfone (300 mg, 1.6 mmol) was dissolved in anhydrous THF (6.5 mL), and the resulting solution was cooled to −78 °C. 2.5 M *n*-BuLi in hexanes (0.63 mL, 1.6 mmol, 1 equiv) was added slowly to the solution, and after the addition, the solution was allowed to warm slowly to 0 °C using an ice bath. The mixture was then cooled again to −78 °C, and 6-azidohexanal (**5**) (244 mg,1.7 mmol, 1.1 equiv) was added. After stirring for 15 min, the cooling bath was removed, and the mixture was allowed to warm. When the mixture became clear, 2 mL of saturated aqueous NH_4Cl was added, and the mixture was allowed to continue warming to ambient temperature. The mixture was diluted with ethyl acetate and washed successively with H_2O (10 mL) and brine (10 mL), and then dried over Na_2SO_4(s) and filtered. The solvent was removed by concentration under reduced pressure. The resulting oil was purified by column chromatography (silica, 0–50% v/v EtOAc in hexanes) to yield 1-(4-chlorophenylsulfonyl)-7-azido-2-heptanol (**3B**) as a pale-yellow oil (189 mg, 0.56 mmol, 36%).

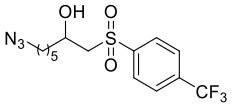
TLC: R_f = 0.4 (30% v/v EtOAc in hexanes).

^{1}H NMR (500 MHz, $CDCl_3$ δ): 7.87 (dd, *J* = 8.7, 2.3 Hz, 3H), 7.57 (dd, *J* = 8.9, 2.3 Hz, 3H), 1.17 (tq, *J* = 6.0, 2.0 Hz, 2H), 3.29–3.11 (m, 7H), 1.62–1.57 (m, 5H), 1.50–1.23 (m, 5H).

^{13}C{^{1}H} NMR (126 MHz, $CDCl_3$ δ): 141.1, 137.9, 130.0, 129.6, 65.94, 62.6, 51.4, 36.5, 28.8, 26.6, 21.7.

HRMS (ESI–TOF): *m*/*z* calcd for $C_{13}ClH_{19}N_3O_3S$ $[M + H]^+$, 332.0830; found, 332.0839.

1-(4-(Trifluoromethyl)phenylsulfonyl)-7-azido-2-heptanol (**3C**)



The following procedure was adapted from the literature.[2] In an oven-dried round-bottom flask under N_2(g), 4-(trifluoromethyl) phenyl methyl sulfone (50 mg, 0.22 mmol) was dissolved in anhydrous THF (2 mL), and the resulting solution was cooled to −78 °C. 2.5 M *n*-BuLi in hexanes (0.10 mL, 0.25 mmol, 1.1 equiv) was added slowly to the solution. After the addition, the solution was allowed to warm slowly to 0 °C using an ice bath. The mixture was then cooled again to −78 °C, and 6-azidohexanal (**5**) (35 mg, 0.25 mmol, 1.1 equiv) was added. After stirring for 15 min, the cooling bath was removed, and the mixture was allowed to warm. When the mixture became clear, 1 mL of saturated aqueous NH_4Cl was added, and the mixture was allowed to continue warming to ambient temperature. The mixture was diluted with ethyl acetate and washed successively with H_2O (10 mL) and brine (10 mL) and then dried over Na_2SO_4(s) and filtered. The solvent was removed by concentration under reduced pressure. The resulting oil was purified by column chromatography (silica, 0–50% v/v EtOAc in hexanes) to yield 1-(4-(trifluoromethyl)phenylsulfonyl)-7-azido-2-heptanol (**3C**) as a transparent oil (47 mg, 0.13 mmol, 58%).

TLC: R_f = 0.4 (30% v/v EtOAc in hexanes).

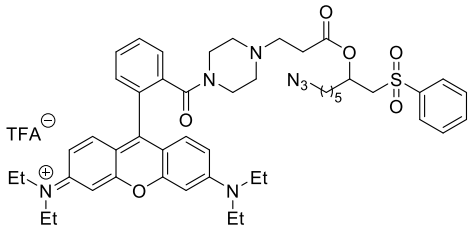
^{1}H NMR (500 MHz, $CDCl_3$, δ): 8.08 (d, *J* = 8.1 Hz, 2H), 7.86 (d, *J* = 8.7 Hz, 2H), 4.25–4.17 (m, 1H), 3.32–3.16 (m, 4H), 1.57 (tdd, *J* = 9.3, 5.8, 3.2 Hz, 3H), 1.50–1.29 (m, 5H).

$^{13}C\{^{1}H\}$ NMR (126 MHz, $CDCl_3$, δ): 143.06, 135.88 (q, J = 33.2 Hz), 128.76, 126.75 (q, J = 3.7 Hz), 123.15 (q, J = 273.2 Hz), 65.92, 62.45, 51.35, 36.51, 28.78, 26.52, 24.68.

$^{19}F\{^{1}H\}$ NMR (471 MHz, $CDCl_3$, δ): −63.24.

HRMS (ESI–TOF): m/z calcd for $C_{14}F_3H_{19}N_3O_3S$ $[M + H]^+$, 366.1074; found, 366.1072.

TFA·rhodamine B 4-(3-((7-Azido-1-(phenylsulfonyl)heptan-2-yl)oxy)-3-oxopropyl)piperazine Amide (**4A**)



The following procedure was adapted from the literature.[2] 1-(Phenylsulfonyl)-7-azido-2-heptanol (**3A**) (21 mg, 71 μmol) was dissolved in 0.6 mL of CH_2Cl_2 in a screw-cap vial, and the resulting solution was stirred. TFA·rhodamine B 4-(3-carboxypropyl) piperazine amide (**2**) (50 mg, 71 μmol, 1 equiv), DMAP (1.5 mg, 35 μmol, 0.5 equiv), and DIC (21.5 μL, 143 μmol, 2 equiv) were added to the solution in that order. After 3 h, the solvent was removed under reduced pressure and the resulting solid was dissolved in CH_2Cl_2. The crude product was purified by column chromatography (silica, 5–20% v/v MeOH in CH_2Cl_2), yielding the TFA salt of fluorophore conjugate **4A** as a dark purple solid (38 mg, 44 μmol, 61%).

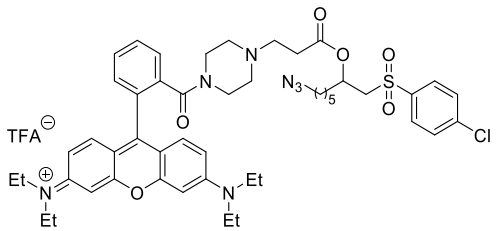
TLC: R_f = 0.6 (10% v/v MeOH in CH_2Cl_2).

^{1}H NMR (500 MHz, $CDCl_3$, δ): 7.87 (dd, J = 7.5, 1.0 Hz, 2H), 7.71–7.62 (m, 3H), 7.61–7.53 (m, 3H), 7.32 (dd, J = 6.4, 2.3 Hz, 1H), 7.23 (dd, J = 8.4, 1.9 Hz, 2H), 6.98 (dd, J = 7.8, 1.4 Hz, 2H), 6.76 (d, J = 2.4 Hz, 2H), 5.30–5.22 (m, 1H), 3.70–3.54 (m, 8H), 3.49–3.30 (m, 5H), 3.25–3.18 (m, 3H), 2.60 (s, 2H), 2.44–2.23 (m, 8H), 1.61 (dh, J = 11.8, 6.7 Hz, 2H), 1.51 (p, J = 7.1 Hz, 2H), 1.31 (t, J = 7.1 Hz, 14H).

$^{13}C\{^{1}H\}$ NMR (126 MHz, $CDCl_3$, δ): 171.1, 167.4, 157.8, 156.1, 155.77, 155.76, 139.4, 135.5, 132.2, 131.2, 130.8, 130.4, 130.3, 130.0, 129.6, 128.2, 127.8, 125.4, 111.4, 113.89, 113.87, 96.4, 67.8, 59.3, 53.0, 52.9, 52.4, 51.3, 47.4, 46.3, 41.6, 31.1, 31.9, 28.7, 26.3, 21.4, 12.8.

HRMS (Q–TOF): m/z calcd for $C_{48}H_{60}N_7O_6S$ $[M]^+$, 862.4326; found, 862.4351.

TFA·rhodamine B 4-(3-((7-Azido-1-((4-chlorophenyl)sulfonyl)heptan-2-yl)oxy)-3-oxopropyl)piperazine Amide (**4B**)



The following procedure was adapted from the literature.[2] 1-(4-Chlorophenylsulfonyl)-7-azido-2-heptanol (**3B**) (18 mg, 54 μmol, 1.5 equiv) was dissolved in 0.3 mL of CH_2Cl_2 in a screw-cap vial, and the resulting solution was stirred.

TFA·rhodamine B 4-(3-carboxypropyl) piperazine amide (**2**) (25 mg, 36 µmol, 1 equiv), DMAP (2 mg, 35 µmol, 0.5 equiv), and DIC (11.1 µL, 72 µmol, 2 equiv) were added to the solution in that order. After 3 h, the solvent was removed under reduced pressure and the obtained solid was dissolved in CH_2Cl_2. The crude product was purified by column chromatography (silica, 5–20% v/v MeOH in CH_2Cl_2), yielding the TFA salt of fluorophore conjugate **4B** as a dark purple solid (23 mg, 26 µmol, 73%).

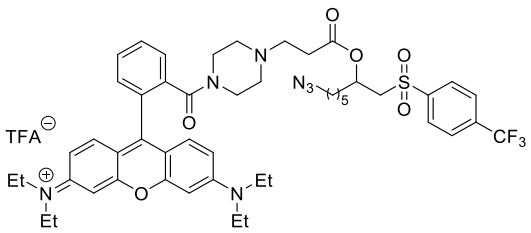
TLC: R_f = 0.6 (10% v/v MeOH in CH_2Cl_2).

^{1}H NMR (500 MHz, $CDCl_3$ δ): 7.84 (dd, J = 8.5, 2.0 Hz, 2H), 7.72–7.64 (m, 2H), 7.58–7.52 (m, 3H), 7.37–7.32 (m, 1H), 7.25 (dd, J = 9.5, 1.6 Hz, 2H), 6.97 (d, J = 9.3 Hz, 2H), 6.80 (d, J = 1.9 Hz, 2H), 5.30–5.22 (m, 1H), 3.71–3.56 (m, 8H), 3.54–3.30 (m, 5H), 3.31–3.21 (m, 3H), 2.60–2.50 (m, 2H), 2.36–2.25 (m, 8H), 1.65 (tt, J = 13.9, 7.2 Hz, 2H), 1.54 (tt, J = 7.2 Hz, 2H), 1.40–1.25 (m, 14H).

^{13}C{^{1}H} NMR (126 MHz, $CDCl_3$, δ): 171.3, 167.4, 157.9, 156.1, 155.8, 140.8, 137.9, 135.5, 132.3, 130.7, 130.3, 130.2, 130.04, 130.00, 129.82, 129.82, 127.8, 111.3, 111.2, 113.9, 96.4, 67.7, 59.4, 53.0, 52.9, 52.5, 51.3, 47.6, 46.2, 41.8, 31.1, 32.1, 28.7, 26.3, 21.4, 12.7.

HRMS (Q–TOF): m/z calcd for $C_{48}ClH_{59}N_7O_6S$ $[M]^+$, 896.3936; found, 896.3930.

TFA·rhodamine B 4-(3-((7-Azido-1-((4-(trifluoromethyl)phenyl)sulfonyl)heptan-2-yl)oxy)-3-oxopropyl)piperazine Amide (**4C**)



The following procedure was adapted from the literature.[2] 1-(4-(Trifluoromethyl)phenylsulfonyl)-7-azido-2-heptanol (**3C**) (20 mg, 54 µmol, 1.5 equiv) was dissolved in 0.3 mL of CH_2Cl_2 in a screw-cap vial, and the resulting solutions was stirred. TFA·rhodamine B 4-(3-carboxypropyl) piperazine amide (**2**) (25 mg, 36 µmol, 1 equiv), DMAP (2 mg, 35 µmol, 0.5 equiv), and DIC (11.1 µL, 72 µmol, 2 equiv) were added to the solution in that order. After 3 h, the solvent was removed under reduced pressure, and the resulting solid was dissolved in CH_2Cl_2. The crude product was purified by column chromatography (silica, 5–20% v/v MeOH in CH_2Cl_2), yielding the TFA salt of fluorophore conjugate **4C** as a dark purple solid (19.7mg, 21 µmol, 59%).

TLC: R_f = 0.6 (10% MeOH/CH_2Cl_2).

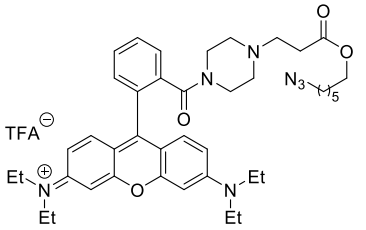
^{1}H NMR (500 MHz, $CDCl_3$, δ): 8.04 (d, J = 8.1 Hz, 2H), 7.82 (d, J = 8.1 Hz, 2H), 7.64 (p, J = 7.4 Hz, 2H), 7.55 (d, J = 7.1 Hz, 1H), 7.30 (d, J = 7.2 Hz, 1H), 7.22 (d, J = 9.5 Hz, 2H), 6.92 (d, J = 9.5 Hz, 2H), 6.74 (d, J = 2.3 Hz, 2H), 5.25 (dd, J = 8.1, 1.2 Hz, 1H), 3.57 (dddq, J = 30.3, 22.9, 11.8, 7.9, 7.5 Hz, 11H), 3.44–3.10 (m, 7H), 2.61–2.08 (m, 8H), 1.68–1.56 (m, J = 7.5 Hz, 2H), 1.50 (p, J = 7.1 Hz, 2H), 1.29 (t, J = 7.2 Hz, 14H).

^{13}C{^{1}H} NMR (126 MHz, $CDCl_3$, δ): 171.3, 167.6, 157.8, 155.8, 155.8, 155.7, 143.0, 135.6 (q, J = 33.9, 33.5 Hz), 135.4, 132.2, 130.4, 130.4, 130.2, 130.0, 129.0, 128.0, 126.6 (q, J = 3.8 Hz), 123.2 (q, J = 273.4 Hz), 111.3, 111.2, 113.8, 96.4, 67.7, 59.1, 52.9, 52.7, 52.3, 51.3, 47.4, 46.2, 41.7, 31.9, 31.0, 28.6, 26.2, 21.3, 12.6.

^{19}F{^{1}H} NMR (471 MHz, $CDCl_3$, δ): −63.15, −75.23 (TFA).

HRMS (Q–TOF): m/z calcd for $C_{49}F_3H_{59}N_7O_6S$ $[M]^+$, 931.4200; found, 931.4202.

