Supporting information

Beyond Traditional SPAAC: Achieving Orthogonality and Rapid Kinetics with Fluoroalkyl Azides

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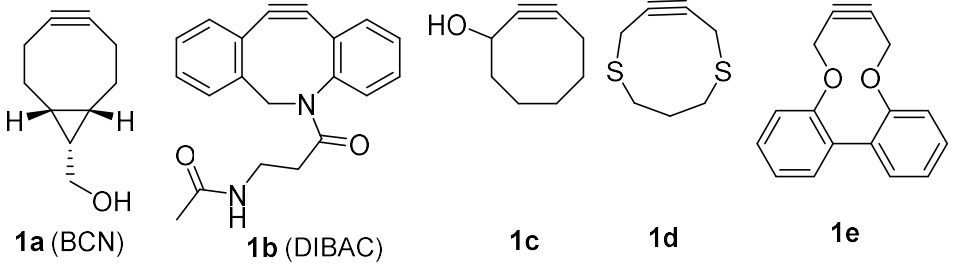
General

Flash column chromatography was performed using silica gel 60 (0.040–0.063 mm). Automated flash column chromatography was performed on Teledyne ISCO CombiFlash Rf+ Lumen Automated Flash Chromatography System with UV/Vis detection. ^{1}H, ^{13}C, and ^{19}F NMR spectra were measured at ambient temperature using 5 mm diameter NMR tubes. ^{13}C spectra were proton decoupled. The chemical shift values (δ) are reported in ppm relative to residual solvents (^{1}H and ^{13}C NMR) and internal standard $CFCl_3$ (0 ppm for ^{19}F NMR). Coupling constants (J) are reported in Hertz. Structural elucidation was aided by additional acquisition of ^{13}C APT and/or 2D spectra (^{1}H-^{1}H COSY, ^{1}H-^{13}C HSQC, ^{1}H-^{13}C HMBC). High resolution MS spectra (HRMS) were recorded on an LTQ Orbitrap XL using electrospray ionization (ESI) ionization. UPLC-MS analyses were performed on Acquity UPLC Instrument (Waters Corporation).

Reagents and chemicals

$PhOCF_2CF_2N_3$ (**2a**)
$CF_3CF_2N_3$ (**2b**)
$BrCF_2CF_2N_3$ (**2c**)
$CF_3(CF_2)_7N_3$ (**2d**)
$HCF_2CF_2N_3$ (**2e**)
$PhSO_2CF_2N_3$ (**2f**)
$PhOCH_2CH_2N_3$ (**4**)



1a (BCN) **1b** (DIBAC) **1c** **1d** **1e**

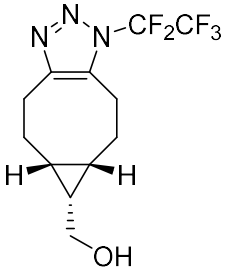
Figure SI1. Starting compounds.

Azides **2** were prepared following literature procedures.[1–5] Azide **4** was prepared according to literature.[6] Cyclooctynes **1a** and **1c** were obtained from commercial vendors, **1b**,[7] **1d**[8] and **1e**[9] were prepared according to literature.

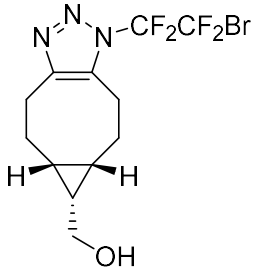
Synthesis of triazoles 3

In a dried screw cap glass vial (2 ml), cyclooctyne (0.03 mmol, 1 equiv.) was dissolved in THF (0.5 ml). Subsequently azide **2** (0.06 mmol, 2 equiv.) was added. The vial was sealed the reaction mixture was stirred at room temperature. The solvent was removed under reduced pressure and the product **3** purified by column chromatography (silica gel, cyclohexane/EtOAc).

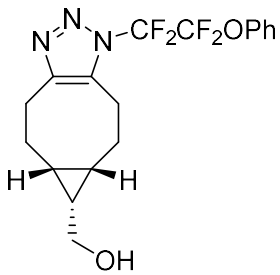
*(1-(Perfluoroethyl)-1,4,5,5a,6,6a,7,8-octahydrocyclopropa[5,6]cycloocta[1,2-d][1,2,3]triazol-6-yl)methanol (**3a**):* Prepared following the general procedure (reaction time 1 h). Yield 10 mg, 98%, yellow oil. ^{1}H NMR (401 MHz, $CDCl_3$) δ 3.79–3.67 (m, 2H), 3.20–2.86 (m, 4H), 2.36–2.22 (m, 2H), 1.75–1.60 (m, 2H), 1.28–1.17 (m, 1H), 1.07–0.90 (m, 2H); ^{13}C {^{1}H} NMR (101 MHz, $CDCl_3$) δ 146.7, 135.4 (t, $^{3}J_{C\text{-}F}$ = 2.4 Hz), 123.8–106.9 (m, 2C), 59.8, 25.3, 22.7 (t, $^{4}J_{C\text{-}F}$ = 3.9 Hz), 22.5, 22.1, 21.2, 19.3, 18.5; ^{19}F NMR (376 MHz, $CDCl_3$) δ –82.16 (s, 3F), –95.17 to –95.21 (m, 2F); HRMS (ESI^+) *m/z* calcd for $C_{12}H_{15}F_5N_3O$ $[M+H]^+$: 312.11298, found 312.11302.



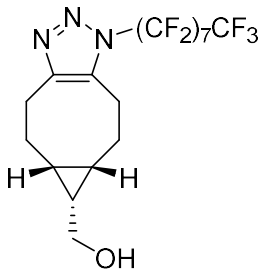
*(1-(2-Bromo-1,1,2,2-tetrafluoroethyl)-1,4,5,5a,6,6a,7,8-octahydrocyclopropa[5,6]cycloocta[1,2-d][1,2,3]triazol-6-yl)methanol (**3b**):* Prepared following the general procedure (reaction time 1 h). Yield 12 mg, 98%, yellow oil. ^{1}H NMR (401 MHz, $CDCl_3$) δ 3.78–3.66 (m, 2H), 3.17–2.82 (m, 4H), 2.35–2.16 (m, 2H), 1.73–1.55 (m, 2H), 1.30–1.13 (m, 1H), 1.05–0.90 (m, 2H); ^{13}C {^{1}H} NMR (101 MHz, $CDCl_3$) δ 146.6, 135.8 (t, $^{3}J_{C\text{-}F}$ = 2.1 Hz), 119.3–104.7 (m, 2C), 59.8, 25.2, 22.9–22.7 (m), 22.7, 22.3, 21.2, 19.3, 18.5; ^{19}F NMR (377 MHz, $CDCl_3$) δ –65.76 (t, $^{3}J_{F\text{-}F}$ = 5.0 Hz, 2F), –92.31 (t, $^{3}J_{F\text{-}F}$ = 5.0 Hz, 2F); HRMS (ESI$^+$) *m/z* calc. for $C_{12}H_{14}BrF_4N_3NaO$ $[M+Na]^+$: 394.01486, found 394.01498.



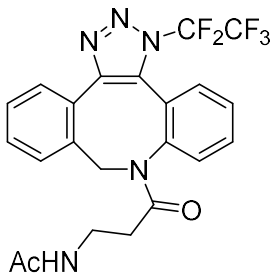
*(1-(1,1,2,2-Tetrafluoro-2-phenoxyethyl)-1,4,5,5a,6,6a,7,8-octahydrocyclopropa[5,6]cycloocta[1,2-d][1,2,3]triazol-6-yl)methanol (**3c**):* Prepared following the general procedure (reaction time 1 h). Yield 13 mg, 97%, white solid. ^{1}H NMR (401 MHz, $CDCl_3$) δ 7.42–7.33 (m, 2H), 7.31–7.25 (m, 1H), 7.21–7.14 (m, 2H), 3.79–3.65 (m, 2H), 3.24–3.14 (m, 1H), 3.13–3.05 (m, 1H), 3.04–2.93 (m, 2H), 2.39–2.22 (m, 2H), 1.74–1.59 (m, 3H), 1.24–1.14 (m, 1H), 1.05–0.90 (m, 2H); ^{13}C {^{1}H} NMR (101 MHz, $CDCl_3$) δ 148.7, 146.3, 135.7 (t, $^{3}J_{C\text{-}F}$ = 2.1 Hz), 129.9 (2C), 127.1, 121.9 (2C), 117.1–107.1 (m, 2C), 59.9, 25.3, 22.8–22.7 (m), 22.7, 22.3, 21.2, 19.4, 18.6; ^{19}F NMR (376 MHz, $CDCl_3$) δ –84.76 to –84.92 (m, 2F), –96.16 to –96.53 (m, 2F); HRMS (ESI$^+$) *m/z* calcd for $C_{18}H_{20}F_4N_3O_2$ $[M+H]^+$: 386.14862 found 386.14876.



*(1-Perfluorooctyl)-1,4,5,5a,6,6a,7,8-octahydrocyclopropa[5,6]cycloocta[1,2-d][1,2,3]triazol-6-yl)methanol (**3d**):* Prepared following the general procedure (reaction time 1 h). Yield 20 mg, 98%, white solid. ^{1}H NMR (401 MHz, $CDCl_3$) δ 3.79–3.66 (m, 2H), 3.16–2.95 (m, 3H), 2.94–2.83 (m, 1H), 2.35–2.22 (m, 2H), 1.75–1.59 (m, 3H), 1.27–1.15 (m, 1H), 1.06–0.88 (m, 2H); ^{13}C {^{1}H} NMR (101 MHz, $CDCl_3$) δ 146.8, 135.9 (t, $^{3}J_{C\text{-}F}$ = 2.2 Hz), 120.0–106.2 (m, 8C), 59.8, 25.2, 22.8–22.7 (m), 22.7, 22.3, 21.2, 19.3, 18.4; ^{19}F NMR (376 MHz, $CDCl_3$) δ –81.22 to –81.33 (m, 3F), –92.91 to –93.09 (m, 2F), –121.75 to –122.55 (m, 8F), –123.05 to –123.26 (m, 2F), –126.47 to –126.72 (m, 2F); HRMS (ESI$^+$) *m/z* calcd for $C_{18}H_{14}F_{17}N_3NaO$ $[M+Na]^+$: 634.07576, found 634.07600.

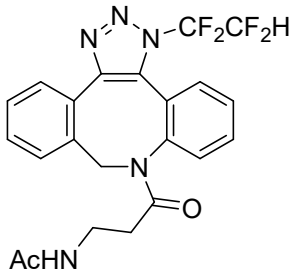


*N-(3-Oxo-3-(3-(perfluoroethyl)-3,9-dihydro-8H-dibenzo[b,f][1,2,3]triazolo[4,5-d]azocin-8-yl)propyl)acetamide (**3e**):* Prepared following the general procedure (reaction time 4 h). Yield 8 mg, 98%, colourless oil. Mixture of regioisomers (73:27) and rotamers. ^{13}C {^{1}H} NMR (101 MHz, $CDCl_3$) δ 172.0, 171.7, 170.0, 147.3, 145.1, 140.4, 139.9, 136.0, 135.0, 133.6, 132.6, 132.2, 132.0, 132.0, 132.0, 131.7, 130.7, 130.4, 130.3, 129.8, 129.8, 129.7, 128.3, 127.7, 127.5, 126.0, 124.0, 123.9, 118.9 – 107.9 (m), 52.9, 51.5, 34.8, 34.8, 34.7, 34.5, 23.4, 23.4; HRMS (ESI$^+$) *m/z* calcd for $C_{22}H_{19}F_5N_5O_2$ $[M+H]^+$: 480.14534, found 480.14547. Major isomer: ^{1}H NMR (401 MHz, $CDCl_3$) δ 7.68–7.10 (m, 8H), 6.05–5.99 (m, 1H), 5.93 (d, $^{2}J_{H\text{-}H}$ = 16.2 Hz, 1H), 4.38 (d, $^{2}J_{H\text{-}H}$ = 16.2 Hz, 1H), 3.34–3.15 (m, 2H), 2.04–1.97 (m, 1H), 1.90 (s, 3H), 1.74–1.66 (m, 1H); ^{19}F NMR (376 MHz, $CDCl_3$) δ –82.94 (s, 3F), –92.78 (dm, $^{2}J_{F\text{-}F}$ = 221.9 Hz, 1F), –96.24 (dm, $^{2}J_{F\text{-}F}$ = 221.9 Hz, 1F). Minor isomer: ^{1}H NMR (401 MHz, $CDCl_3$) δ 7.67–7.10 (m, 8H), 6.24–6.19 (m, 1H), 5.78 (d, $^{2}J_{H\text{-}H}$ = 16.0 Hz, 1H), 4.45 (d, $^{2}J_{H\text{-}H}$ = 16.0 Hz, 1H), 3.41–3.34 (m, 1H), 3.33–3.16 (m, 1H), 2.24–2.14 (m, 1H), 1.90 (s, 3H),

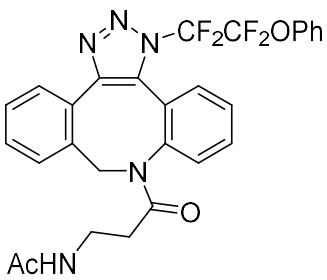


1.74–1.66 (m, 1H); ^{19}F NMR (376 MHz, $CDCl_3$) δ –81.37 (s, 3F), –89.54 (dm, $^2J_{F\text{-}F}$ = 221.2 Hz, 1F), –95.03 (d, $^2J_{F\text{-}F}$ = 221.2 Hz, 1F).

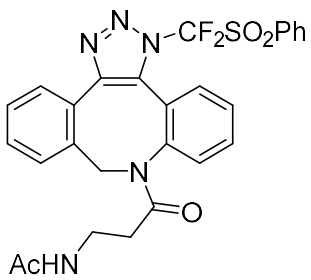
*N-(3-Oxo-3-(3-(1,1,2,2-tetrafluoroethyl)-3,9-dihydro-8H-dibenzo[b,f][1,2,3]triazolo[4,5-d]azocin-8-yl)propyl)acetamide (**3f**):* Prepared following the general procedure (reaction time 4 h). Yield 7.2 mg, 98%, pale-yellow oil. Mixture of regioisomers (64:36) and rotamers. ^{13}C {^{1}H} NMR (101 MHz, $CDCl_3$) *δ* 172.0, 171.5, 170.0, 169.9, 147.0, 144.6, 140.3, 139.8, 135.5, 134.7, 133.6, 132.6, 132.5, 131.9–131.8 (m), 131.5, 130.8–130.7 (m), 130.5, 130.2, 130.1, 129.7, 129.6, 129.5, 129.3, 129.2, 128.8, 128.1, 127.7, 127.4, 127.2, 125.9, 123.8, 123.8, 116.0–102.8 (m), 52.6, 51.5, 34.7, 34.7, 34.5, 23.3, 23.2; HRMS (ESI$^+$) *m/z* calcd for $C_{22}H_{20}F_4N_5O_2$ $[M+H]^+$: 462.15476, found 462.15493. Major isomer: ^{1}H NMR (401 MHz, $CDCl_3$) *δ* 7.71–7.19 (m, 8H), 6.96–6.63 (m, 1H), 6.10–6.03 (m, 1H), 5.96 (d, $^2J_{H\text{-}H}$ = 16.2 Hz, 1H), 4.40 (d, $^2J_{H\text{-}H}$ = 16.3 Hz, 1H), 3.40–3.14 (m, 2H), 2.04–1.96 (m, 1H), 1.89 (s, 3H), 1.72–1.63 (m, 1H); ^{19}F NMR (376 MHz, $CDCl_3$) *δ* –88.14 to –88.85 (m, 1F), –103.62 to –104.37 (m, 1F), –136.29 to –139.37 (m, 2F). Minor isomer: ^{1}H NMR (401 MHz, $CDCl_3$) *δ* 7.71–7.18 (m, 8H), 7.15–6.82 (m, 1H), 6.18–6.12 (m, 1H), 5.94 (d, $^2J_{H\text{-}H}$ = 16.4 Hz, 1H), 4.43 (d, $^2J_{H\text{-}H}$ = 16.4 Hz, 1H), 3.40–3.15 (m, 2H), 2.17–2.09 (m, 1H), 1.89 (s, 3H), 1.78–1.70 (m, 1H); ^{19}F NMR (376 MHz, $CDCl_3$) *δ* –86.72 to –87.47 (m, 1F), –103.26 to –103.94 (m, 1F), –136.29 to –139.37 (m, 2F).



*N-(3-Oxo-3-(3-(1,1,2,2-tetrafluoro-2-phenoxyethyl)-3,9-dihydro-8Hdibenzo[b,f][1,2,3]triazolo[4,5-d]azocin-8-yl)propyl)acetamide (**3g**):* Prepared following the general procedure (reaction time 4 h). Yield 8 mg, 92%, pale-yellow oil. Mixture of regioisomers (65:35) and rotamers. ^{13}C {^{1}H} NMR (101 MHz, $CDCl_3$) *δ* 171.8, 171.7, 170.0, 169.9, 148.5, 146.7, 145.1, 140.2, 139.9, 136.0, 134.8, 133.6, 132.2, 132.1, 132.0, 131.4, 130.3, 130.3, 130.0, 129.9, 129.9, 129.7, 129.5, 129.5, 129.1, 129.0, 128.4, 128.1, 127.9, 127.4, 127.3, 127.1, 124.9, 122.0, 121.9, 119.7–106.8 (m), 52.8, 51.6, 34.7, 34.6, 34.4, 23.2, 23.1; HRMS (ESI$^+$) *m/z* calcd for $C_{28}H_{24}F_4N_5O_3$ $[M+H]^+$: 554.18098, found 554.18084. Major isomer: ^{1}H NMR (401 MHz, CDCl3) *δ* 7.64–7.16 (m, 13H), 6.04–5.95 (m, 1H), 5.84 (d, $^2J_{H\text{-}H}$ = 15.8 Hz, 1H), 4.35 (d, $^2J_{H\text{-}H}$ = 15.8 Hz, 1H), 3.30–3.10 (m, 2H), 2.03–1.93 (m, 1H), 1.78 (s, 3H), 1.76–1.65 (m, 1H); ^{19}F NMR (376 MHz, $CDCl_3$) *δ* –84.25 to –84.91 (m, 1F), –84.93 to –85.66 (m, 1F), –94.16 to –94.84 (m, 1F), –94.87 to –95.56 (m, 1F). Minor isomer: ^{1}H NMR (401 MHz, $CDCl_3$) *δ* 7.67–7.12 (m, 13H), 6.37–6.19 (m, 1H), 5.77 (d, $^2J_{H\text{-}H}$ = 16.0 Hz, 1H), 4.45 (d, $^2J_{H\text{-}H}$ = 16.0 Hz, 1H), 3.41–3.30 (m, 1H), 3.30–3.09 (m, 1H), 2.25–2.16 (m, 1H), 1.82 (s, 3H), 1.76–1.68 (m, 1H); ^{19}F NMR (376 MHz, $CDCl_3$) *δ* –82.93 (dd, $^2J_{F\text{-}F}$ = 140.3 Hz, $^3J_{F\text{-}F}$ = 6.9 Hz, 1F), –83.43 (dd, $^2J_{F\text{-}F}$ = 140.5 Hz, $^3J_{F\text{-}F}$ = 7.0 Hz, 1F), –90.96 (dd, $^2J_{F\text{-}F}$ = 217.5 Hz, $^3J_{F\text{-}F}$ = 7.0 Hz, 1F), –94.31 (dd, $^2J_{F\text{-}F}$ = 217.5 Hz, $^2J_{F\text{-}F}$ = 6.9 Hz, 1F).



*N-(3-(3-(Difluoro(phenylsulfonyl)methyl)-3,9-dihydro-8H-dibenzo[b,f][1,2,3]triazolo[4,5-d]azocin-8-yl)-3-oxopropyl)acetamide (**3h**):* Prepared following the general procedure (reaction time 4 h). Yield 8 mg, 92%, white solid. Mixture of regioisomers (72:28) and rotamers. ^{13}C {^{1}H} NMR (101 MHz, $CDCl_3$) *δ* 171.9, 171.7, 170.4, 170.1, 147.3, 145.4, 142.2, 140.3, 140.1, 136.9, 136.9, 136.7, 135.6, 133.7, 132.9, 132.2, 132.2, 132.1, 131.9, 131.6, 131.3, 131.2, 131.1, 130.5, 130.4, 130.3, 130.0, 130.0, 129.9, 129.7, 129.7, 129.3, 129.1, 128.3, 128.1, 127.2, 126.5, 126.2, 124.6, 119.8–114.5 (m), 52.9, 51.8, 35.0, 34.8, 34.8, 34.7; HRMS (ESI$^+$) *m/z* calcd for $C_{27}H_{24}F_2N_5O_4S$ $[M+H]^+$: 552.15116, found 552.15125.



Major isomer: ^{1}H NMR (401 MHz, $CDCl_3$) δ 7.99–7.95 (m, 2H), 7.95–7.92 (m, 1H), 7.85–7.81 (m, 1H), 7.73–7.68 (m, 1H), 7.60–7.56 (m, 2H), 7.54–7.41 (m) and 7.37–7.19 (m) (6H), 6.27–6.22 (m, 1H), 6.17 (d, $^2J_{H\text{-}H}$ = 15.9 Hz, 1H), 4.39 (d, $^2J_{H\text{-}H}$ = 15.9 Hz, 1H), 3.41–3.26 (m, 2H), 2.03–1.96 (m, 1H), 1.92 (s, 3H), 1.77–1.70 (m, 1H); ^{19}F NMR (376 MHz, $CDCl_3$) δ –85.97 (dm, $^2J_{F\text{-}F}$ = 192.6, 1F), –87.74 (d, $^2J_{F\text{-}F}$ = 192.6 Hz, 1F). Minor isomer: ^{1}H NMR (401 MHz, $CDCl_3$) δ 8.08–8.03 (m, 2H), 7.95–7.92 (m, 1 H), 7.89–7.85 (m, 1H), 7.60–7.56 (m, 2H), 7.54–7.41 (m) and 7.37–7.19 (m) (7H), 6.17–6.13 (m, 1H), 5.63 (d, $^2J_{H\text{-}H}$ = 15.8 Hz, 1H), 4.52 (d, $^2J_{H\text{-}H}$ = 15.8 Hz, 1H), 3.41–3.26 (m, 2H), 2.26–2.18 (m, 1H), 1.86 (s, 3H), 1.85–1.79 (m, 1H); ^{19}F NMR (376 MHz, $CDCl_3$) δ –84.54 (dm, $^2J_{F\text{-}F}$ = 195.3 Hz, 1F), –87.50 (dm, $^2J_{F\text{-}F}$ = 195.3 Hz, 1F).

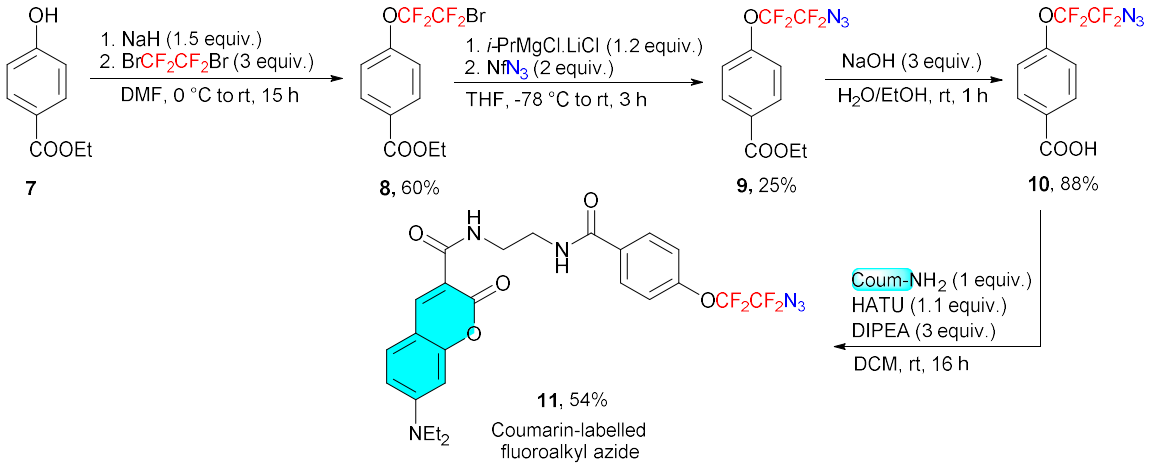
*1-(1,1,2,2-tetrafluoro-2-phenoxyethyl)-4,5,6,7,8,9-hexahydro-1H-cycloocta[d][1,2,3]triazol-4-ol (**3i**):* In a round bottom flask, alkyne **1c** (50 mg, 0.4 mmol, 1 equiv.) and azide **2a** (188 mg, 0.8 mmol, 2 equiv.) were dissolved in THF (1 mL). The reaction mixture was stirred at room temperature for 16 h and then quenched with water (5 mL). The water layer was extracted with EtOAc (2 × 10 ml), the combined organic layers were washed with brine and dried over $MgSO_4$. The solvent was evaporated under reduced pressure and the crude product was purified by flash chromatography (silica gel, cyclohexane/EtOAc) yielding triazole **3j** as a colorless oil. Yield 72 mg, 50%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39 (t, J = 7.7 Hz, 2H), 7.30 (t, J = 7.3 Hz, 1H), 7.17–7.21 (m, 2H), 5.15 (dd, J = 9.1, 4.1 Hz, 1H), 3.20–3.28 (m, 1H), 2.96–3.02 (m, 1H), 2.10–2.16 (m, 1H), 1.90–1.99 (m, 1H), 1.79–1.86 (m, 2H), 1.58–1.64 (m, 2H), 1.34–1.53 (m, 2H), 1.26 (s, 1H); ^{13}C {^{1}H} NMR (100 MHz, $CDCl_3$) δ 148.5, 148.3, 134.5, 129.8, 127.0, 121.7, 115.9 (tt, $J_{C\text{-}F}$= 271.6, 37.1 Hz), 112.3 (tt, $J_{C\text{-}F}$= 268.1, 41.5 Hz), 66.8, 37.4, 27.2, 25.0, 21.8, 21.0; ^{19}F NMR (376 MHz, $CDCl_3$) δ –84.72 (s, 2F), –96.81 (s, 2F); HRMS (ESI$^+$) *m/z* calcd for $C_{16}H_{18}O_2N_3F_4$ $[M+H]^+$: 360.13297, found 360.13272.

*1-(1,1,2,2-tetrafluoro-2-phenoxyethyl)-4,7,8,10-tetrahydro-1H,6H-[1,5]dithionino[7,8-d][1,2,3]triazole (**3j**):* In a round bottom flask, alkyne **1d** (47.5 mg, 0.3 mmol, 1 equiv.) and azide **2a** (141 mg, 0.6 mmol, 2 equiv.) were dissolved in DMF (2 ml). The reaction mixture was stirred at 100 °C for 16 h and then quenched with water (5 ml). The water layer was extracted with EtOAc (2 × 10 ml), the combined organic layers were washed with brine and dried over $MgSO_4$. The solvent was evaporated under reduced pressure and the crude product was purified by flash chromatography (silica gel, cyclohexane/EtOAc) yielding triazole **3k** as a yellow oil. Yield 102 mg, 86%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36–7.41 (m, 2H), 7.29–7.32 (m, 1H), 7.18 (d, J = 8.8 Hz, 2H), 4.02 (s, 2H), 3.97 (s, 2H), 2.76–2.80 (m, 2H), 2.59–2.63 (m, 2H), 1.89–1.96 (m, 2H); ^{13}C {^{1}H} NMR (100 MHz, $CDCl_3$) δ 148.4, 144.2, 135.0, 129.8, 127.1, 121.7, 115.9 (tt, $J_{C\text{-}F}$= 275.2, 37.3 Hz), 112.4 (tt, $J_{C\text{-}F}$ = 269.4, 41.5 Hz), 32.4, 31.2, 28.9, 26.9, 25.1; ^{19}F NMR (376 MHz, $CDCl_3$) δ –84.71 (s, 2F), –97.16 (s, 2F); HRMS (ESI$^+$) *m/z* calcd for $C_{15}H_{16}ON_3F_4S_2$ $[M+H]^+$: 394.06654, found 394.06608.

*1-(1,1,2,2-tetrafluoro-2-phenoxyethyl)-4,15-dihydro-1H-dibenzo[7,8:9,10][1,6]dioxecino[3,4-d][1,2,3]triazole (**3k**):* In a round bottom flask, alkyne **1e** (110 mg, 0.47 mmol, 1 equiv.) and azide **2a** (219 mg, 0.93 mmol, 2 equiv.) were dissolved in DMF (5 ml). The reaction mixture was stirred at 100 °C for 16 h and then quenched with water (10 ml). The water layer was extracted with EtOAc (2 × 20 ml), the combined organic layers were washed with brine and dried over $MgSO_4$. The solvent was evaporated under reduced pressure and the crude product was purified by flash chromatography (silica gel, cyclohexane/EtOAc) yielding

triazole **3l** as a colourless oil. Yield 211 mg, 96%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.01–7.34 (m, 13H), 5.56–5.49 (m, 4H); ^{13}C {^{1}H, ^{19}F} NMR (100 MHz, $CDCl_3$) δ 156.4, 155.7, 148.3, 145.7, 134.5, 127.1–130.9 (m, 4C), 123.5, 122.6, 121.6, 115.1, 114.5, 62.6, 60.2; ^{19}F NMR (376 MHz, $CDCl_3$) δ –84.91 to –85.98 (m, 2F), –96.15 to –98.11 (m, 2F); HRMS (ESI$^+$) *m/z* calcd for $C_{24}H_{18}O_3N_3F_4$ $[M+H]^+$: 472.12788, found 472.12766.

Synthesis of coumarin-labelled fluoroalkyl azide 11



Scheme SI1. Synthesis of coumarin-labelled fluoroalkyl azide **11**.

*Ethyl 4-(2-bromo-1,1,2,2-tetrafluoroethoxy)benzoate (**8**):* Prepared according to a modified reported procedure.[10] In a dried round-bottomed flask NaH (722 mg, 18 mmol, 1.5 equiv., 60% w/w in mineral oil) was dissolved in dry DMF (10 ml). The reaction mixture was cooled to 0 °C and then a solution of ethyl 4-hydroxybenzoate **7** (2 g, 12 mmol) in dry DMF (10 ml) was slowly added over 20 min. After one hour, 1,2-dibromo-1,1,2,2-tetrafluoroethane (9.35 g, 36 mmol, 3 equiv.) was slowly added. The reaction mixture was left to stir overnight and quenched with water (20 ml) and the aqueous phase was extracted with Et_2O (2 × 20 ml). The combined organic layers were washed with brine (3 × 15 ml) and dried over $MgSO_4$. The solvent was evaporated under vacuum and the crude product was purified by flash column chromatography (silica gel, cyclohexane/EtOAc), yielding **8** as pale-yellow liquid. Yield 2.50 g (60%); ^{1}H NMR (401 MHz, $CDCl_3$) δ 8.12–8.06 (m, 2H), 7.31–7.26 (m, 2H), 4.39 (q, $^{3}J_{H-H}$ = 7.1 Hz, 2H), 1.40 (t, $^{3}J_{H-H}$ = 7.1 Hz, 3H); ^{19}F NMR (376 MHz, $CDCl_3$) δ –68.69 (t, $^{3}J_{F-F}$ = 4.7 Hz, 1F), –86.49 (t, $^{3}J_{F-F}$ = 4.6 Hz, 1F).

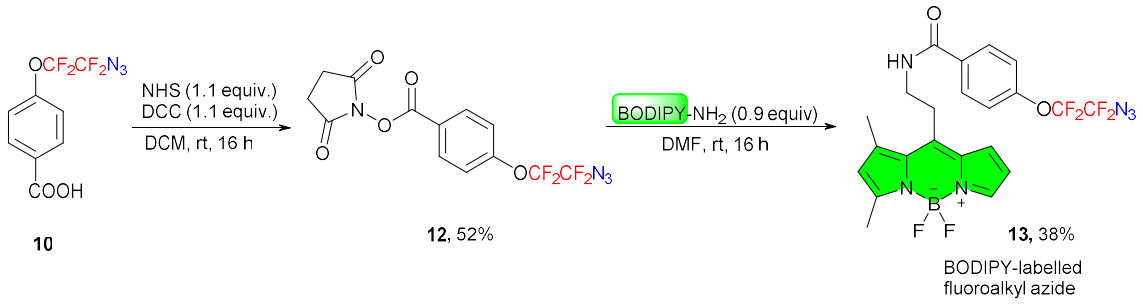
*Ethyl 4-(2-azido-1,1,2,2-tetrafluoroethoxy)benzoate (**9**):* In a dried round-bottomed flask, bromide **8** (2.5 g, 7.2 mmol, 1 equiv.) was dissolved in anhydrous THF (20 ml). The solution was cooled to –78 °C and *i*-PrMgCl.LiCl (1.3 M in THF, 6.7 ml, 8.7 mmol, 1.2 equiv.) was slowly added dropwise. After 1 hour, a solution of NfN_3 (4.7 g, 14.5 mmol, 2 equiv.) in anhydrous THF (5 ml) was slowly added and the reaction mixture was allowed to warm to room temperature and left to stir for 3 hours. The reaction mixture was quenched with sat. NH_4Cl solution (20 ml) and the aqueous phase was extracted with Et_2O (2 × 30 ml). The combined organic layers were washed with brine (2 × 15 ml) and dried over $MgSO_4$. The solvent was removed on vacuum and the crude product was purified by flash column chromatography (silica gel, cyclohexane/EtOAc), yielding **9** as pale-yellow liquid. Yield 560 mg, 25%; ^{1}H NMR (401 MHz, $CDCl_3$) δ 8.13–8.03 (m, 2H), 7.31–7.22 (m, 2H), 4.38 (q, $^{3}J_{H-H}$ = 7.1 Hz, 2H), 1.40 (t, $^{3}J_{H-H}$ = 7.1 Hz, 3H); ^{13}C {^{1}H} NMR (101 MHz, $CDCl_3$) δ 165.6, 152.3, 131.6 (2C), 129.1, 121.1 (2C), 114.3 (tt, $^{1}J_{C-F}$ = 272.0 Hz, $^{2}J_{C-F}$ = 39.9 Hz), 116.2

(tt, $^{1}J_{C\text{-}F}$ = 277.4 Hz, $^{2}J_{C\text{-}F}$ = 39.0 Hz), 61.4, 14.4; ^{19}F NMR (376 MHz, $CDCl_3$) δ –87.28 (t, $^{3}J_{F\text{-}F}$ = 3.0 Hz, 2F), –93.98 (t, $^{3}J_{F\text{-}F}$ = 3.0 Hz, 2F); HRMS (EI$^+$) *m/z* calcd for $C_{11}H_9F_4N_3O_3$ $[M]^+$: 307.0575, found 307.0569.

*4-(2-Azido-1,1,2,2-tetrafluoroethoxy)benzoic acid (**10**):* In a glass vial, NaOH (117 mg, 2.93 mmol, 3 equiv.) was dissolved in water (2 ml). Ester **9** (300 mg, 0.98 mmol, 1 equiv.) in EtOH (2 ml) was added and the reaction mixture was stirred for 1 h at room temperature. The mixture was diluted with water (2 ml) and was acidified with conc. HCl to pH ~ 2. The formed crystals were extracted with Et_2O (2 × 10 ml). The combined organic layers were dried over $MgSO_4$ and evaporated to yield **10** as of off-white crystals. Yield 240 mg, 88%, m. p. 119–123 °C; ^{1}H NMR (401 MHz, $CDCl_3$) δ 8.24–8.09 (m, 2H), 7.33 (m, 2H); ^{13}C NMR {^{1}H} (101 MHz, $CDCl_3$) δ 171.3, 153.2, 132.4 (2C), 127.7, 121.3 (2C), 119.1–111.6 (m, 2C); ^{19}F NMR (376 MHz, $CDCl_3$) δ –87.36 (t, $^{3}J_{F\text{-}F}$ = 2.9 Hz, 2F), –93.91 (t, $^{3}J_{F\text{-}F}$ = 2.9 Hz, 2F); HRMS (EI$^+$) *m/z* calcd for $C_9H_5F_4N_3O_3$ $[M]^+$: 279.0262, found 279.0259.

*N-(2-(4-(2-Azido-1,1,2,2-tetrafluoroethoxy)benzamido)ethyl)-7-(diethylamino)-2-oxo-2H-chromene-3-carboxamide (**11**):* In a round bottom flask **10** (100 mg, 0.37 mmol, 1 equiv.) and HATU (153 mg, 0.40 mmol, 1.1 equiv.) were dissolved in DCM (5 ml). DIPEA (191 µl, 1.10 mmol, 3.0 equiv.) was added. After 10 minutes, 2-(7-(diethylamino)-2-oxo-2*H*-chromene-3-carboxamido)ethan-1-aminium 2,2,2-trifluoroacetate[11] (152 mg, 0.37 mmol, 1.0 equiv.) was added. The reaction mixture was left to stir overnight and quenched with 5% aqueous HCl (10 ml) and extracted with DCM (2 × 10 ml). The combined organic layers were washed with sat. $NaHCO_3$ solution (10 ml), brine (10 ml), dried over $MgSO_4$ and the solvent was removed on vacuum. The crude product was purified by flash column chromatography (silica gel, DCM/MeOH, 95:5) to yield **11** as a yellow solid. Yield 135 mg, 54%; m. p. 219–224 °C; ^{1}H NMR (401 MHz, $CDCl_3$) δ 9.27 (t, $^{3}J_{H\text{-}H}$ = 5.9 Hz, 1H), 8.70 (s, 1H), 8.01 (t, $^{3}J_{H\text{-}H}$ = 4.2 Hz, 1H), 7.98–7.86 (m, 2H), 7.44 (d, $^{3}J_{H\text{-}H}$ = 9.0 Hz, 1H), 7.32–7.23 (m, 2H), 6.66 (dd, $^{3}J_{H\text{-}H}$ = 9.0 Hz, $^{4}J_{H\text{-}H}$ = 2.5 Hz, 1H), 6.49 (d, $^{4}J_{H\text{-}H}$ = 2.5 Hz, 1H), 3.75–3.69 (m, 2H), 3.68–3.62 (m, 2H), 3.46 (q, $^{3}J_{H\text{-}H}$ = 7.1 Hz, 4H), 1.24 (t, $^{3}J_{H\text{-}H}$ = 7.1 Hz, 6H); ^{13}C {^{1}H} NMR (101 MHz, $CDCl_3$) δ 166.2, 165.8, 162.8, 157.9, 153.0, 150.9, 148.4, 133.0, 131.4, 129.1 (2C), 121.3 (2C), 115.2 (m, 2C), 110.3, 109.4, 108.4, 96.7, 45.3 (2C), 43.1, 39.3, 12.5 (2C); ^{19}F NMR (376 MHz, $CDCl_3$) δ –87.18 (t, $^{3}J_{F\text{-}F}$ = 3.2 Hz, 2F), –94.07 (t, $^{3}J_{F\text{-}F}$ = 3.2 Hz, 2F); HRMS (ESI$^+$) *m/z* calcd for $C_{25}H_{25}F_4N_6O_5$ $[M+H]^+$: 565.18171, found 565.18149.

Synthesis of BODIPY-labelled fluoroalkyl azide 13



Scheme SI2. Synthesis of BODIPY-labelled fluoroalkyl azide **13**.

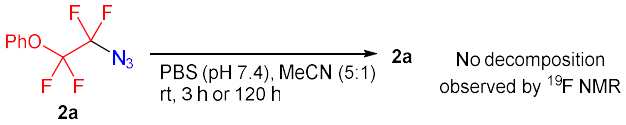
*2,5-dioxopyrrolidin-1-yl 4-(2-azido-1,1,2,2-tetrafluoroethoxy)benzoate (**12**):* In a round bottom flask **10** (50 mg, 0.18 mmol, 1 equiv.) and DCC (41 mg, 0.2 mmol, 1.1 equiv.) were dissolved in DCM (5 ml). *N-*

hydroxysuccinimide (23 mg, 0.2 mmol, 1.1 equiv.) was added. The reaction mixture was left to stir overnight and quenched with 5% aqueous HCl (10 ml) and extracted with DCM (2 × 10 ml). The combined organic layers were washed with sat. $NaHCO_3$ solution (10 ml), brine (10 ml), dried over $MgSO_4$ and the solvent was removed on vacuum. The crude product was purified by flash column chromatography (silica gel, cyclohexane/EtOAc) to yield **11** as an off-white amorphous solid. Yield 35 mg, 52%; ^{1}H NMR (401 MHz, $CDCl_3$) δ 8.23–8.17 (m, 2H), 7.40–7.32 (m, 2H), 2.92 (s, 4H); ^{13}C {^{1}H} NMR (101 MHz, $CDCl_3$) δ 169.2, 161.0, 153.9 (t, $^3J_{C\text{-}F}$ = 1.2 Hz), 132.8, 123.6, 121.6, 116.1 (tt, $^1J_{C\text{-}F}$ = 278.8, $^2J_{C\text{-}F}$ = 39.2 Hz), 112.9 (tt, $^1J_{C\text{-}F}$ = 272.2, $^2J_{C\text{-}F}$ = 39.4 Hz), 25.8.; ^{19}F NMR (376 MHz, $CDCl_3$) δ –87.44 (t, $^3J_{F\text{-}F}$ = 3.1 Hz, 2F), –93.77 (t, $^3J_{F\text{-}F}$ = 3.1 Hz, 2F); HRMS (ESI$^+$) *m/z* calcd for $C_{13}H_8F_4NaN_4O_5$ $[M+Na]^+$: 399.03230, found 399.03238.

*4-(2-azido-1,1,2,2-tetrafluoroethoxy)-N-(2-(5,5-difluoro-1,3-dimethyl-5H-5λ^4,6λ^4-dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-10-yl)ethyl)benzamide (**13**):* In a round bottom flask (9*H*-fluoren-9-yl)methyl (2-(5,5-difluoro-1,3-dimethyl-5*H*-5λ^4,6λ^4-dipyrrolo[1,2-c:2',1'-*f*][1,3,2]diazaborinin-10-yl)ethyl)carbamate (24 mg, 0.05 mmol, 0.9 equiv.) was dissolved in DMF (2 ml). Piperidine (4.2 mg, 0.05 mmol, 1 equiv.) was added and the reaction mixture was stirred at ambient temperature in the dark. After 2 hours **12** (25 mg, 0.06 mmol, 1 equiv.) was added and the reaction mixture was stirred overnight in the dark. The reaction mixture was diluted with EtOAc (20 ml), washed with brine (2 × 20 ml). The organic layer was dried over $MgSO_4$ and evaporated. The crude product was purified by flash column chromatography (silica gel, cyclohexane/EtOAc) to yield **13** as a red-orange amorphous solid. Yield 10 mg (38%); ^{1}H NMR (401 MHz, $CDCl_3$) δ 7.74–7.68 (m, 2H), 7.63 (s, 1H), 7.29–7.24 (m, 2H), 7.14 (d, $^3J_{H\text{-}H}$ = 4.0 Hz, 1H), 6.42 (dd, $^3J_{H\text{-}H}$ = 4.1, 2.1 Hz, 1H), 6.32 (t, $^3J_{H\text{-}H}$ = 6.2 Hz, 1H), 6.20 (s, 1H), 3.78 (q, $^3J_{H\text{-}H}$ = 6.8 Hz, 2H), 3.32 (t, $^3J_{H\text{-}H}$ = 6.8 Hz, 2H), 2.60 (s, 3H), 2.51 (s, 3H); ^{13}C {^{1}H} NMR (101 MHz, $CDCl_3$) δ 166.7, 161.6, 151.4, 145.4, 142.6, 138.6, 134.2, 134.2, 132.6, 128.9, 124.3, 124.1, 121.6, 116.3, 118.9–111.8 (m), 41.9, 29.6, 16.5, 15.2; ^{19}F NMR (376 MHz, $CDCl_3$) δ –87.30 (s, 2F), –93.93 (s, 2F), –146.82 (d, $^1J_{B\text{-}F}$ = 30.2 Hz, 1F), –146.95 (d, $^1J_{B\text{-}F}$ = 30.0 Hz, 1F); ^{11}B NMR (160 MHz, $CDCl_3$) δ 0.35 (t, $^1J_{B\text{-}F}$ = 30.8 Hz); HRMS (ESI$^-$) *m/z* calcd for $C_{22}H_{18}BF_6N_6O_2$ $[M-H]^-$: 523.14940, found 523.14928.

Control experiments

*Stability of **2a** in PBS (Scheme SI3):* Azide **2a** (75 mg) was dissolved in MeCN/PBS buffer pH 7.4 (1:2 vol/vol, 9 ml). The solution was stirred at ambient temperature, periodically monitored by ^{19}F NMR and no decomposition was observed. After 5 days, the solution was extracted with Et_2O (10 ml), the organic layer was washed with brine (10 ml), dried with $MgSO_4$ and evaporated. Pure azide **2a** (73 mg) was recovered. No other fluorine-containing compounds were observed in the aqueous layer after extraction (Figure SI2).



Scheme SI3. Monitoring stability of **2a** in phosphate buffer pH 7.4.

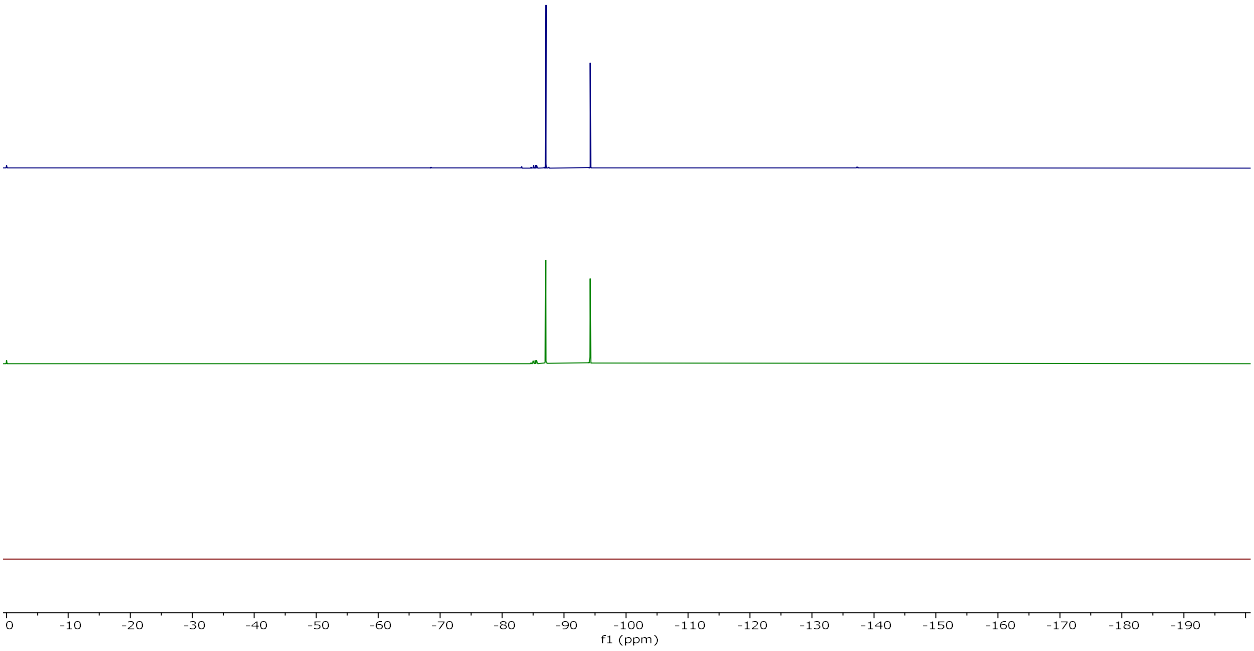
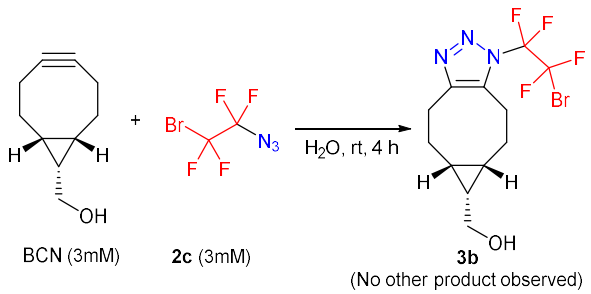


Figure SI2**.** ^{19}F NMR (376 MHz) spectra of **2a** (top), **2a** after 5 days incubation in PBS and extraction (middle) and aqueous layer after extraction (bottom).

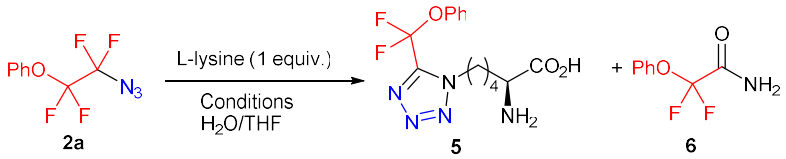
*SPAAC reaction of **2c** in water:* In a round bottom flask BCN (20 mg, 0.13 mmol) was dissolved in H_2O (30 ml) (concentration 3 mM). Azide **2c** (95 mg, solution in THF) was added and the reaction mixture was stirred at ambient temperature. After 4 hours, the reaction mixture was extracted with DCM (20 ml). The organic layer was dried with $MgSO_4$ and evaporated, yielding pure triazole **3b** as a white solid. Yield: 45 mg (91%) (Scheme SI4).



Scheme SI4. Testing reactivity of BCN with azide **2c**.

*Reactivity of **2a** with lysine (Scheme SI5):* In a round bottom flask *L*-lysine (28 mg, 0.19 mmol, 1 equiv.) and **2a** (45 mg, 0.19 mmol, 1 equiv.) were dissolved in water (2 ml) and THF (0.5 ml) (Scheme SI5). The reaction mixture was stirred at ambient temperature and analysed by NMR after 24 h. Products **5** and **6** were not observed. The same experiment repeated with NEt_3 (3 equiv.) as an additive. The reaction was stirred at 40 °C and analysed by NMR after 72 h. The formation of products **5** and **6** was observed (Figures SI3 and SI4). Product **5** was further confirmed by HRMS analysis of the reaction mixture. HRMS (ESI$^-$) *m/z* calcd for $C_{14}H_{16}F_2N_5O_3$ $[M–H]^-$: 340.12267, found 340.12292. Amides like **6** are known to be the byproduct of the reaction between fluorinated azides and primary amines,[5] moreover the chemical shift matches the

published spectra.[12] The ^{19}F NMR signal at –119 ppm was assigned to inorganic fluorides formed during the reaction.



Scheme SI5. Reactivity of azide **2a** with lysine.

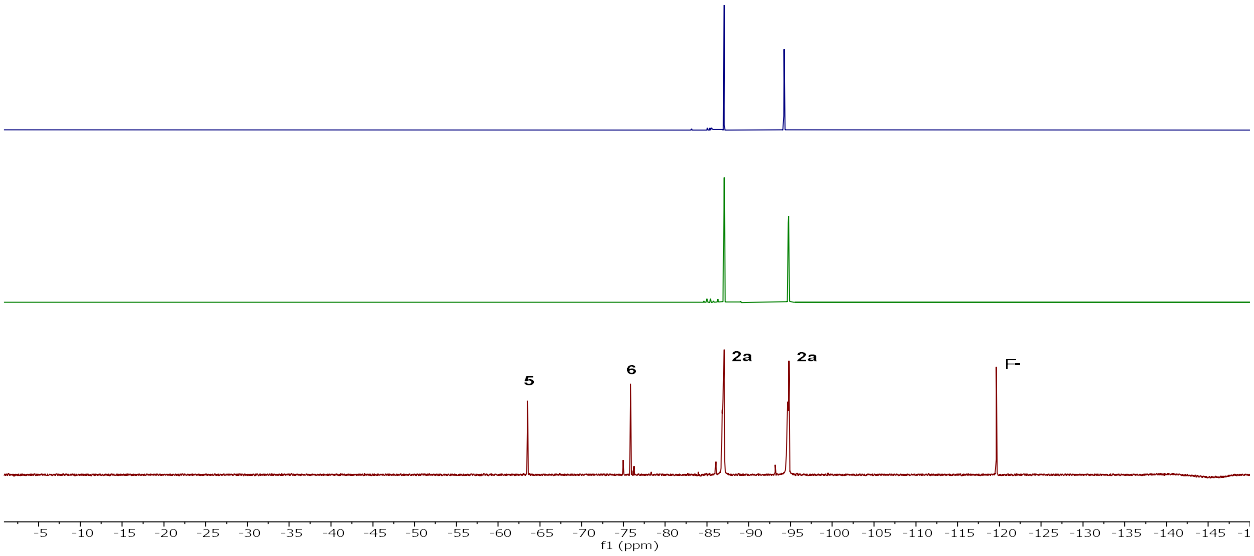


Figure SI3. ^{19}F NMR (376 MHz) spectra of **2a** (top), reaction mixture of **2a** and lysine after 24 h at ambient temperature (middle), reaction mixture of **2a** and lysine in the presence of NEt_3 after 3 days at 40 °C (bottom).

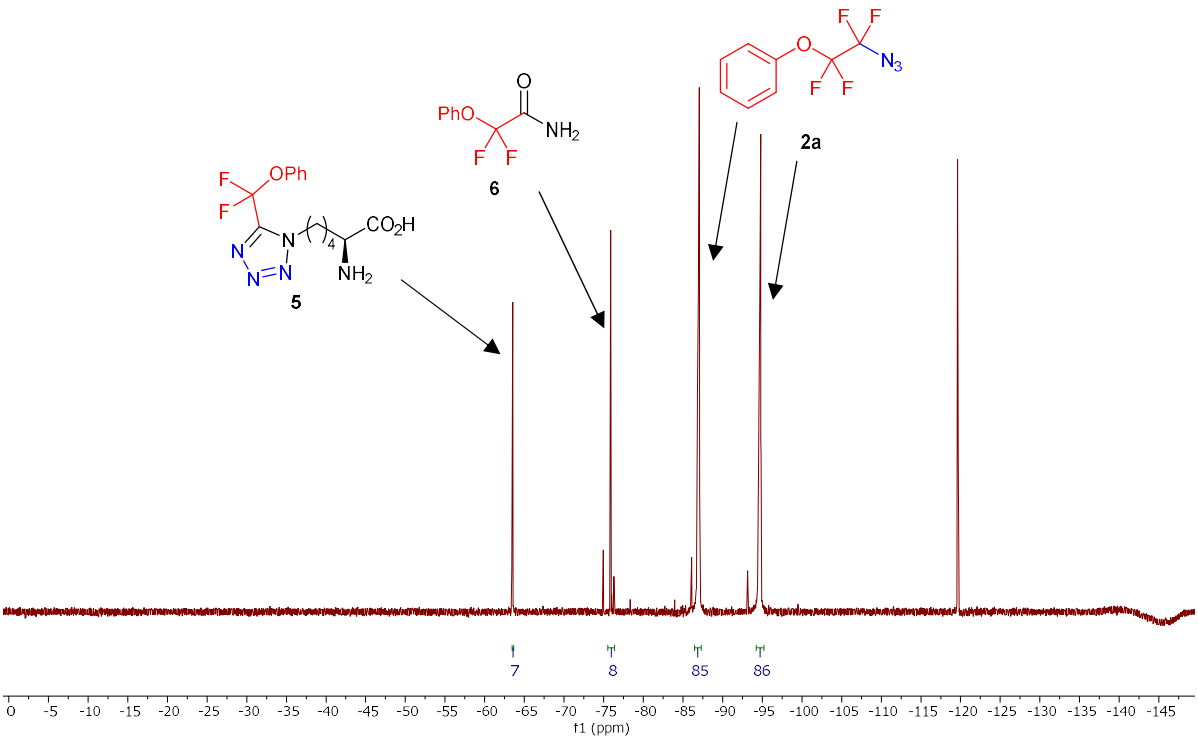
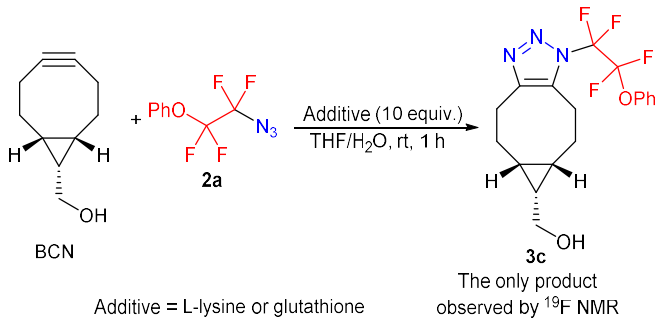


Figure SI4. Integrated ^{19}F NMR (376 MHz) spectrum of the reaction of azide **2a** with lysine and NEt_3 after 72 h at 40 °C.

SPAAC in the presence of lysine and glutathione (Scheme SI6): Azide **2a** (9 mg, 38 µmol) and BCN (5.8 mg, 38 µmol) were dissolved in THF/H_2O (5:1 vol/vol, 3 ml). Subsequently glutathione (117 mg, 383 µmol, 10 equiv.) or lysine (56 mg, 383 µmol, 10 equiv.) were added. The reaction mixture was stirred for 1 h and analyzed by ^{19}F NMR. Only the expected product of SPAAC **3c** was observed (Figure SI5).



Scheme SI6. Reactivity of BCN with **2a** in the presence of lysine or glutathione.

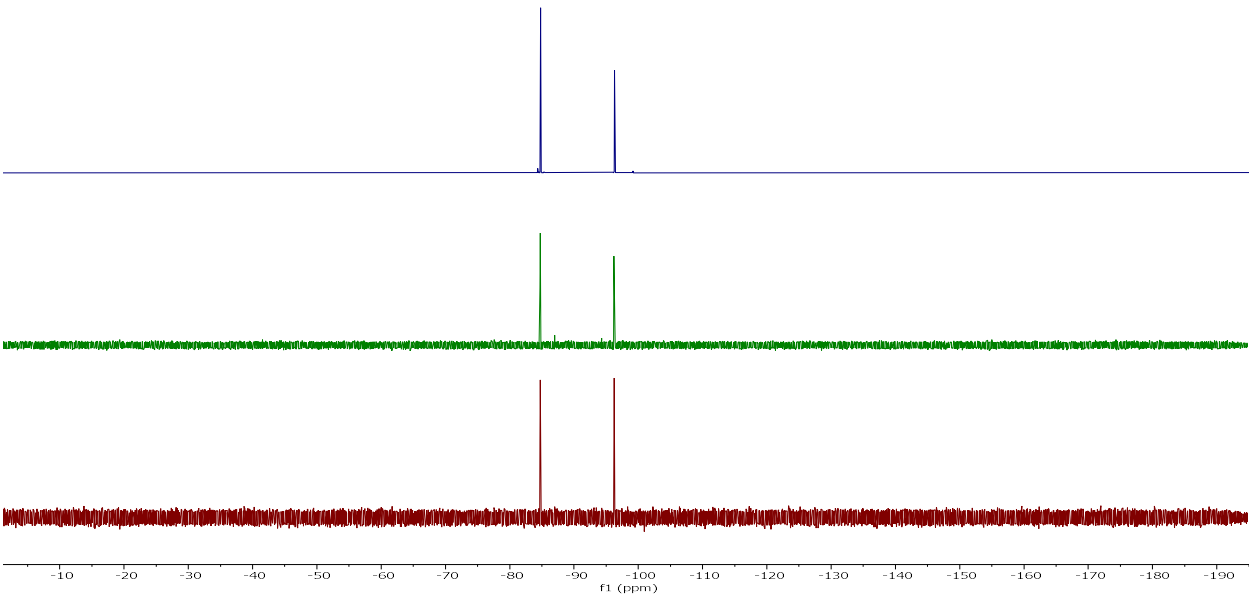


Figure SI5. ^{19}F NMR (376 MHz) spectra of **3c** (top), reaction mixture of **2a**, BCN and glutathione (middle) and reaction mixture of **2a**, BCN and lysine (bottom).

Determination of reaction kinetics

All starting materials and kinetic measurements were measured by infrared spectroscopy on IR spectrometer Nicolet 6800. For sample characterization the following experimental setup was used: DTGS detector, 2 cm^{-1} spectral resolution, Happ-Genzel apodization function, 64 scans, KBr cell (0.1 mm path length). $CHCl_3$ was used as solvent and subtracted as background.

For kinetic measurements the following experimental setup was used: MCT detector, 2 cm^{-1} resolution, Happ-Genzel apodization function, 32 scans within time interval of 60 seconds, BaF_2 cell (0.05 mm path length) (SpeCaC). THF/H_2O (9:1) was used as solvent and subtracted as background. The kinetics study was adapted from literature.[13] Compounds DIBAC, BCN, **2a** and **4** were dissolved in THF/H_2O (9:1) to obtain 15 mM stock solutions. Additionally, 75 mM stock solutions of **2a** and DIBAC were prepared. The 15 mM solution of **2a** (50 µl) was diluted with THF/H_2O (9:1) (100 µl) to obtain 5 mM solution. IR spectrum of this solution was measured to obtain the height of the azide vibration peak. The height of azide vibration of **4** was obtained the same way.

Table SI1. Azide stretching vibration peaks of azides **2a** and **4** and their heights in 5 mM solutions.

Compound	N_3 vibration peak (cm^{-1})	Peak height in 5 mM solution
2a	2161	0.0136
4	2111	0.0133

For the kinetic measurements, alkyne solution (100 µl) was mixed with the azide solution (50 µl) and quickly transferred to BaF_2 cell. IR spectra series acquisition was started immediately and collected until most of the azide reacted according to the observed peak height. All measurements were executed two

times. Typical IR kinetic spectra are shown in Figure SI6. The reaction between **2a** and DIBAC proved to be very slow when using the 15 mM stock solutions of reactants. Therefore, this reaction was monitored with 5-times higher concentration of reactants using the 75 mM stock solutions. From the obtained plots of peak height, azide concentration was calculated based on the known peak height at 5 mM concentration. Concentration of alkyne at the start of the reaction was assumed to be 10 mM (or 50 mM for the reaction of DIBAC and **2a**). Product concentration was assumed to be equal to the difference between starting and current concentration of the azide. The reaction was assumed to be of 2nd order and the obtained data were linearized according to the equation SI1 to obtain the values of reaction rate constants (Figures SI7 and SI8).

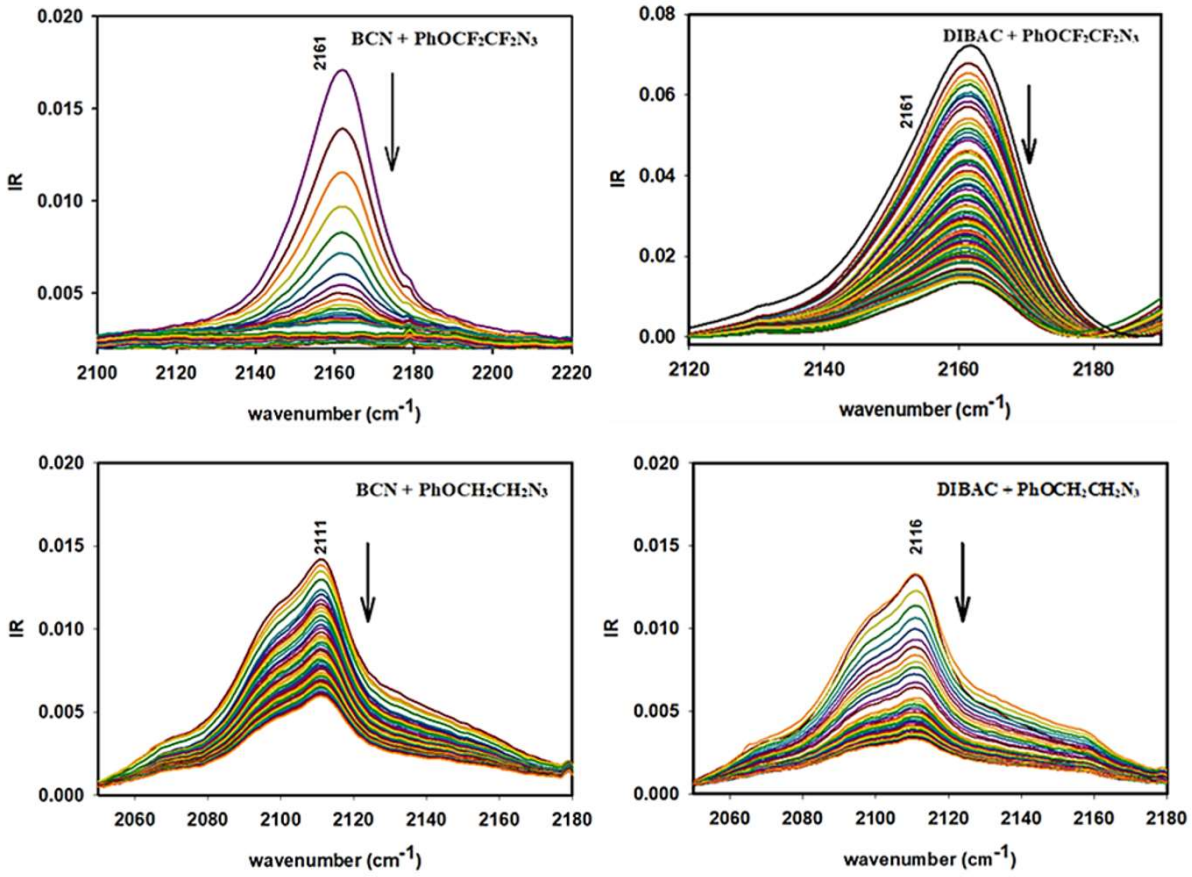


Figure SI6. Typical IR kinetics of reactions of BCN or DIBAC with azides **2a** or **4**.

Equation SI1. $$k \cdot t = \frac{1}{[alkyne]_0 - [azide]_0} \ln \frac{[azide]_0([alkyne]_0 - [product])}{[alkyne]_0([azide]_0 - [product])}$$

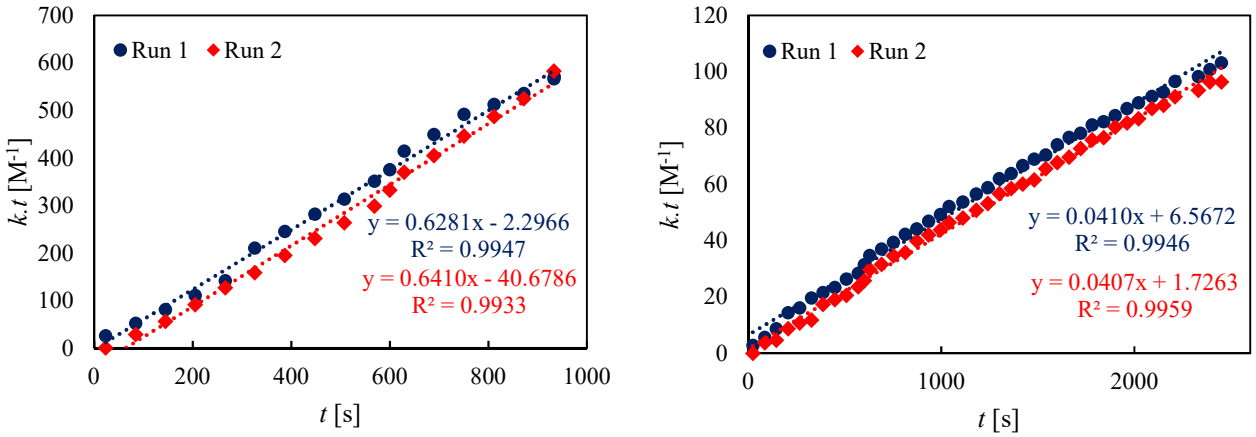


Figure SI7. Linearization of obtained kinetic data of the reaction of BCN with **2a** (left) and **4** (right)

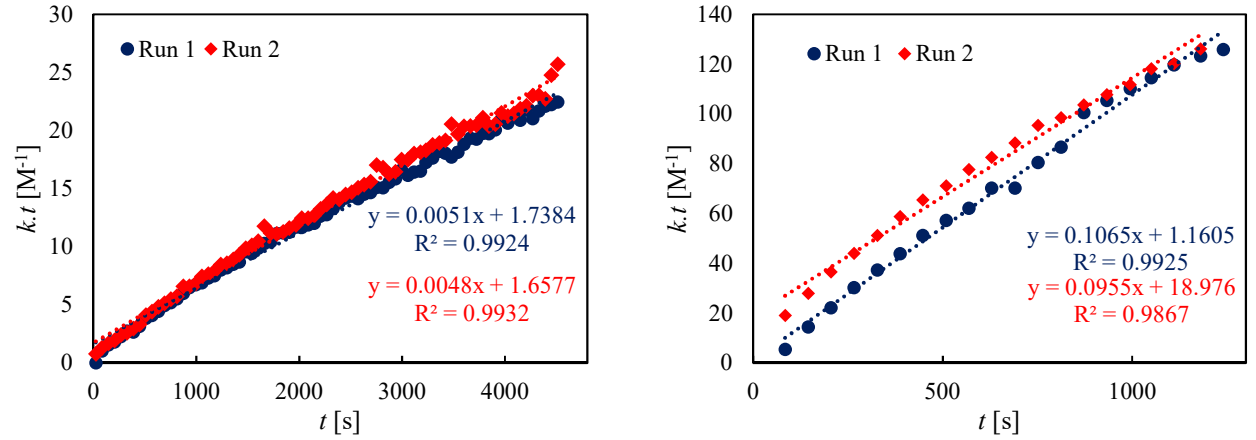


Figure SI8. Linearization of obtained kinetic data of the reaction of DIBAC with **2a** (left) and **4** (right)

Quantum chemical calculations

Pair natural orbital (PNO) extrapolation: We used the domain-based local pair natural orbital (DLPNO) coupled cluster theory with single, double and perturbative triple excitations (CCSD(T)), as it is implemented in the ORCA 6.0.1 software.[14] We extrapolated the DLPNO-CCSD(T)/cc-pVTZ energies to the complete PNO space within the TightPNO setting using two calculations with T_{cut}PNO being 1e−6 and 1e−7, utilizing a published extrapolation scheme.[15]

Entropic and solvation effects: We evaluated thermal corrections at the level of the geometry optimization, i.e., CAM-B3LYP/6-31g*, in order to calculate the activation Gibbs free energies for each azide–alkyne pair. The results are shown in Table SI2. These activation Gibbs free energies are qualitatively still in line with an observation that BCN reacts preferentially with **2a**, whereas DIBAC prefers **4**.

Table SI2. Activation Gibbs free energies ($\Delta G^{\#}$) with electronic energies calculated at the DLPNO-CCSD(T)/cc-pVTZ level with extrapolation to a complete PNO space and thermal corrections evaluated at the CAM-B3LYP/6-31g* level at 300 K.

Reactants	$\Delta G^{\#}$ (eV)
BCN + **4**	1.06
BCN + **2a**	1.03
DIBAC + **4**	0.93
DIBAC + **2a**	1.07

To involve solvent effects, we calculated solvation Gibbs free energies in THF for each species, utilizing the polarizable continuum model (PCM). Three approaches were chosen:

- conductor-like PCM (CPCM) with the DLPNO-CCSD(T)/cc-pVTZ energies (without PNO extrapolation),
- CPCM with the M05-2X/6-31g* energies,
- CPCM in combination with the solvation model based on solute electron density (SMD) with the M05-2X/6-31g* energies.

The resulting activation Gibbs free energies in a liquid phase utilizing all of the three approaches are in Table SI3. We observe that for DIBAC, the above-described trend in a preference in azides still holds, whereas for BCN, the activation Gibbs free energies are either the same for both azides (CPCM/DLPNO-CCSD(T)) or inverted relatively to the observed order (CPCM/M05-2X and SMD/M05-2X). The explanation of this discrepancy might be some of possible specific solute–solvent interactions which cannot be captured within simple PCM-based approaches. We also see that even in a gas phase (Table SI2), the activation Gibbs free energies differ by 0.03 eV for BCN and the two azides. Then even a small variation in solvation energy of the order of 10^{-2} eV would lead to a reversal of the trend. Therefore, the solvation energy needs to be described more accurately.