TFA·rhodamine B 4-(3-((6-Azidohexyl)oxy)-3-oxopropyl)piperazine Amide (**4D**)



The following procedure was adapted from the literature.[2] 6-Chloro-1-hexanol (5.0 g, 41 mmol) was mixed with 200 mL of H_2O. Sodium azide (6.5 g, 100 mmol, 2.45 equiv) was added, and the mixture was stirred at 95 °C for 24 h. The mixture was allowed to cool to ambient temperature and extracted three times with ethyl acetate. The combined extracts were washed with brine, dried over $MgSO_4$(s), filtered, and concentrated to give 6-azido hexanol as a transparent oil (5.2 g, 180 mmol, 97%) (**3D**). 6-Azido hexanol (**3D**) (21 mg, 140 µmol, 2 equiv) was dissolved in 0.6 mL of CH_2Cl_2 in a screw-cap vial, and the resulting solution was stirred at ambient temperature. TFA·rhodamine B 4-(3-carboxypropyl) piperazine amide (**3**) (50 mg, 72 µmol, 1 equiv), DMAP (1.5 mg, 35 µmol, 0.5 equiv), and DIC (21.5 µL, 140 µmol, 2 equiv) were added to the solution in that order. After 3 h, the solvent was removed under reduced pressure, and the resulting solid was dissolved in CH_2Cl_2. The crude product was purified by column chromatography (silica, 5–20% v/v MeOH in CH_2Cl_2), yielding the TFA salt of fluorophore conjugate **4D** as a dark purple solid (35 mg, 49 µmol, 68%).

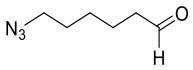
TLC: R_f = 0.6 (10% MeOH/CH_2Cl_2).

^{1}H NMR (500 MHz, $CDCl_3$, δ): δ = 7.69–7.60 (m, 2H), 7.55–7.48 (m, 1H), 7.34–7.27 (m, 1H), 7.20 (d, J = 9.6 Hz, 2H), 6.92 (dd, J = 9.6, 2.5 Hz, 2H), 6.75 (d, J = 2.4 Hz, 2H), 1.01 (t, J = 6.7 Hz, 2H), 3.60 (qd, J = 7.4, 1.2 Hz, 8H), 3.35 (d, J = 36.4 Hz, 4H), 3.23 (t, J = 6.9 Hz, 2H), 2.61 (t, J = 7.2 Hz, 2H), 2.42 (t, J = 7.2 Hz, 2H), 2.29 (s, 4H), 1.63–1.51 (m, 4H), 1.38–1.26 (m, 16H).

^{13}C{^{1}H} NMR (126 MHz, $CDCl_3$, δ): 172.2, 167.4, 157.8, 156.0, 155.7, 135.4, 132.2, 130.7, 130.3, 130.2, 130.0, 127.7, 113.8, 111.2, 96.4, 61.5, 53.3, 53.0, 52.3, 51.4, 47.4, 46.2, 41.6, 32.0, 28.8, 28.5, 26.4, 25.5, 12.7.

HRMS (Q–TOF): m/z calcd for $C_{41}H_{54}N_7O_4$ $[M]^+$, 708.4237; found, 708.4202.

6-Azido Hexanal (**5**)



The following procedure was adapted from the literature.[2] 6-Chloro-1-hexanol (5.0 g, 41 mmol) was mixed with 200 mL of H_2O. Sodium azide (6.5 g, 100 mmol, 2.45 equiv) was added, and the mixture was stirred at 95 °C for 24 h. The solution was allowed to cool to ambient temperature and extracted three times with ethyl acetate. The combined extracts were washed with brine, dried over $MgSO_4$(s), filtered, and concentrated to give 6-azido hexanol as a transparent oil (5.2 g, 180 mmol, 97%) (**3D**), which was used without further purification. Trichloroisocyanuric acid (3.6 g, 15 mmol, 1.05 equiv) was added in small portions to a vigorously stirred mixture of 6-azido-1-hexanol (**3D**) (2.1 g, 15 mmol) in dichloromethane (30 mL) and the solution was cooled to 0 °C. TEMPO (23 mg, 0.15 mmol, 0.01 equiv) was dissolved in dichloromethane (1 mL) and slowly added to the solution. The mixture was stirred for 15 min at 0 °C and then allowed to warm to ambient temperature. After 30 min at ambient temperature, the suspension was filtered through a pad of celite, and the filtrate was washed with 1.0 M Na_2CO_3 (30 mL, 2×), H_2O (30 mL, 1×), 1.0 M HCl (30 mL, 1×), and brine (30 mL, 1×). The combined organic layers were dried over Na_2SO_4(s) and filtered, and the solvent was removed by concentration under reduced pressure. The resulting yellow oil was purified by

column chromatography (silica, 0–20% v/v EtOAc in hexanes) to yield 6-azidohexanal (**5**) as a colorless oil (1.20 g, 58%, 8.5 mmol).

TLC: R_f = 0.6 (30% v/v EtOAc in hexanes).

^{1}H NMR (500 MHz, $CDCl_3$, δ): 9.77 (d, J = 1.8 Hz, 1H), 3.28 (t, J = 6.9 Hz, 2H), 2.46 (td, J = 7.2, 1.7 Hz, 2H), 1.69–1.57 (m, 4H), 1.41 (dd, J = 7.8, 7.1 Hz, 2H).

^{13}C{^{1}H} NMR (126 MHz, $CDCl_3$, δ): 202.3, 51.3, 43.8, 28.8, 26.4, 21.6.

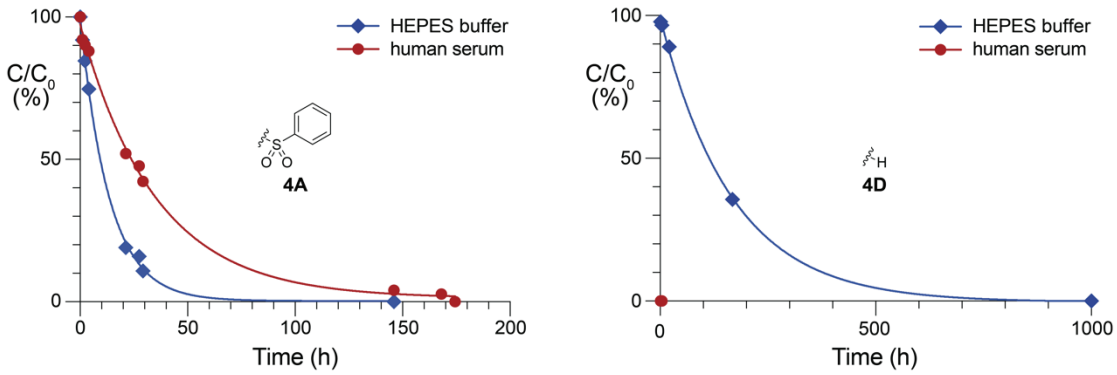
HRMS (ESI–TOF): m/z calcd for $C_6H_{12}N_3O$ $[M + H]^+$, 142.0975; found, 142.0982.

β-Elimination Kinetics of Conjugates 4A–4D

For all kinetic assays, solutions containing 21 μM of conjugate were prepared starting from a 2.1 mM stock solution in CH_3CN and mixing 10 μL of the stock solution with 990 μL of the appropriate buffer solution. The concentration of the conjugate in the CH_3CN solution was determined by mixing 10 μL of the CH_3CN stock solution with 990 μL of 10 mM sodium phosphate buffer, pH 7.4, and measuring the absorbance at 567 nm. By using the reported extinction coefficient of ε_{567} = 96,100 $M^{-1}cm^{-1}$, the concentrations of compounds were determined with the Beer–Lambert equation.[3] Kinetic assays were performed in 100 mM sodium acetate buffer, pH 5.0, 100 mM sodium phosphate buffer, pH 6.0 or 7.0, and 100 mM Tris–HCl buffer, pH 8.0. At appropriate time intervals, 25 μL aliquots of the sample in buffer were mixed with 25 μL of 0.2 M acetic acid solution in water, and the resulting solution was stored at −20 °C for up to 5 days prior to UPLC measurement. The progression of the reaction was monitored by quantifying the relative absorbance at 567 nm of the fluorophore-conjugates (R_t = 7.5–8.7 min) to the one of the released fluorophore (R_t = 5.5 min). Rate constants for cleavage (k_{obs}) were calculated by fitting the percentage of reaction completion (A_{567} reactant/[A_{567} reactant + A_{567} product]) versus time to a one-phase decay equation.

Stability of Conjugates 4A and 4D in Mouse and Human Serum

For all serum stability studies, 29 μM solutions of fluorophore conjugates in 50 mM HEPES–NaOH buffer, pH 7.4, were prepared starting from a 2.9 mM stock solution in CH_3CN. Afterward, 600 μL of the conjugate solutions were mixed with 400 μL of mouse serum or human serum, giving a final conjugate concentration of 21 μM. As a control, 600 μL of the conjugate solutions were mixed with 400 μL of 50 mM HEPES–NaOH buffer, pH 7.4. The samples were then incubated at 37 °C for 1 h after which and at appropriate time points, 50 μL of each sample was mixed with 50 μL of 0.2 M AcOH in CH_3CN. The resulting mixture was subjected to centrifugation at 13,000 rpm for 10 min. The supernatant was then isolated and stored at −20 °C for up to 3 days prior to measurement. The supernatants were then analysed by UPLC chromatography. The progression of the reaction was monitored by quantifying the relative absorbance at 567 nm of the fluorophore conjugates (R_t = 7.5–8.7 min) to that of the released fluorophore (R_t= 5.5 min). Release rates (k_{obsd}) were calculated by fitting the percentage of reaction completion ($A_{567\ nm}$ reactant/[$A_{567\ nm}$ reactant + $A_{567\ nm}$ product]) versus time to a one-phase decay equation. All the conjugates showed complete hydrolysis after 1 h of incubation in mouse serum, and thus, the curve of the cleavage kinetics is not shown. The cleavage kinetics curve of the fluorophore conjugates **4A** and **4D** in human serum is reported in Figure S1.

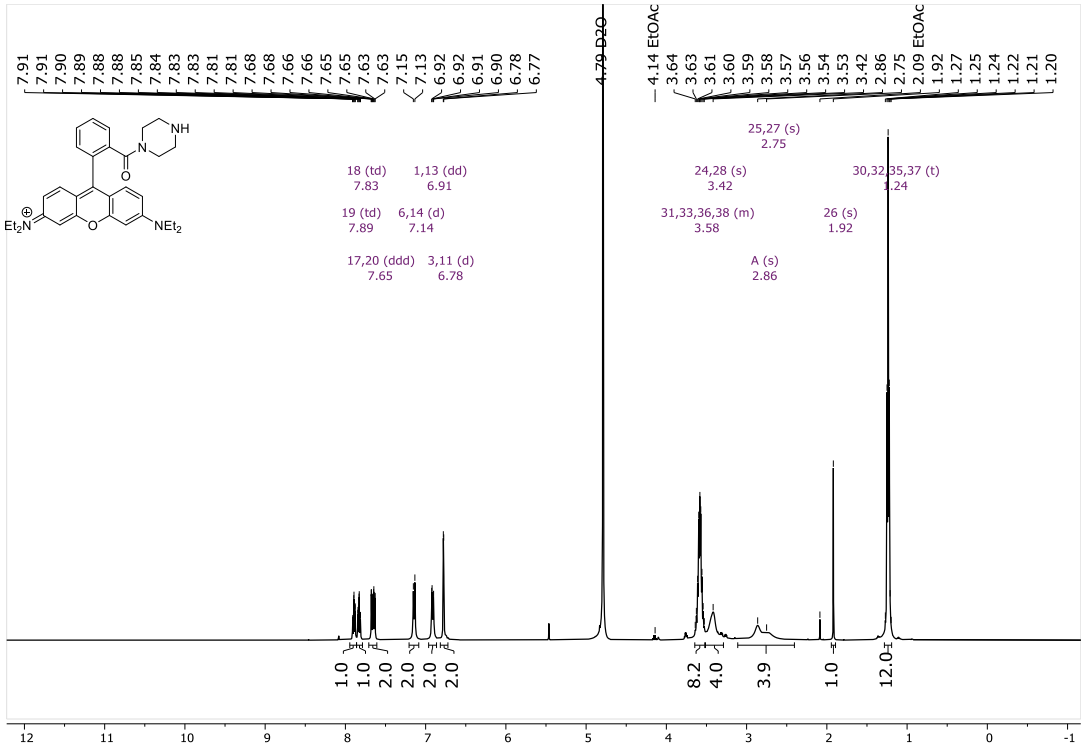


Modulator	Condition	$t_{1/2}$ (h)	k_{obs} (s^{-1})
$-SO_2Ph$ (**4A**)	HEPES–NaOH buffer	9.3	2.1×10^{-5}
	human serum	24.4	7.9×10^{-6}
–H (**4D**)	HEPES–NaOH buffer	115.6	1.7×10^{-6}
	human serum	<1	$>1.9 \times 10^{-4}$

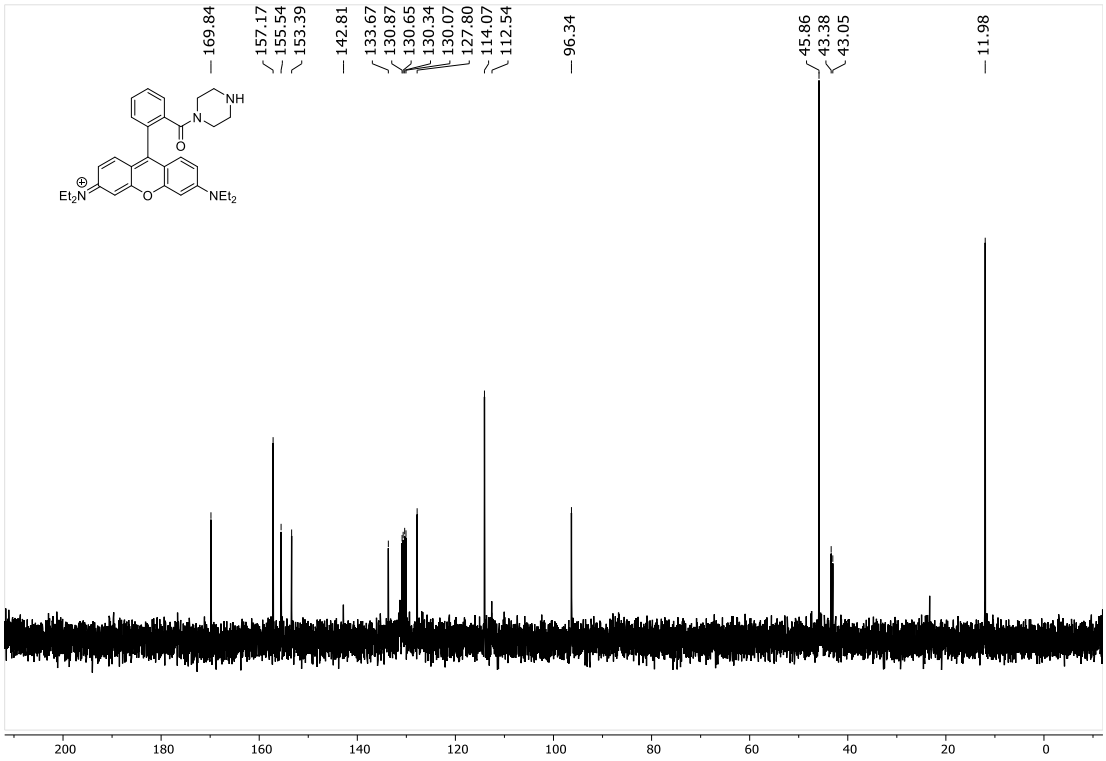
Fig S1 Cleavage kinetics of conjugates **4A** (left) and **4D** (right) in 50 mM HEPES–NaOH buffer, pH 7.4, or 40% v/v human serum in 50 mM HEPES–NaOH buffer, pH 7.4.