Table SI3. Activation Gibbs free energies ($\Delta G^{\#}$) in solution with gas-phase electronic energies calculated at the DLPNO-CCSD(T)/cc-pVTZ level with extrapolation to a complete PNO space, solvation energies evaluated at different levels and thermal corrections calculated at the CAM-B3LYP/6-31g* level at 300 K.

Reactants	$\Delta G^{\#}$ (eV)		
	CPCM DLPNO-CCSD(T)	CPCM M05-2X	SMD M05-2X
BCN + **4**	1.12	1.10	1.06
BCN + **2a**	1.12	1.11	1.08
DIBAC + **4**	1.01	1.01	0.98
DIBAC + **2a**	1.17	1.16	1.12

Deformation energies: For each isolated molecule of alkyne and azide we took a deformed structure corresponding to the respective transition state. We then calculated a deformation energy given as an energy difference between an energy in the transition-state geometry and the optimal geometry. We evaluated these deformation energies at the DLPNO-CCSD(T)/cc-pVTZ level with the PNO extrapolation

described above. The results are listed in Table SI4. We observe that the azide controls the deformation energy more than the alkyne,

Table SI4. Deformation energies calculated at the DLPNO-CCSD(T)/cc-pVTZ level with extrapolation to a complete PNO space.

Transition state	Deformation energy (in eV)	
	Azide	Alkyne
BCN + **4**	0.63	0.06
BCN + **2a**	0.70	0.04
DIBAC + **4**	0.61	0.07
DIBAC + **2a**	0.72	0.05

Analysis of the HOMO–LUMO gaps: A computational protocol allowing one to predict a reactivity of azide and alkyne in the strain-promoted cycloadditions is described in literature.[13,16] In Ref.[16] the energies of the Hartree–Fock (HF) lowest unoccupied molecular orbitals (LUMOs) of the considered alkynes (for geometries in the respective transition states) are compared and the one with a lower LUMO energy is predicted to be more reactive. This approach does not consider the reacting azide and therefore it cannot explain a different reactivity of an alkyne with different azides as in our case.

In Ref.[13] the procedure is adapted to account for the azide as well. Here, the HOMO(azide)–LUMO(alkyne) and HOMO(alkyne)–LUMO(azide) energy gaps are compared in the geometries of the transition states. We calculated the HF orbital energies (as Ref.[16] shows, the HF orbitals predict the reactivity more accurately than the DFT ones) for geometries in the transition states of our systems; the results are shown in Figure SI9.

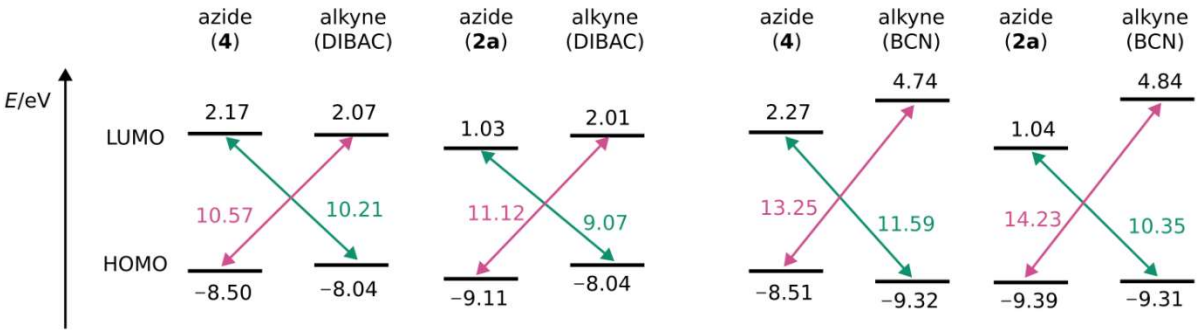


Figure SI9. HOMO and LUMO energies calculated at the HF/6-31g* level for geometries taken from the respective transition states optimized at the CAM-B3LYP/6-31g* level.

For BCN, the lowest HOMO–LUMO gap is found to be the HOMO(BCN)–LUMO(**2a**) combination, predicting BCN to react preferentially with **2a**, in line with the experimental findings. This situation is described as the inverse electron-demand cycloaddition, as it is azide's LUMO is accepting the electrons from alkyne.

However, if we focus on the systems with DIBAC, employing the same protocol, we also arrive at the conclusion that the preferred reaction would be an inverse electron-demand cycloaddition of **2a** with DIBAC. This conclusion does not correspond to the experiment where a reaction of DIBAC with **4** was

found to be 20 times faster than the one with **2a**. When we performed this analysis at the ZORA/OLYP/def2-TZVP (with the relativistically re-contracted basis set) level of theory, similarly to Ref.[13] the results were qualitatively the same.

Cartesian coordinates of the geometries: Each of the geometry was optimized at the CAM-B3LYP/6-31g* level and a frequency analysis confirmed the character of the respective stationary point.

DIBAC-**2a** TS

63

C	-6.010224	0.985080	-1.517731
C	-6.108060	0.452126	-0.239605
C	-7.275464	0.558494	0.502806
C	-8.370062	1.212987	-0.044445
C	-8.295207	1.755693	-1.323188
C	-7.117239	1.638383	-2.050181
O	-5.073579	-0.228122	0.402626
C	-3.861318	-0.349690	-0.178765
C	-2.915472	-1.111153	0.772063
N	-1.603772	-1.270564	0.239781
N	-1.421422	-2.268801	-0.506982
N	-0.545987	-2.865025	-0.970374
C	1.125735	-1.922903	-0.084654
C	2.283875	-2.707403	-0.439825
C	2.215410	-3.966658	-1.042296
C	3.369821	-4.683761	-1.312604
C	4.612057	-4.156265	-0.977465
C	4.695447	-2.911468	-0.370983
C	3.545574	-2.178363	-0.101878
N	3.653399	-0.890474	0.517827
C	4.403410	0.066469	-0.133454
O	4.880452	-0.139409	-1.239101
C	0.698837	-0.938585	0.527571

C	0.813127	0.206794	1.380953
C	-0.168917	1.185499	1.539418
C	0.037393	2.247213	2.408256
C	1.222327	2.340520	3.128275
C	2.214459	1.382574	2.950392
C	2.038829	0.320330	2.070641
C	3.130219	-0.716357	1.888931
C	4.634996	1.394051	0.570956
C	5.561093	2.293921	-0.250294
N	4.998988	2.690178	-1.521907
C	4.338322	3.845019	-1.827892
O	3.773591	3.986019	-2.899397
C	4.362369	4.955260	-0.792497
H	3.957239	-0.484208	2.568881
H	2.751489	-1.696763	2.185676
H	1.246387	-4.374645	-1.296543
H	3.298980	-5.657831	-1.785582
H	5.519034	-4.713576	-1.188243
H	5.659715	-2.485890	-0.119775
H	3.148505	1.464633	3.500024
H	1.379926	3.161856	3.819922
H	-0.734393	3.000805	2.525691
H	-1.090271	1.106890	0.977883
F	-2.793299	-0.388217	1.899245
F	-3.493452	-2.292219	1.077558
H	5.073517	1.216563	1.561033
H	3.678149	1.894987	0.740669
H	5.800343	3.187622	0.328905
H	6.501459	1.767509	-0.435877

H	4.890252	1.943903	-2.197727
H	3.807552	5.797866	-1.203068
H	3.896658	4.646041	0.148737
H	5.385759	5.274605	-0.570977
F	-3.302313	0.856539	-0.449433
F	-3.920002	-1.020591	-1.353729
H	-7.306996	0.125928	1.496470
H	-9.284920	1.297808	0.533211
H	-9.151227	2.267140	-1.750647
H	-7.049505	2.058425	-3.048597
H	-5.096228	0.898788	-2.089303

BCN-**4** TS

46

O	-1.210942	-2.092125	-0.993324
C	-2.242729	-2.006567	-1.951824
C	-2.575646	-3.421830	-2.389532
N	-3.225145	-4.206706	-1.351396
N	-2.531034	-4.687274	-0.424121
N	-2.570773	-5.519606	0.380893
C	-4.406452	-6.490184	-0.263338
C	-4.544076	-7.583826	0.722401
C	-5.543520	-8.665948	0.245530
C	-6.757983	-8.011781	-0.374639
C	-7.802794	-8.630819	-1.272953
C	-7.781358	-10.080749	-1.662192
O	-8.409909	-10.203155	-2.928928
C	-4.807393	-5.836465	-1.226190
C	-5.677108	-5.493213	-2.366259

C	-7.013455	-6.276914	-2.302061
C	-6.774692	-7.694945	-1.843564
H	-5.155122	-5.744442	-3.298572
H	-7.694221	-5.779212	-1.601876
H	-7.490801	-6.245286	-3.288388
H	-5.999690	-8.207488	-2.414197
H	-7.162259	-7.216769	0.251395
H	-8.810326	-8.238599	-1.148188
H	-5.810395	-9.297045	1.102257
H	-5.050558	-9.314695	-0.486471
H	-4.898813	-7.151900	1.666656
H	-3.571862	-8.033224	0.946286
H	-5.873131	-4.417018	-2.404011
H	-3.294718	-3.370236	-3.211618
H	-1.669366	-3.911526	-2.764898
H	-6.744119	-10.445207	-1.700645
H	-8.309549	-10.677819	-0.903128
H	-8.471854	-11.144240	-3.142667
H	-1.914559	-1.417816	-2.819967
H	-3.132395	-1.530838	-1.520679
C	-0.784177	-0.950577	-0.383935
C	0.187858	-1.120243	0.603993
C	0.689490	-0.018568	1.275498
C	0.231686	1.263542	0.974545
C	-0.733911	1.424206	-0.007501
C	-1.248542	0.325292	-0.693416
H	0.527431	-2.126130	0.825719
H	1.444141	-0.160786	2.043104
H	0.625987	2.125139	1.503093

H	-1.100352	2.416542	-0.252809
H	-2.000849	0.476226	-1.457931

BCN-**2a** TS

46

C	-4.804052	-1.005769	0.701285
C	-3.937786	-0.191736	-0.014755
C	-3.747261	1.140516	0.318137
C	-4.441516	1.672609	1.396920
C	-5.307119	0.873069	2.136015
C	-5.481591	-0.461090	1.786726
O	-3.250463	-0.635129	-1.146928
C	-2.629457	-1.837006	-1.134927
C	-1.654606	-1.897562	-2.328888
N	-0.620666	-0.924620	-2.288707
N	-0.933259	0.282511	-2.479240
N	-0.515681	1.338900	-2.275426
C	1.185053	0.823302	-1.011199
C	1.148289	-0.397287	-0.867444
C	1.547044	-1.750335	-0.445190
C	2.609811	-1.661394	0.681205
C	3.574616	-0.531036	0.411973
C	4.461053	0.142210	1.420519
C	5.862986	0.521491	1.038371
O	6.671636	-0.639405	1.142028
C	1.767777	2.145153	-0.690945
C	3.195396	2.004471	-0.107152
C	3.246413	0.860373	0.881503
H	1.963046	-2.279357	-1.310174

H	2.109768	-1.491730	1.641522
H	3.125998	-2.625054	0.755789
H	4.013364	-0.568104	-0.585504
H	2.430669	0.901358	1.603018
H	4.377823	-0.194390	2.452067
H	3.476229	2.958499	0.355515
H	3.901243	1.826948	-0.925275
H	1.117787	2.637163	0.043286
H	1.776675	2.796642	-1.569955
H	0.680898	-2.330049	-0.115917
F	-1.042626	-3.096270	-2.278943
F	-2.387331	-1.832320	-3.461981
H	5.879053	0.923000	0.014198
H	6.228479	1.316161	1.706636
H	7.588791	-0.382516	0.974740
F	-3.506111	-2.866691	-1.230777
F	-1.931229	-2.032799	0.006316
H	-3.058480	1.736537	-0.270341
H	-4.299899	2.715393	1.662284
H	-5.845919	1.289330	2.980884
H	-6.158412	-1.089211	2.357099
H	-4.947282	-2.039456	0.415079

DIBAC-**4** TS

63

C	-0.636150	-1.481322	-0.762348
C	0.254370	-0.418109	-0.925265
C	1.337045	-0.549200	-1.820412
C	1.445029	-1.711512	-2.576802

C	0.534015	-2.752688	-2.435099
C	-0.496802	-2.642328	-1.509942
C	0.160470	0.830316	-0.223086
C	0.642217	1.905345	0.152795
C	1.777405	2.789084	0.204915
C	1.715181	4.106522	0.669366
C	2.838215	4.917739	0.651141
C	4.042977	4.427462	0.158745
C	4.119728	3.125528	-0.313689
C	3.002055	2.298933	-0.290737
N	3.106261	0.953533	-0.774976
C	2.346881	0.570087	-1.983428
C	4.037511	0.132947	-0.176292
C	4.259086	-1.249872	-0.769966
C	5.403995	-1.972694	-0.057771
N	5.117586	-2.265509	1.328180
C	4.722288	-3.448858	1.879457
C	4.718415	-4.670385	0.977223
O	4.682591	0.497638	0.795499
O	4.400329	-3.524586	3.053529
N	-1.054679	2.952132	1.113327
N	-1.855718	2.172551	0.826491
N	-2.018459	1.025389	0.351751
C	-3.212502	0.802272	-0.463955
C	-4.395294	0.381178	0.395726
O	-5.450947	0.092793	-0.493799
H	3.046491	0.303386	-2.783409
H	1.833545	1.477202	-2.308664
H	0.772872	4.481625	1.046272

H	2.772926	5.935814	1.021359
H	4.925927	5.058283	0.142417
H	5.057967	2.730631	-0.685075
H	2.265489	-1.807617	-3.283334
H	0.638949	-3.650885	-3.035064
H	-1.196579	-3.460159	-1.370889
H	-1.428638	-1.389242	-0.029304
H	-2.963145	-0.006673	-1.152716
H	-3.471866	1.682739	-1.060174
H	4.483987	-1.163286	-1.840445
H	3.340839	-1.838064	-0.691049
H	5.620438	-2.904341	-0.583053
H	6.306179	-1.356219	-0.106949
H	5.045453	-1.459908	1.937269
H	4.382140	-5.516906	1.574518
H	4.043541	-4.543859	0.124444
H	5.717894	-4.884379	0.585479
H	-4.128453	-0.499638	0.993925
H	-4.672726	1.189864	1.085027
C	-6.646499	-0.316289	0.021410
C	-7.649523	-0.576914	-0.913662
C	-8.897381	-0.999132	-0.487529
C	-9.162132	-1.166682	0.870836
C	-8.160461	-0.905308	1.793458
C	-6.899027	-0.479623	1.380671
H	-7.423781	-0.440480	-1.965734
H	-9.671630	-1.199583	-1.221834
H	-10.140736	-1.497603	1.202276
H	-8.351937	-1.031189	2.854762

H -6.132556 -0.281940 2.120133

BCN

25

C 1.310060 0.738300 -1.175566

C 1.676618 2.118439 -0.839714

C 2.918369 2.073222 0.103166

C 2.794715 0.926974 1.089147

C 3.879566 0.277754 1.917968

C 5.311335 0.728783 1.880666

O 6.129770 -0.386871 2.196000

C 1.267050 -0.462203 -1.069570

C 1.610571 -1.801334 -0.580600

C 2.370653 -1.639588 0.771751

C 3.299681 -0.444898 0.733677

H 2.257980 -2.304806 -1.308775

H 1.641337 -1.505053 1.578704

H 2.917130 -2.565386 0.985270

H 3.970331 -0.454397 -0.126064

H 1.828106 0.920705 1.591890

H 3.566367 -0.058184 2.904731

H 3.009316 3.041374 0.610526

H 3.818174 1.945646 -0.507430

H 0.840818 2.606316 -0.323510

H 1.894982 2.728588 -1.722414

H 0.733810 -2.443992 -0.448397

H 5.555057 1.126847 0.884430

H 5.463918 1.544098 2.604450

H 7.045389 -0.080698 2.251098

4

21

H	6.043386	-0.699115	-1.259932
H	6.191870	-2.796587	-2.586764
H	4.146387	-4.148660	-3.002853
H	1.973876	-3.383745	-2.084438
H	1.827736	-1.312597	-0.773935
N	0.560973	4.118501	0.564148
N	1.259421	3.385214	1.074232
N	1.943933	2.610145	1.744302
C	3.120695	2.056065	1.060339
H	3.564688	2.775711	0.363911
C	2.764759	0.772940	0.323573
H	2.029146	0.975262	-0.467113
O	3.968629	0.284572	-0.225401
C	3.937943	-0.878206	-0.938557
C	2.787050	-1.627669	-1.166104
C	2.874590	-2.802933	-1.910359
C	4.088581	-3.232402	-2.424776
C	5.234113	-2.473230	-2.190561
C	5.163767	-1.303510	-1.453233
H	2.325413	0.050852	1.022856
H	3.857562	1.835595	1.833875

2a

21

H	5.136427	0.061093	-1.843921
H	5.394967	-1.748331	-3.543794

H	4.188044	-3.893317	-3.251831
H	2.727996	-4.234213	-1.278274
H	2.480804	-2.412348	0.410645
N	2.176519	4.633452	-0.210291
N	2.048626	3.741811	0.465606
N	1.771515	2.833899	1.266097
C	2.760253	1.802090	1.368101
F	4.008578	2.319504	1.449751
C	2.736848	0.824772	0.176932
F	2.915631	1.554387	-0.958126
O	3.701929	-0.103219	0.337295
C	3.794767	-1.097702	-0.647048
C	3.110207	-2.287982	-0.462881
C	3.256518	-3.295512	-1.409104
C	4.075807	-3.102945	-2.516761
C	4.753853	-1.899590	-2.681401
C	4.616204	-0.884164	-1.742127
F	1.509433	0.273363	0.086331
F	2.499594	1.121153	2.488011

DIBAC

42

C	-2.637321	0.643146	2.030399
C	-1.365414	1.258872	2.090464
C	-0.574929	1.142378	3.231988
C	-1.038112	0.424097	4.326518
C	-2.290148	-0.177322	4.283776
C	-3.079129	-0.064970	3.142012
C	-1.021874	1.960905	0.899551

C	-1.230904	2.341241	-0.229237
C	-1.936082	2.344228	-1.465752
C	-1.765930	3.260834	-2.503747
C	-2.557340	3.179839	-3.638984
C	-3.529475	2.189152	-3.738631
C	-3.713477	1.277390	-2.706916
C	-2.922053	1.338492	-1.566773
N	-3.095225	0.384134	-0.506339
C	-3.552396	0.841849	0.827210
C	-2.952788	-0.948647	-0.825970
C	-3.229588	-1.972326	0.264644
C	-3.090463	-3.396660	-0.275476
N	-1.743825	-3.720165	-0.688472
C	-0.798400	-4.431788	-0.009253
C	-1.231830	-5.114402	1.275984
O	-2.624624	-1.310508	-1.946042
O	0.343166	-4.528217	-0.427563
H	-4.516508	0.371761	1.049138
H	-3.748610	1.910090	0.715878
H	-1.009154	4.031635	-2.407520
H	-2.418322	3.890799	-4.446831
H	-4.149836	2.123579	-4.626584
H	-4.459588	0.496306	-2.791962
H	-4.058767	-0.535370	3.118472
H	-2.656499	-0.735132	5.139614
H	-0.420449	0.335262	5.214326
H	0.399205	1.618587	3.254052
H	-4.241552	-1.824598	0.662782
H	-2.540669	-1.819599	1.100289

H	-3.409196	-4.103395	0.492374
H	-3.759322	-3.524410	-1.131252
H	-1.406476	-3.225004	-1.504602
H	-0.362689	-5.630969	1.681167
H	-1.597135	-4.394742	2.015788
H	-2.028162	-5.843305	1.094956

4 in a geometry of TS BCN-**4**

21

H	-1.100352	2.416542	-0.252809
C	-0.733911	1.424206	-0.007501
H	0.625987	2.125139	1.503093
C	0.231686	1.263542	0.974545
H	1.444141	-0.160786	2.043104
C	0.689490	-0.018568	1.275498
C	0.187858	-1.120243	0.603993
H	0.527431	-2.126130	0.825719
C	-0.784177	-0.950577	-0.383935
C	-1.248542	0.325292	-0.693416
H	-2.000849	0.476226	-1.457931
O	-1.210942	-2.092125	-0.993324
C	-2.242729	-2.006567	-1.951824
H	-1.914559	-1.417816	-2.819967
H	-3.132395	-1.530838	-1.520679
C	-2.575646	-3.421830	-2.389532
H	-1.669366	-3.911526	-2.764898
H	-3.294718	-3.370235	-3.211618
N	-3.225145	-4.206706	-1.351396
N	-2.531034	-4.687274	-0.424121

N -2.570773 -5.519606 0.380893

BCN in a geometry of TS BNC-**4**

25

C -4.406452 -6.490184 -0.263338

H -4.898813 -7.151900 1.666656

C -4.544077 -7.583826 0.722401

H -3.571863 -8.033224 0.946286

C -4.807394 -5.836465 -1.226190

H -5.155123 -5.744441 -3.298572

C -5.677109 -5.493213 -2.366259

H -5.873132 -4.417018 -2.404011

C -7.013456 -6.276914 -2.302061

C -6.774693 -7.694944 -1.843564

C -5.543521 -8.665948 0.245530

H -5.999691 -8.207488 -2.414197

C -6.757984 -8.011780 -0.374639

C -7.802795 -8.630818 -1.272953

H -7.694221 -5.779211 -1.601876

H -7.490802 -6.245286 -3.288388

H -7.162260 -7.216768 0.251395

H -5.810396 -9.297045 1.102257

H -5.050559 -9.314695 -0.486471

H -8.810327 -8.238598 -1.148188

H -8.471856 -11.144239 -3.142667

O -8.409910 -10.203154 -2.928928

H -8.309550 -10.677818 -0.903128

C -7.781359 -10.080748 -1.662192

H -6.744120 -10.445206 -1.700645

2a in a geometry of TS BCN-**2a**

21

H	-4.947282	-2.039456	0.415079
C	-4.804052	-1.005769	0.701285
H	-6.158412	-1.089211	2.357099
C	-5.481591	-0.461090	1.786726
C	-5.307119	0.873069	2.136015
H	-5.845919	1.289330	2.980884
C	-4.441516	1.672609	1.396920
H	-4.299899	2.715393	1.662284
C	-3.747261	1.140516	0.318137
H	-3.058480	1.736537	-0.270341
C	-3.937786	-0.191736	-0.014755
O	-3.250463	-0.635129	-1.146928
F	-3.506111	-2.866691	-1.230777
C	-2.629457	-1.837006	-1.134927
F	-1.931229	-2.032800	0.006316
F	-2.387332	-1.832320	-3.461981
F	-1.042627	-3.096271	-2.278944
C	-1.654606	-1.897562	-2.328888
N	-0.620666	-0.924621	-2.288707
N	-0.933259	0.282510	-2.479240
N	-0.515681	1.338899	-2.275426

BCN in a geometry of TS BCN-**2a**

25

C	1.148289	-0.397288	-0.867444
C	1.185053	0.823301	-1.011199

H	0.680898	-2.330050	-0.115917
H	1.963046	-2.279358	-1.310174
H	1.117788	2.637162	0.043286
H	1.776676	2.796641	-1.569955
C	1.767778	2.145152	-0.690945
C	1.547044	-1.750336	-0.445190
C	2.609811	-1.661395	0.681204
C	3.574616	-0.531037	0.411972
C	3.246414	0.860372	0.881503
C	3.195397	2.004470	-0.107152
H	2.430670	0.901357	1.603018
H	3.901244	1.826946	-0.925276
H	4.013364	-0.568106	-0.585505
H	3.476230	2.958498	0.355515
H	2.109768	-1.491731	1.641522
H	3.125998	-2.625055	0.755788
H	4.377824	-0.194392	2.452066
C	4.461054	0.142208	1.420518
C	5.862987	0.521489	1.038370
H	5.879054	0.922998	0.014197
H	6.228480	1.316159	1.706635
O	6.671636	-0.639407	1.142027
H	7.588792	-0.382518	0.974739

DIBAC in a geometry of TS DIBAC-**4**

42

H	-1.428638	-1.389242	-0.029304
C	0.160470	0.830316	-0.223086
C	0.642217	1.905344	0.152796

C	0.254370	-0.418109	-0.925265
C	1.337045	-0.549200	-1.820412
C	2.346881	0.570087	-1.983428
N	3.106261	0.953533	-0.774976
C	3.002055	2.298932	-0.290736
C	1.777405	2.789083	0.204916
C	1.715181	4.106521	0.669367
C	2.838215	4.917738	0.651142
C	4.042977	4.427462	0.158746
C	4.119728	3.125528	-0.313688
H	5.057967	2.730631	-0.685074
H	4.925927	5.058283	0.142418
H	2.772926	5.935813	1.021361
H	0.772872	4.481624	1.046273
H	1.833545	1.477202	-2.308664
C	-0.636150	-1.481322	-0.762348
C	1.445029	-1.711512	-2.576802
C	0.534015	-2.752688	-2.435099
C	-0.496802	-2.642328	-1.509942
H	-1.196579	-3.460159	-1.370889
H	0.638948	-3.650885	-3.035065
H	2.265488	-1.807617	-3.283334
H	3.046491	0.303386	-2.783409
C	4.037511	0.132946	-0.176292
O	4.682591	0.497637	0.795499
C	4.259086	-1.249873	-0.769967
H	3.340839	-1.838065	-0.691050
H	4.483987	-1.163286	-1.840446
C	5.403995	-1.972695	-0.057772

H	5.620438	-2.904342	-0.583054
H	4.043540	-4.543860	0.124442
C	4.718415	-4.670386	0.977221
H	5.717894	-4.884380	0.585477
N	5.117586	-2.265511	1.328179
C	4.722288	-3.448860	1.879455
O	4.400329	-3.524588	3.053527
H	5.045453	-1.459910	1.937268
H	4.382140	-5.516907	1.574516
H	6.306179	-1.356220	-0.106950

4 in a geometry of TS DIBAC-**4**

21

C	-7.649523	-0.576914	-0.913662
H	-7.423781	-0.440480	-1.965734
C	-8.897381	-0.999132	-0.487529
H	-9.671630	-1.199583	-1.221834
C	-9.162132	-1.166682	0.870836
H	-10.140736	-1.497603	1.202276
C	-8.160461	-0.905308	1.793458
H	-8.351937	-1.031189	2.854762
C	-6.899027	-0.479623	1.380671
H	-6.132556	-0.281940	2.120133
C	-6.646499	-0.316289	0.021410
O	-5.450947	0.092793	-0.493799
C	-4.395294	0.381178	0.395726
H	-4.672726	1.189863	1.085027
H	-4.128453	-0.499639	0.993925
C	-3.212502	0.802272	-0.463955

H	-3.471866	1.682739	-1.060173
H	-2.963145	-0.006673	-1.152716
N	-2.018459	1.025388	0.351752
N	-1.855718	2.172550	0.826492
N	-1.054679	2.952131	1.113328

2a in a geometry of TS DIBAC-**2a**

21

C	-6.010224	0.985080	-1.517731
H	-5.096228	0.898788	-2.089303
H	-7.049505	2.058425	-3.048597
C	-7.117239	1.638383	-2.050181
C	-8.295207	1.755693	-1.323188
H	-9.151227	2.267140	-1.750647
C	-8.370062	1.212987	-0.044445
H	-9.284920	1.297808	0.533211
C	-7.275464	0.558494	0.502806
H	-7.306996	0.125928	1.496470
C	-6.108060	0.452126	-0.239605
O	-5.073579	-0.228122	0.402626
F	-3.920002	-1.020591	-1.353729
C	-3.861318	-0.349690	-0.178765
F	-3.302313	0.856539	-0.449433
F	-3.493452	-2.292219	1.077558
C	-2.915472	-1.111153	0.772063
F	-2.793299	-0.388217	1.899245
N	-1.603772	-1.270564	0.239781
N	-1.421422	-2.268801	-0.506982
N	-0.545987	-2.865024	-0.970374

DIBAC in a geometry of TS DIBAC-**2a**

42

C	0.698837	-0.938584	0.527571
C	1.125735	-1.922902	-0.084654
C	2.283875	-2.707402	-0.439825
C	3.545574	-2.178362	-0.101878
N	3.653399	-0.890473	0.517827
C	3.130219	-0.716356	1.888931
C	2.038829	0.320331	2.070641
C	0.813127	0.206795	1.380953
C	-0.168917	1.185500	1.539418
C	0.037393	2.247214	2.408256
C	1.222327	2.340521	3.128275
C	2.214459	1.382575	2.950391
H	3.148505	1.464634	3.500023
H	1.379926	3.161857	3.819922
H	-0.734393	3.000806	2.525691
H	-1.090271	1.106891	0.977883
H	1.246388	-4.374644	-1.296543
C	2.215411	-3.966657	-1.042296
H	3.298981	-5.657830	-1.785581
C	3.369822	-4.683760	-1.312604
C	4.612058	-4.156264	-0.977465
H	5.519035	-4.713575	-1.188243
C	4.695448	-2.911467	-0.370983
H	5.659716	-2.485889	-0.119775
H	2.751489	-1.696762	2.185676
H	3.678149	1.894988	0.740669

H	3.957239	-0.484207	2.568881
C	4.403410	0.066470	-0.133454
O	4.880452	-0.139408	-1.239101
C	4.634996	1.394052	0.570956
H	5.073517	1.216564	1.561033
N	4.998988	2.690179	-1.521908
H	4.890252	1.943904	-2.197728
C	5.561093	2.293922	-0.250294
H	6.501459	1.767510	-0.435877
H	5.800343	3.187623	0.328905
C	4.338322	3.845020	-1.827893
O	3.773591	3.986020	-2.899398
C	4.362369	4.955261	-0.792498
H	5.385759	5.274606	-0.570978
H	3.807552	5.797867	-1.203069
H	3.896658	4.646042	0.148736

SPAAC reaction with fluorescent probe 11

For UPLC-MS analysis of the selective SPAAC reaction: 10mM stock solutions of **11**, BCN and DIBAC in DMSO were prepared. Solution of **11** (10 µl) was transferred to a vial, diluted with MeCN (90 µl) and analysed to obtain UPLC chromatogram of the pure azide (Figure 4A).

For the reactions of **11** with BCN or DIBAC: A solution of **11** (10 µl) was mixed with the corresponding alkyne solution (20 µl) in a vial. After 30 minutes, the solutions were diluted with MeCN (70 µl) and analysed (Figures 4B and 4C).

The SPAAC reaction of **11** in the presence of both BCN and DIBAC was conducted by first mixing solution of BCN (20 µl) and solution of DIBAC (20 µl). To this solution was subsequently added solution of **11** (10 µl). The reaction mixture was incubated for 1 hour, diluted with MeCN (50 µl) and analysed by UPLC (Figure 4D).

Bioconjugation experiments

General information

Cell culture: U2OS (purchased from Sigma Aldrich; UNSPSC Code: 41106514) and SKBR3 (obtained from Dr. Karel Souček (Masaryk University, Brno) in 2024) cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Biowest L0103-500) supplemented with 10% FBS (Gibco A5256701). The cells were cultured at 37 °C in a 5% CO_2 atmosphere and passaged – using trypsin-EDTA (0.25%; Gibco 25200-072) – every 2–3 days. Both cell lines were authenticated using AmpFLSTR Identifiler PCR Amplification Kit.

Confocal microscopy: Pictures from the confocal microscope ZEISS LSM 980 (objective: 40× W) were processed in ZEN (3.3) blue edition software from ZEISS or in FIJI-win64.

Intact protein mass analysis: The (2.5 mg/ml) sample was diluted 10 times with pure LC-MS grade H_2O. The trastuzumab antibody was deglycosylated by addition of 100 Units of PNGaseF. Samples were incubated for 1 hour at 37 °C. Then, TCEP was added to the "reduced" sample to a final concentration of 10 mM, and samples were again incubated at 37 °C for 30 minutes. 4 μg of the protein was injected into the LC-MS for analysis (acquired on Synapt G2 mass spectrometer). Method details: MS Range: 400–4000 or 400–2500 *m/z*. Source parameters: ESI Voltage 3000 V, Source Temp: 120 °C, Desolvation Temp: 400 °C, Desolvation Gas: 800 l/h, Sampling Cone: 60 V or 40 V, Extraction Cone: 4.5 V, Trap Collision Energy: 15 V or 20 V. The sample was concentrated, desalted, and eluted into MS analysis using MassPREP Micro Desalting Column (Waters, SKU: 186004032) using the following LC gradients:

Gradient (Trastuzumab): Solvent A: 0.1% FA in H_2O. Solvent B: 0.1% FA in MeCN. 0–1.5 min – 200 μl/min 20% B (desalting step), 1.5–6 min – 200 μl/min 20–60% B gradient, 6–10 min – 200 μl/min 90% B for column washing.

Gradient (ConA): Solvent A: 0.1% FA in H_2O. Solvent B: 0.1% FA in MeCN. 0–0.5 min – 400 μl/min 10% B (desalting step), 0.5–3 min – 150 μl/min 10–70% B gradient, 3–7 min – 400 μl/min 90% B for column washing.

Degree of labeling analysis: DOL calculation was performed for each chain (Light Chain and Heavy Chain) of the reduced and deglycosylated antibodies individually using UniDec Processing Pipeline. Light chain and heavy chain masses were defined in the pipeline alongside mass increments expected from the drug molecules and software searched the spectra for masses belonging to modified antibody chains and calculated their ratios. Exact processing pipeline settings and the deconvolution configuration file used for the processing alongside raw data are provided in PRIDE submission. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE[17] partner repository with the dataset identifier PXD066546.

2025-05-02-DAR-Analysis-Pipeline.csv belongs to:

- 25-04-25-ConA-F0272-Reduced-0CE.raw
- 25-04-25-ConA-F0257-Reduced-0CE.raw
- 25-05-02-CONFIG.dat

2025-04-23-DAR-Analysis-Pipeline.csv belongs to:

- 25-04-15-F0272-Deglyko-Reduced-2.raw
- 25-04-15-F0257-Deglyko-Reduced-2.raw
- 25-04-15-DAR_conf.dat

SDS-PAGE protein analysis: Non-reducing glycine-SDS-PAGE at 4–15% precast acrylamide gel (BIO RAD: cat. #456-1085) was performed following standard lab procedures. A broad-range MW marker Precision Plus Protein™ Kaleidoscope™ Standards (10–250 kDa, BIO RAD: cat #1610375) was co-run to estimate protein weights. Loading was performed in 4 × Laemmli SDS sample loading buffer (BIO RAD: cat. #1610747). The resulting gel was imaged using Typhoon™ scanner using Cy3 (λ_{ex} = 532 nm) or Cy2 (λ_{ex} = 488 nm) channel with the software provided by the manufacturer. Images were saved under default brightness, contrast, and gamma settings. The samples were stained by the InstantBlue® Coomassie Protein Stain (ISB1L) (Abcam: cat. #ab119211) following a standard manufacturer's protocol. Densitometry was performed using ImageJ. Brightness and contrast settings were auto-adjusted within the software.

Source of material: Herceptin (Trastuzumab, 150 mg, Roche) monoclonal antibody was purchased from Všeobecná Fakultní Nemocnice v Praze. Concanavalin A protein (CAS 11028-71-0) was purchased from Merck. *endo*-BCN-PEG3-NHS (CAS 2101206-94-2) was obtained from SiChem and DIBAC-PEG5-NHS (CAS 2144395-59-3), and Sulfo-DIBAC-PEG3-NHS (catalog number BP-25142) from BroadPharm. Azide **14** (CAS# 1386385-76-7) was obtained from Merck, and azide **15** (CAS# 825651-66-9) from Lumiprobe.