NMR Spectra

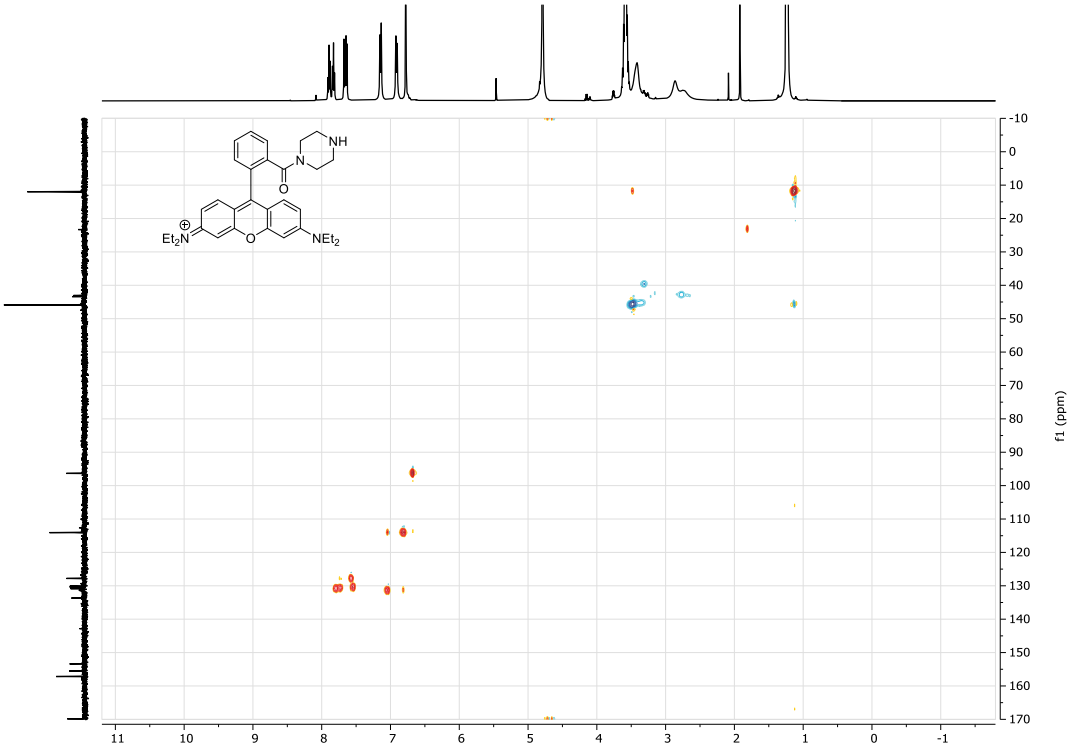
^{1}H NMR Spectrum (500 MHz, $CDCl_3$) of Rhodamine B Piperazine Amide (1)



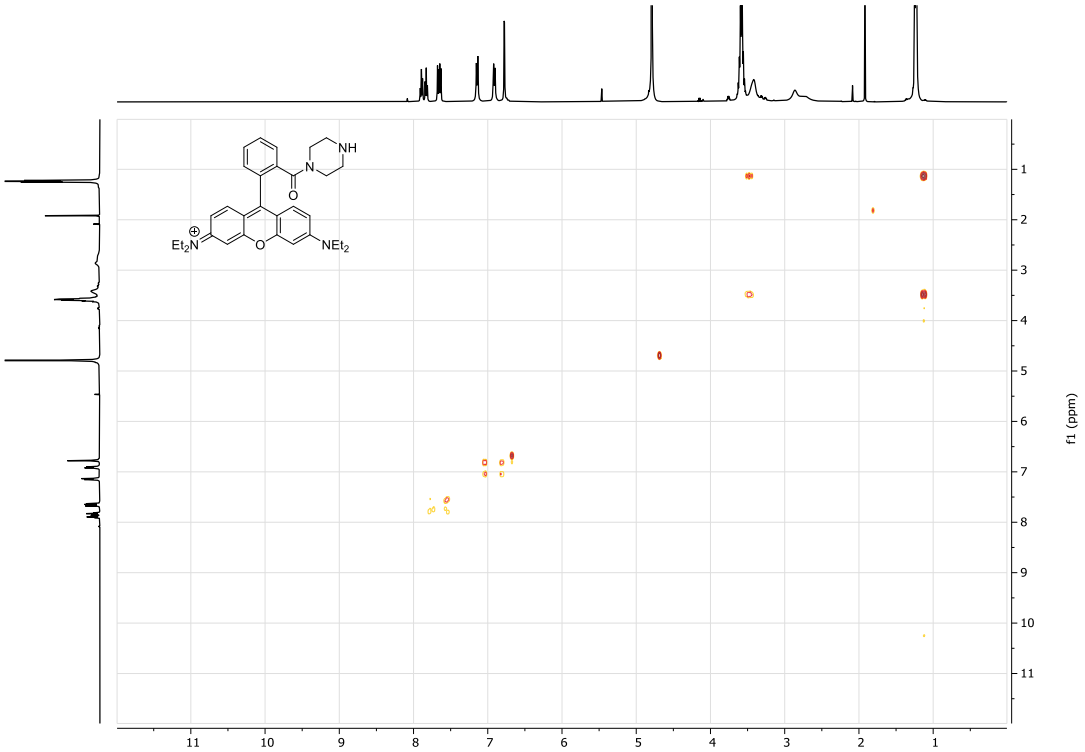
^{13}C{^{1}H} NMR Spectrum (126 MHz, $CDCl_3$) of Rhodamine B Piperazine Amide (1)



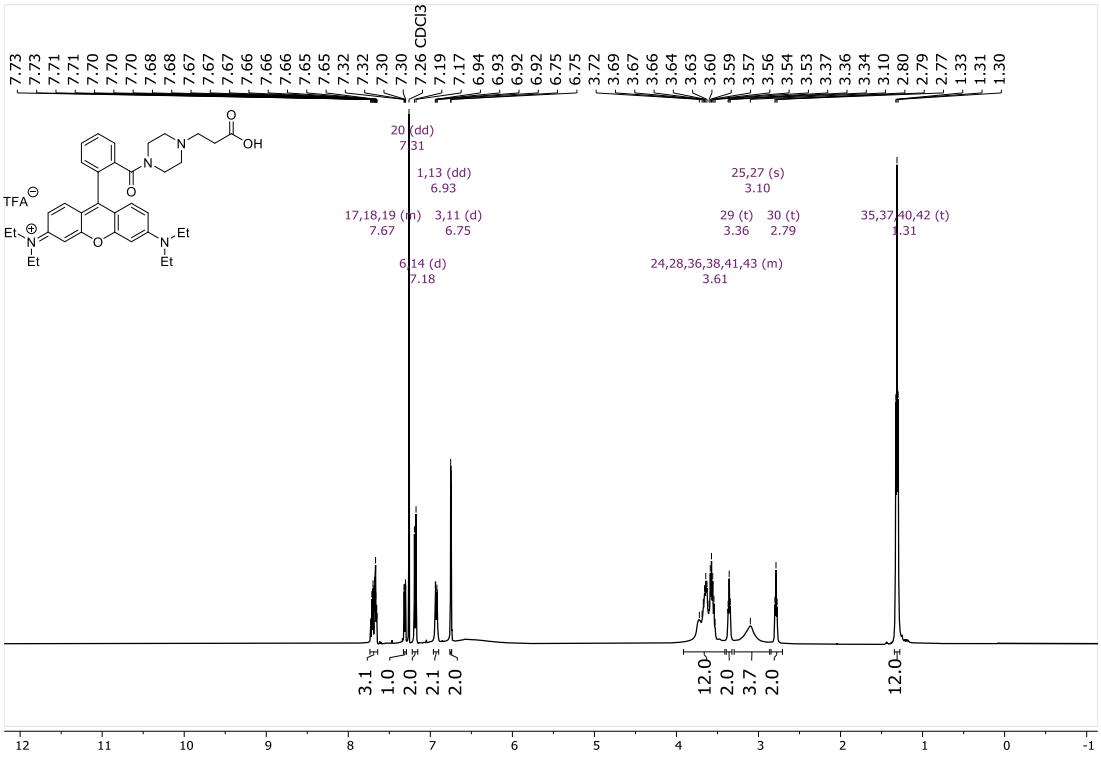
HSQC NMR Spectrum (126 MHz, $CDCl_3$) of Rhodamine B Piperazine Amide (1)



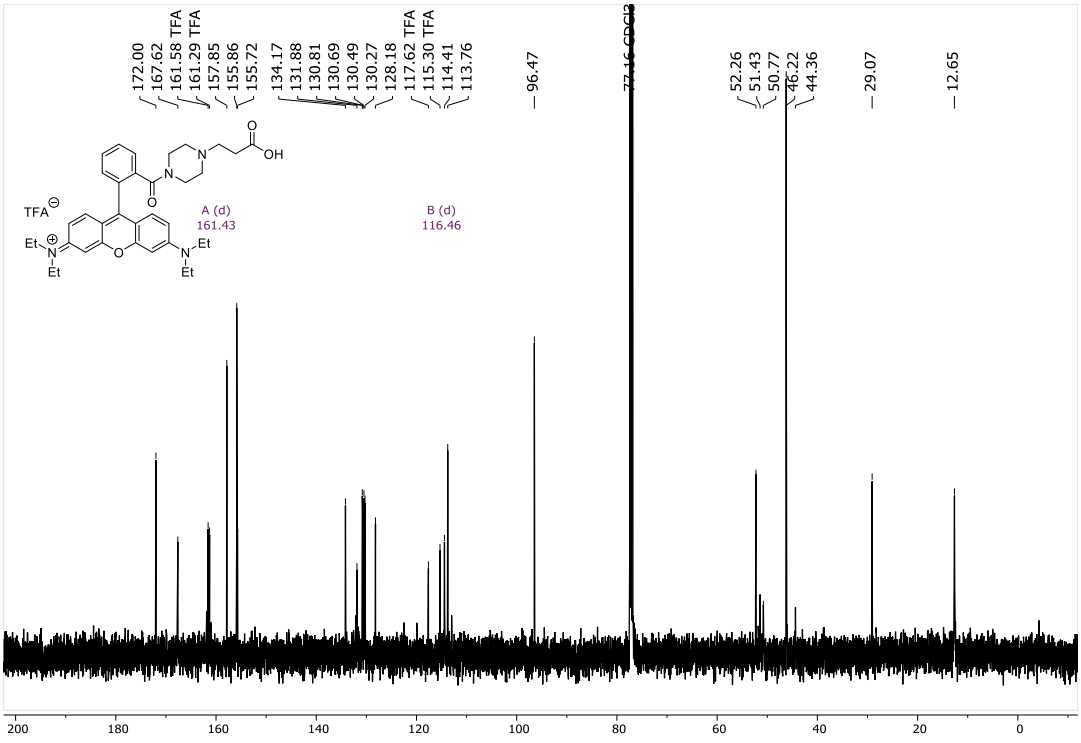
COSY NMR Spectrum (126 MHz, $CDCl_3$) of Rhodamine B Piperazine Amide (1)



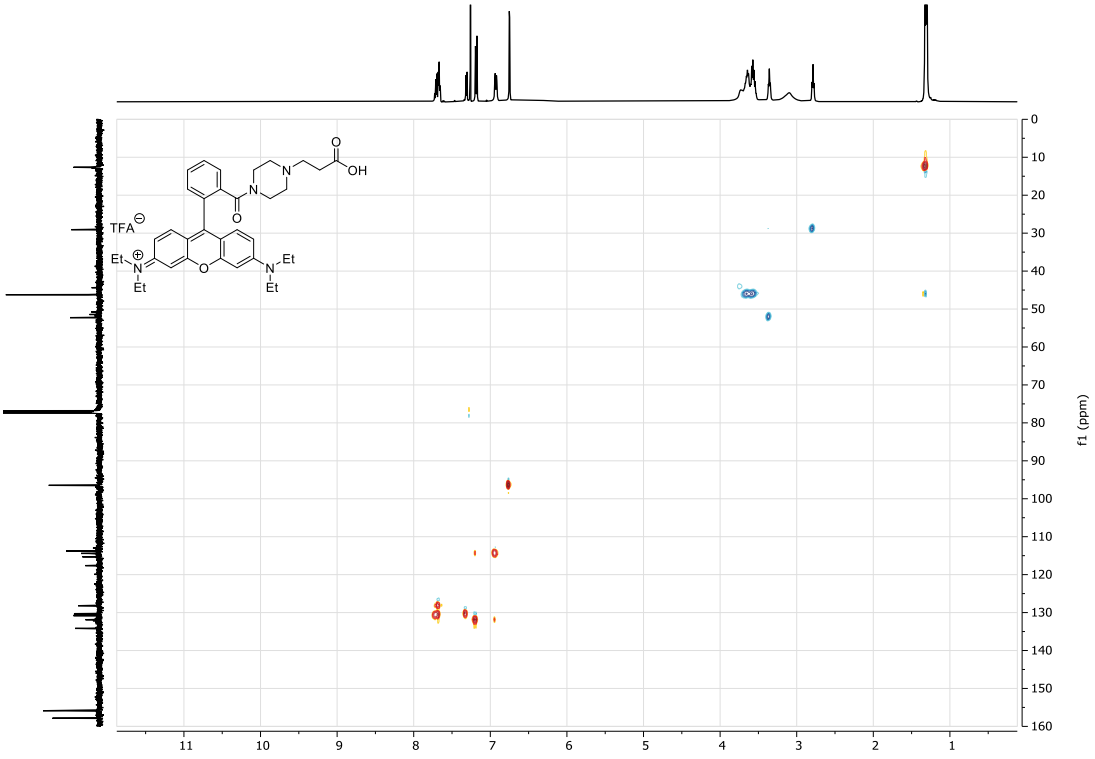
^{1}H NMR Spectrum (500 MHz, $CDCl_3$) of TFA·rhodamine B 4-(3-Carboxypropyl) Piperazine Amide (2)



^{13}C{^{1}H} NMR Spectrum (126 MHz, $CDCl_3$) of TFA·rhodamine B 4-(3-Carboxypropyl) Piperazine Amide (2)

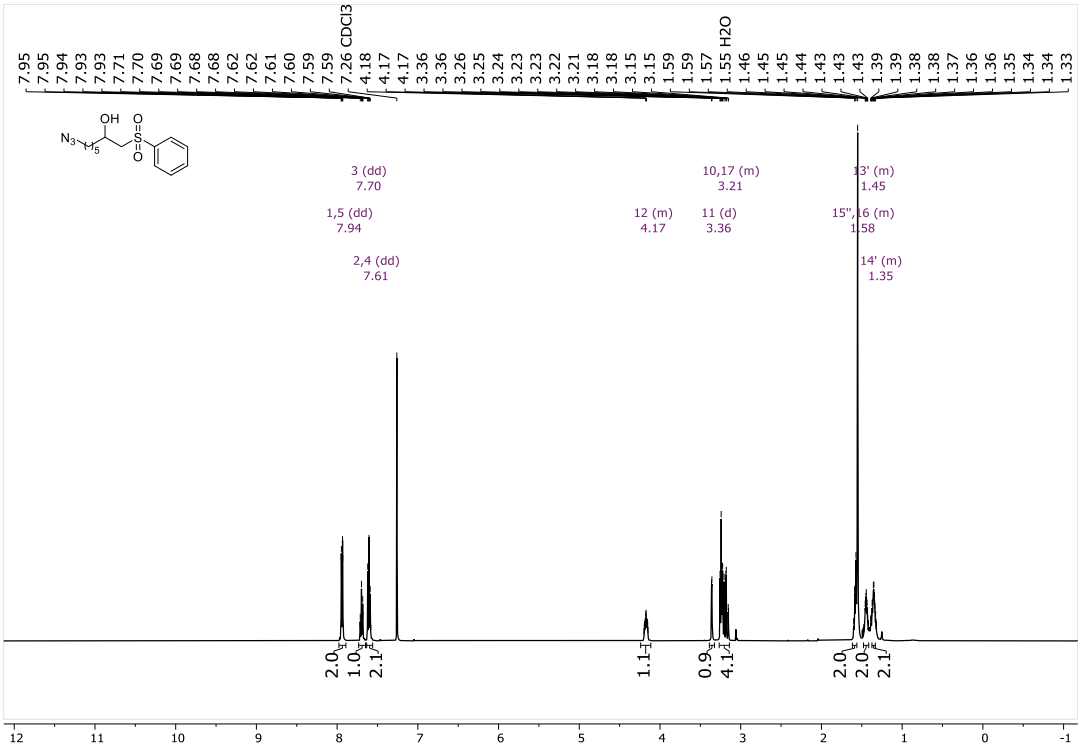


HSQC NMR Spectrum (500 MHz, $CDCl_3$) of TFA·rhodamine B 4-(3-Carboxypropyl) Piperazine (2)

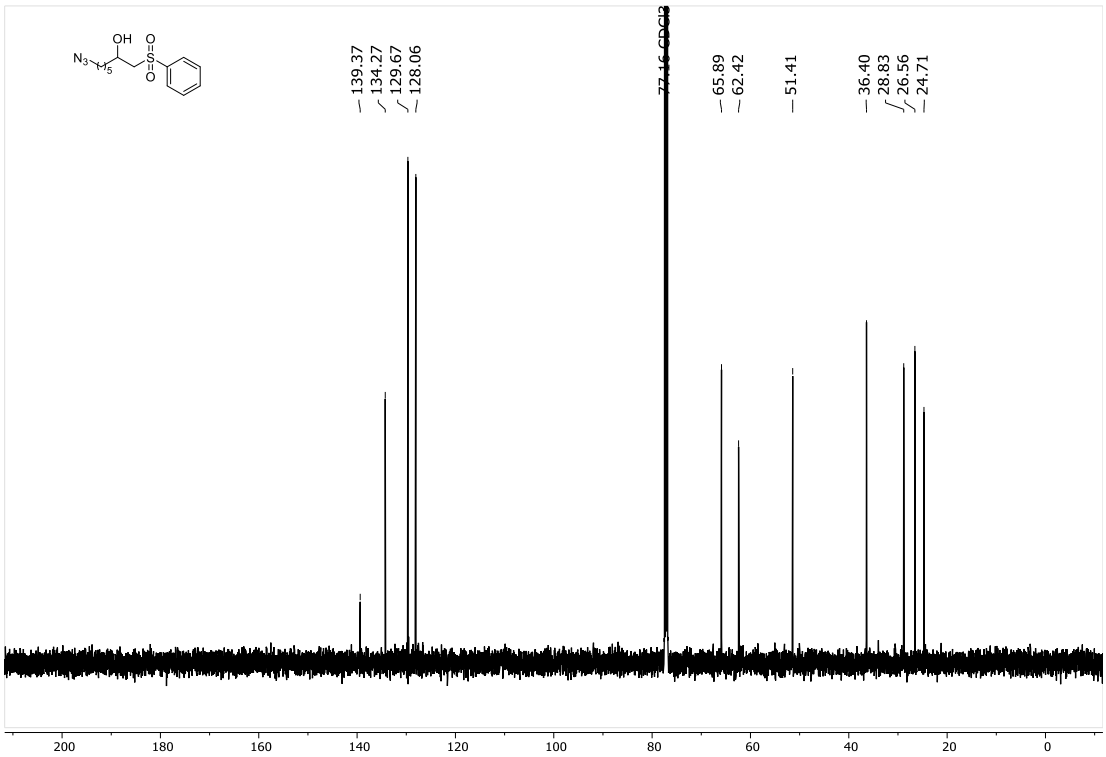


COSY NMR Spectrum (126 MHz, $CDCl_3$) of TFA·rhodamine B 4-(3-Carboxypropyl) Piperazine (2)

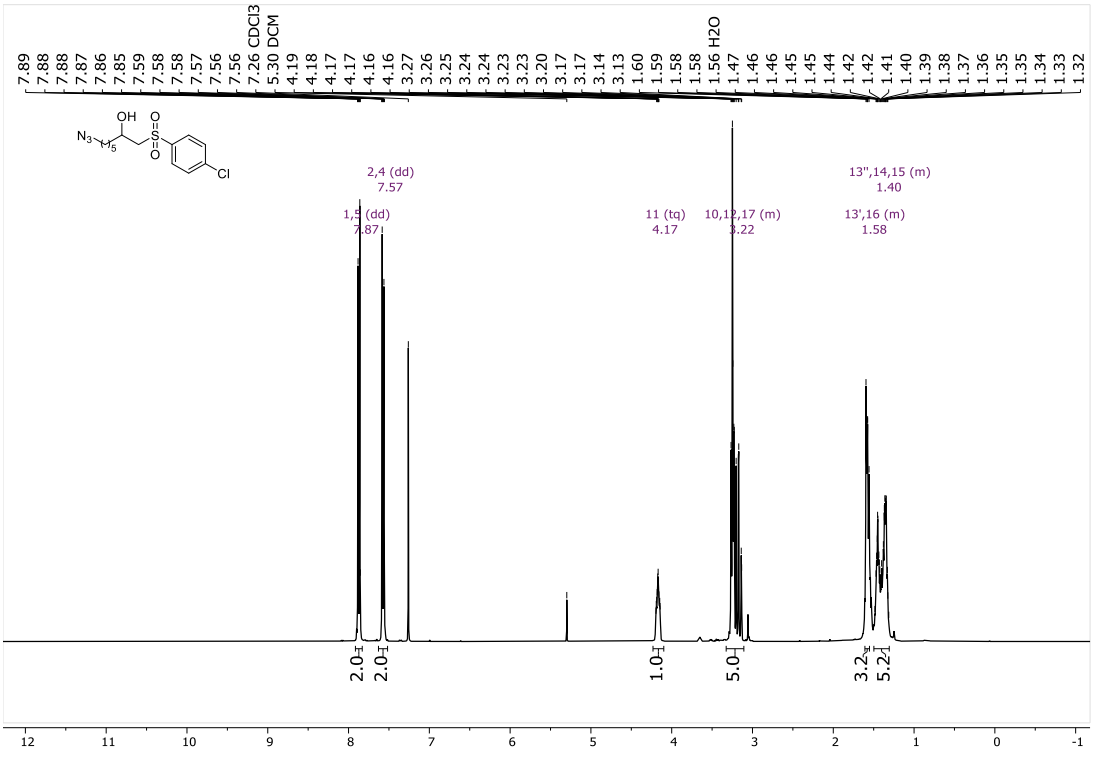
^{1}H NMR Spectrum (500 MHz, $CDCl_3$) of 1-(Phenylsulfonyl)-7-azido-2-heptanol (3A)



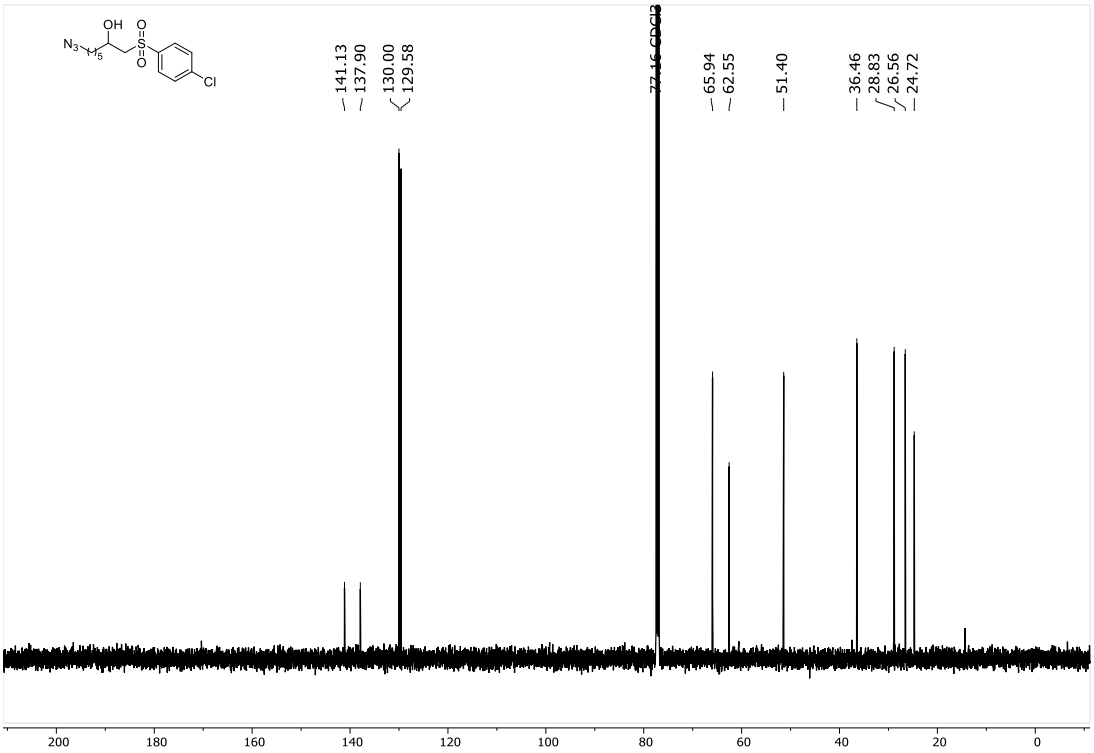
^{13}C{^{1}H} NMR Spectrum (126 MHz, $CDCl_3$) of 1-(Phenylsulfonyl)-7-azido-2-heptanol (3A)



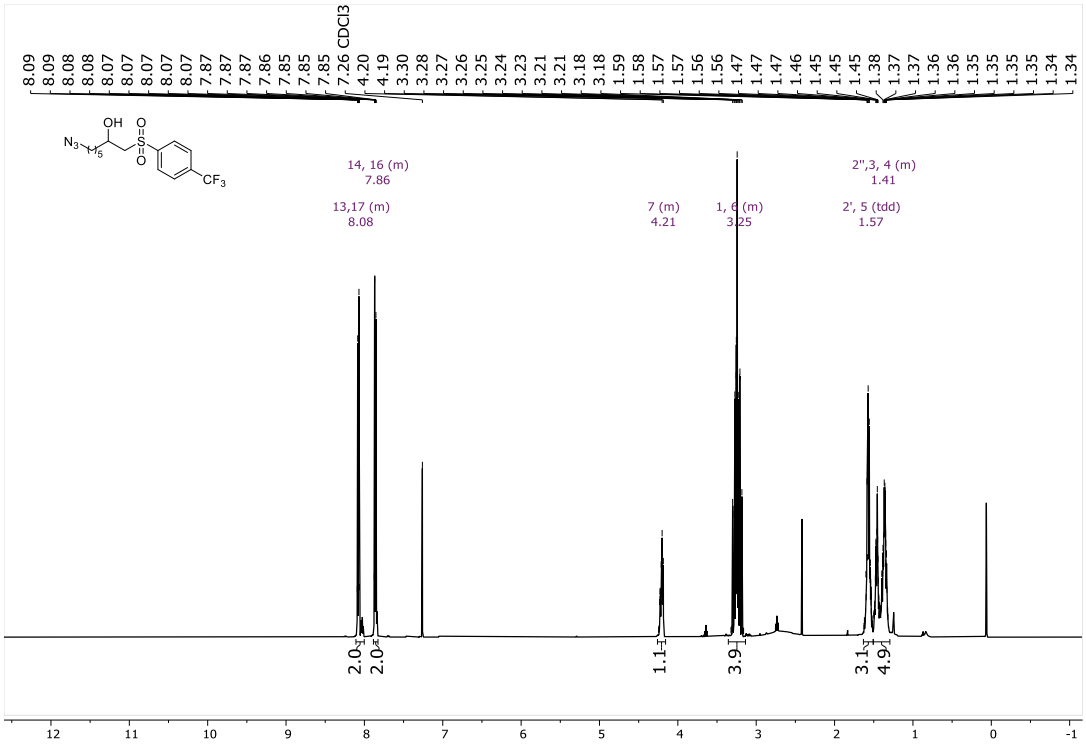
^{1}H NMR Spectrum (500 MHz, $CDCl_3$) of 1-(4-Chlorophenylsulfonyl)-7-azido-2-heptanol (3B)