Competition experiment on proteins

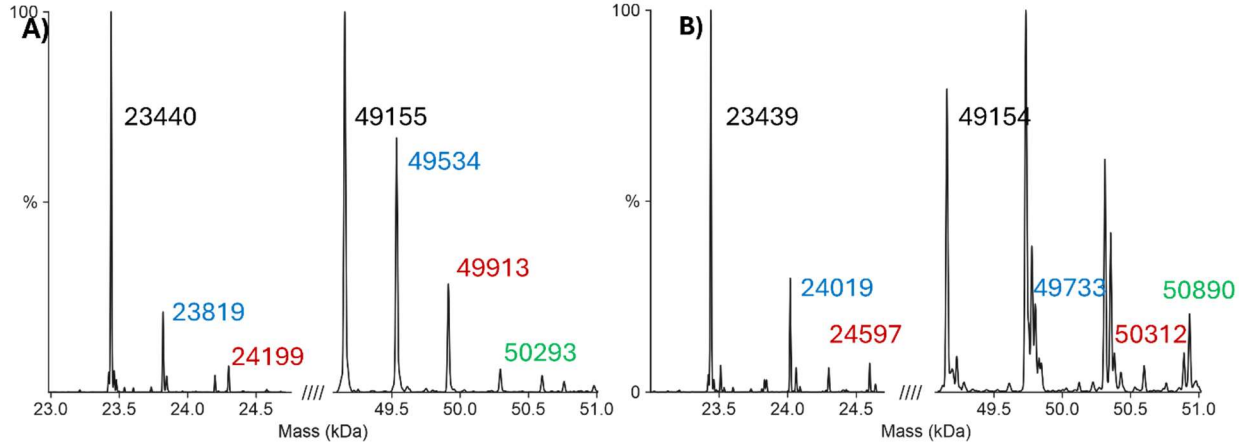
Modification of proteins

*Modification of **Trastuzumab** mAb with **endo-BCN-PEG3-NHS / DIBAC-PEG5-NHS:*** 35 µl of 15 mg/ml solution of Trastuzumab (Herceptin) was diluted to a concentration of 5 mg/ml with buffer containing: 25 mM NaCl, 50 mM HEPES-NaOH (pH 8.3), 0.5 mM EDTA, (2%) DMSO. It was buffer exchanged by (0.5 ml) Zeba (Thermo®) spin column into the indicated buffer containing: 25 mM NaCl, 50 mM HEPES-NaOH (pH 8.3), 0.5 mM EDTA, (2%) DMSO. The resulting 105 µl of 5.0 mg/ml solution of Trastuzumab was treated with freshly dissolved **endo-BCN-PEG3-NHS** (1.05 µl,10 mM in DMSO, 2.94 equiv.) or **DIBAC-PEG5-NHS** (2.1 µl,10 mM in DMSO, 5.88 equiv.). The reaction was incubated at rt for 1 h. After this time, a second portion of **endo-BCN-PEG3-NHS** (1.05 µl,10 mM in DMSO, 2.94 equiv.) or **DIBAC-PEG5-NHS** (2.1 µl,10 mM in DMSO, 5.88 equiv.) was added to the mixture, and the incubation continued for 1 additional hour. Excess of the labeling reagent was removed by desalting using (0.5 ml) Zeba (Thermo®) spin column into PBS (pH 7.4).

*Modification of **ConA** protein with **endo-BCN-PEG3-NHS / DIBAC-PEG5-NHS:*** 100 µl of 5.0 mg/ml solution of ConA protein was buffer exchanged by (0.5 ml) Zeba (Thermo®) spin column into buffer containing: 50 mM HEPES-NaOH (pH 8.3), 100 mM NaCl, with 3 mM $CaCl_2$, 3 mM $MgCl_2$. The resulting solution of ConA protein was treated with freshly dissolved **endo-BCN-PEG3-NHS** (2.35 µl,10 mM in DMSO, 5.0 equiv.) or **DIBAC-PEG5-NHS** (9.4 µl,10 mM in DMSO, 20.0 equiv.). The reaction was incubated at rt for 2 h. Excess reagent was removed by desalting using (0.5 ml) Zeba (Thermo®) spin column into buffer containing: 50 mM HEPES-NaOH (pH 8.3), 100 mM NaCl, with 3 mM $CaCl_2$, 3 mM $MgCl_2$.

*Intact mass analysis of modified **ConA** / **Trastuzumab** proteins:* Based on the indicated intact mass analyses, the dipolarophile-protein ratio (degree of labeling = DOL) was determined for each protein as follows:

A) Trastuzumab-PEG3-*endo*-BCN: 1.9
B) Trastuzumab-PEG5-DIBAC: 2.6
C) ConA-PEG3-*endo*-BCN: 0.9
D) ConA-PEG5-DIBAC: 3.9



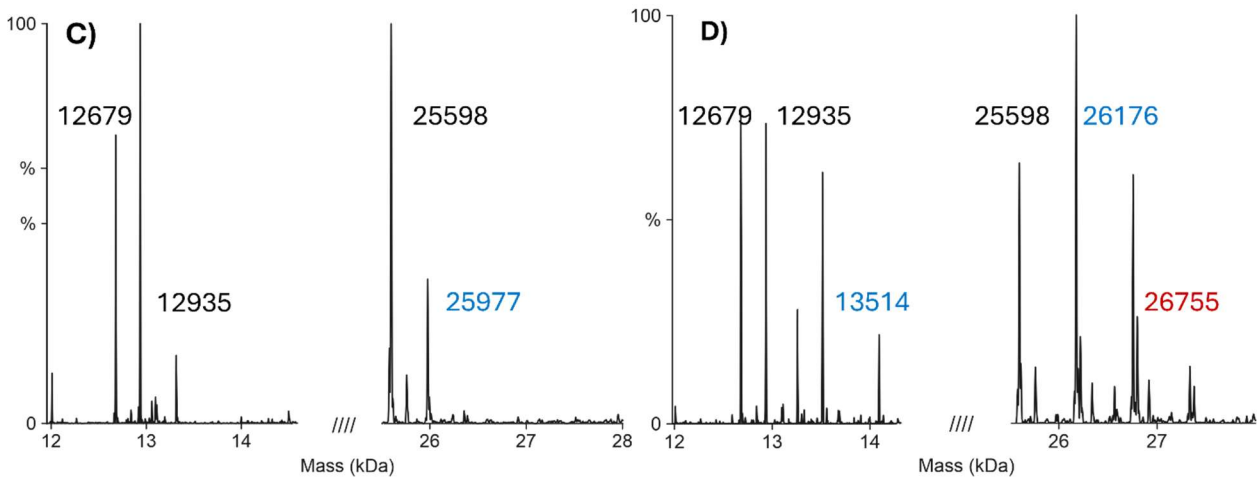


Figure SI10. Intact mass analysis of A) reduced Trastuzumab-PEG3-BCN B) reduced Trastuzumab-PEG5-DIBAC conjugate, C) reduced ConA-PEG3-BCN, D) reduced ConA-PEG5-DIBAC conjugate. Depicted is a deconvoluted zoomed spectral region of interest with main peaks. Parent Trastuzumab / ConA mass is assigned in black, single protein conjugate mass is in blue, double protein conjugate mass is in red, and triple protein conjugate mass is in green.

Click SPAAC labeling of proteins-dipolarophiles with azides: 2.5 mg/ml (17 µM) solution of Trastuzumab and 1.8 mg/ml (17 µM) solution of ConA protein were used. The amount of each protein was adjusted based on the determined DOL to ensure a comparable quantity of each dipolarophile modification in the mixture. The final volume of each protein mixture was always filled up to 10 µl.

Based on the indicated DOL, the amount of each protein used for a click labeling was determined as follows:

A) Trastuzumab-PEG3-*endo*-BCN: 2.6 µl
B) Trastuzumab-PEG5-DIBAC: 1.9 µl
C) ConA-PEG3-*endo*-BCN: 5.5 µl
D) ConA-PEG5-DIBAC: 1.3 µl

The resulting protein mixture was treated with an azido click reagent (0.34 µl of 250 µM in DMSO (100%); 1.0 equiv. per one modification of one protein) at rt for 24 h.

Calculation of azido click reagent for ConA / Trastuzumab protein competition:

Concentration of each protein in the mixture: 8.5 µM = 8.5 pmol/µl.

The total volume of our mixture is 10 µl.

=> The total protein amount in our mixture: 8.5*10 = 0.085 nmol.

1.0 equiv. of the azido click reagent: 1.0 x more is 0.085*1.0 = 0.085 nmol.

We label with 250 µM parent solution of azide = 250 pmol/1 µl = 0.25 nmol/µl.

Add 0.085/0.25 = 0.34 µl of 250 µM azide reagent per 0.085 nmol of one protein from the mixture.

SDS-PAGE analysis of the competition experiment: Non-reducing glycine-SDS-PAGE at 4–15% precast acrylamide gel was used following standard lab procedures. A broad-range MW marker was co-run to estimate protein weights. The resulting protein mixture was treated with an azido click reagent (0.34 µl of 250 µM in DMSO (100%); 1.0 equiv. per one modification of one protein) at rt for 24 h. The resulting samples (5.0 µl of ~9.4–4.3 µM of total mAb/ConA protein) were mixed with 2.5 µl of 4 × Laemmli SDS sample loading buffer and diluted with H_2O up to 10 µl. The samples were heated at 80 °C for 5 min at constant shaking (600 rpm). The gel was run at constant voltage (300 V) for 20 min in 1× Tris/Glycine/SDS running buffer. The resulting gel was imaged using Typhoon™ scanner using Cy3 / Cy2 channel (λ_{ex} = 532 / 488 nm) and then stained by the InstantBlue® Coomassie Protein Stain following a standard manufacturer's protocol (Figures SI11, SI12, and SI13).

Note: ConA protein is a tetramer. Denaturated ConA runs on the gel in different forms, mainly as a monomer (ca. 26 kDa) and dimer (ca. 52 kDa).

Densitometry was used to quantify all resulting protein bands. These values are summarized in Table SI5-SI7. Unfortunately, the different fluorescence properties and intensities of the two fluorophores do not allow direct comparison and determination of yields of the click products form SDS PAGE. However, the qualitative information if a mixture of products or single product is formed can be deduced from the analysis.

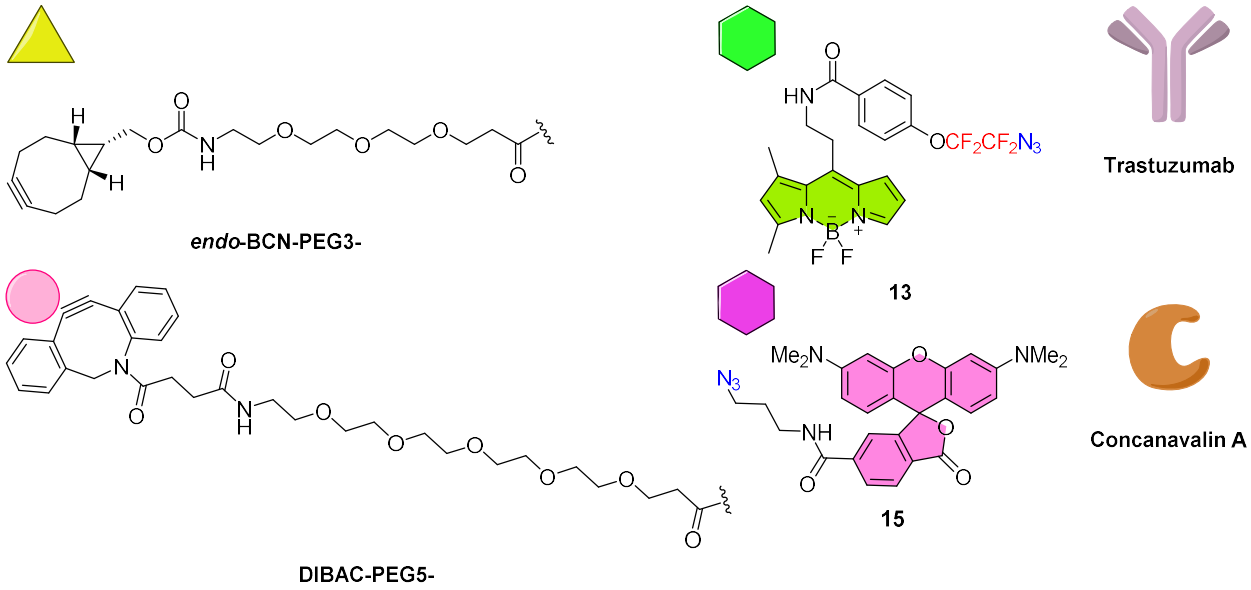


Figure SI11. Structures of dipolarophile tags exposed on Trastuzumab or ConA protein and their complementary fluorophore-azide click labels.

(*A) Control samples for SDS-PAGE:*

Well (02): Trastuzumab-PEG5-DIBAC. **15** label.
Well (03): Trastuzumab-PEG3-*endo*-BCN. **13** label.
Well (04): ConA-PEG5-DIBAC. **15** label.
Well (05): ConA-PEG3-*endo*-BCN. **13** label.

(*A) Competition samples for SDS-PAGE:*

Well (06): Trastuzumab-PEG5-DIBAC. F0266 + **13** label.

Well (07): Trastuzumab-PEG3-*endo*-BCN. **15** + **13** label.

Well (08): ConA-PEG3-*endo*-BCN. **15** + **13** label.

Well (09): ConA-PEG5-DIBAC. F0266 + **13** label.

Well (10): Trastuzumab-PEG5-DIBAC + ConA-PEG3-*endo*-BCN. **13** + **15** label.

Well (11): Trastuzumab-PEG3-*endo*-BCN + ConA-PEG5-DIBAC. **13** + **15** label.

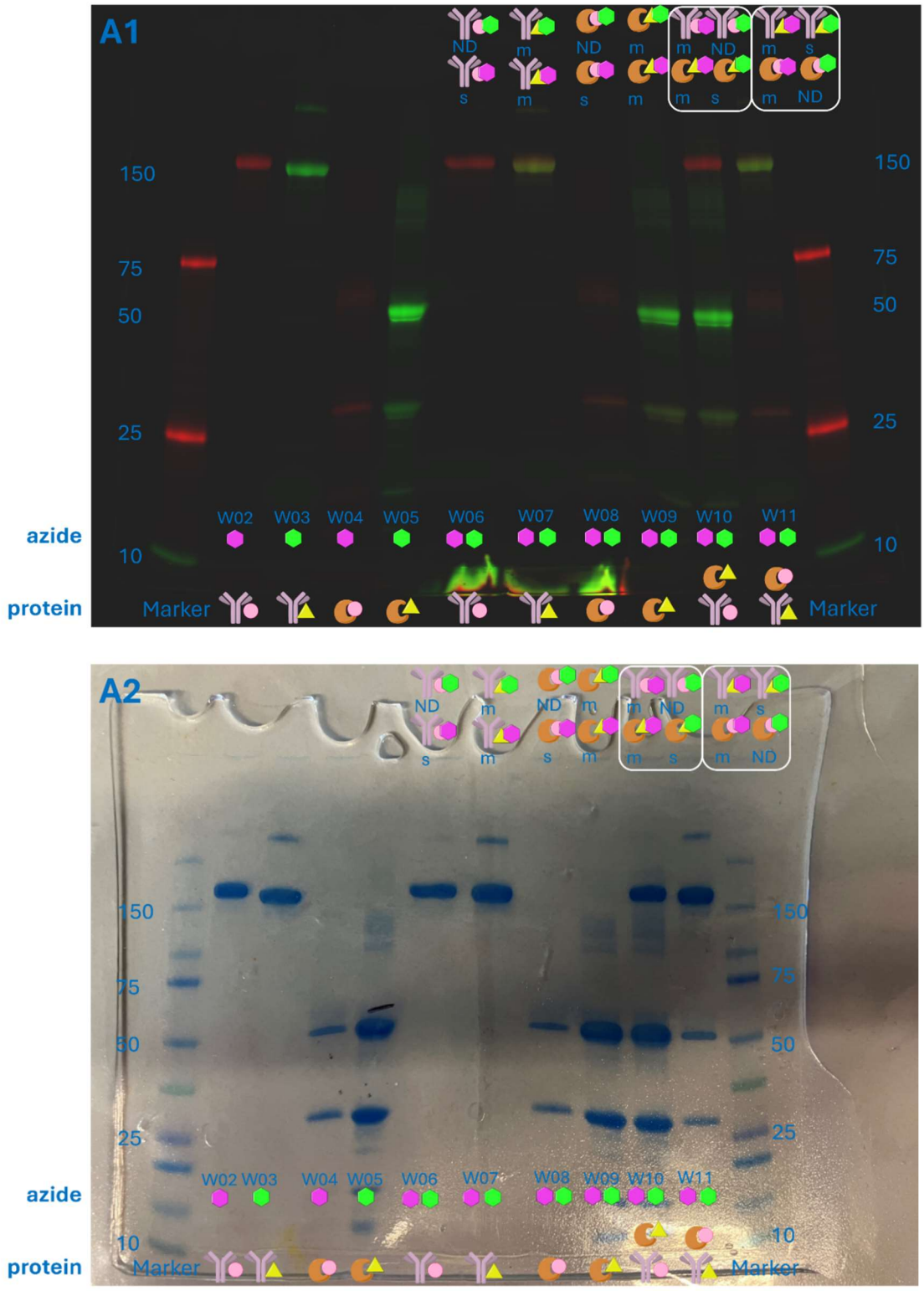


Figure SI12. A1) Typhoon scanned (λ_{em} = 488 and 532) or A2) Coomassie stained SDS-PAGE analysis of W2) W3) W6) W7) **Trastuzumab-PEG-dipolarophile** and W4) W5) W8) W9) **ConA-PEG-dipolarophile** or W10) W11) their various combinations. These proteins were labeled using SPAAC click reaction with W2) W4)

W6) W8) W10 W11) **15** or W3) W5) W7) W9) W10) W11) **13**. Marker: protein ladder. 's' indicates formation of single product, 'm' = multiple products, ND = not determined.

Table SI5.**:** Summary of SDS-PAGE bands quantification (integrated areas) by densitometry from modified Trastuzumab mAb under Coomassie or fluorescent (λ_{em} = 488 or 532 nm) visualization.

Coomassie SDS-PAGE			**Typhoon scan (λ_{em} = 488 nm)**			**Typhoon scan (λ_{em} = 532 nm)**	
Entry	**Trastuz.**	**ConA (total)**	**Entry**	**Trastuz.**	**ConA (total)**	**Trastuz.**	**ConA (total)**
Well 2	14111	-	Well 2	-	-	13276	-
Well 3	16882	-	Well 3	19415	-	-	-
Well 4	-	11924	Well 4	-	-	-	16332
Well 5	-	32520	Well 5	-	24817	-	-
Well 6	15249	-	Well 6	0	-	14335	-
Well 7	19528	-	Well 7	11570	-	13353	-
Well 8	-	9341	Well 8	-	0	-	13664
Well 9	-	33985	Well 9	-	17761	-	12837
Well 10	17372	38290	Well 10	0	20240	14080	9806
Well 11	16389	12762	Well 11	11257	0	8561	13771

(*B) Control samples for SDS-PAGE:*

Well (02): Trastuzumab-PEG5-DIBAC. **13** label.
Well (03): Trastuzumab-PEG3-*endo*-BCN. **15** label.
Well (04): ConA-PEG5-DIBAC. **13** label.
Well (05): ConA-PEG3-*endo*-BCN. **15** label.

(*B) Competition samples for SDS-PAGE:*

Well (06): Trastuzumab-PEG5-DIBAC + ConA-PEG3-*endo*-BCN. **15** label.
Well (07): Trastuzumab-PEG3-*endo*-BCN + ConA-PEG5-DIBAC. **15** label.
Well (08): Trastuzumab-PEG5-DIBAC + ConA-PEG3-*endo*-BCN. **13** label.
Well (09): Trastuzumab-PEG3-*endo*-BCN + ConA-PEG5-DIBAC. **13** label.
Well (10): Trastuzumab-PEG5-DIBAC + ConA-PEG3-*endo*-BCN. **13** + **15** label.
Well (11): Trastuzumab-PEG3-*endo*-BCN + ConA-PEG5-DIBAC. **13** + **15** label.

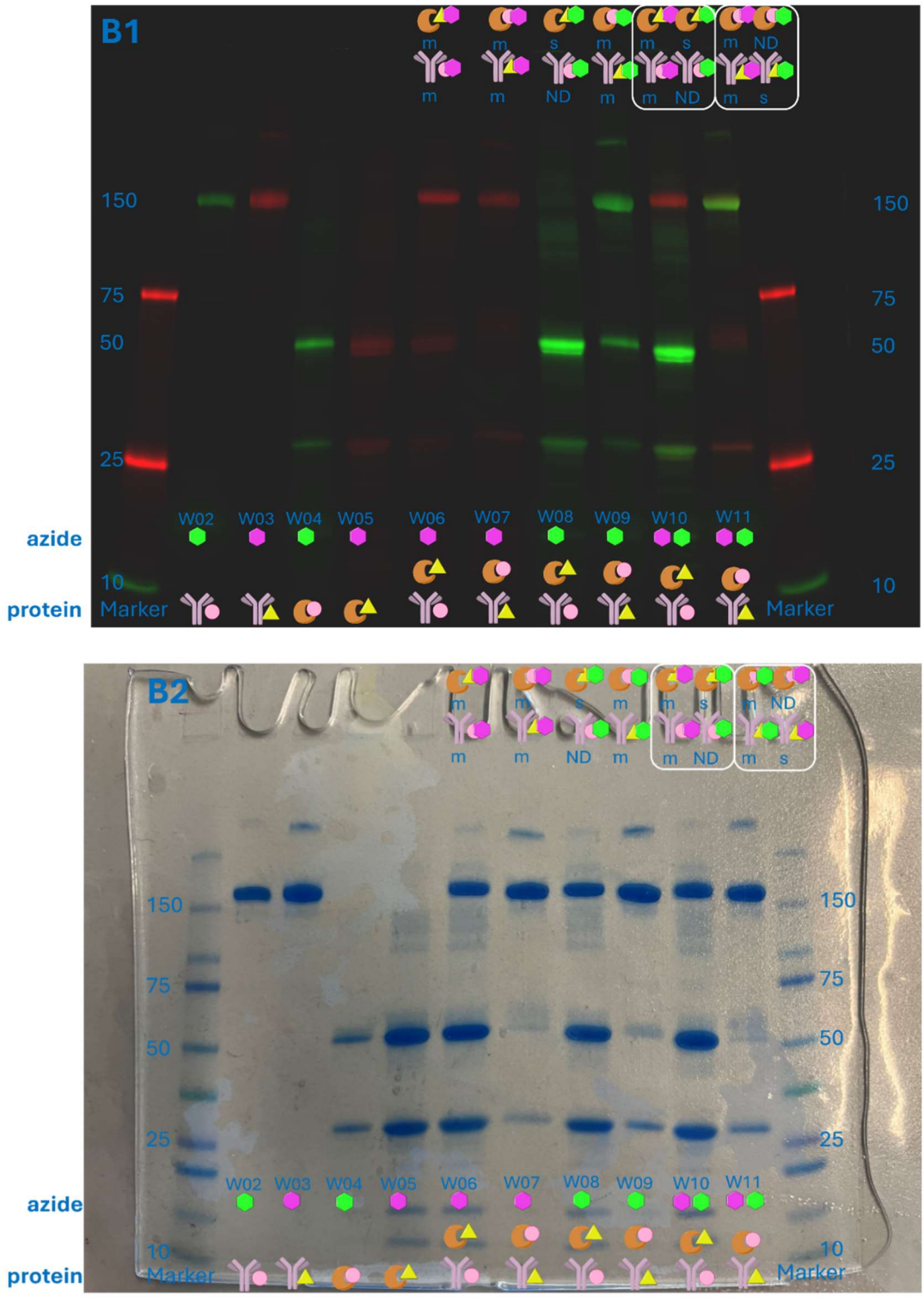


Figure SI13. A1) Typhoon scanned (λ_{em} = 488 and 532) or A2) Coomassie stained SDS-PAGE analysis of W2) W3) **Trastuzumab-PEG-dipolarophile** and W4) W5) **ConA-PEG-dipolarophile** or W6) W7) W8) W9) W10) W11) their various combinations. These proteins were labeled using SPAAC click reaction with W3) W5)

W6) W7) W10 W11) **15** or W2) W4) W8) W9) W10) W11) **13**. Marker: protein ladder. 's' indicates formation of single product, 'm' = multiple products, ND = not determined.

Table S6. Summary of SDS-PAGE bands quantification (integrated areas) by densitometry from modified Trastuzumab mAb under Coomassie or fluorescent (λ_{em} = 488 or 532 nm) visualization.

Coomassie SDS-PAGE			**Typhoon scan (λ_{em} = 488 nm)**			**Typhoon scan (λ_{em} = 532 nm)**	
Entry	**Trastuz.**	**ConA (total)**	**Entry**	**Trastuz.**	**ConA (total)**	**Trastuz.**	**ConA (total)**
Well 2	14246	-	Well 2	7911	-	-	-
Well 3	20803	-	Well 3	-	-	22449	-
Well 4	-	12256	Well 4	-	28950	-	-
Well 5	-	38030	Well 5	-	-	-	43418
Well 6	22698	35375	Well 6	-	-	19374	22750
Well 7	20629	6216	Well 7	-	-	13797	12737
Well 8	23691	32954	Well 8	0	18379	-	-
Well 9	23278	13825	Well 9	22155	29520	-	-
Well 10	20044	35274	Well 10	0	20645	21108	10882
Well 11	19233	11877	Well 11	20978	0	11032	25525

(*C) Control samples for SDS-PAGE:*

Well (02): Trastuzumab-PEG5-DIBAC. **15** label.
Well (03): Trastuzumab-PEG3-*endo*-BCN. **13** label.
Well (04): ConA-PEG5-DIBAC. **15** label.
Well (05): ConA-PEG3-*endo*-BCN. **13** label.
Well (06): ConA-PEG3-*endo*-BCN. **15** label.

(*C) Competition samples for SDS-PAGE:*

Well (07): Trastuzumab-PEG5-DIBAC + ConA-PEG3-*endo*-BCN.

- **13** label added immediately.
- **15** label added after 12 h incubation.

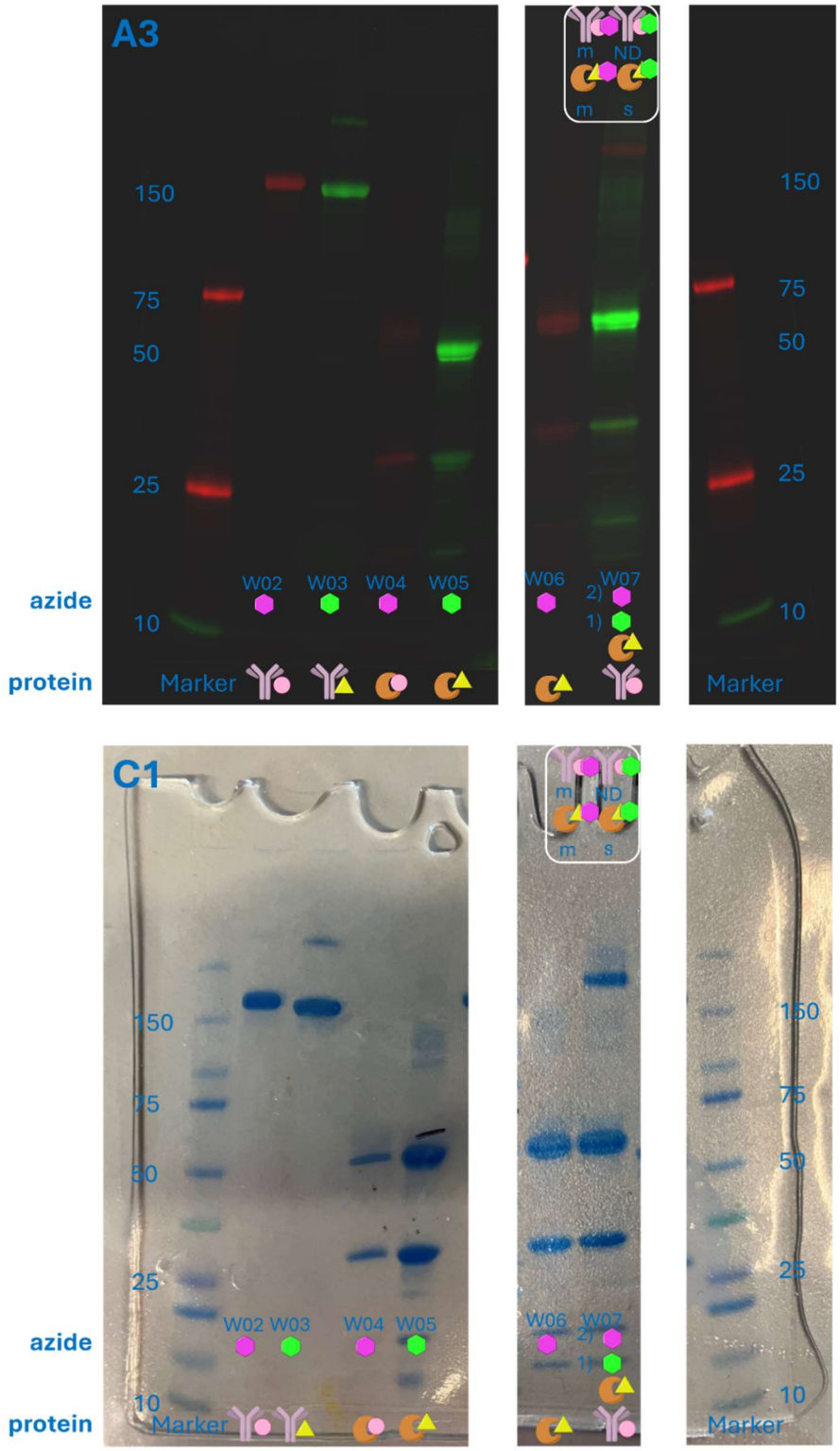


Figure SI14. A1) Typhoon scanned (λ_{em} = 488 and 532) or A2) Coomassie stained SDS-PAGE analysis of W2) W3) Trastuzumab-PEG-dipolarophile and W4) W5) W6) ConA-PEG-dipolarophile or W7) Trastuzumab-PEG3-endo-BCN + ConA-PEG5-DIBAC. These proteins were labeled using SPAAC click reaction with W2)

W4) W6) W7) **15** or W3) W5) W7) **13**. Marker: protein ladder. 's' indicates formation of single product, 'm' = multiple products, ND = not determined.

Table SI7. Summary of SDS-PAGE bands quantification (integrated areas) by densitometry from modified Trastuzumab mAb under Coomassie or fluorescent (λ_{em} = 488 or 532 nm) visualization.

Coomassie SDS-PAGE			**Typhoon scan (λ_{em} = 488 nm)**			**Typhoon scan (λ_{em} = 532 nm)**	
Entry	**Trastuz.**	**ConA (total)**	**Entry**	**Trastuz.**	**ConA (total)**	**Trastuz.**	**ConA (total)**
Well 2	14111	-	Well 2	-	-	13276	-
Well 3	16882	-	Well 3	19415	-	-	-
Well 4	-	11924	Well 4	-	-	-	16332
Well 5	-	32520	Well 5	-	24817	-	-
Well 6	-	68537	Well 6	-	-	-	60806
Well 7	22860	70793	Well 7	0	45930	9945	9317

Confocal microscopy analysis of competition experiment:

SKBR3 cells were seeded on a 96-well plate at a density of 30000 cells per well in a complete DMEM medium. After 48h incubation, the cells were incubated with the indicated mixture of proteins in DPBS (Biowest) with 1mg/mL of BSA, Ca^{2+}, Mg^{2+} for 30 min. Sample preparation is described in the previous chapter Click SPAAC labeling of proteins-dipolarophiles with azides.

Competition samples for confocal microscopy:

Sample (01): Trastuzumab-PEG5-DIBAC. **15** label.
Sample (02): ConA-PEG3-*endo*-BCN. **13** label.
Sample (03): Trastuzumab-PEG5-DIBAC + ConA-PEG3-*endo*-BCN. **13** + **15** label.
Sample (04): Trastuzumab-PEG5-DIBAC + ConA-PEG3-*endo*-BCN.

- **13** label added immediately.
- **15** label added after 12 h incubation.

The cells were washed 3 times with a complete L-15 (Leibovitz's) medium (W / O Phenol Red; Gibco 21083-027). Then the cells were imaged on a confocal microscope (Figure SI15).

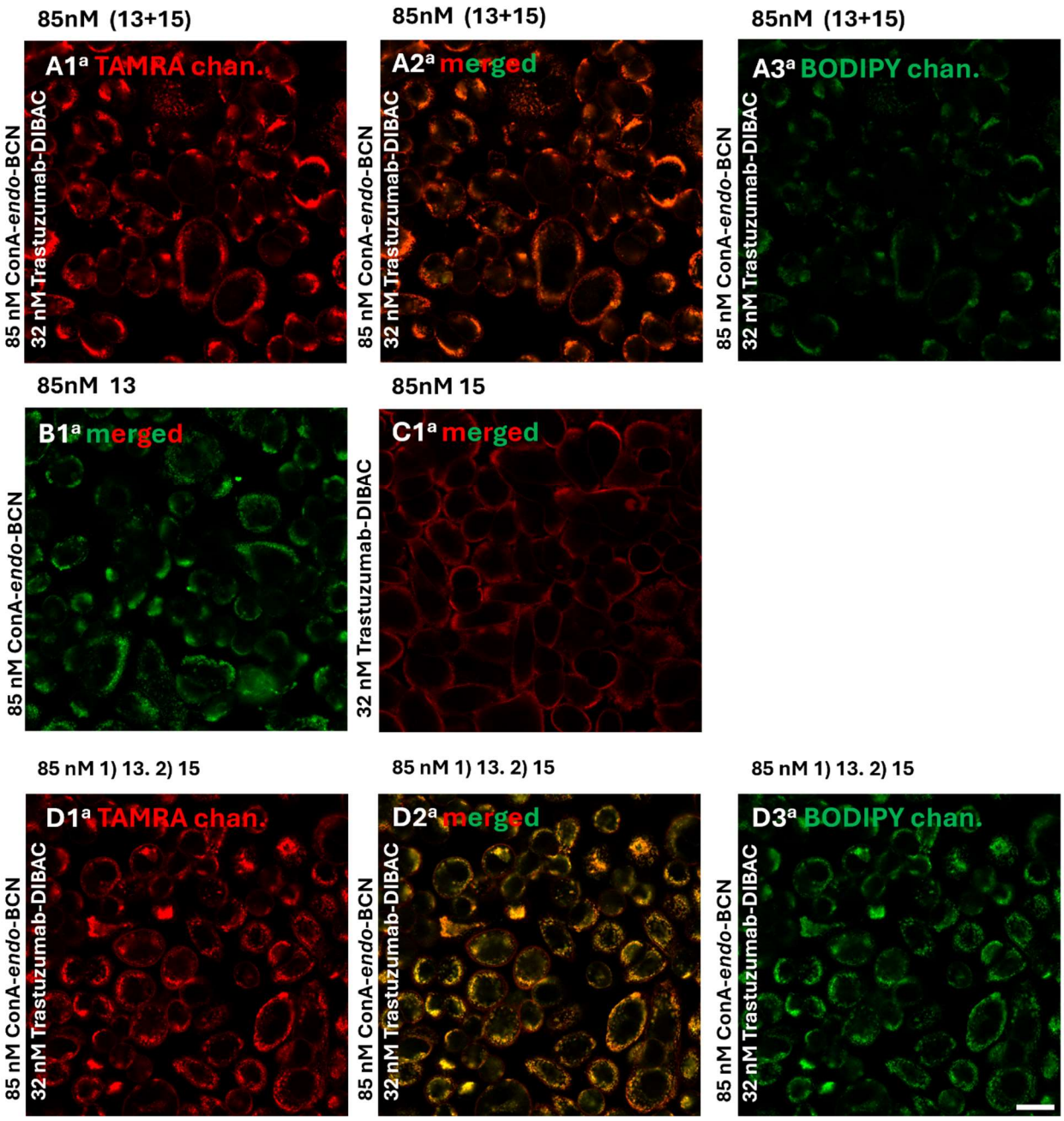


Figure SI15. Confocal microscope images of SKBR3 cells treated with A) D) ConA-PEG3-endo-BCN + Trastuzumab-PEG5-DIBAC, B1) ConA-endo-BCN, C1) Trastuzumab-PEG5-DIBAC labeled with A) **13** + **15**, B) **13**, C) **15**, D) sequentially: D.1) **13**, D.2) **15**. Click labeling is shown in green (**13**) or red (**15**). Nuclei were not stained. [a]Microscope set-up: BODFl (488 nm) laser intensity: 5.0%, and emission (490–534 nm) detector: 750 V. [c]Microscope set-up: TAMRA (561 nm) laser intensity: 2.0%, and emission (552–694 nm) detector: 700 V. Scale bar is 25 µm.

Flow cytometry analysis of competition experiment: After the confocal microscopy experiment, all the cellular samples were washed 3 times with PBS. The cells were detached (after ca 10 min of incubation) from the 96-well plate using 50 µl of Accutase (StemPro® Gibco A11105-01) solution. Each well was additionally washed 2 times with 50 µl of PBS (+ 1 mg/ml BSA) and centrifuged at 300 ×g for 3 min. Supernatant was removed and samples were resuspended to 150 µl of PBS (+ 1 mg/ml BSA). Then the cellular samples were measured by a flow cytometer (LSR Fortessa (BD Biosciences)) and the results are represented as histograms or median fluorescence intensity (Figure SI16 and SI17).

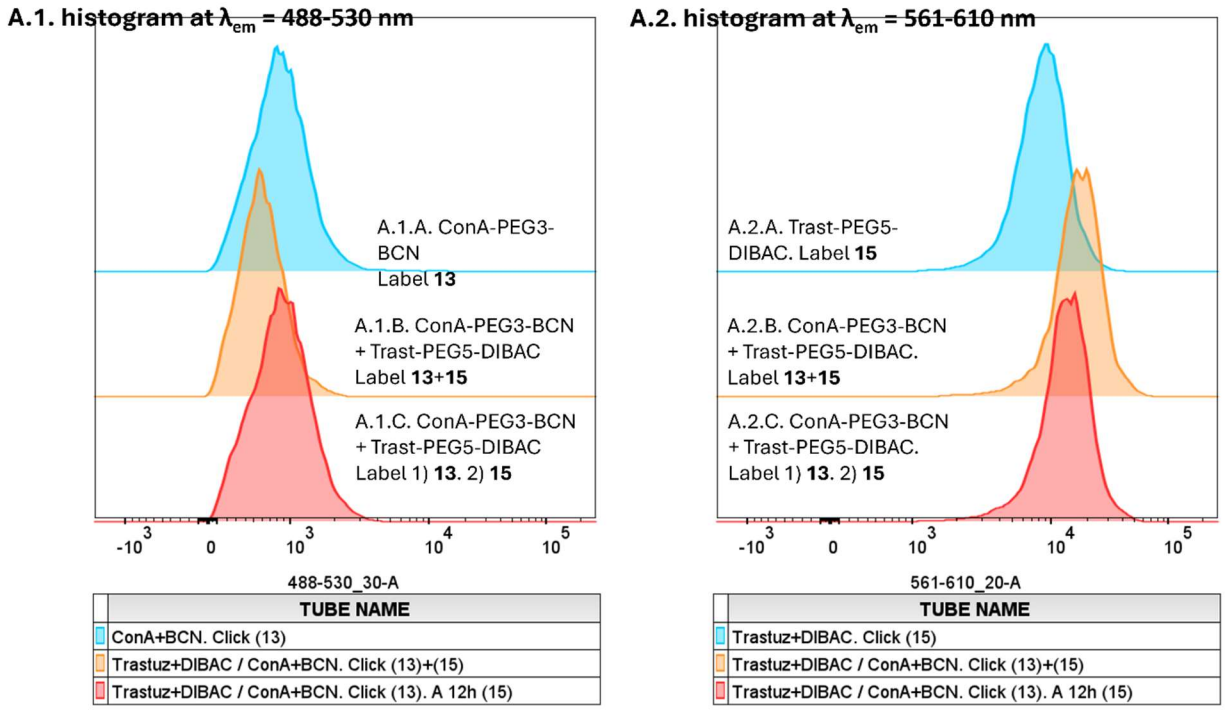


Figure SI16. Graphical representation (histograms) of flow cytometry analyses of SKBR3 cells treated with A.1.A) **ConA-endo-BCN,** A.1.B) A.1.C) B.1.B) B.1.C) **ConA-PEG3-endo-BCN + Trastuzumab-PEG5-DIBAC**, A.2.A) **Trastuzumab-PEG5-DIBAC** labeled with A.1.A) **13**, A.1.B) A.2.B) **13** + **15**, A.1.C) A.2.C) sequentially: 1) **13**, 2) **15**, A.2.A) **15**.

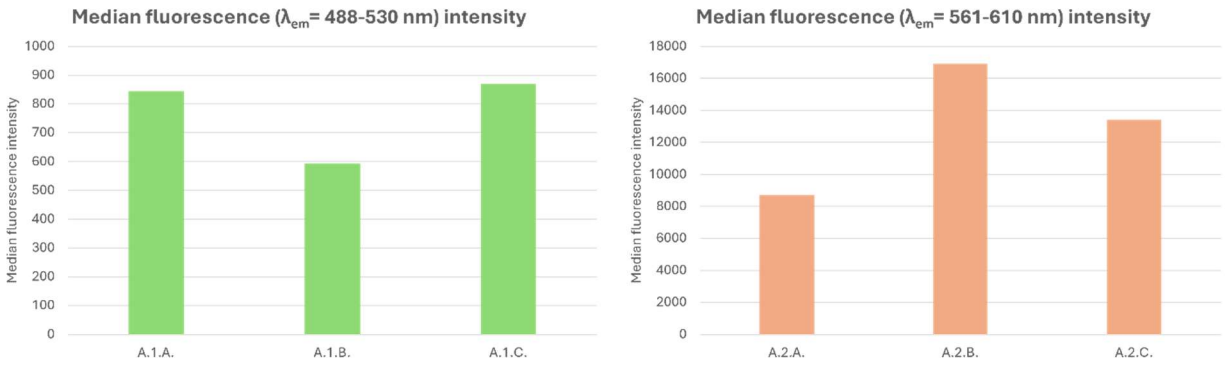


Figure SI17. Median fluorescence intensity from flow cytometry analyses of SKBR3 cells treated with A.1.A) **ConA-endo-BCN,** A.1.B) A.1.C) B.1.B) B.1.C) **ConA-PEG3-endo-BCN + Trastuzumab-PEG5-DIBAC**, A.2.A) **Trastuzumab-PEG5-DIBAC** labeled with A.1.A) **13**, A.1.B) A.2.B) **13** + **15**, A.1.C) A.2.C) sequentially: 1) **13**, 2) **15**, A.2.A) **15**.

Living cell competition labeling

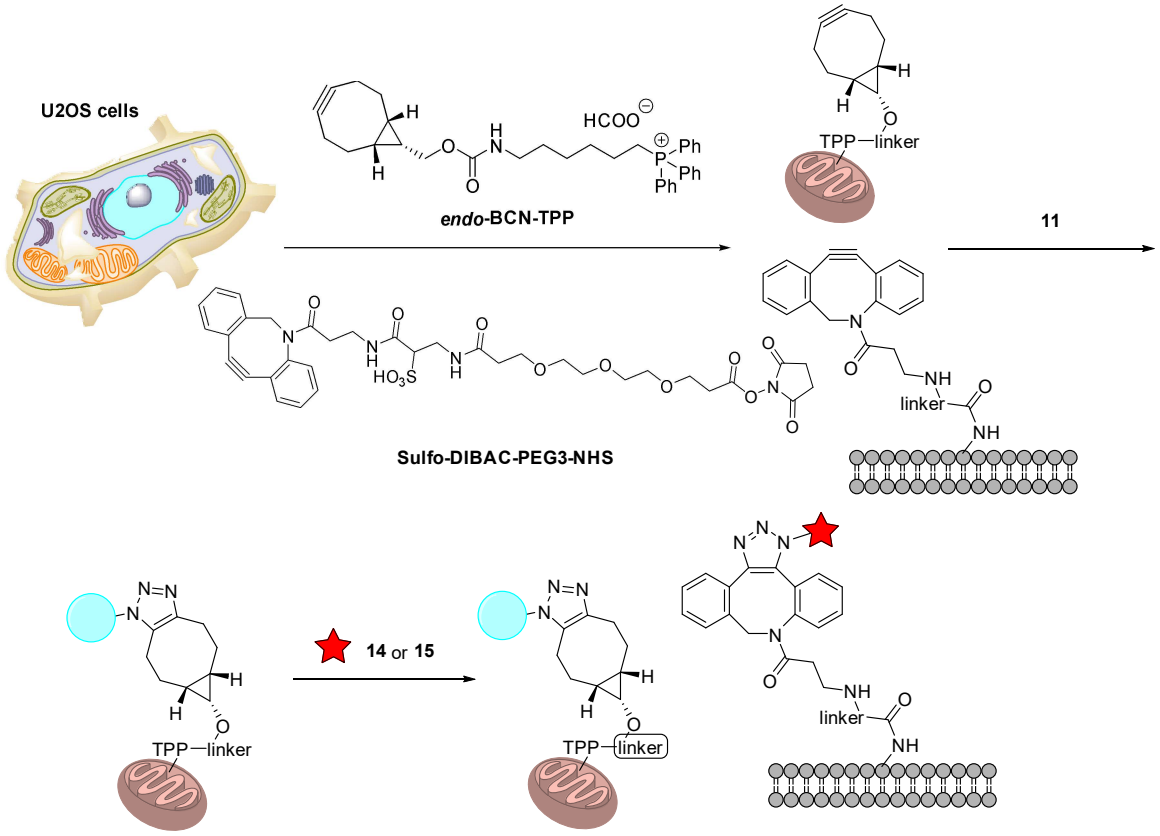
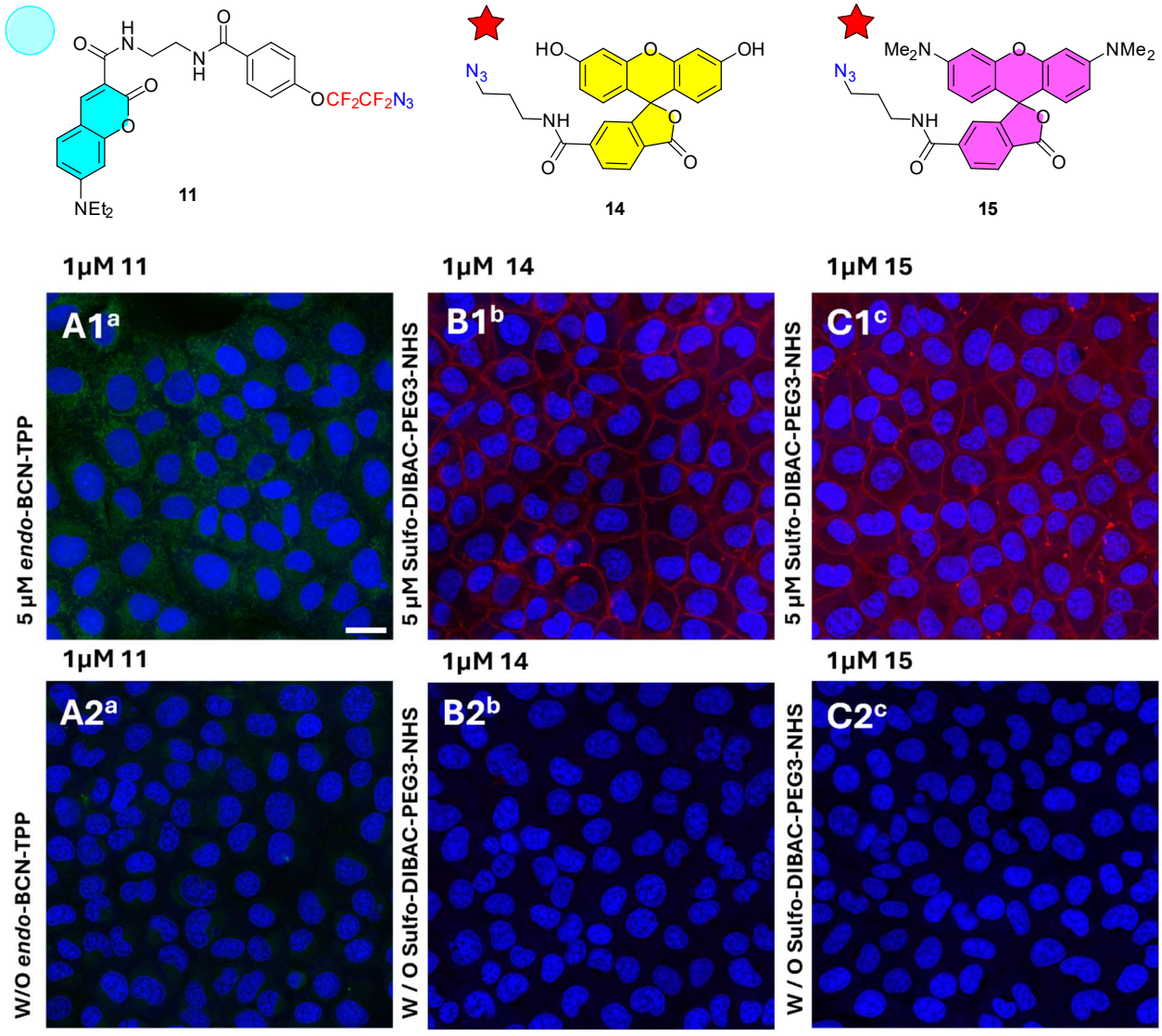


Figure SI18. Schematic depiction of competition experiment between coumarin-fluorinated azide conjugate (**11**) and non-fluorinated analogue (**14** or **15**) in SPAAC with **endo-BCN-TPP** (mitochondrial labeling) or **Sulfo-DIBAC-PEG3-NHS** (cellular membrane labeling).

U2OS cells were seeded on a 96-well plate at a density of 12500 cells per well in a complete DMEM medium. After 48 h incubation, the cells were incubated with 5 µM dipolarophile(s)[18] in DPBS (Biowest) for 15 min. The cells were washed 3 times with a complete L-15 (Leibovitz's) medium (W / O Phenol Red; Gibco 21083-027). After that, 1 µM of **11** was added to the cells in a complete L-15 medium for 1 h. The cells were washed 3 times (after 1, 5, and 15 min) with a complete L-15 medium. After that, 1 µM of **14** or **15** was added to the cells in a complete L-15 medium with DRAQ5 (5 µg/ml) for 1 h. The cells were briefly washed 3 times with a complete L-15 medium. Then the cells were imaged on a confocal microscope (Figures SI19 and SI20).



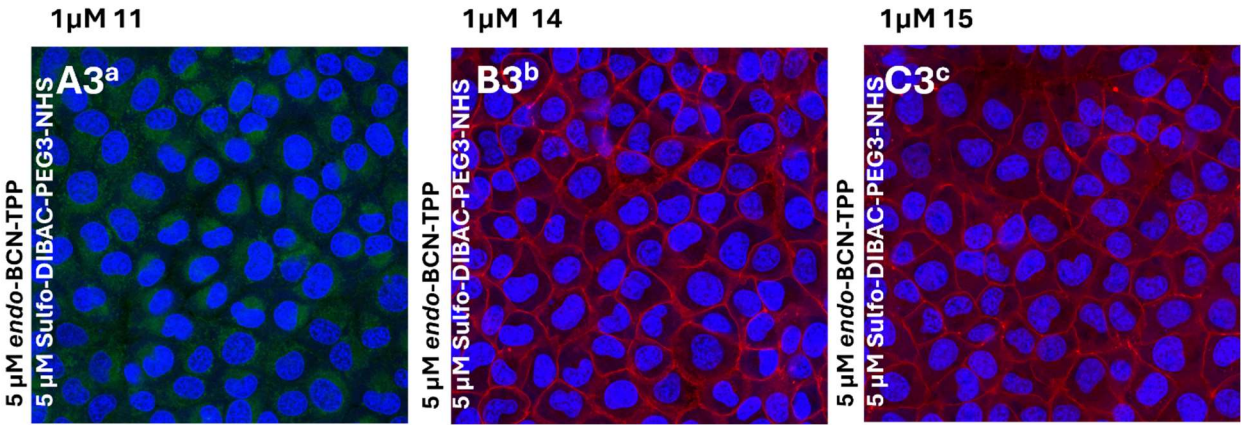


Figure SI19. Structures of fluorophore labels. Confocal microscope images of U2OS cells treated with A1) **endo-BCN-TPP**, B1) C1) **Sulfo-DIBAC-PEG3-NHS** or A3) B3) C3) their combination and labeled with A1) A3) **11**, B1) B3) **14**, C1) C3) **15**. A2) B2) C2) are the corresponding controls (cells treated only with probe). Click labeling is shown in green (**11**) or red (**14**/**15**). Nuclei were stained with DRAQ5 and are shown in blue. [a]Microscope set-up: Coum1 (405 nm) laser intensity: 3.5 %, and emission (410 – 676 nm) detector: 700 V. [b]Microscope set-up: Fcein (488 nm) laser intensity: 3.0 %, and emission (490 – 658 nm) detector: 750[b] or 700[c] V. [c]Microscope set-up: TAMRA (561 nm) laser intensity: 2.0 %, and emission (490 – 658 nm) detector: 700 V. DRAQ5 – laser 639 nm. Scale bar is 25 µm.

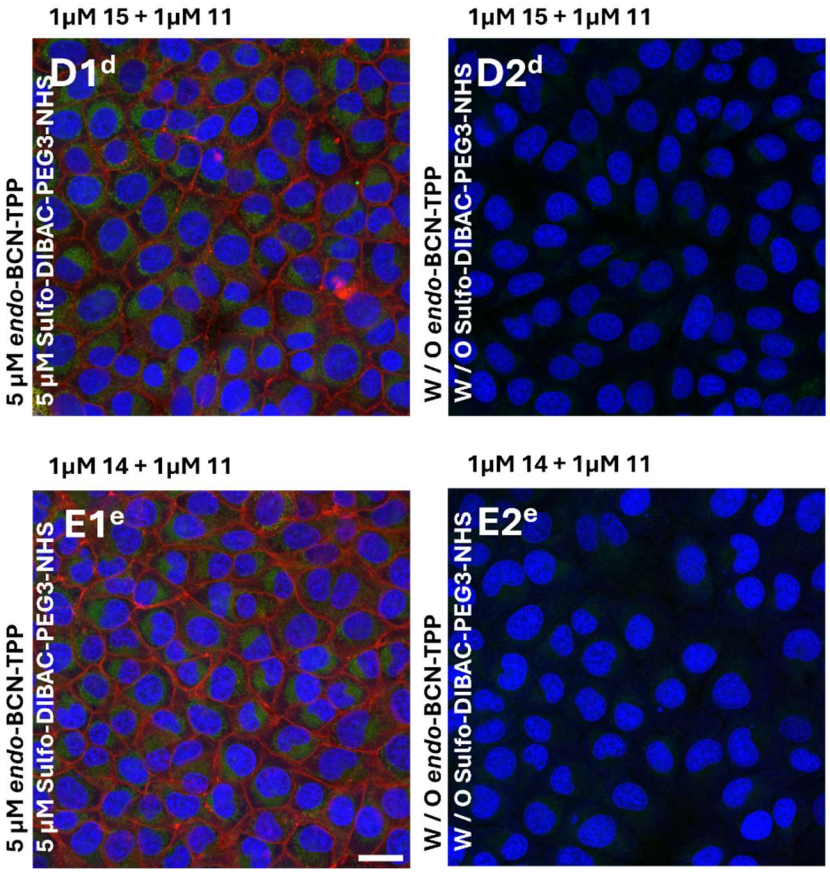


Figure S20. Confocal microscope images of U2OS cells treated with D1) E1) **endo-BCN-TPP + Sulfo-DIBAC-PEG3-NHS** labeled with D1) **11 + 15**, E1) **11 + 14**. D2) E2) are the corresponding controls (cells treated only with probes). Click labeling is shown in green (**11**) or red (**14**/**15**). Nuclei were stained with DRAQ5 and are shown in blue. [d,e]Microscope set-up: Coum1 (405 nm) laser intensity: 3.5 %, and emission (410 – 676 nm) detector: 750 V. [e]Microscope set-up: Fcein (488 nm) laser intensity: 2.5 %, and emission (490 – 658 nm) detector: 750 V. [d]Microscope set-up: TAMRA (561 nm) laser intensity: 3.0 %, and emission (490 – 658 nm) detector: 650 V. DRAQ5 – laser 639 nm. Scale bar is 25 µm.

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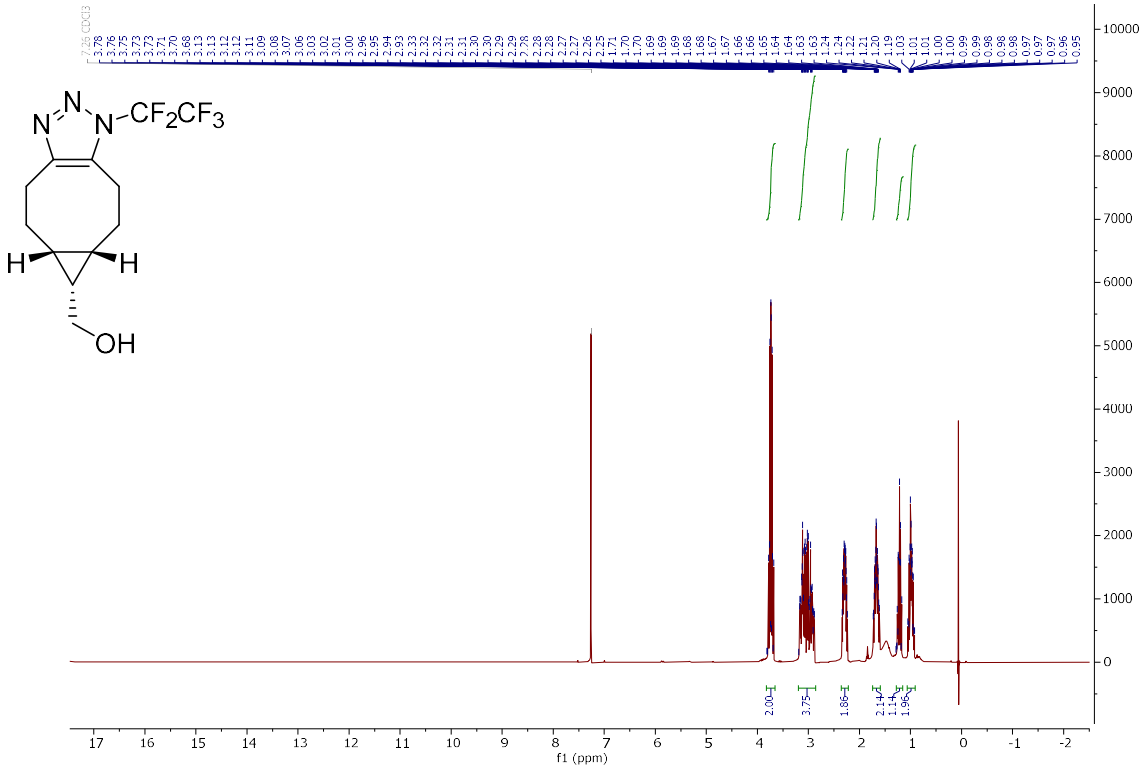
Copies of NMR spectra

^{1}H NMR (401 MHz, $CDCl_3$) spectrum of **3a**



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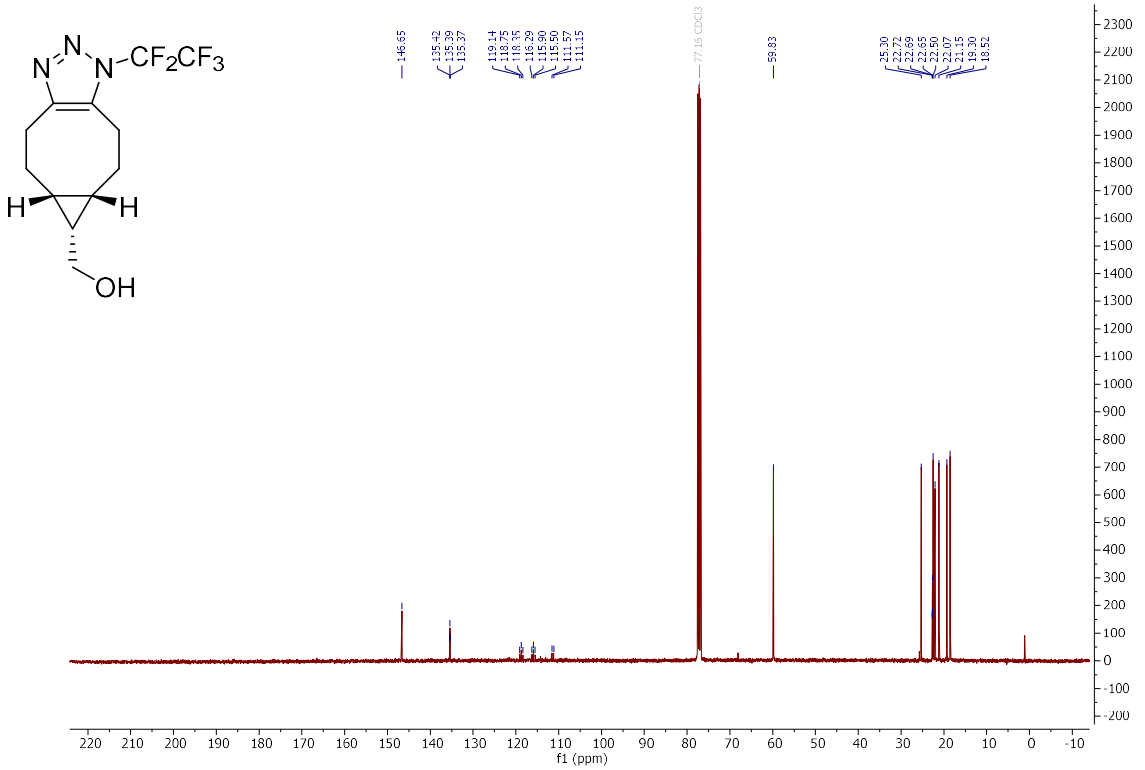
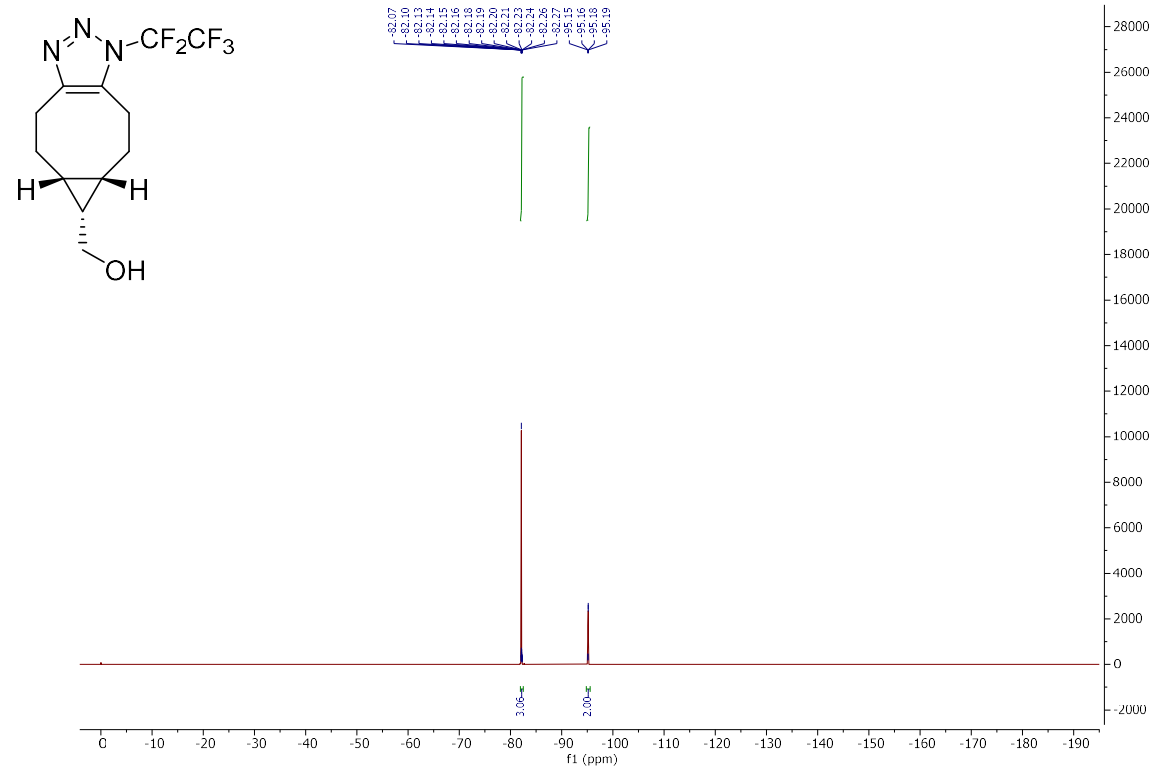


^{19}F NMR (376 MHz, $CDCl_3$) spectrum of **3a**



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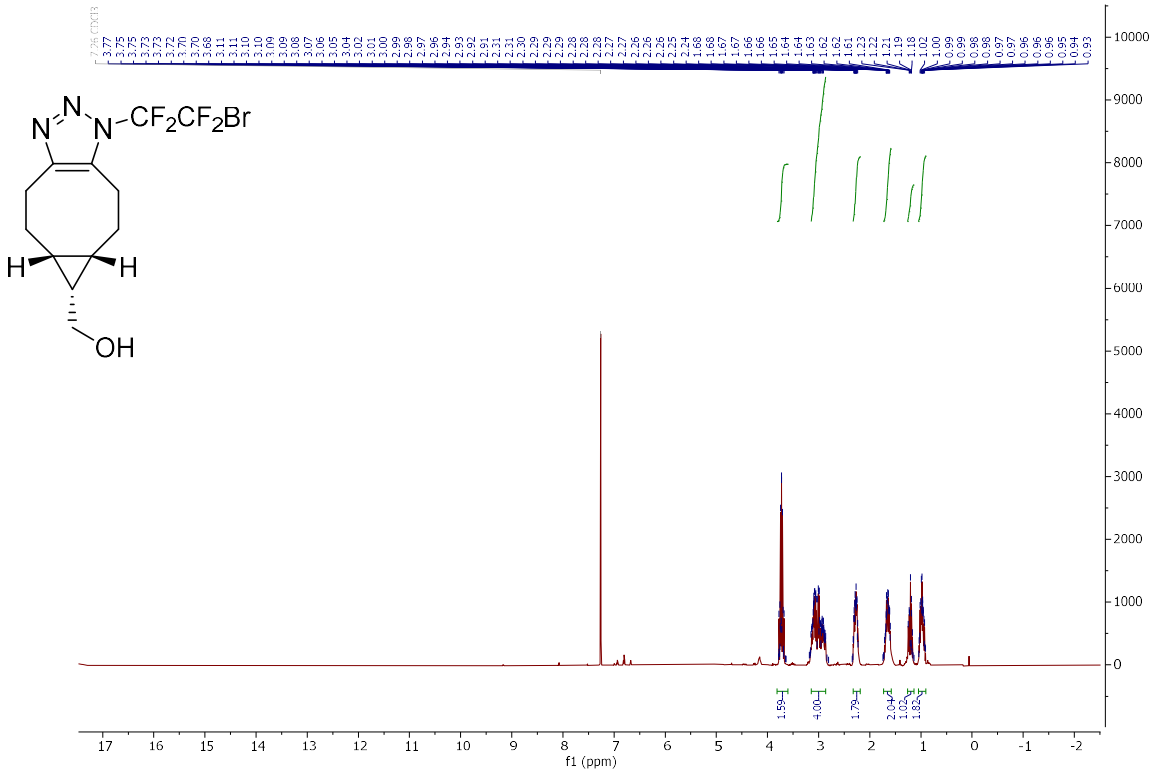

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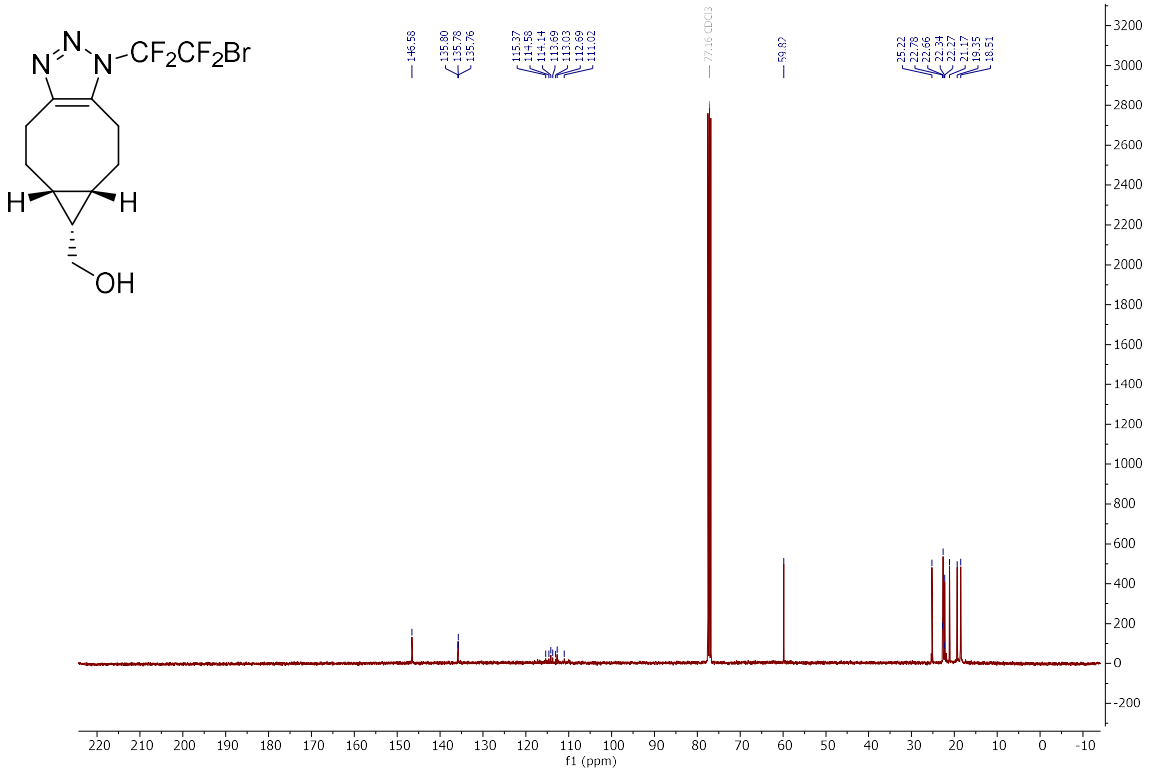

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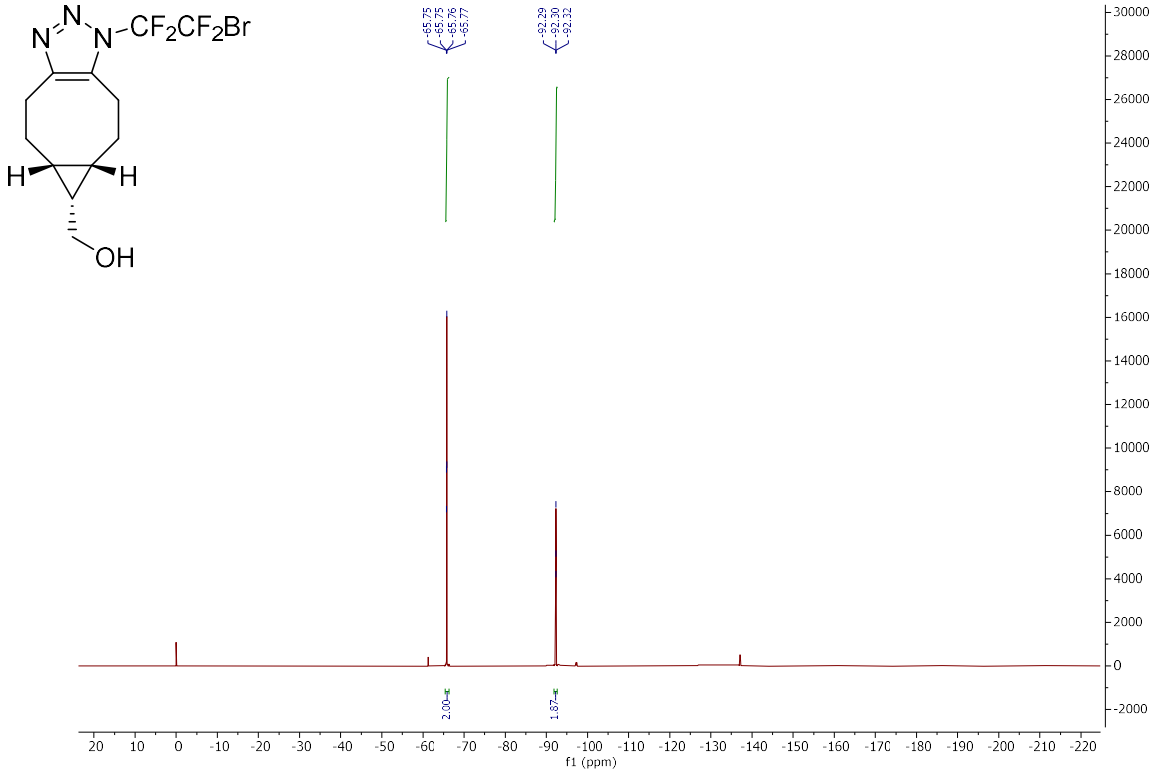