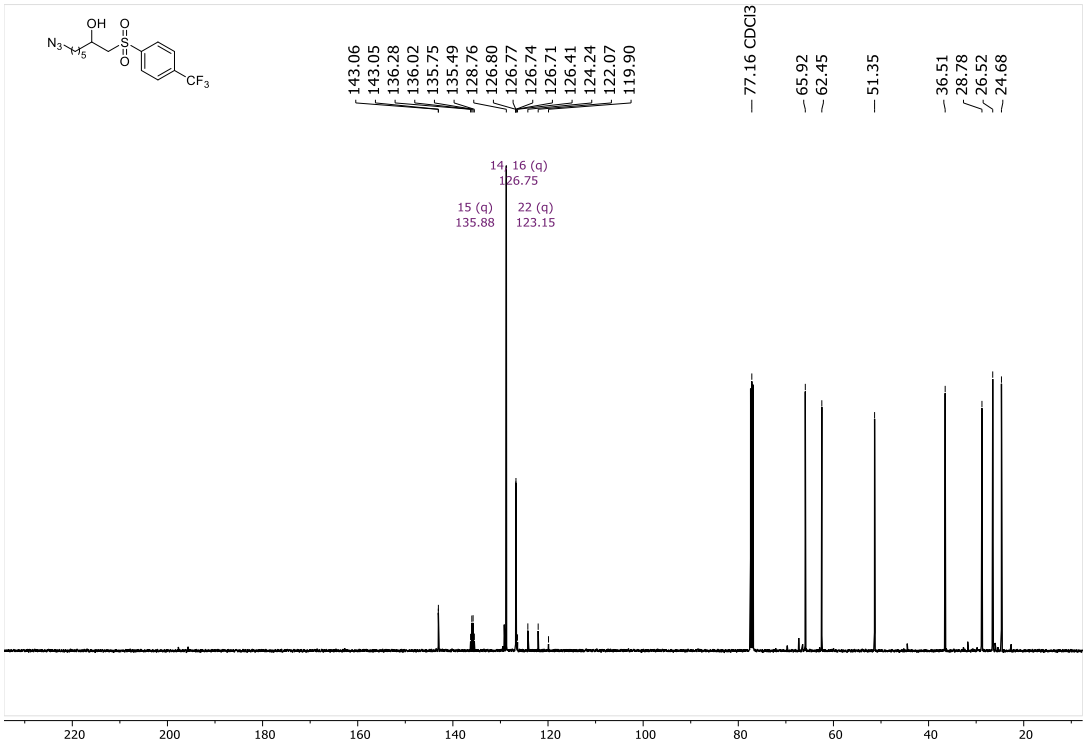
^{13}C{^{1}H} NMR Spectrum (126 MHz, $CDCl_3$) of 1-(4-Chlorophenylsulfonyl)-7-azido-2-heptanol (3B)



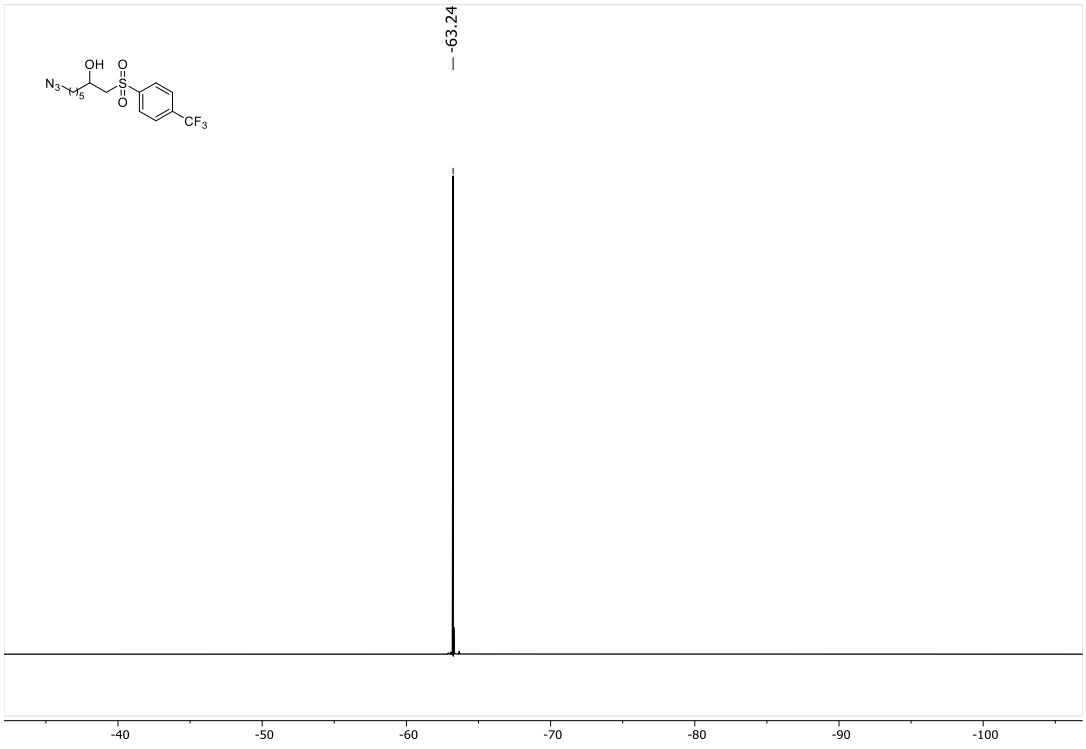
^{1}H NMR Spectrum (500 MHz, $CDCl_3$) of 1-(4-(Trifluoromethyl)phenylsulfonyl)-7-azido-2-heptanol (3C)



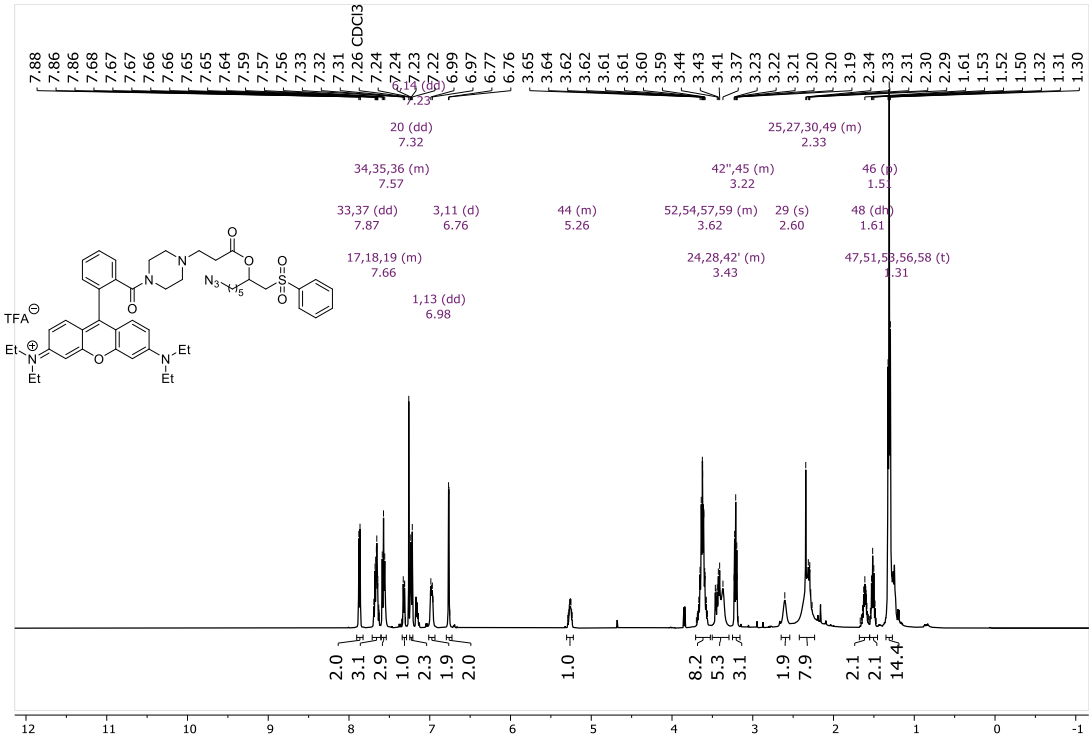
^{13}C{^{1}H} NMR Spectrum (126 MHz, $CDCl_3$) 1-(4-(Trifluoromethyl)phenylsulfonyl)-7-azido-2-heptanol (3C)



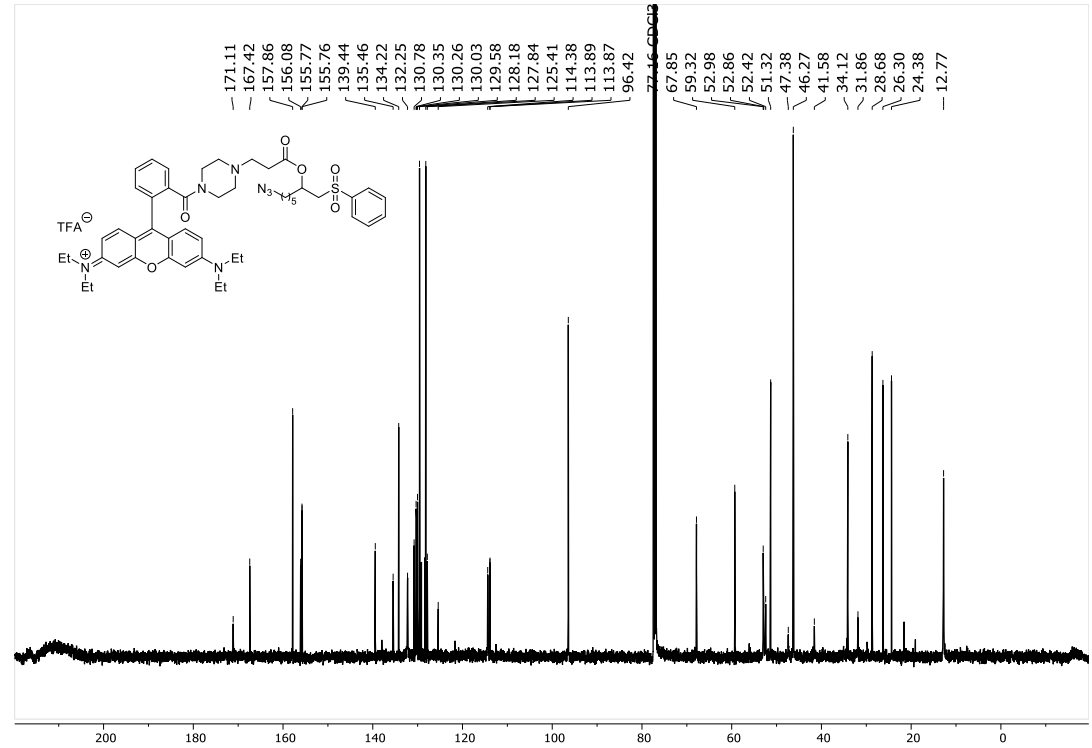
$^{19}F\{^{1}H\}$ NMR Spectrum (471 MHz, $CDCl_3$) of 1-(4-(Trifluoromethyl)phenylsulfonyl)-7-azido-2-heptanol (3C)



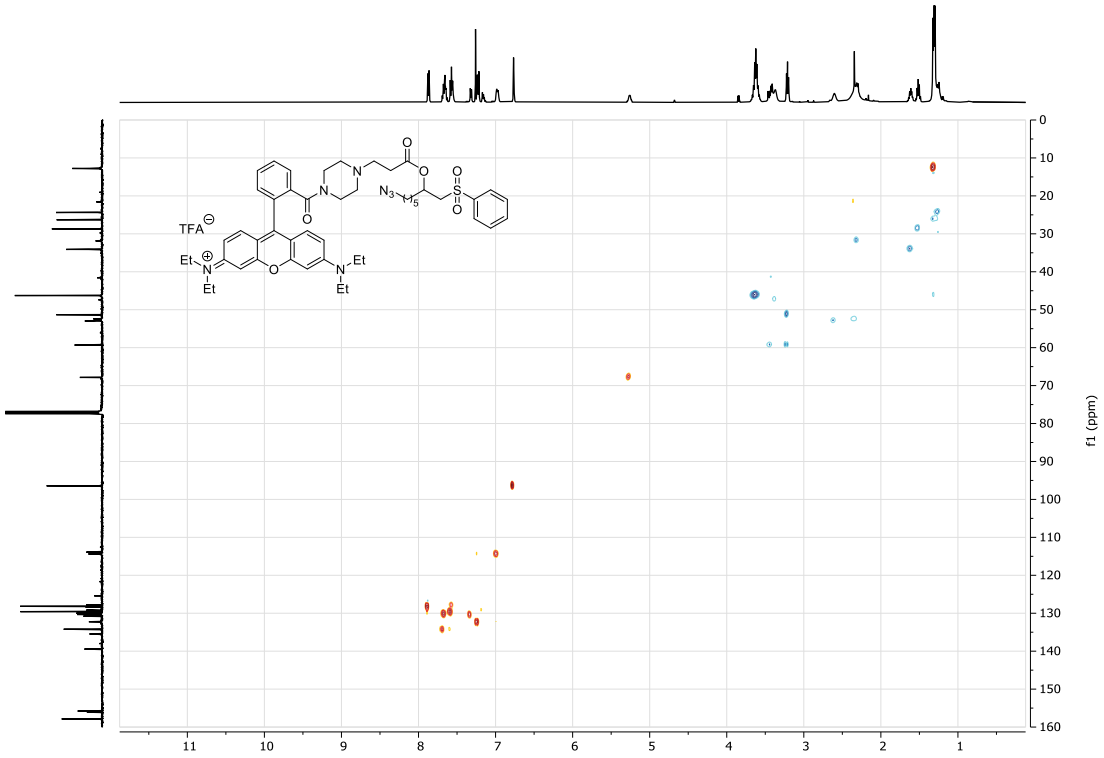
^{1}H NMR Spectrum (500 MHz, $CDCl_3$) of TFA·rhodamine B 4-(3-((7-Azido-1-(phenylsulfonyl)heptan-2-yl)oxy)-3-oxopropyl)piperazine Amide (4A)



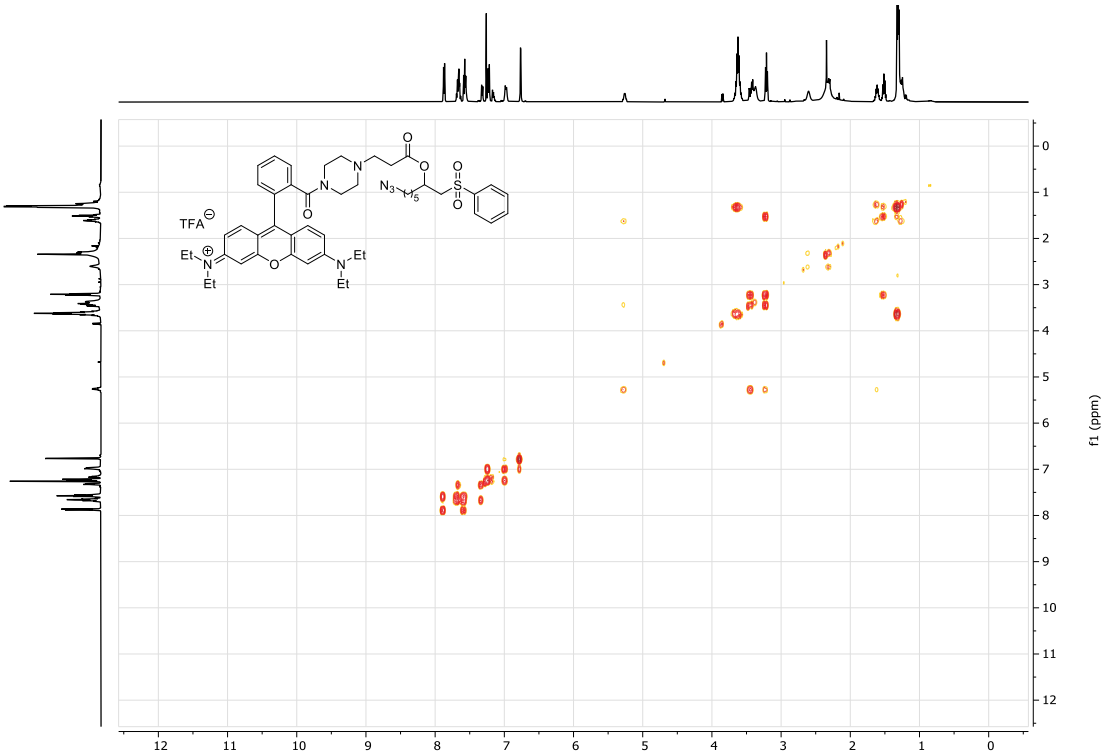
^{13}C{^{1}H} NMR Spectrum (126 MHz, $CDCl_3$) of TFA·Rhodamine B 4-(3-((7-Azido-1-(phenylsulfonyl)heptan-2-yl)oxy)-3-oxopropyl)piperazine Amide (4A)