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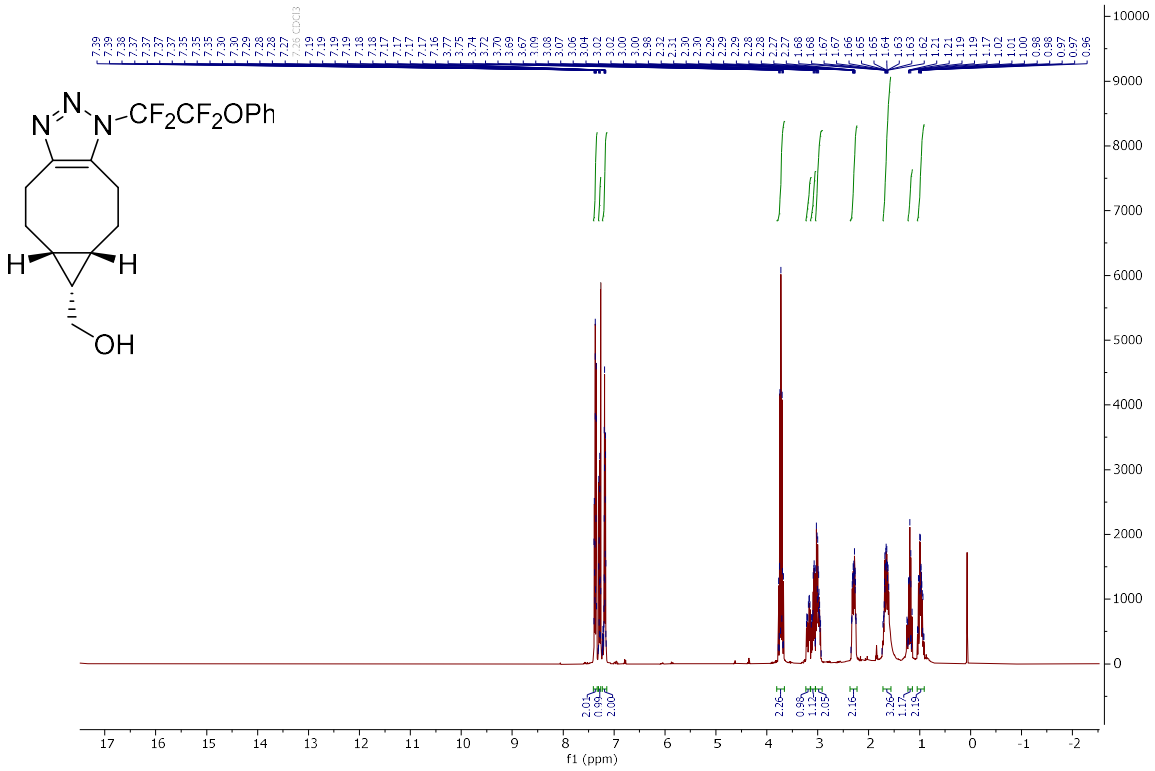

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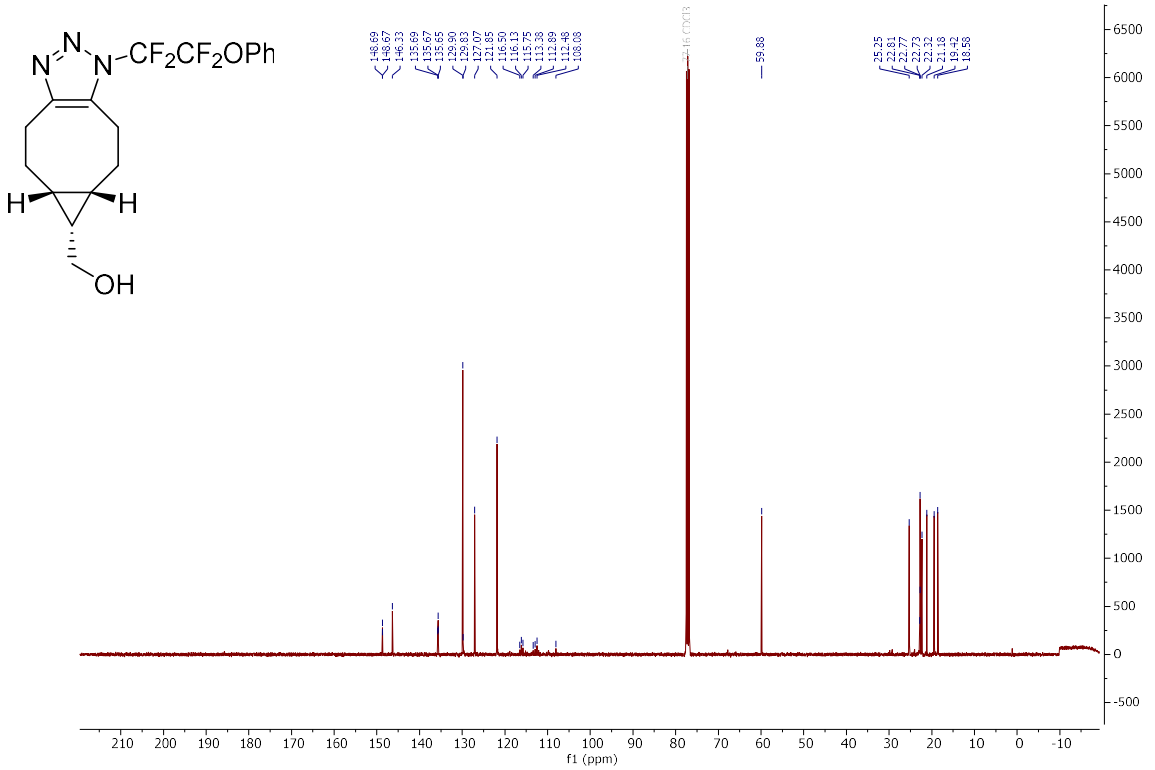
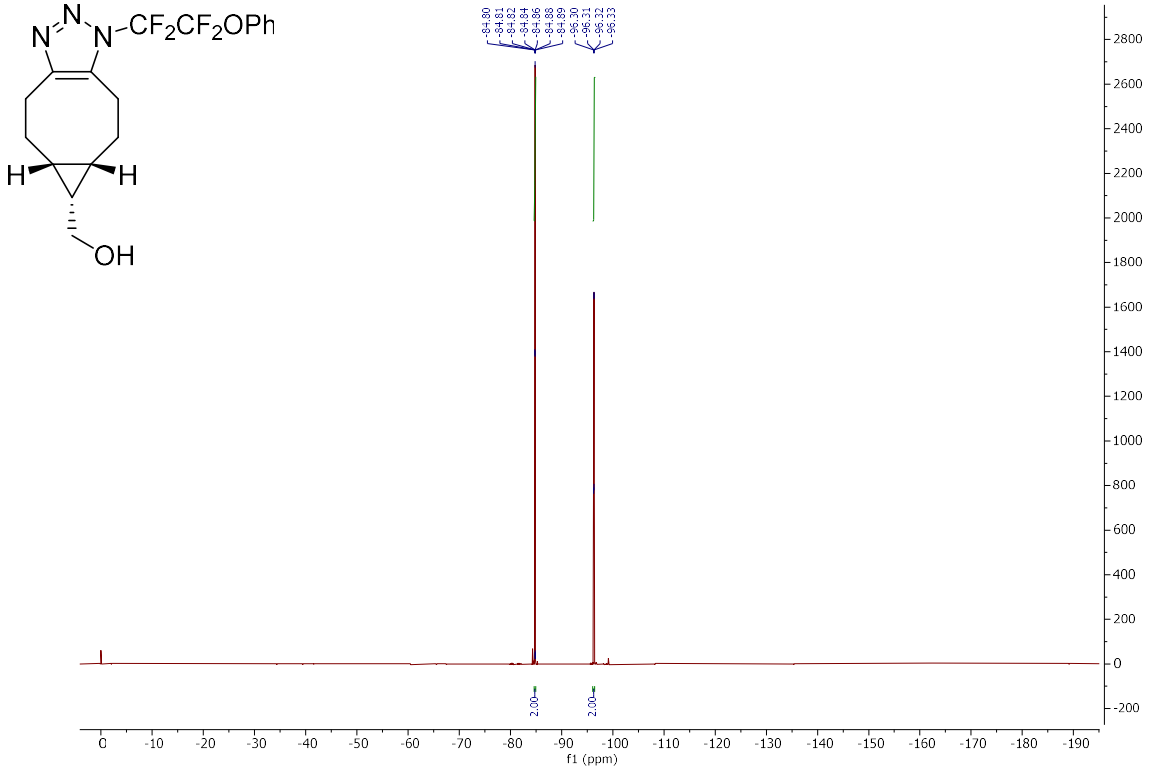


^{19}F NMR (376 MHz, $CDCl_3$) spectrum of **3c**



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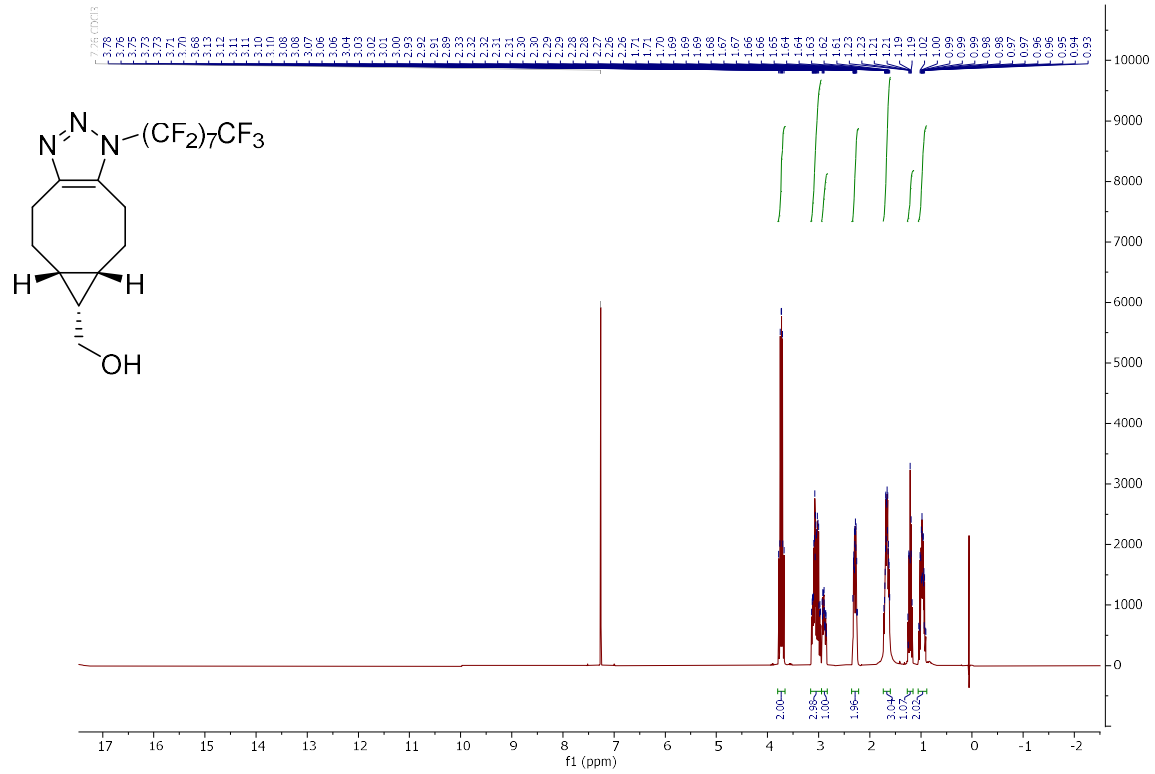

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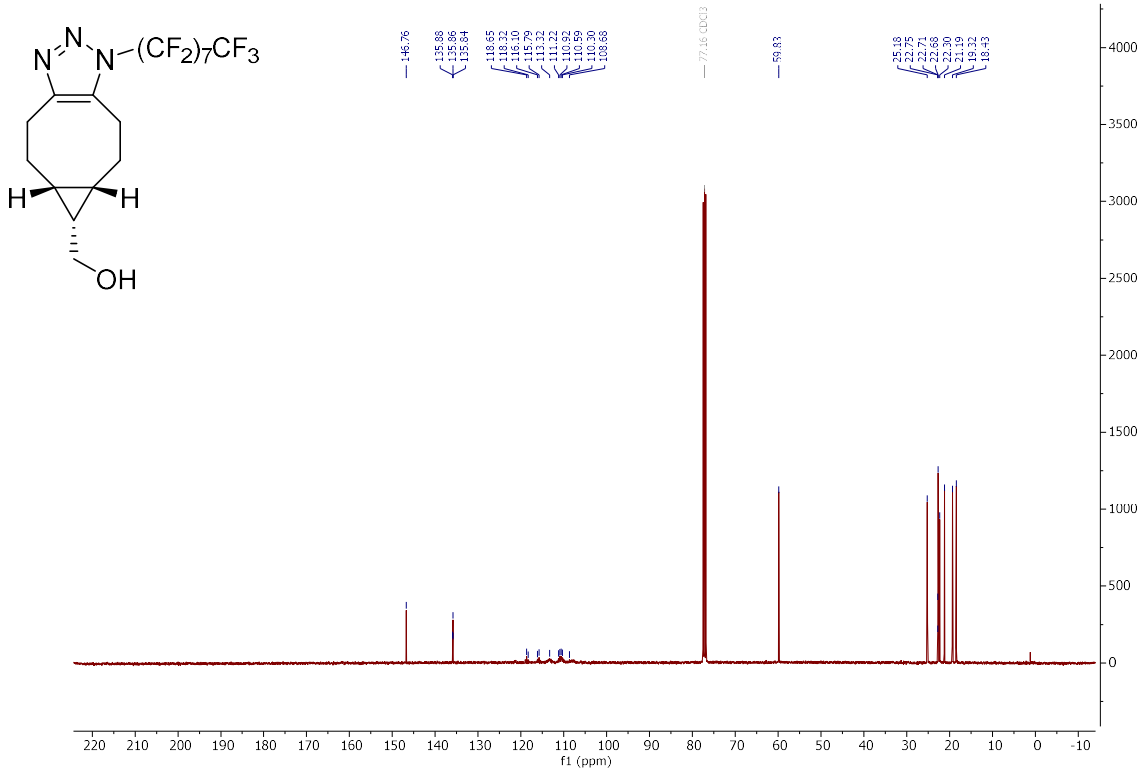

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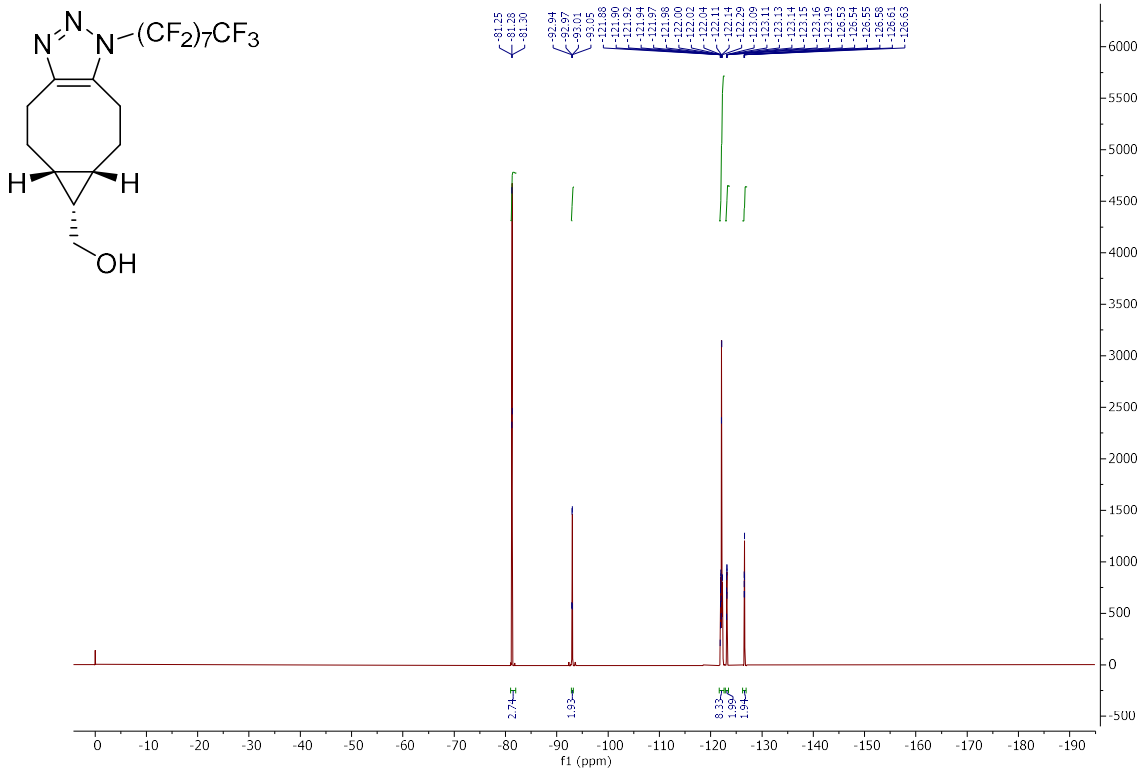

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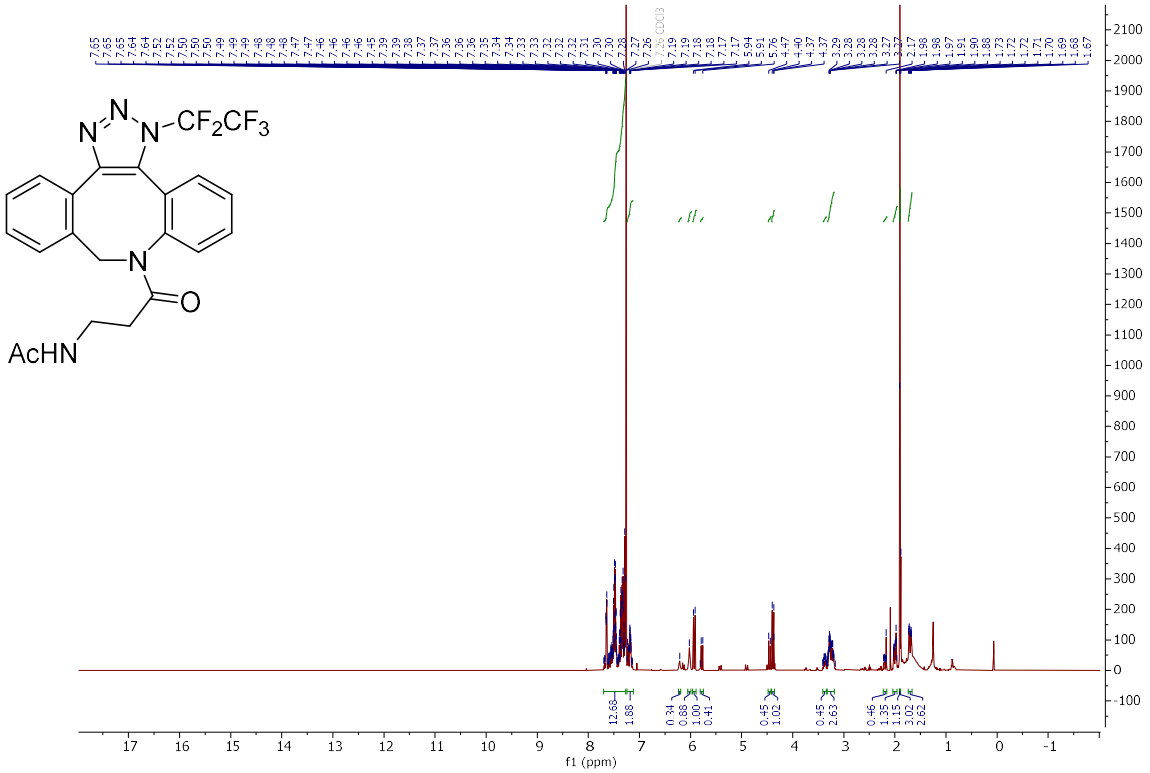

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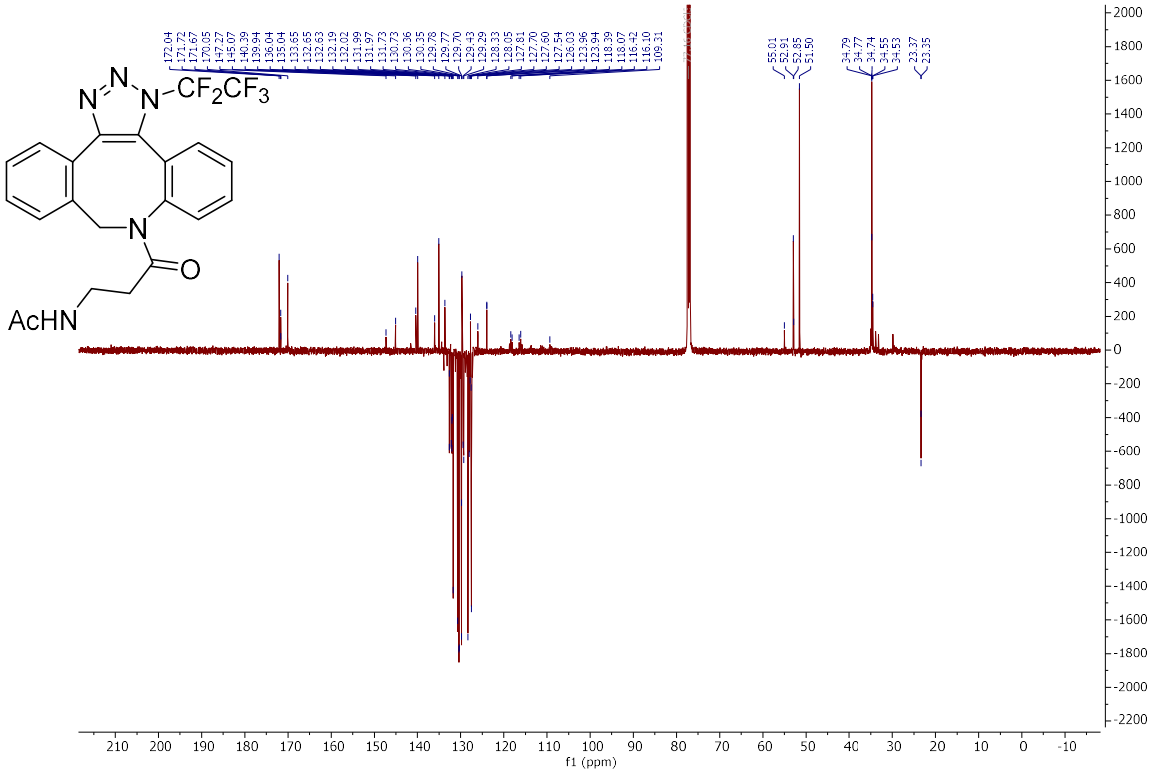

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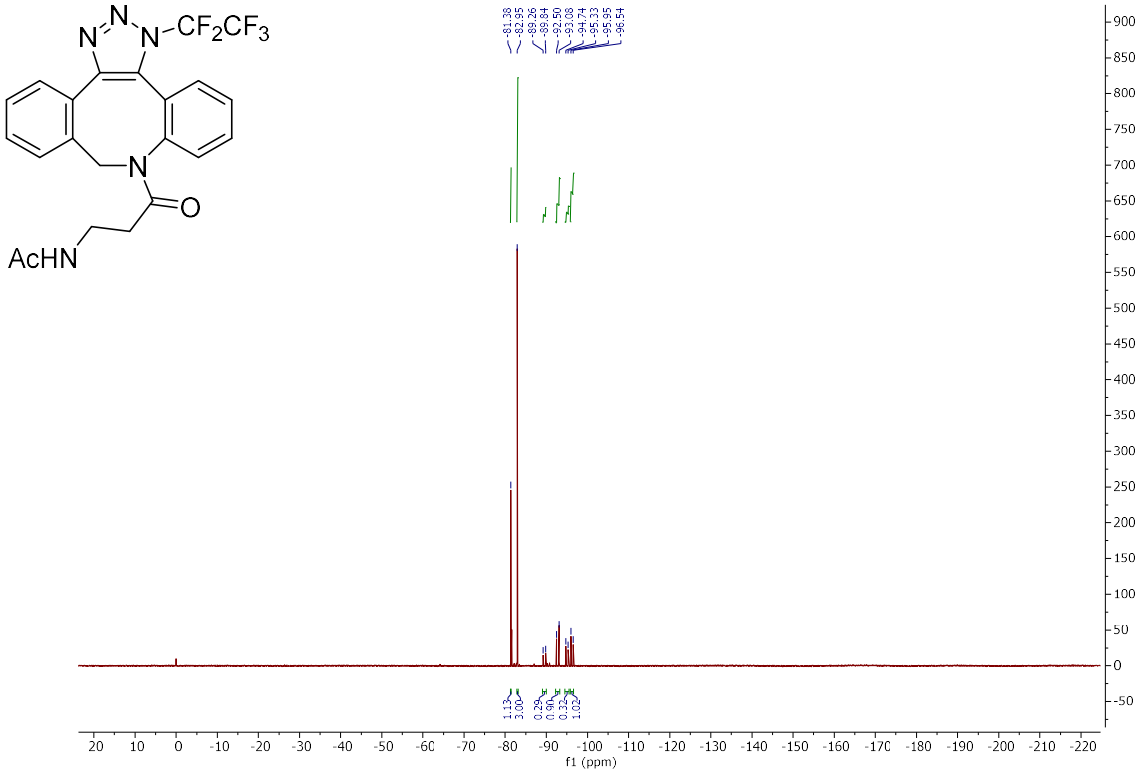

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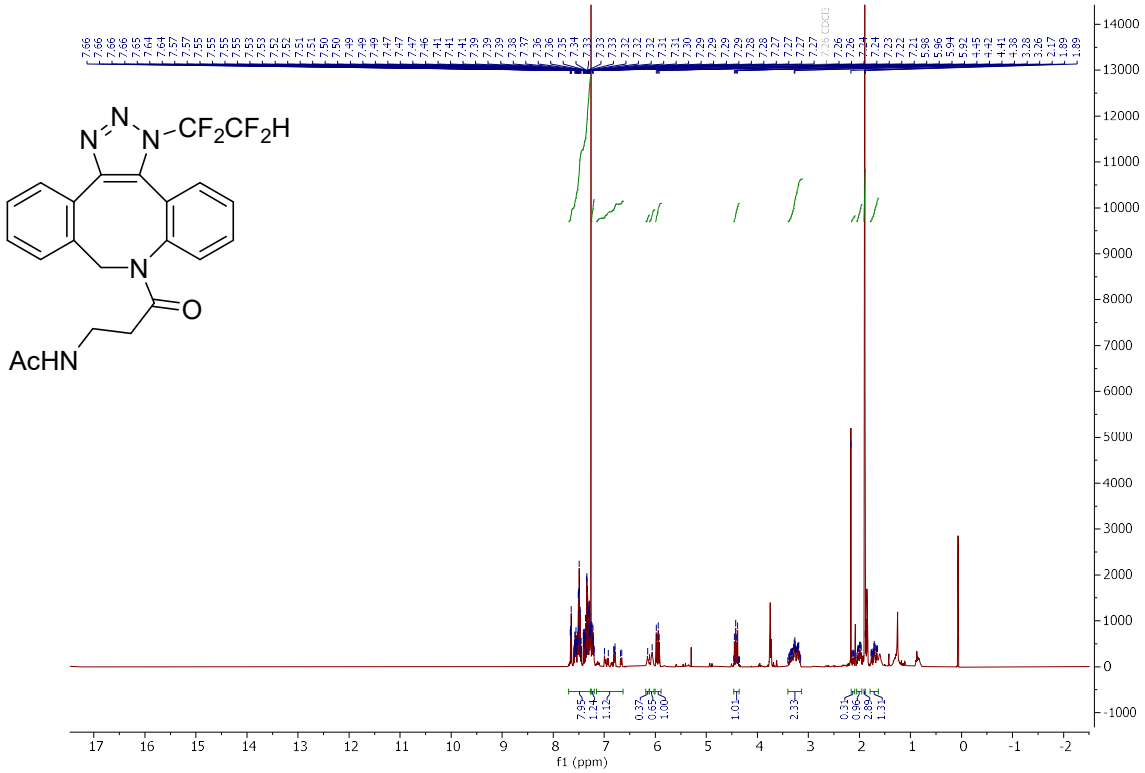

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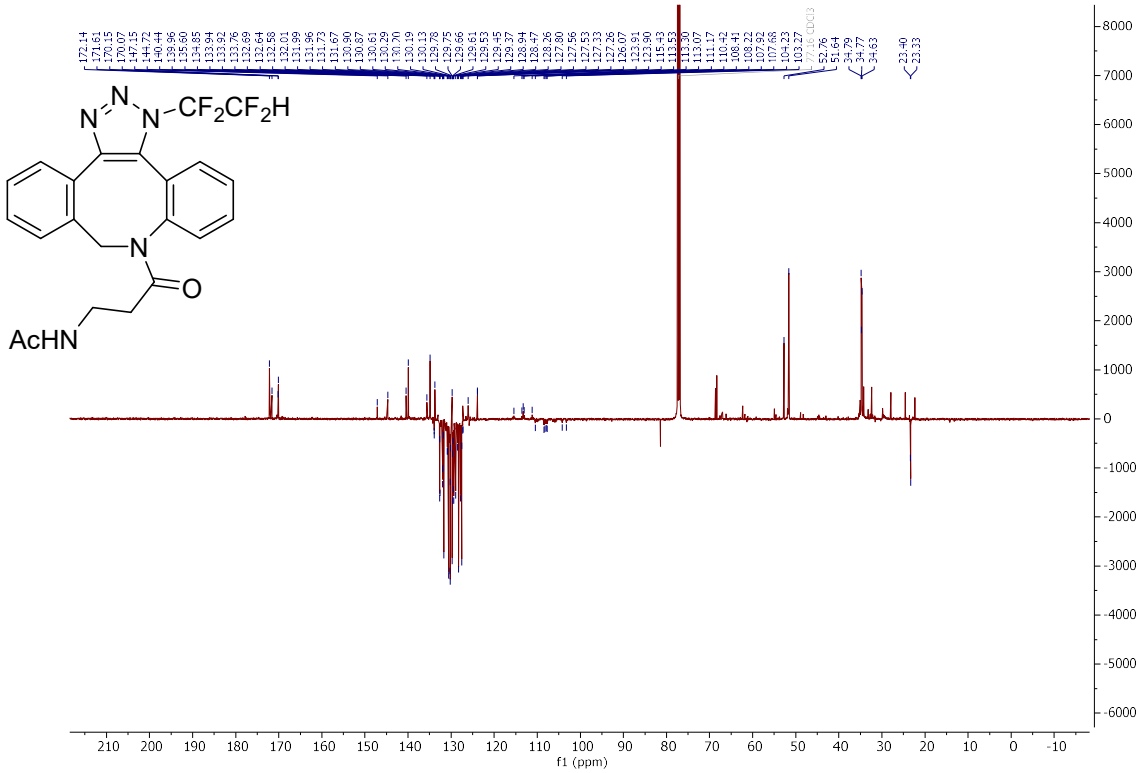

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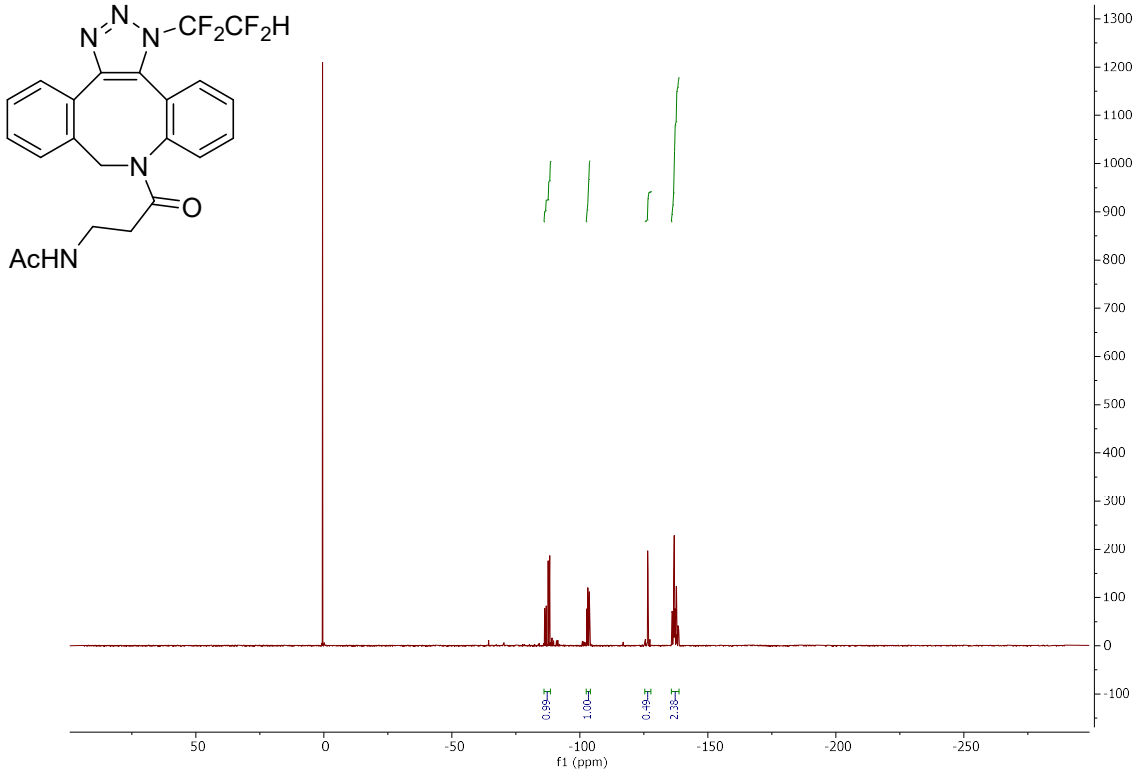