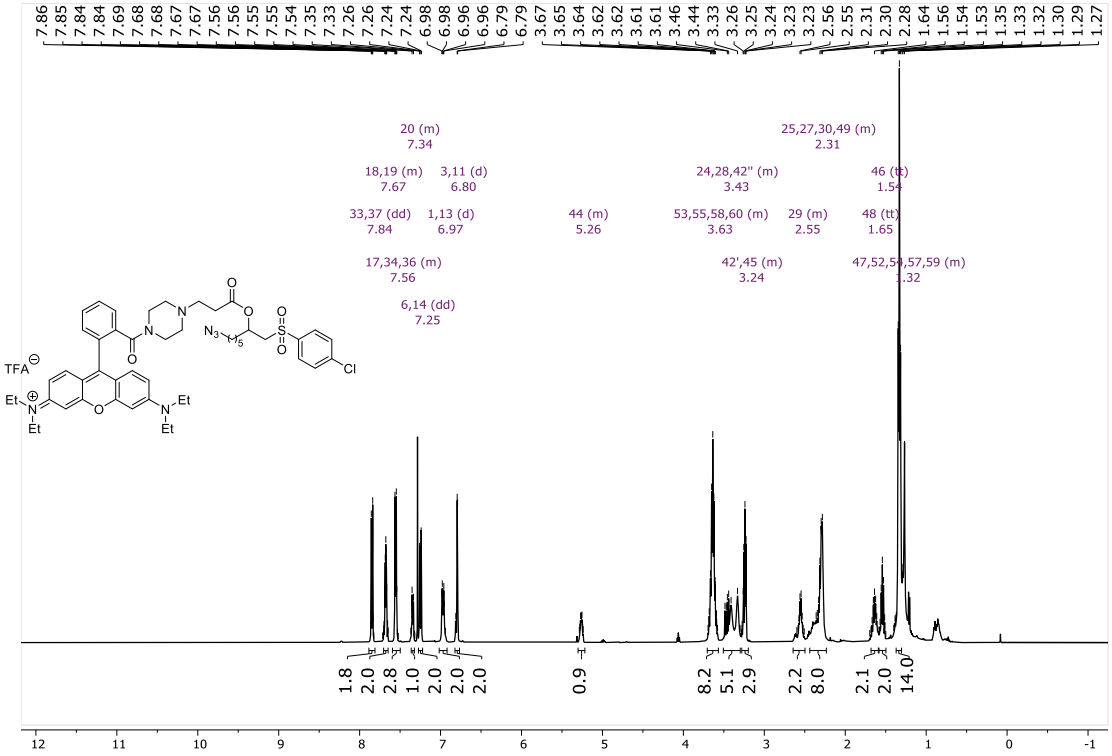
HSQC Spectrum (500 MHz, $CDCl_3$) of TFA·rhodamine B 4-(3-((7-Azido-1-(phenylsulfonyl)heptan-2-yl)oxy)-3-oxopropyl)piperazine Amide (4A)



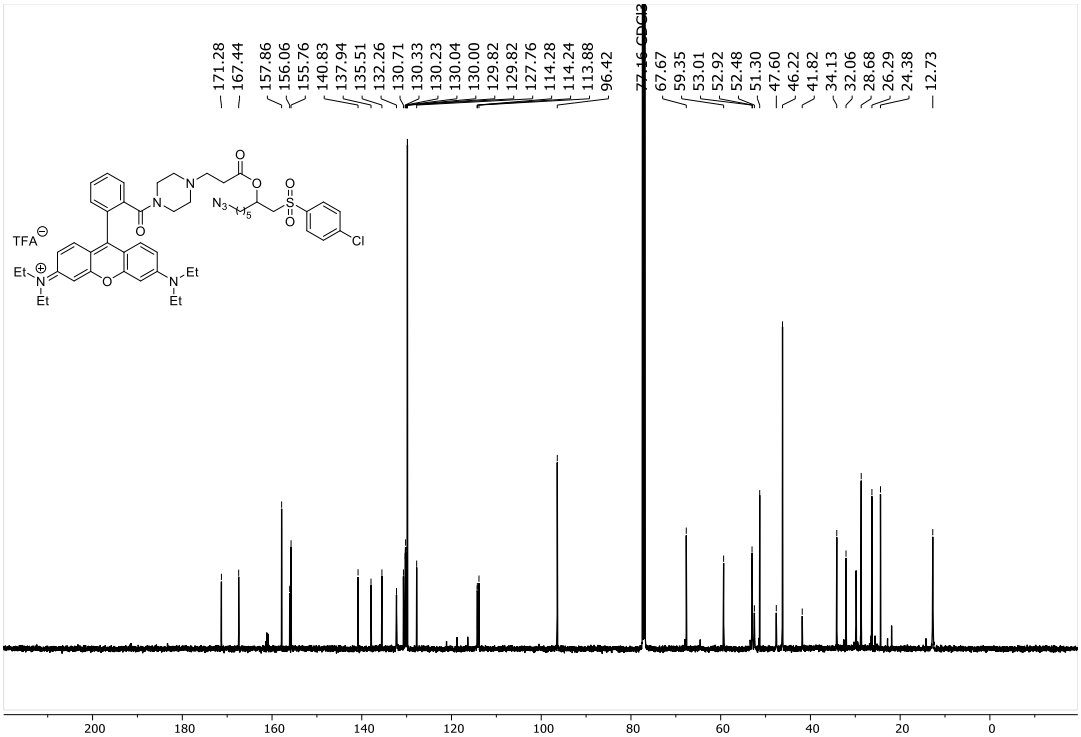
COSY Spectrum (126 MHz, $CDCl_3$) of TFA·rhodamine B 4-(3-((7-Azido-1-(phenylsulfonyl)heptan-2-yl)oxy)-3-oxopropyl)piperazine Amide (4A)



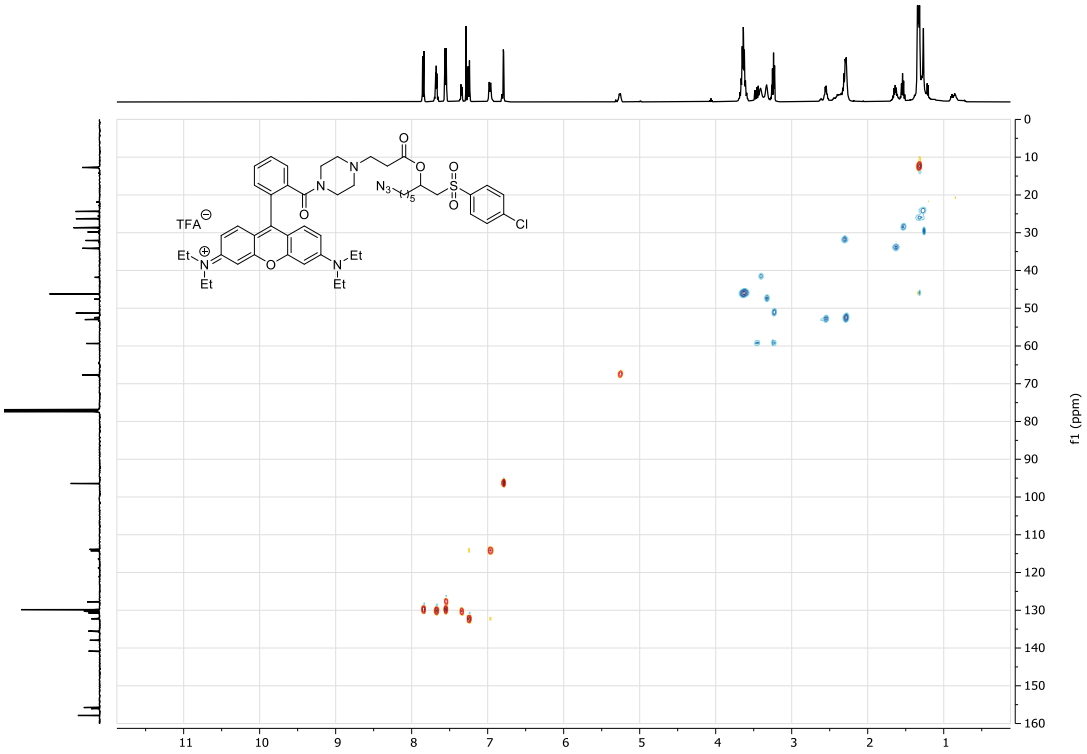
^{1}H NMR Spectrum (500 MHz, $CDCl_3$) of Rhodamine B 4-(3-((7-Azido-1-((4-chlorophenyl)sulfonyl)heptan-2-yl)oxy)-3-oxopropyl)piperazine Amide (4B)



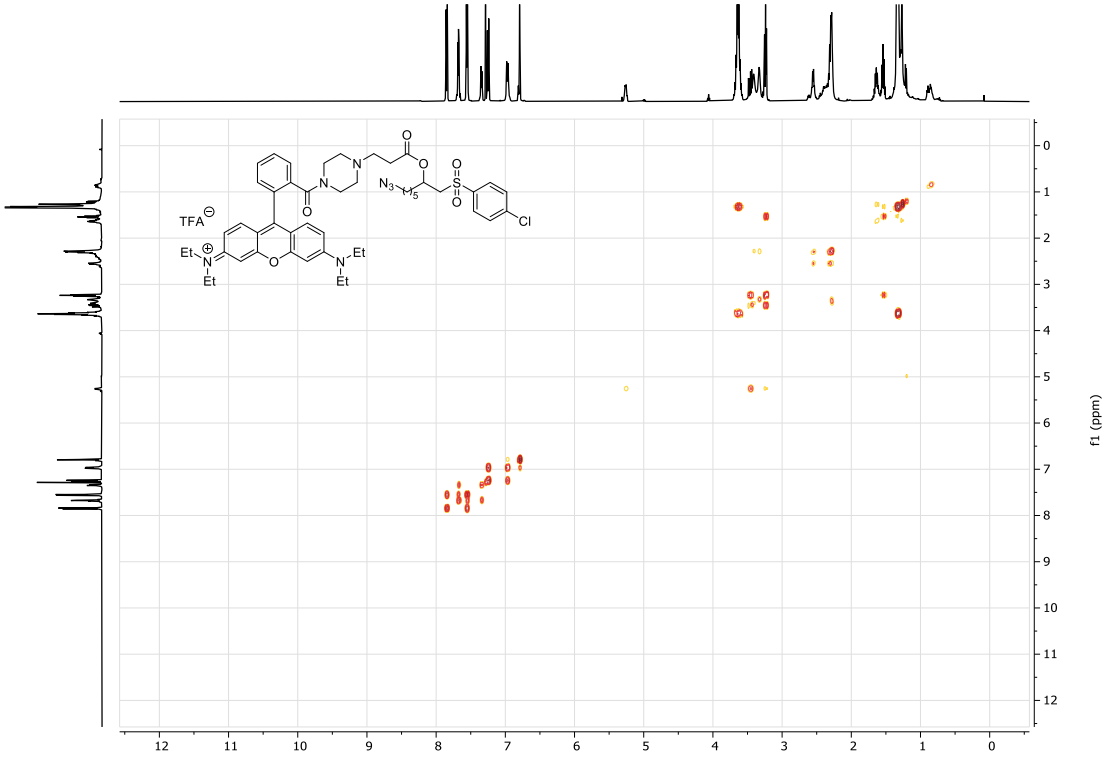
^{13}C{^{1}H} NMR Spectrum (126 MHz, $CDCl_3$) of TFA·rhodamine B 4-(3-((7-Azido-1-((4-chlorophenyl)sulfonyl)heptan-2-yl)oxy)-3-oxopropyl)piperazine Amide (4B)



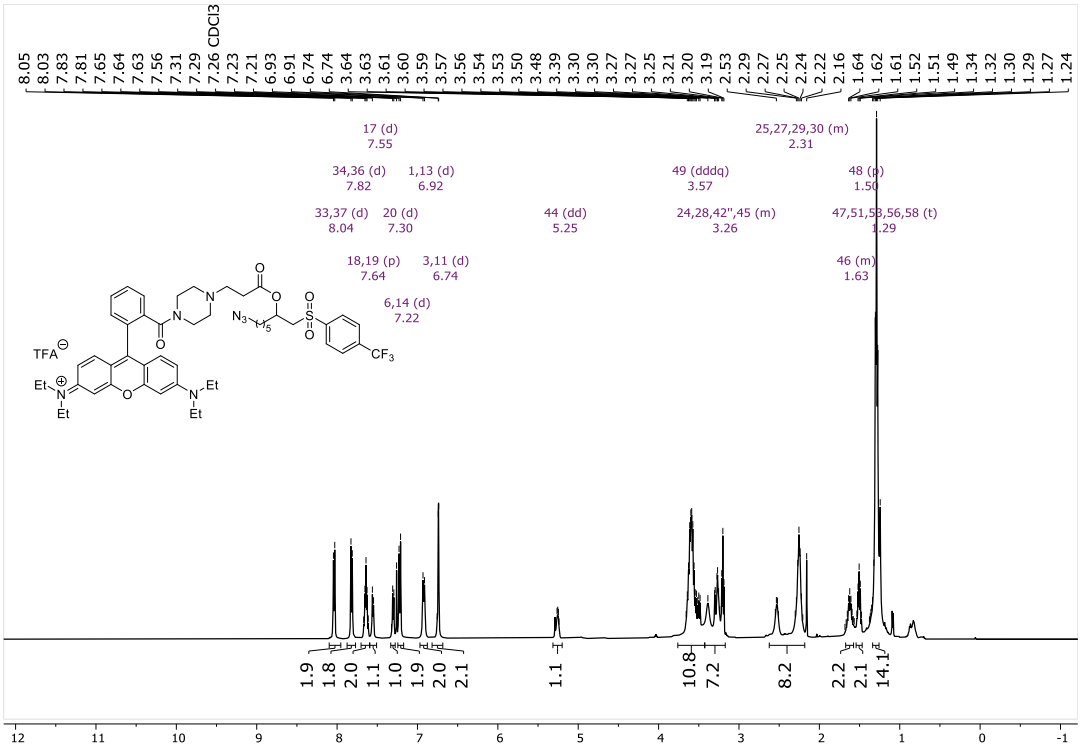
HSQC NMR Spectrum (500 MHz, $CDCl_3$) of TFA·rhodamine B 4-(3-((7-Azido-1-((4-chlorophenyl)sulfonyl)heptan-2-yl)oxy)-3-oxopropyl)piperazine Amide (4B)