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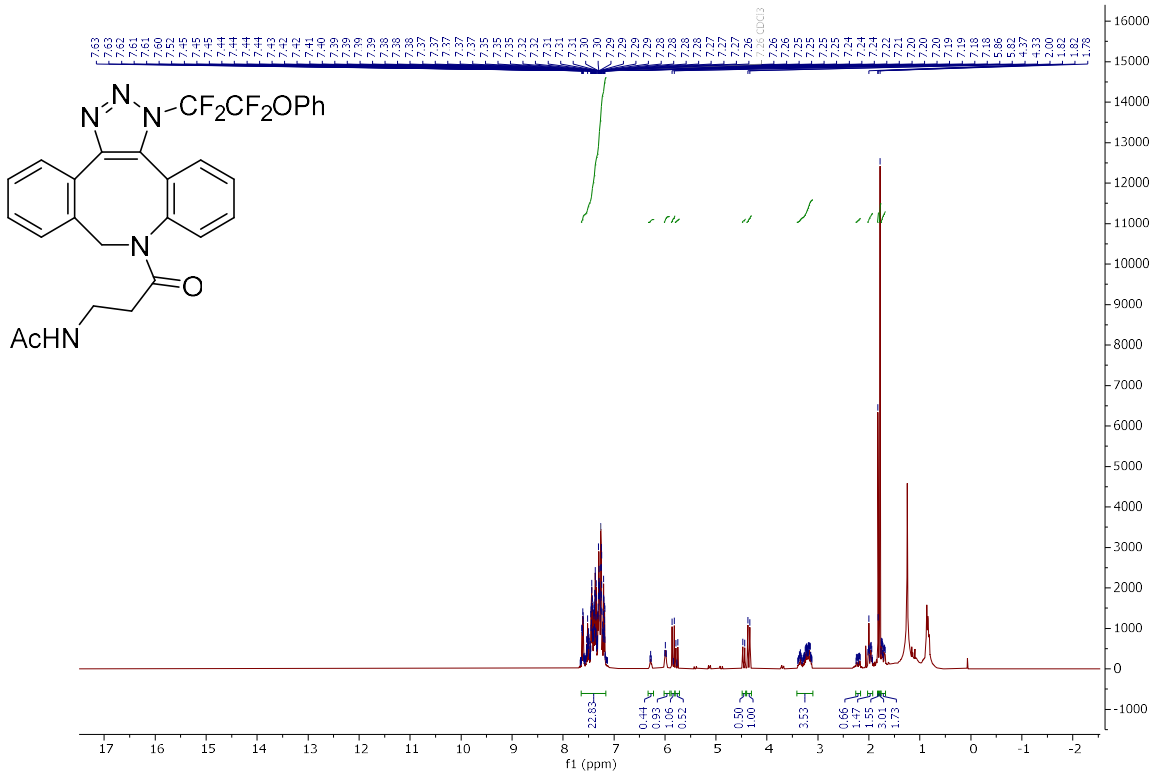

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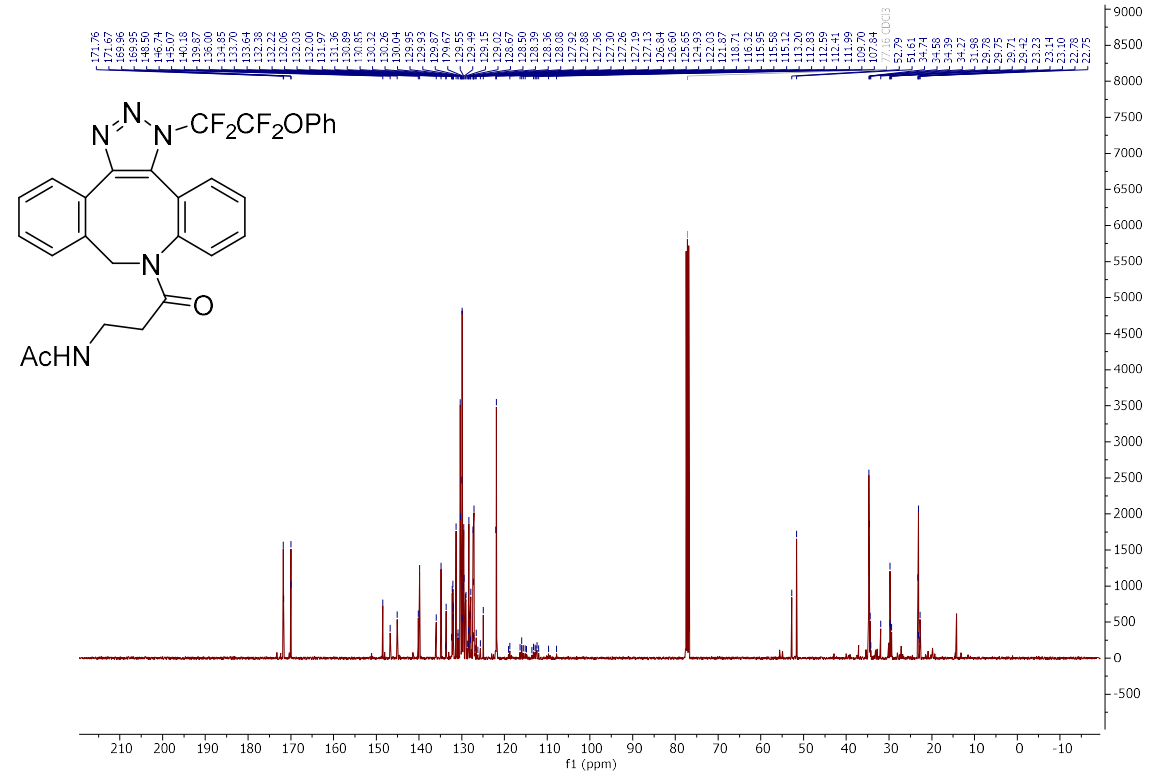
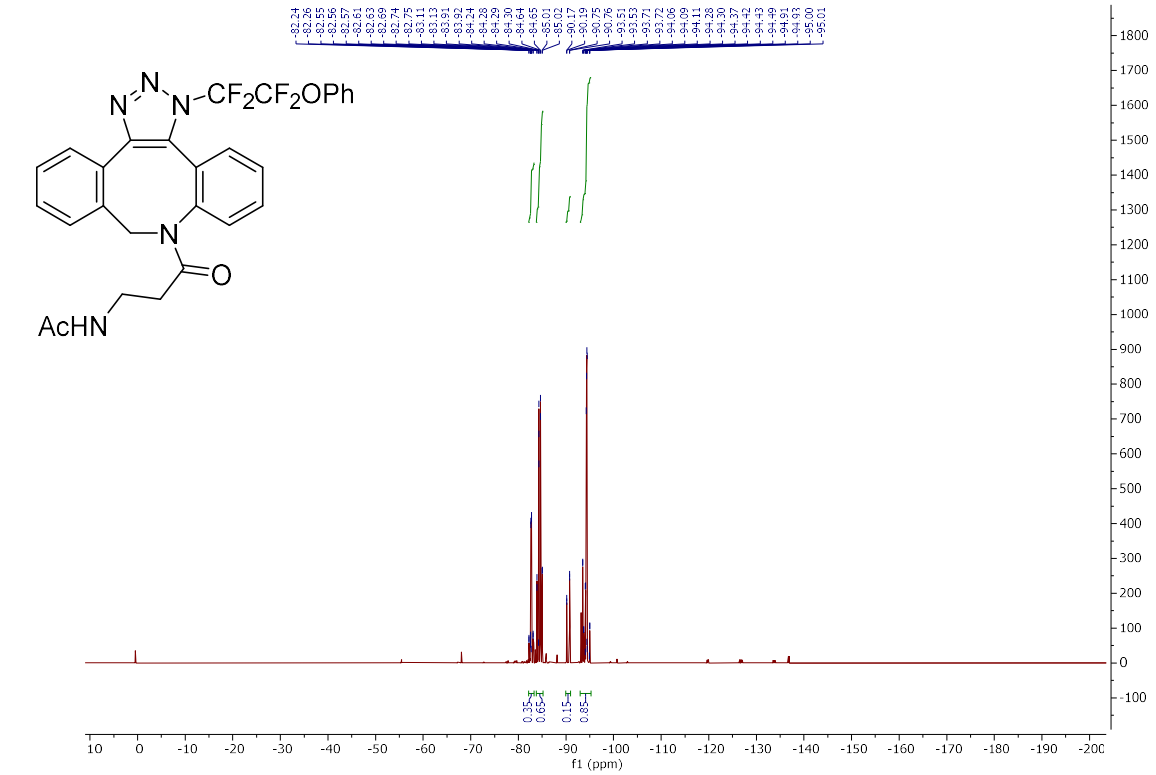


^{19}F NMR (376 MHz, $CDCl_3$) spectrum of **3g**



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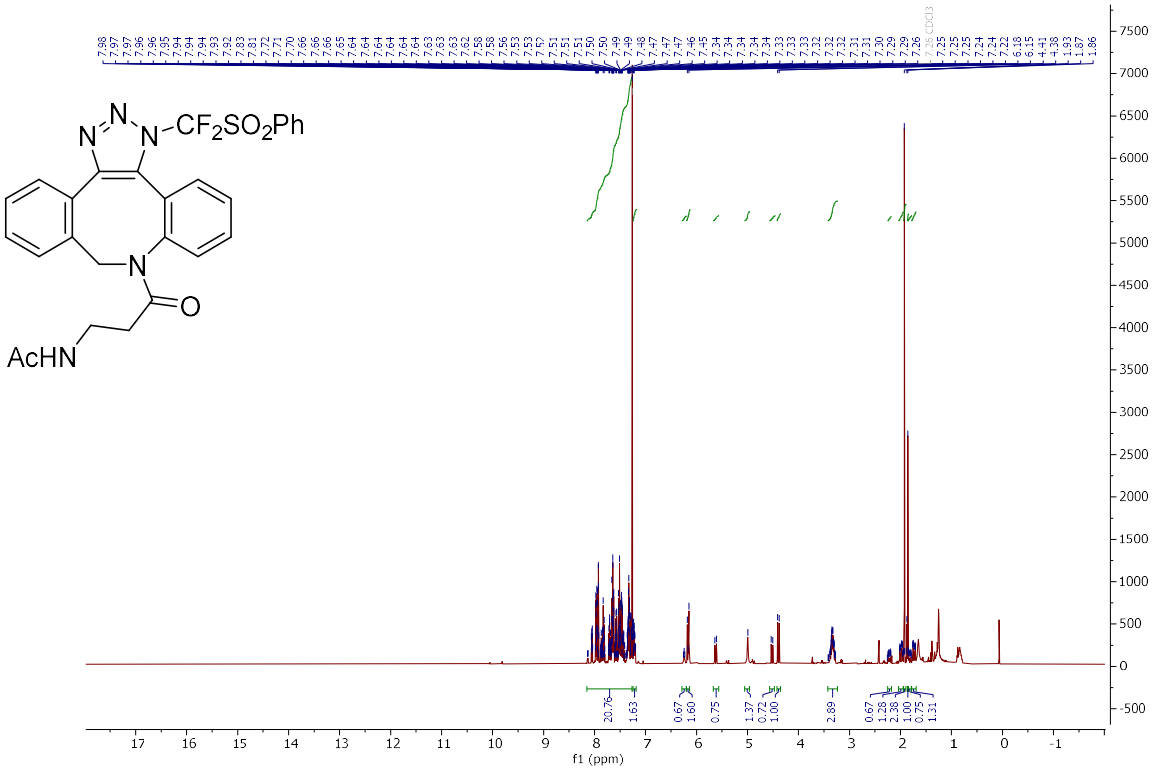

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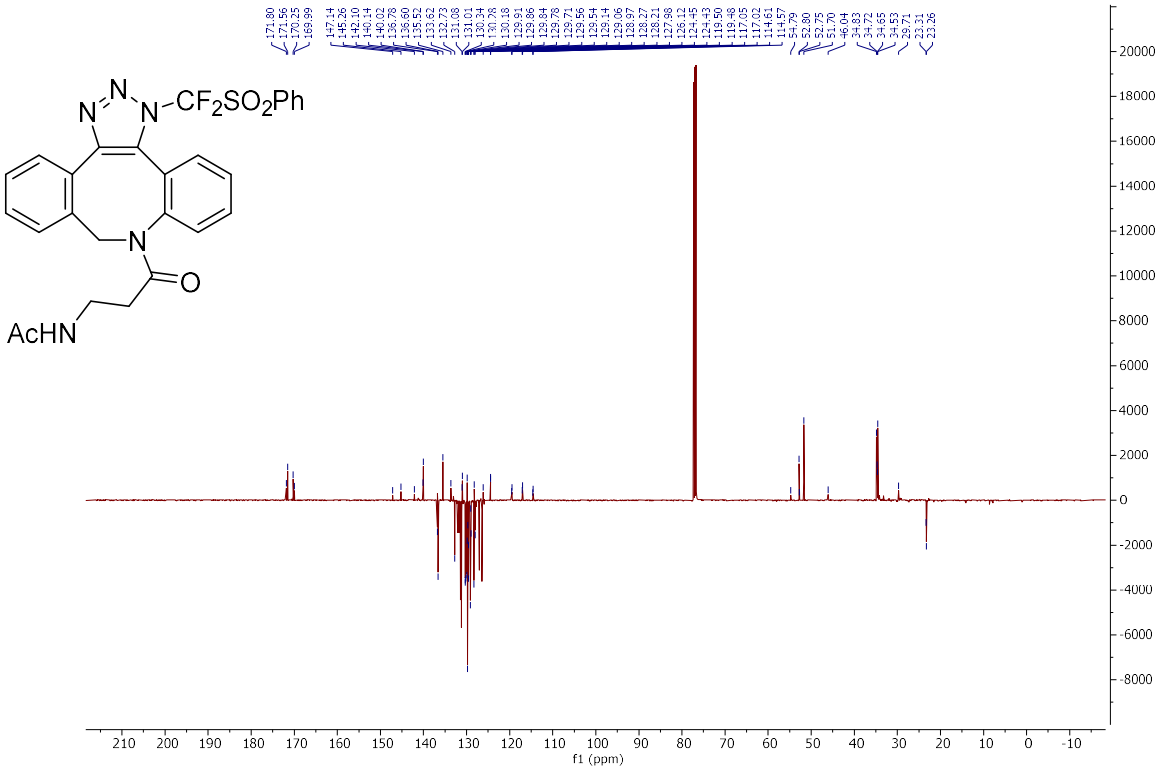

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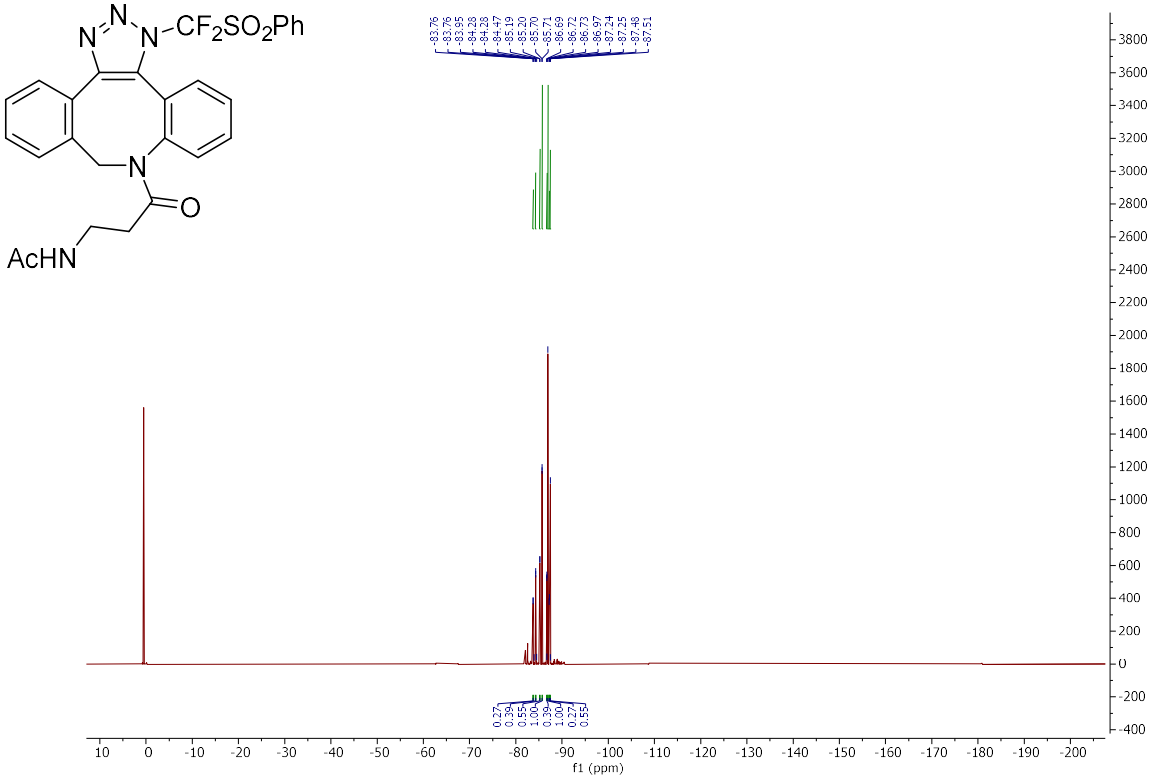

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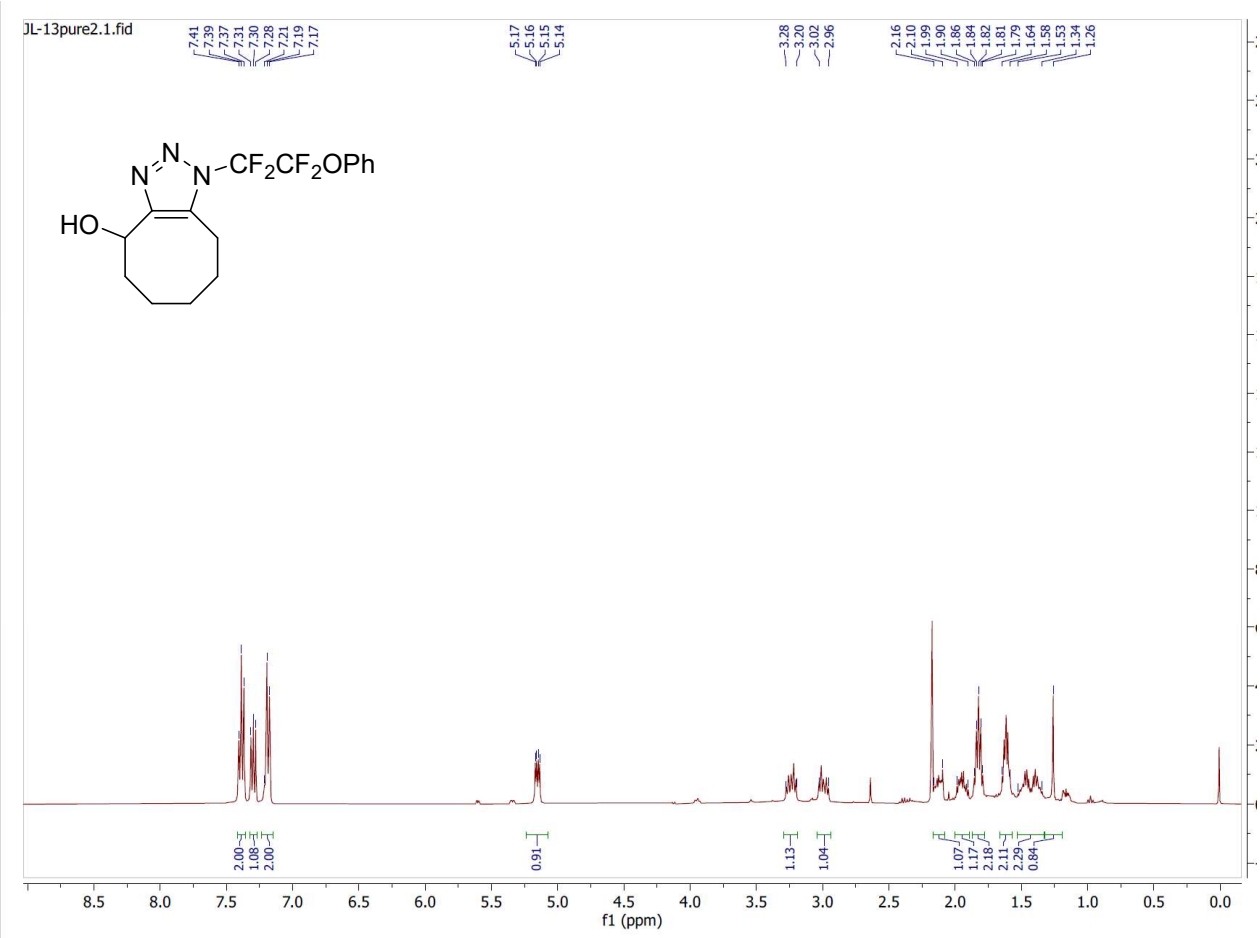

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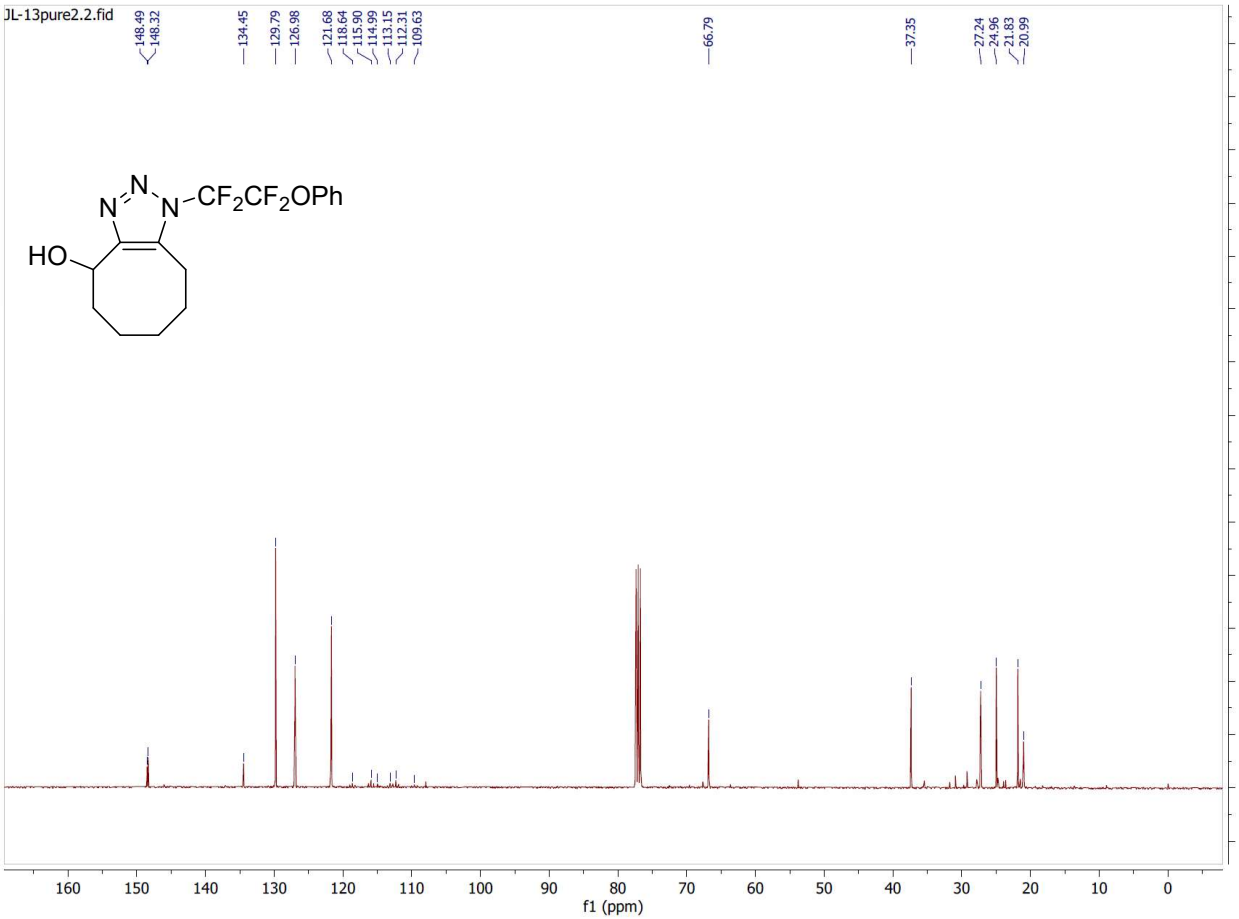

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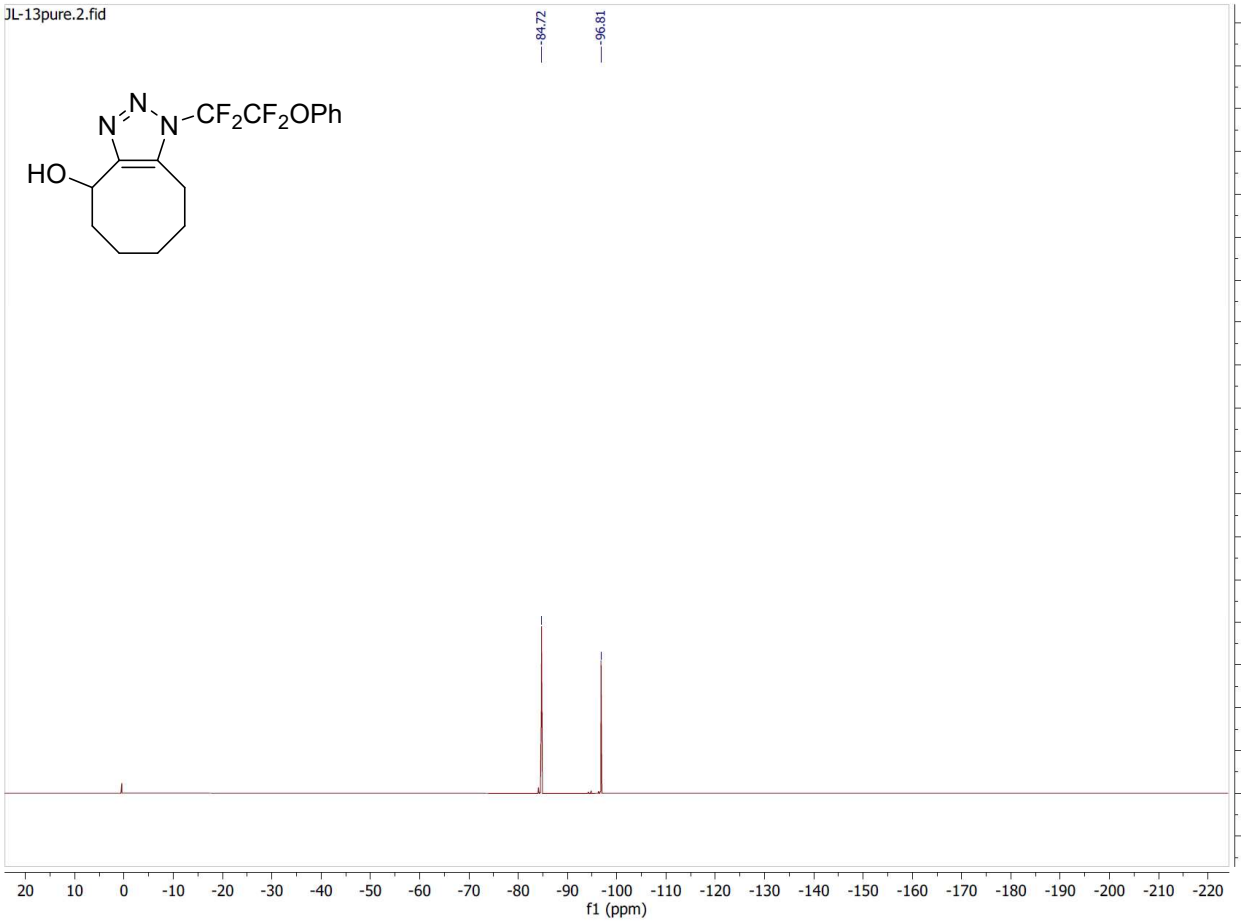

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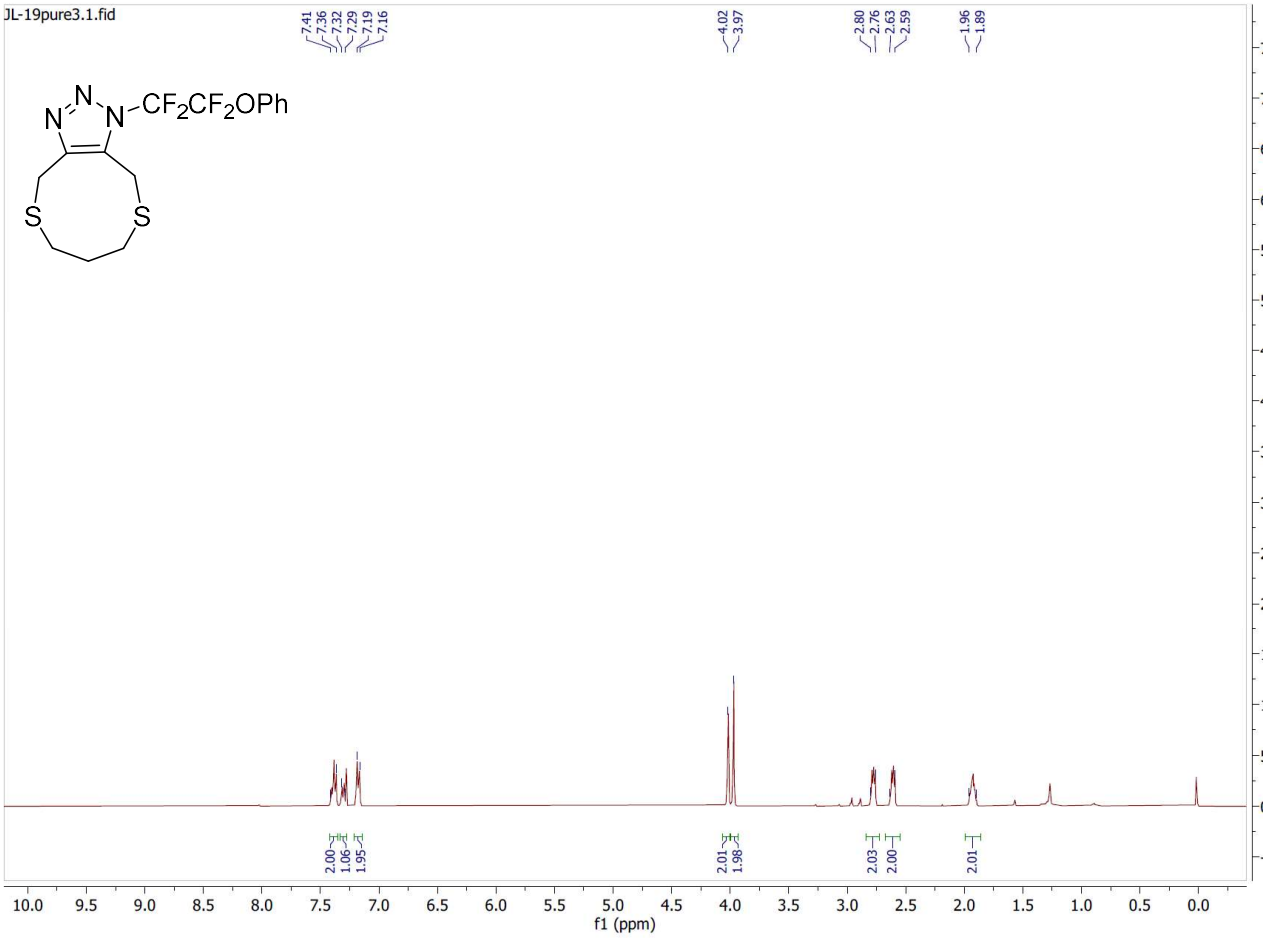

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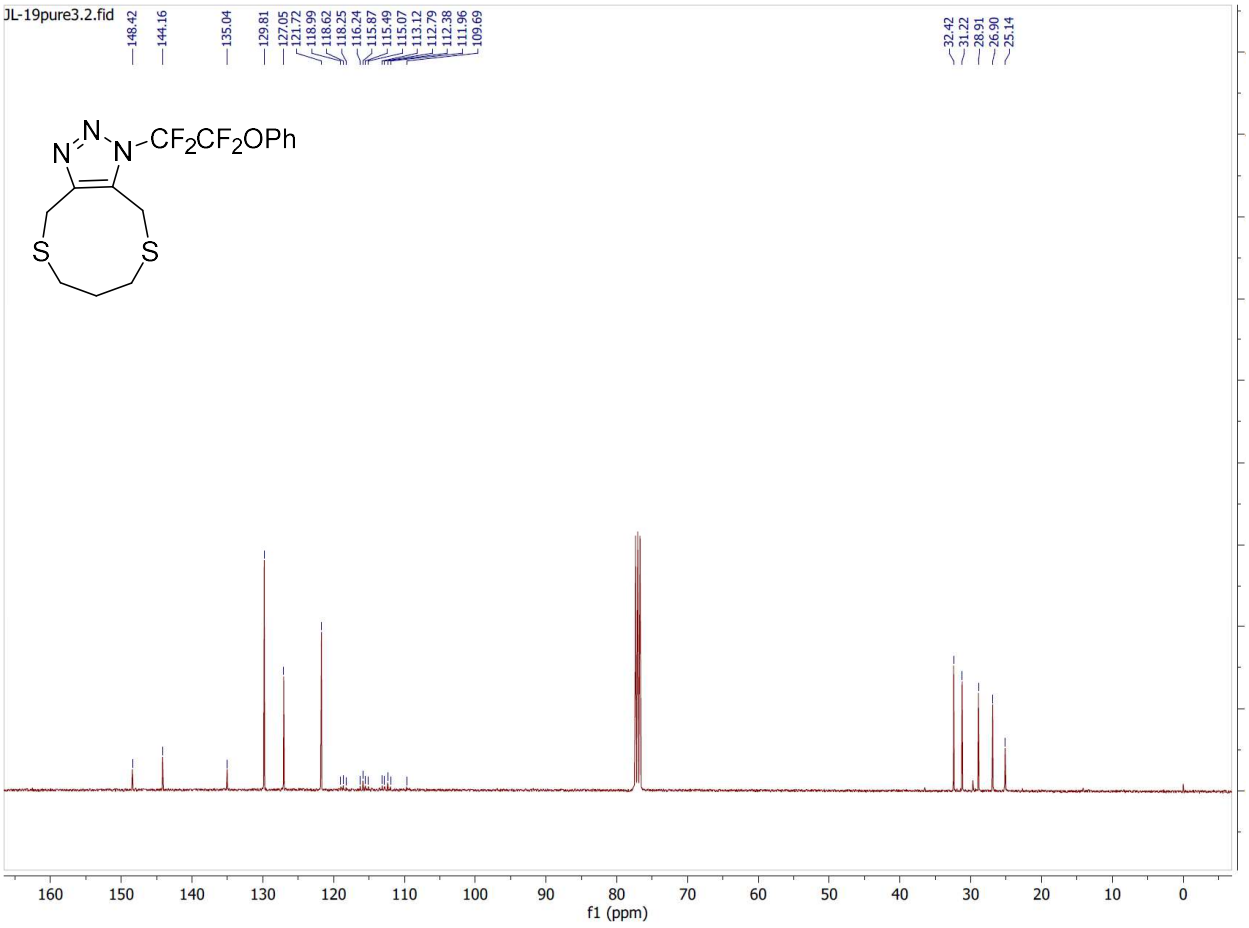