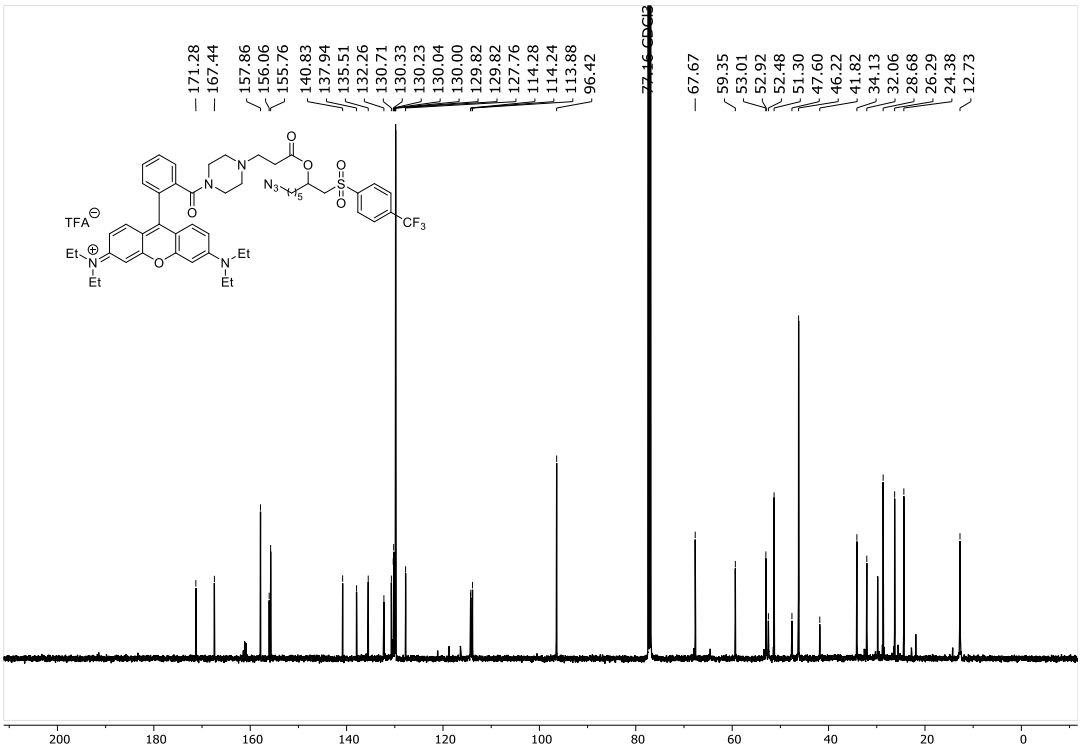
COSY NMR Spectrum (126 MHz, $CDCl_3$) of TFA·rhodamine B 4-(3-((7-Azido-1-((4-chlorophenyl)sulfonyl)heptan-2-yl)oxy)-3-oxopropyl)piperazine Amide (4B)



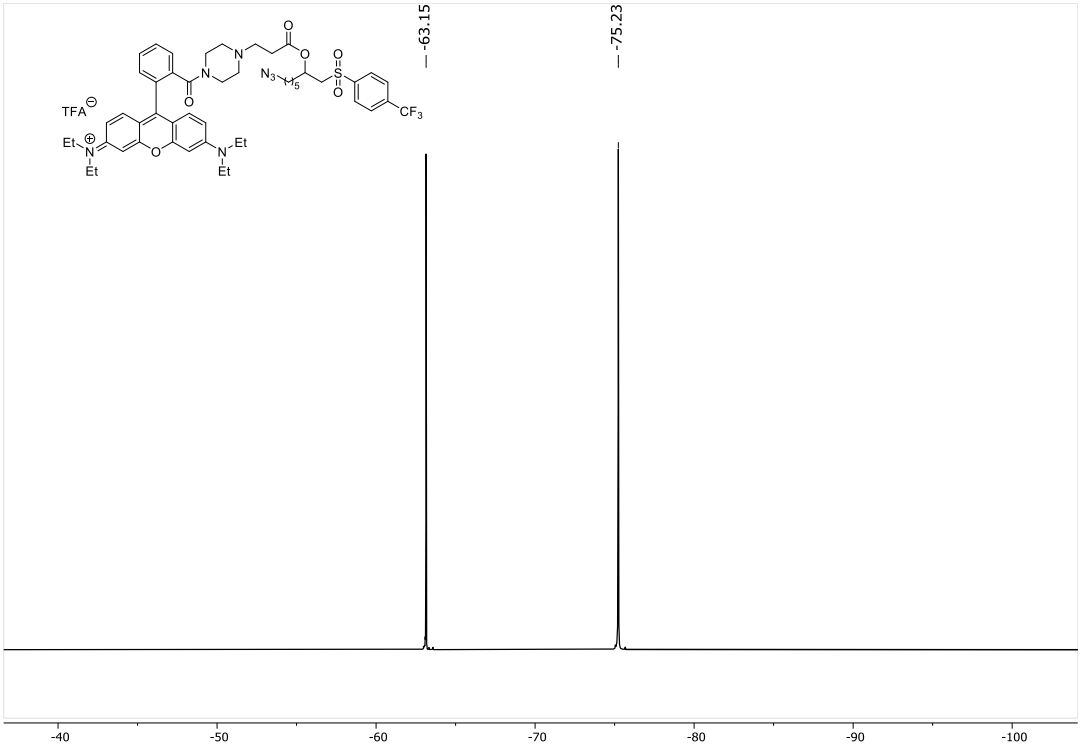
^{1}H NMR Spectrum (500 MHz, $CDCl_3$) of TFA·rhodamine B 4-(3-((7-Azido-1-((4-(trifluoromethyl)phenyl)sulfonyl)heptan-2-yl)oxy)-3-oxopropyl)piperazine Amide (4C)



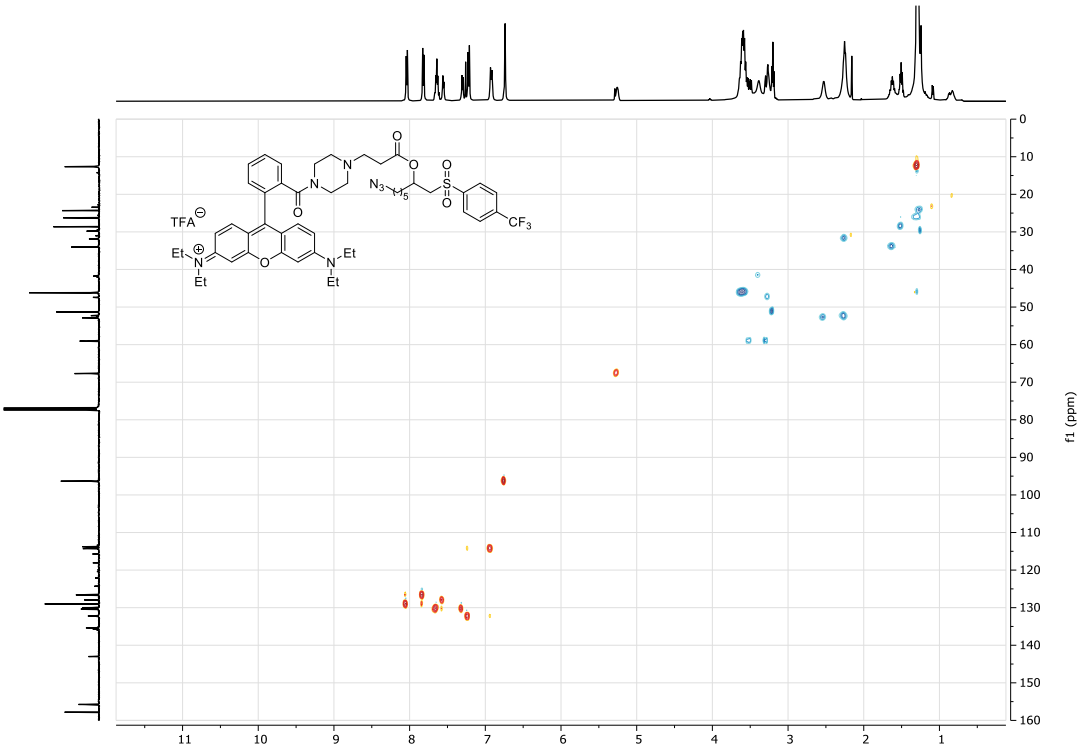
^{13}C{^{1}H} NMR Spectrum (126 MHz, $CDCl_3$) of TFA·rhodamine B 4-(3-((7-Azido-1-((4-(trifluoromethyl)phenyl)sulfonyl)heptan-2-yl)oxy)-3-oxopropyl)piperazine Amide (4C)



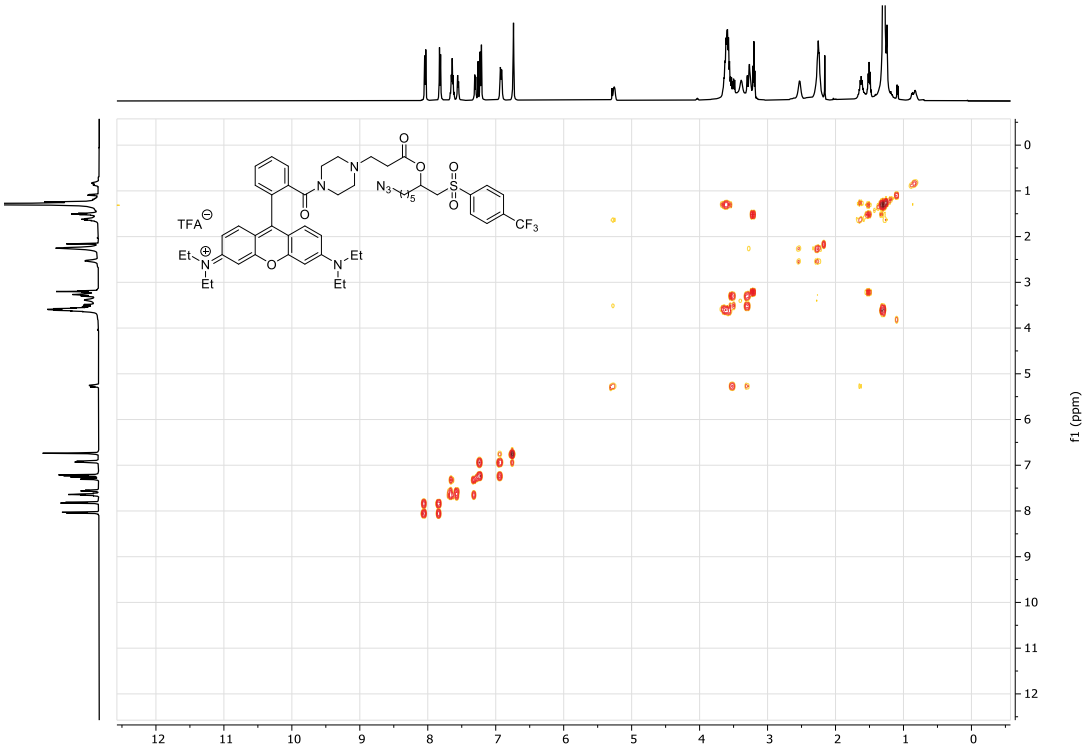
$^{19}F\{^{1}H\}$ NMR Spectrum (471 MHz, $CDCl_3$) of TFA·rhodamine B 4-(3-((7-Azido-1-((4-(trifluoromethyl)phenyl)sulfonyl)heptan-2-yl)oxy)-3-oxopropyl)piperazine Amide (4C)



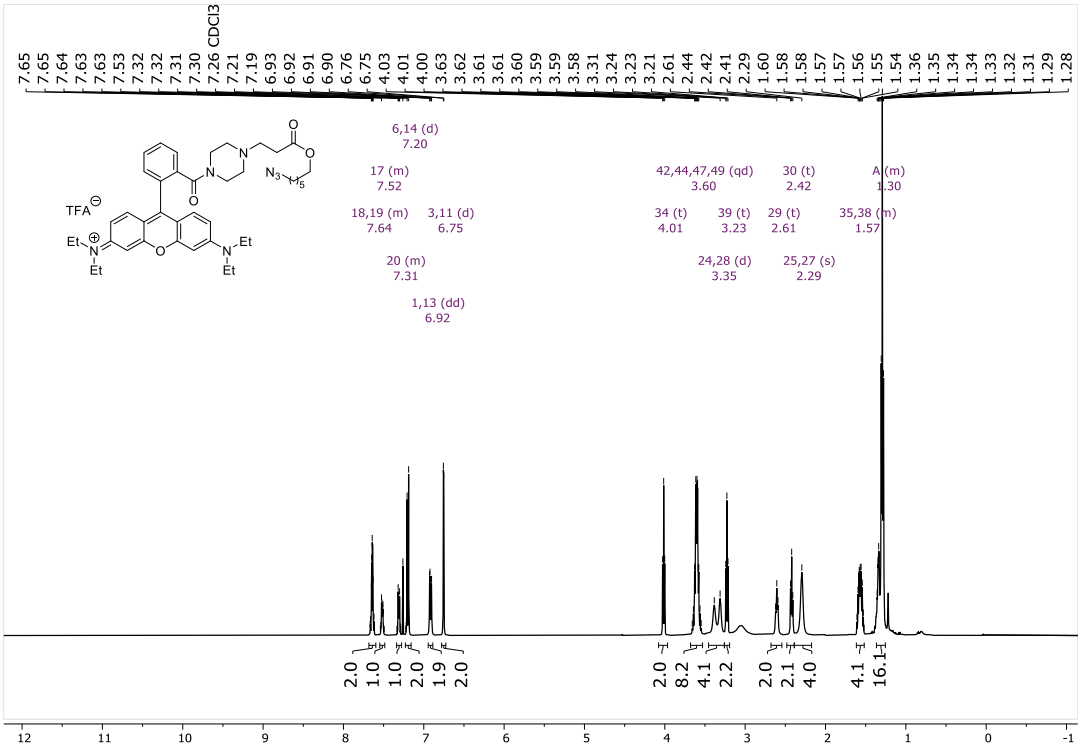
HSQC Spectrum (500 MHz, $CDCl_3$) of TFA·rhodamine B 4-(3-((7-Azido-1-((4-(trifluoromethyl)phenyl)sulfonyl)heptan-2-yl)oxy)-3-oxopropyl)piperazine Amide (4C)



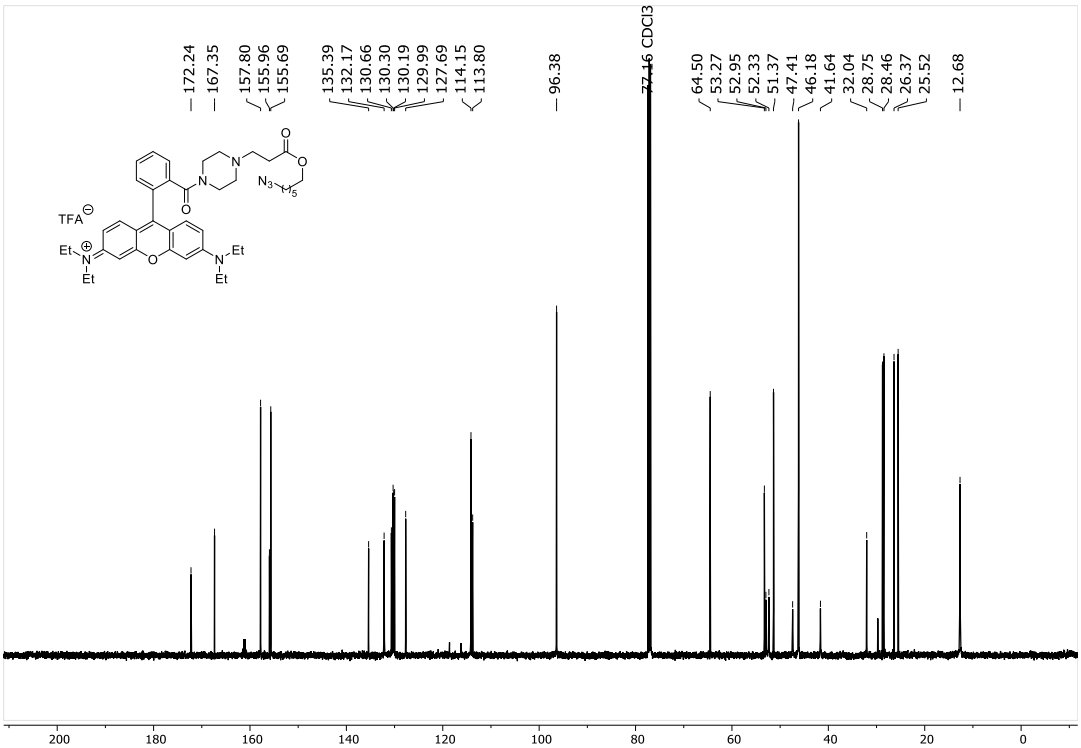
COSY NMR Spectrum (126 MHz, $CDCl_3$) of TFA·rhodamine B 4-(3-((7-Azido-1-((4-(trifluoromethyl)phenyl)sulfonyl)heptan-2-yl)oxy)-3-oxopropyl)piperazine Amide (4C)



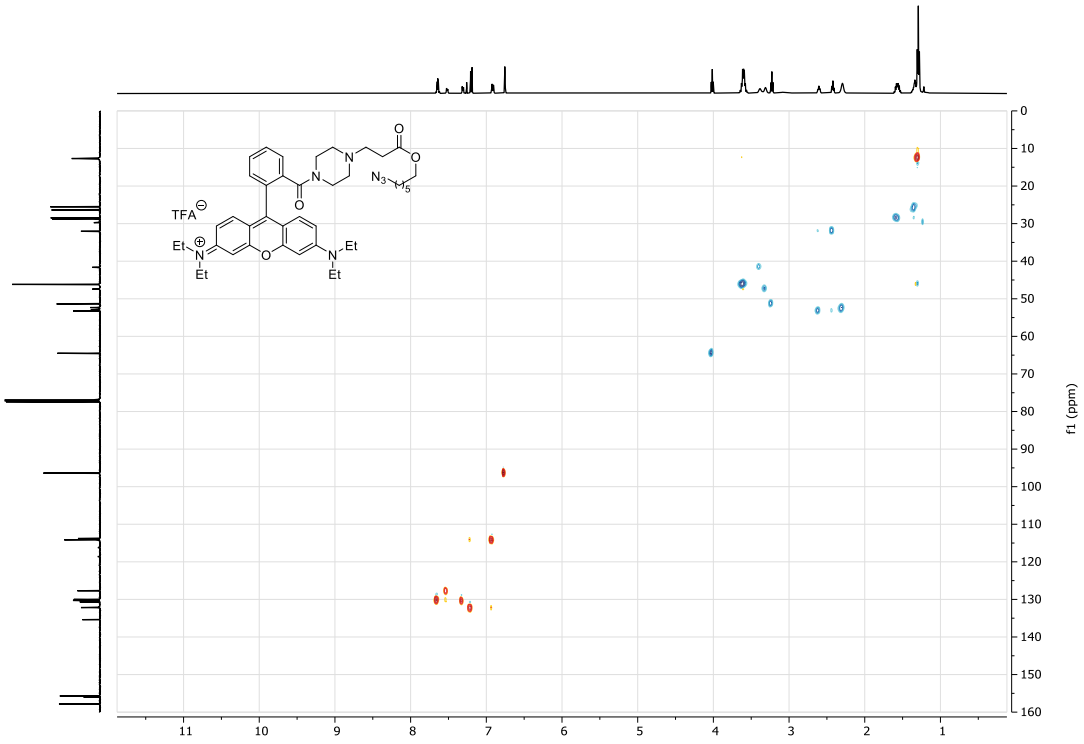
^{1}H NMR Spectrum (500 MHz, $CDCl_3$) of TFA·rhodamine B 4-(3-((6-Azidohexyl)oxy)-3-oxopropyl)piperazine Amide (4D)



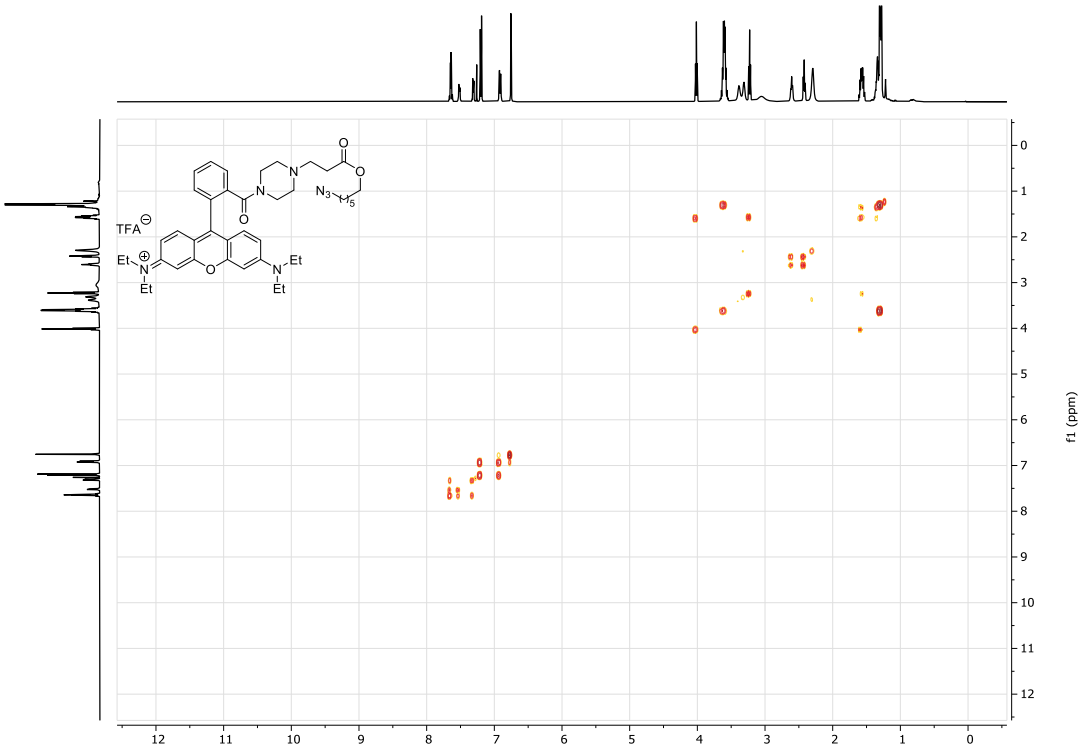
^{13}C{^{1}H} NMR Spectrum (126 MHz, $CDCl_3$) of TFA·rhodamine B 4-(3-((6-Azidohexyl)oxy)-3-oxopropyl)piperazine Amide (4D)



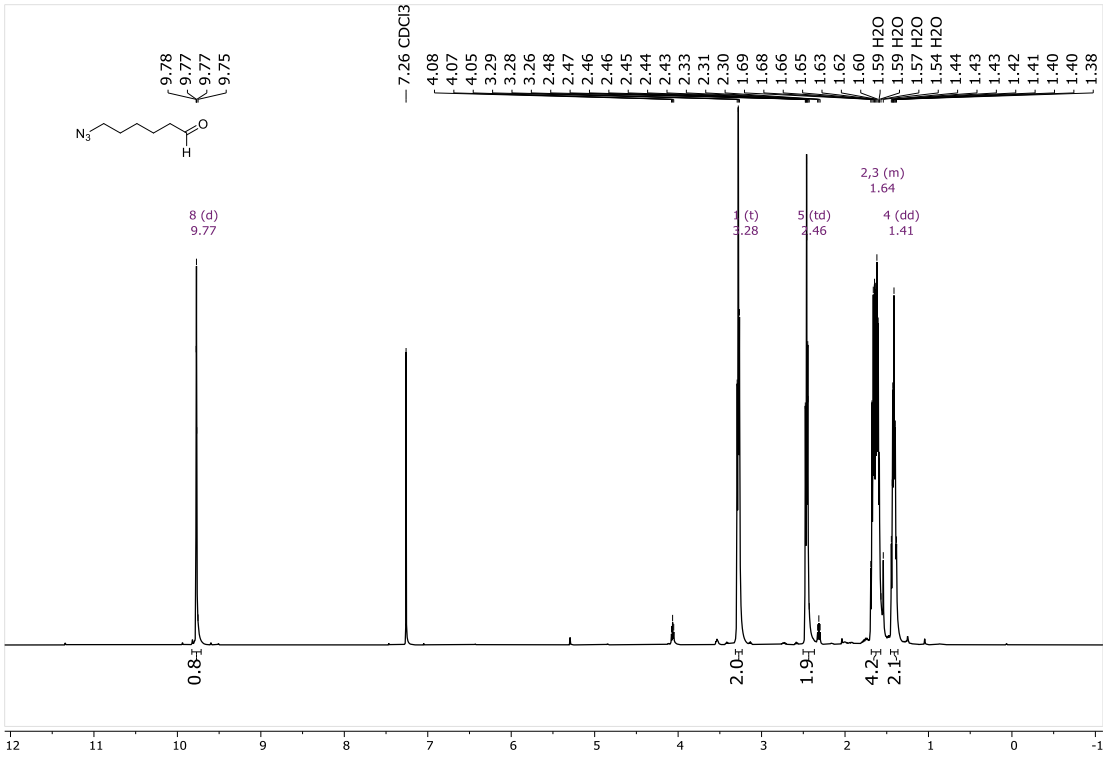
HSQC Spectrum (500 MHz, $CDCl_3$) of TFA·rhodamine B 4-(3-((6-Azidohexyl)oxy)-3-oxopropyl)piperazine Amide (4D)



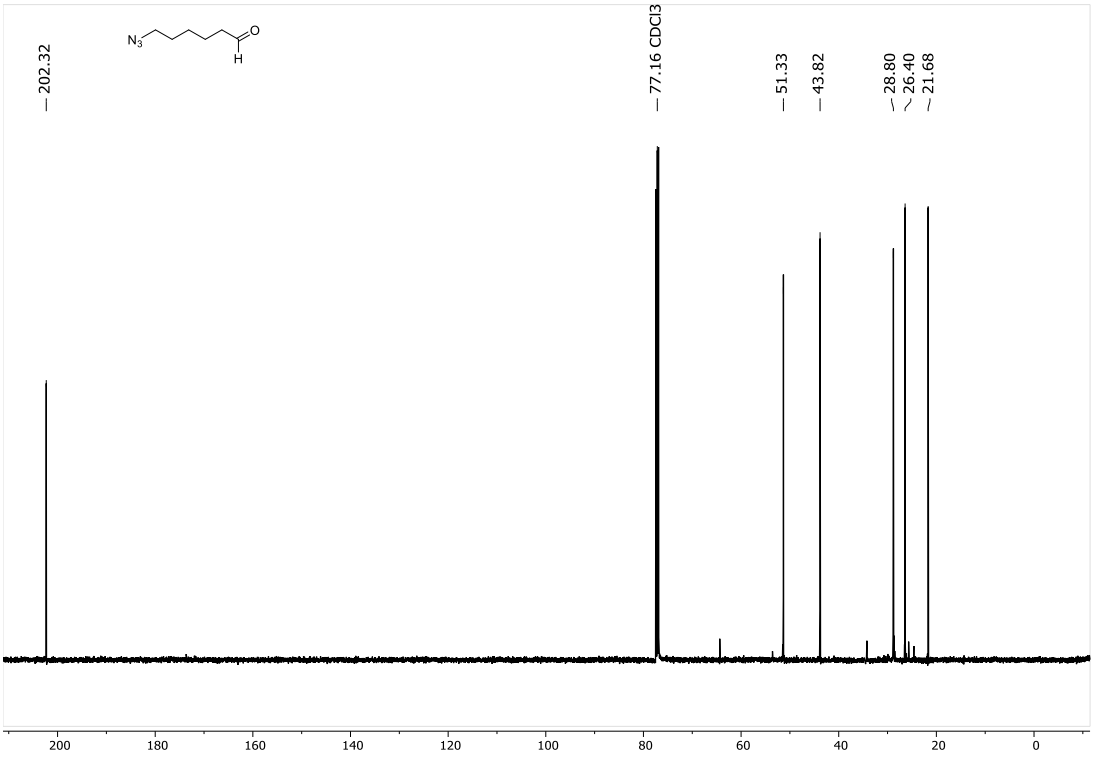
COSY NMR Spectrum (126 MHz, $CDCl_3$) of TFA·rhodamine B 4-(3-((6-Azidohexyl)oxy)-3-oxopropyl)piperazine Amide (4D)



^{1}H NMR Spectrum (500 MHz, $CDCl_3$) of 6-Azido Hexanal (5)



^{13}C{^{1}H} NMR Spectrum (126 MHz, $CDCl_3$) of 6-Azido Hexanal (5)



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