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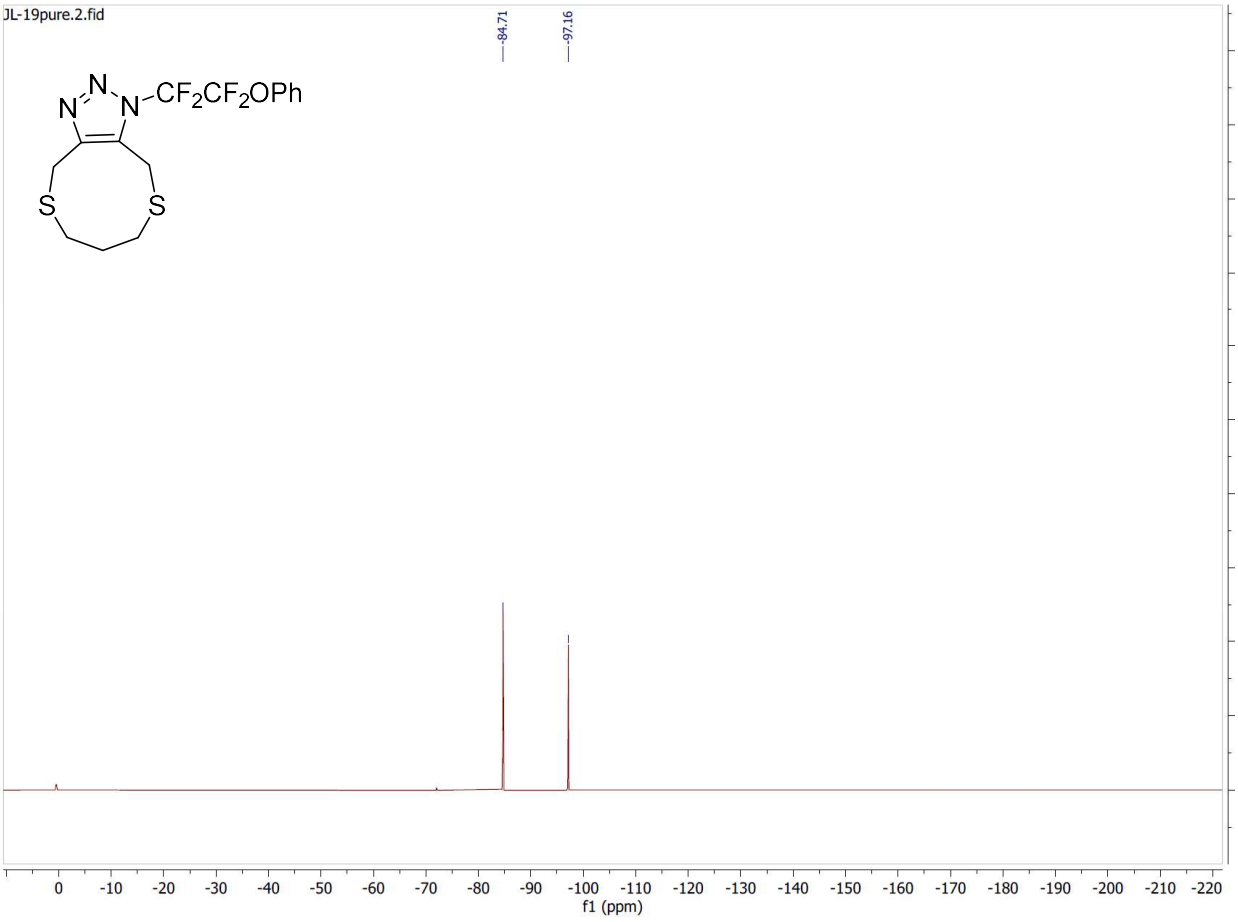

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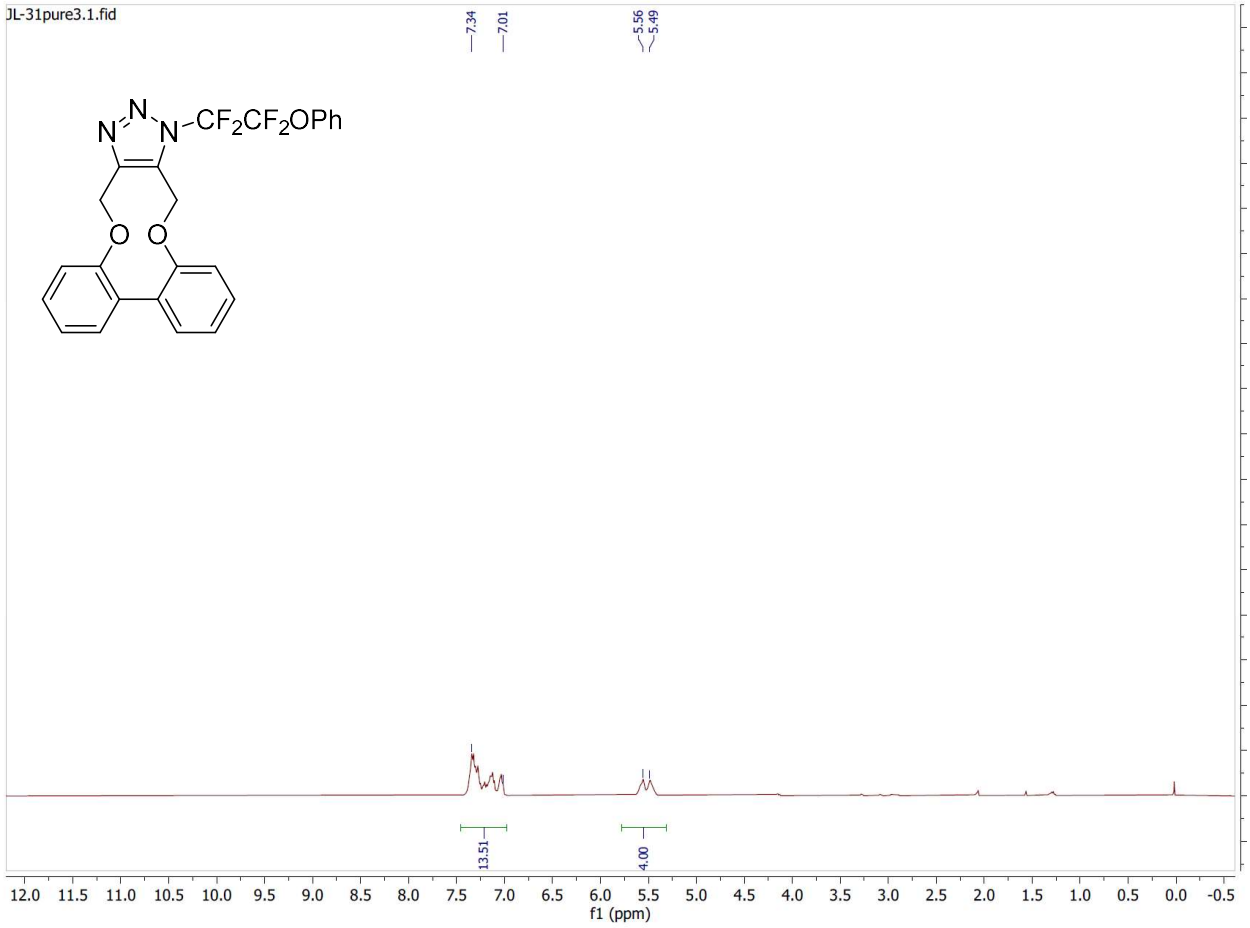

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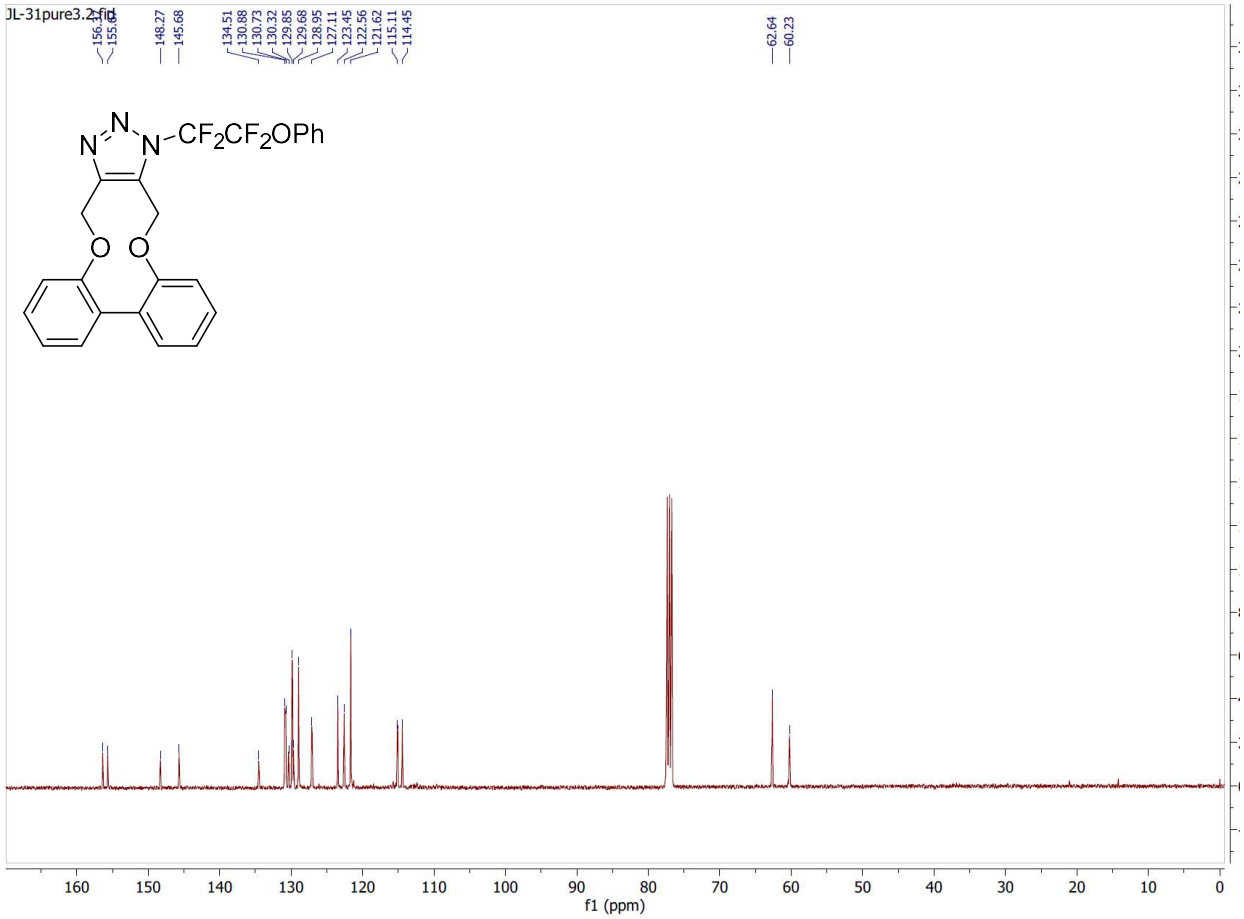

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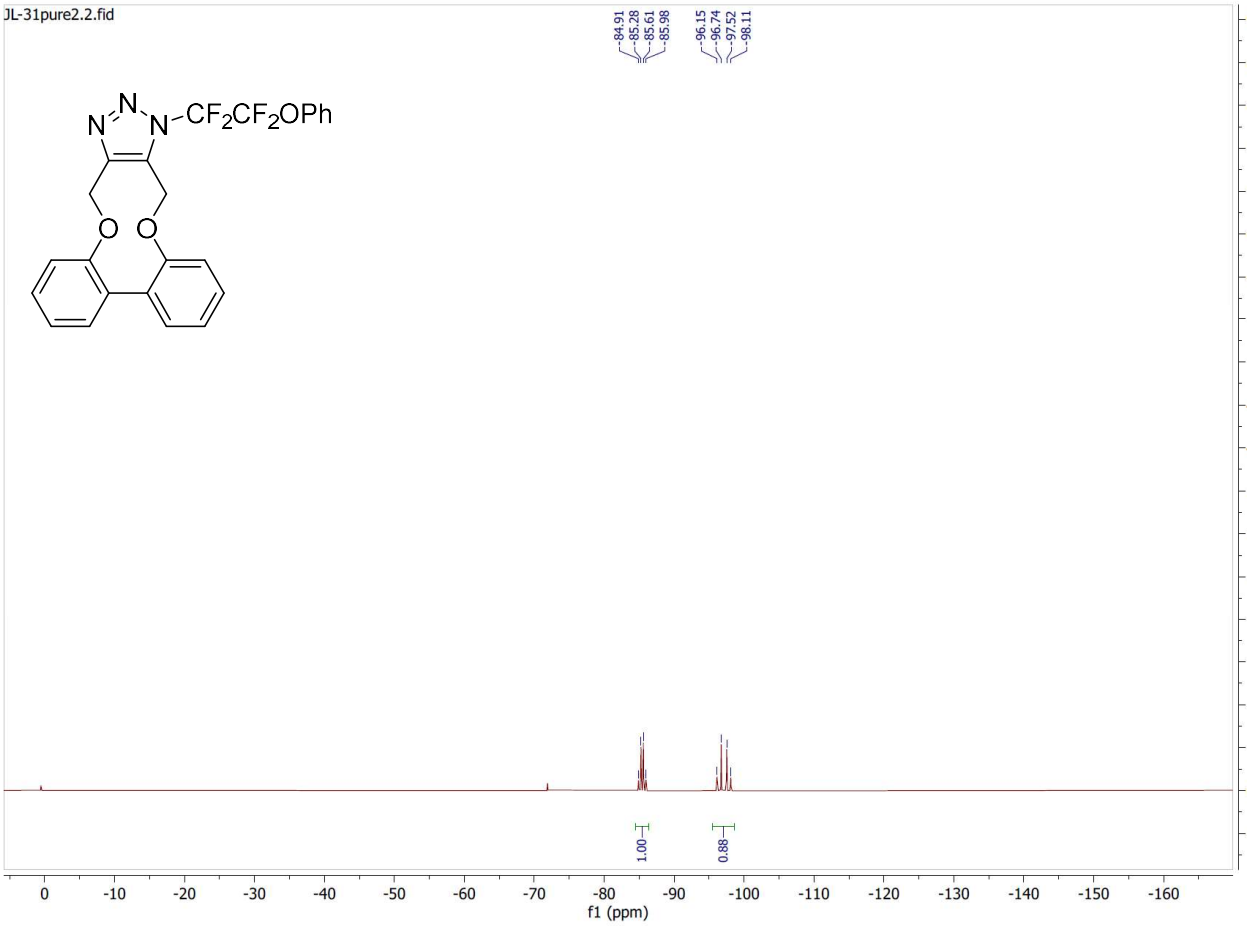

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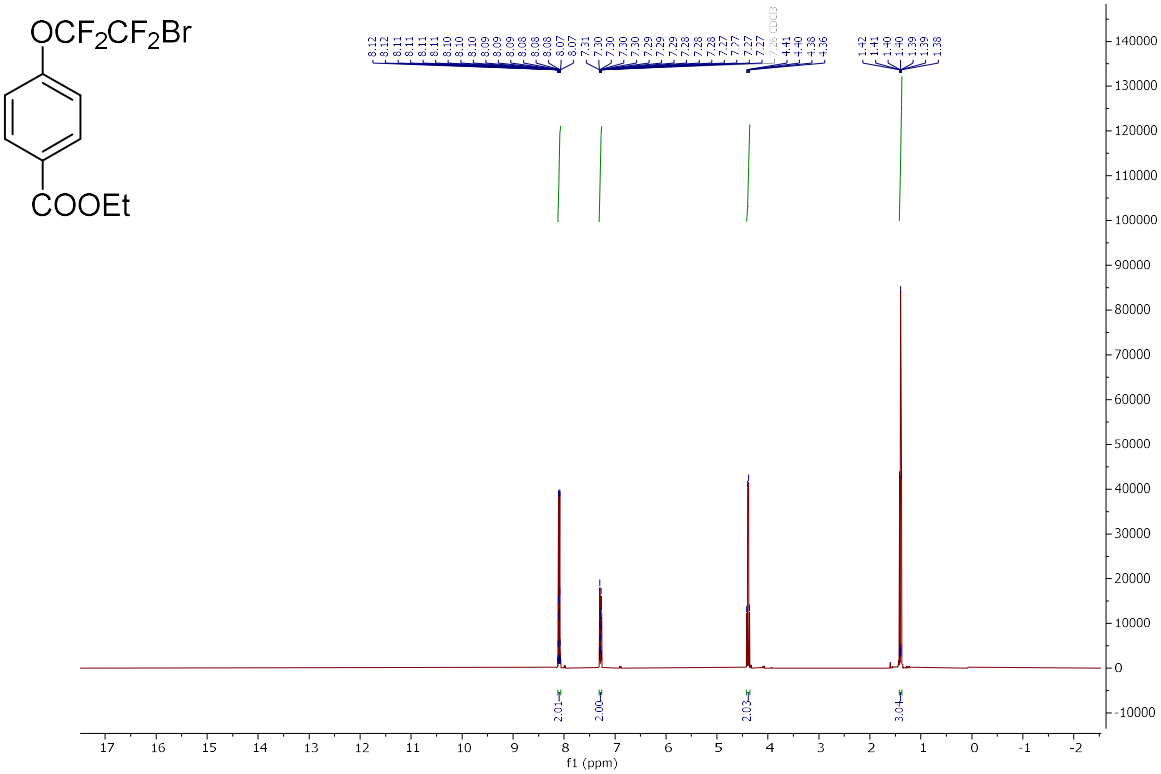

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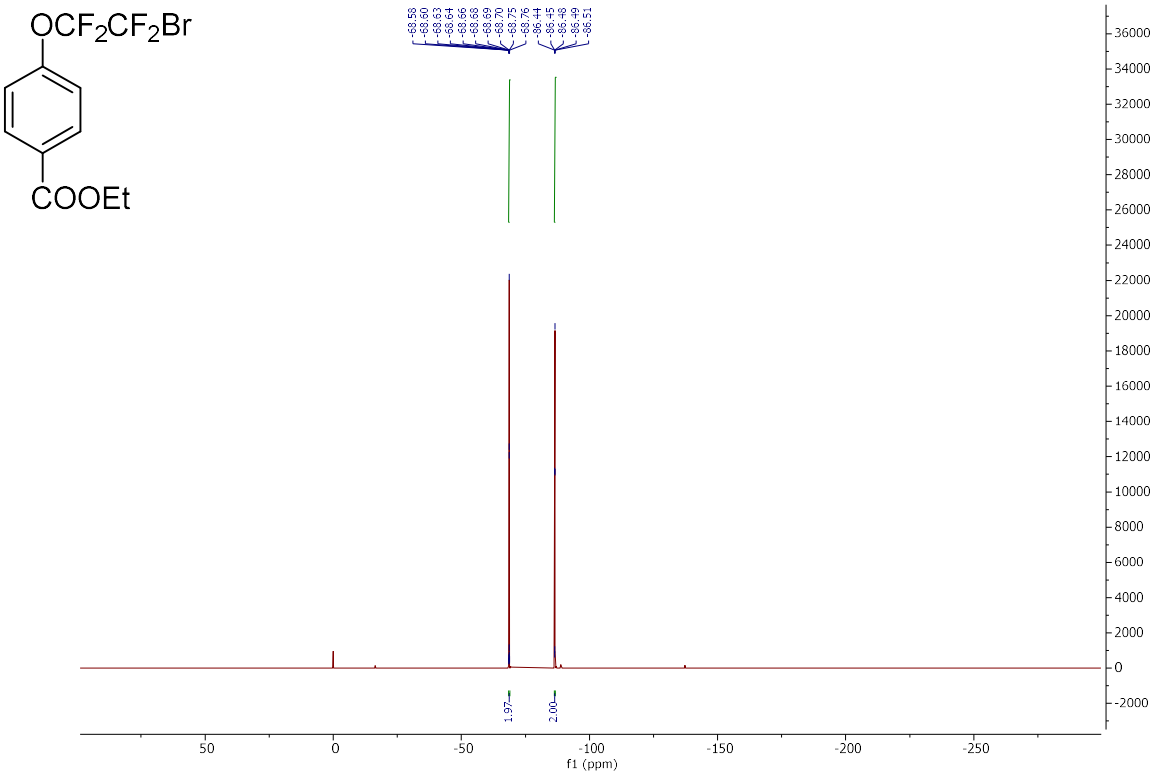

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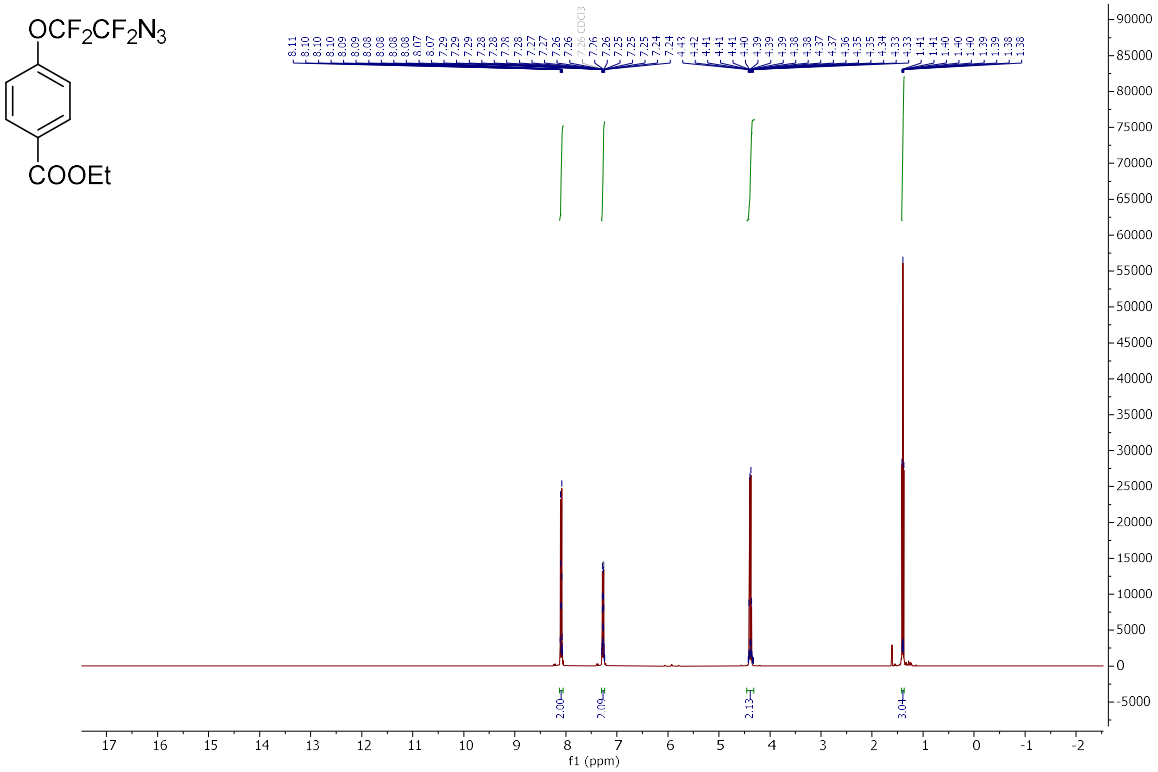

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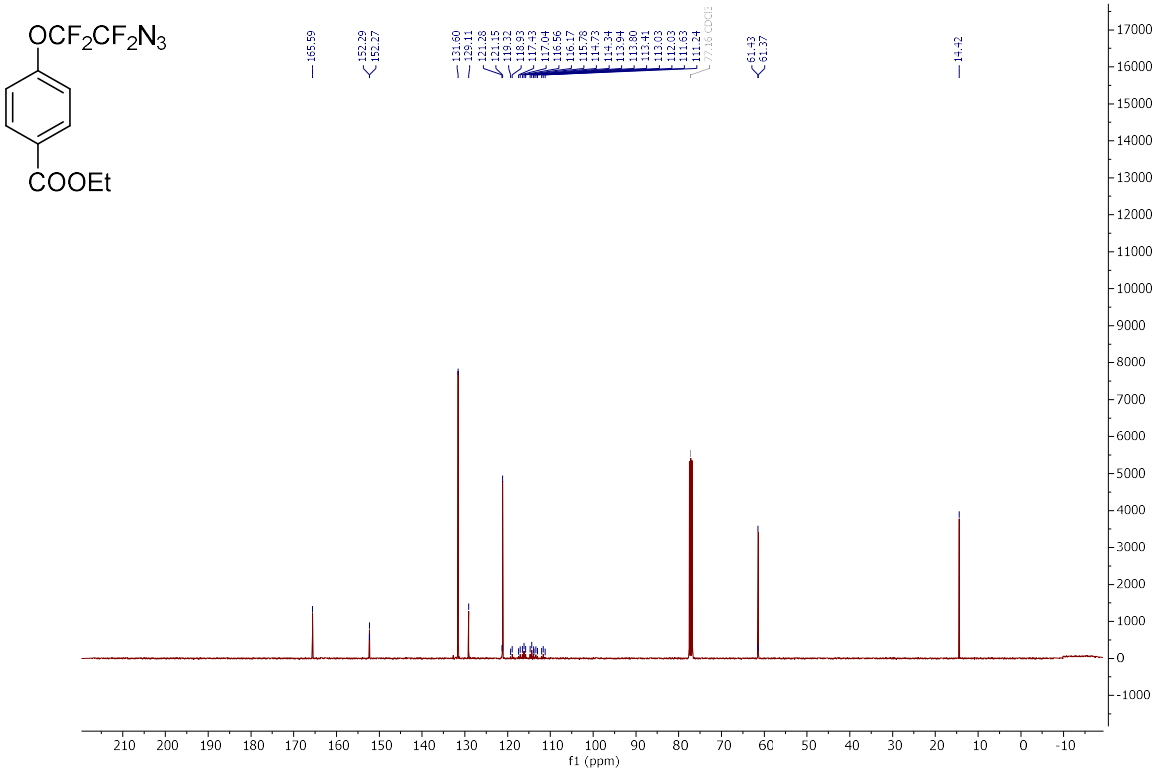

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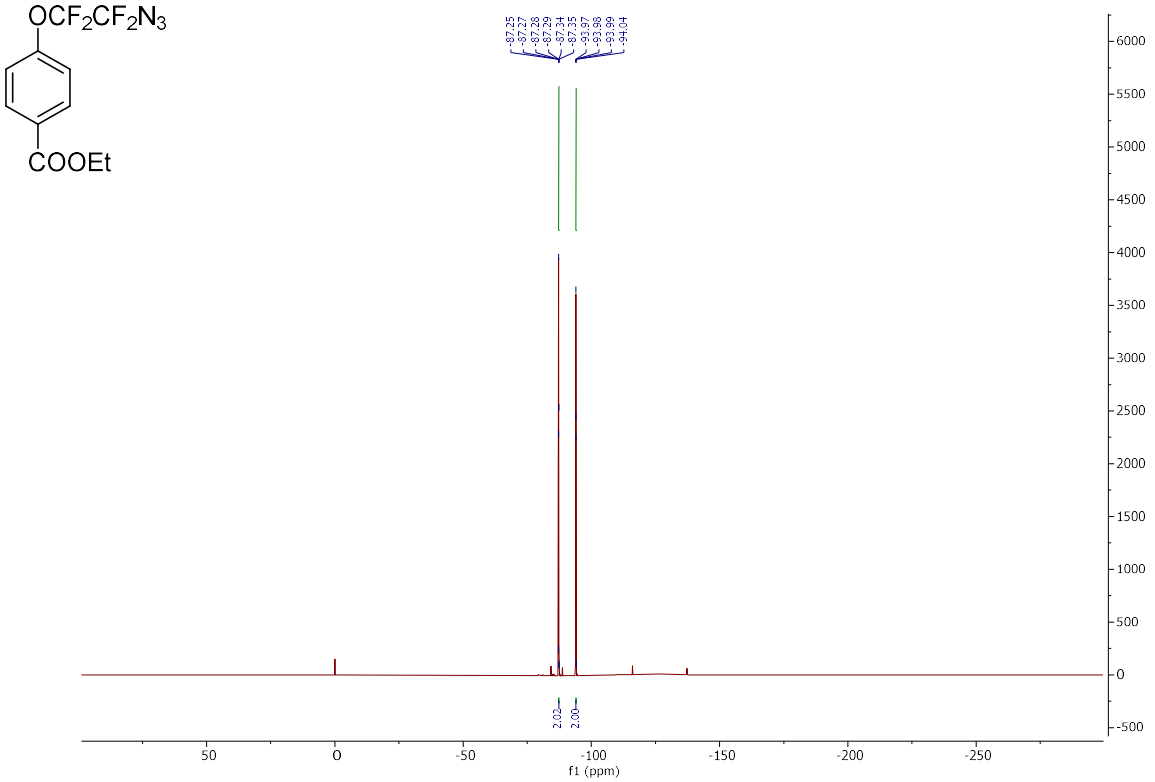

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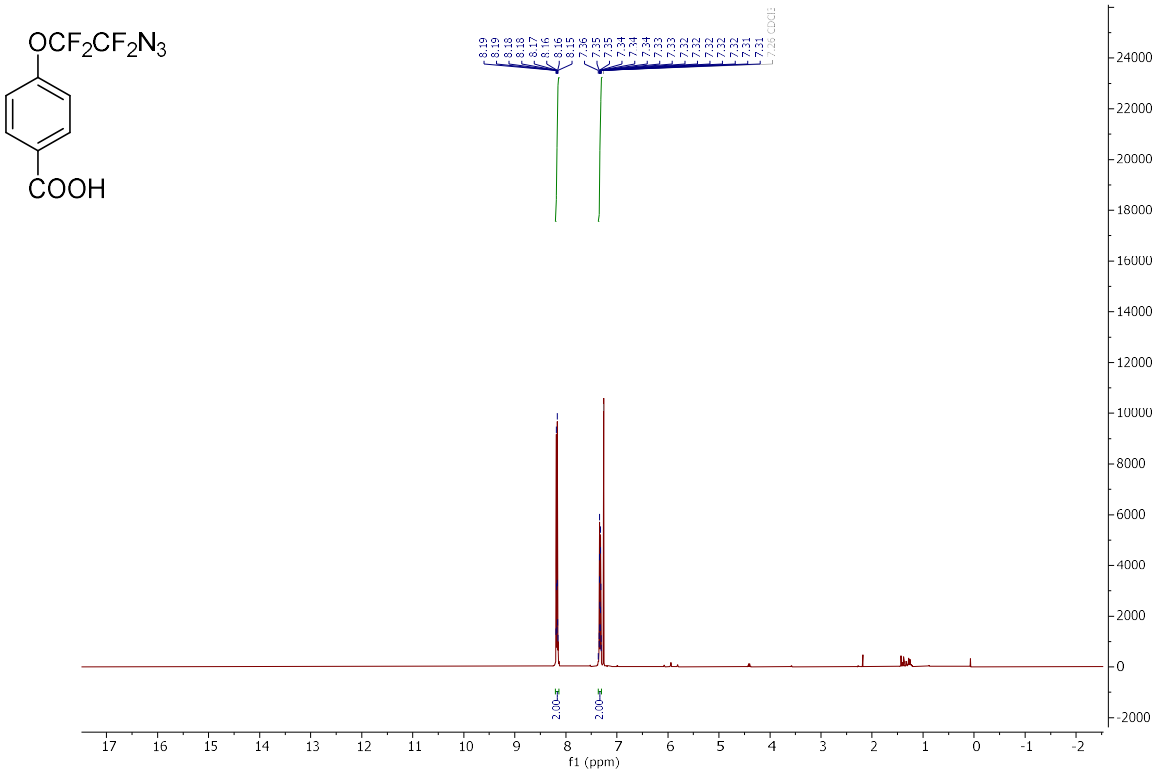

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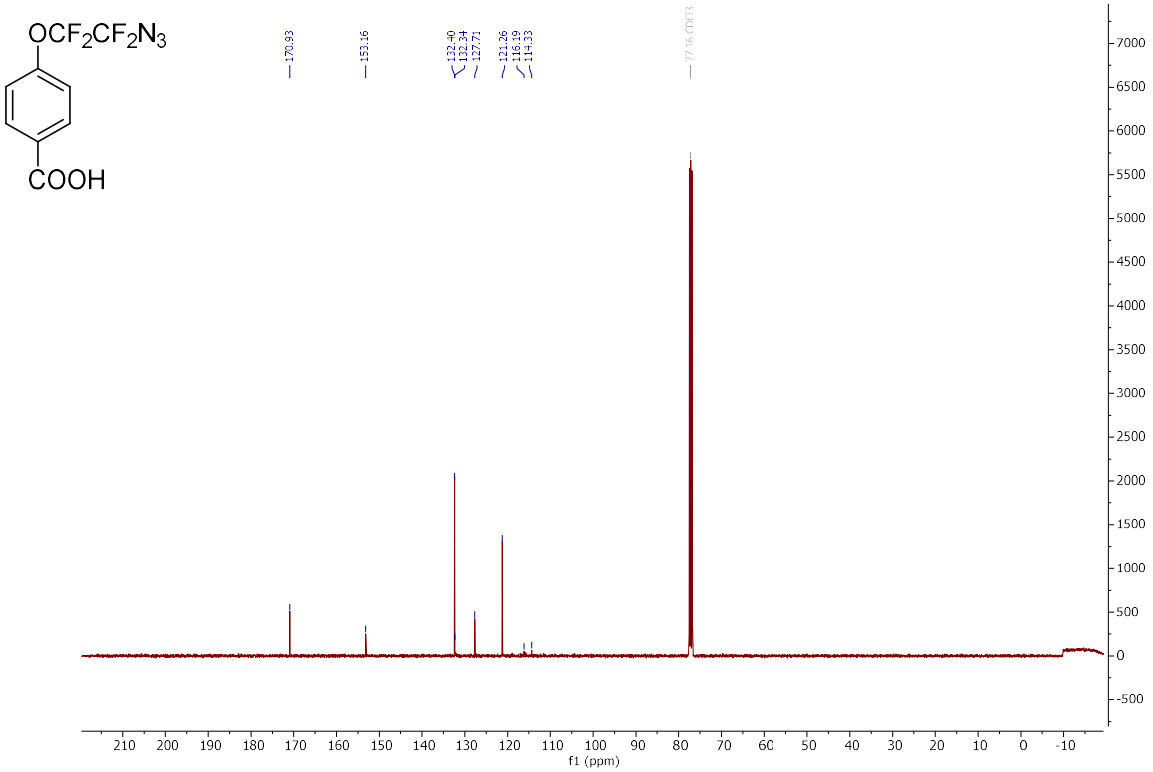
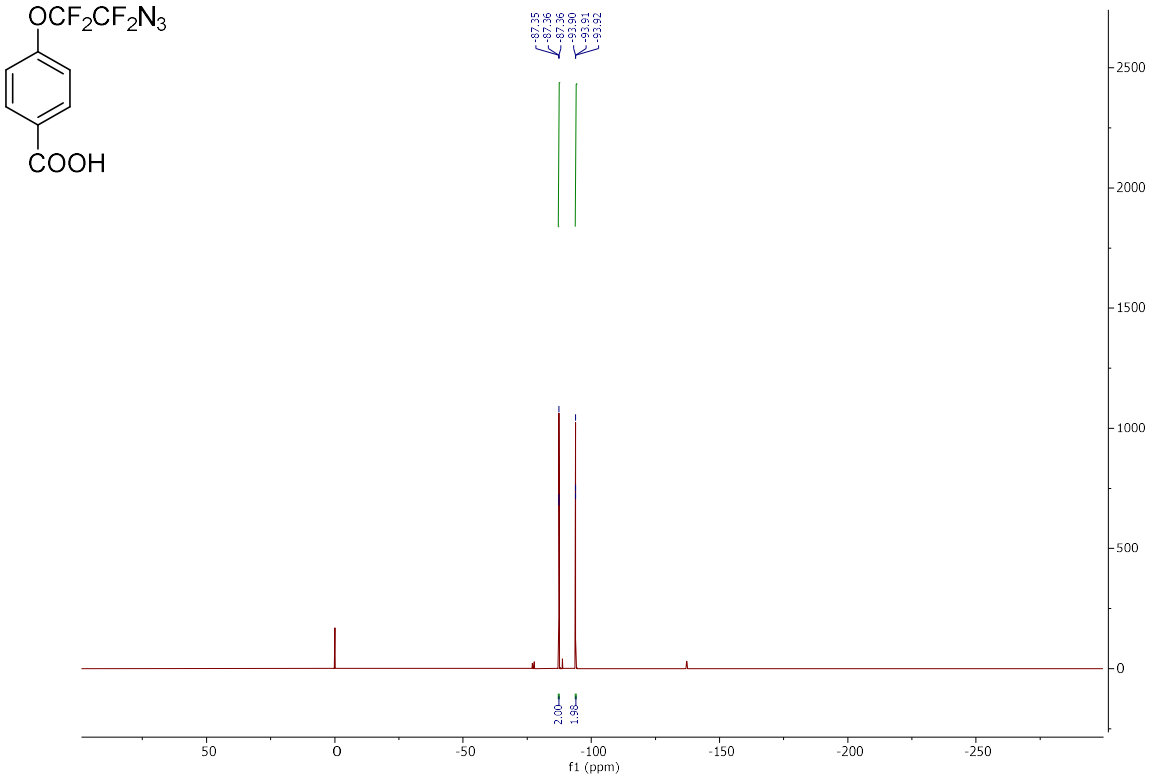
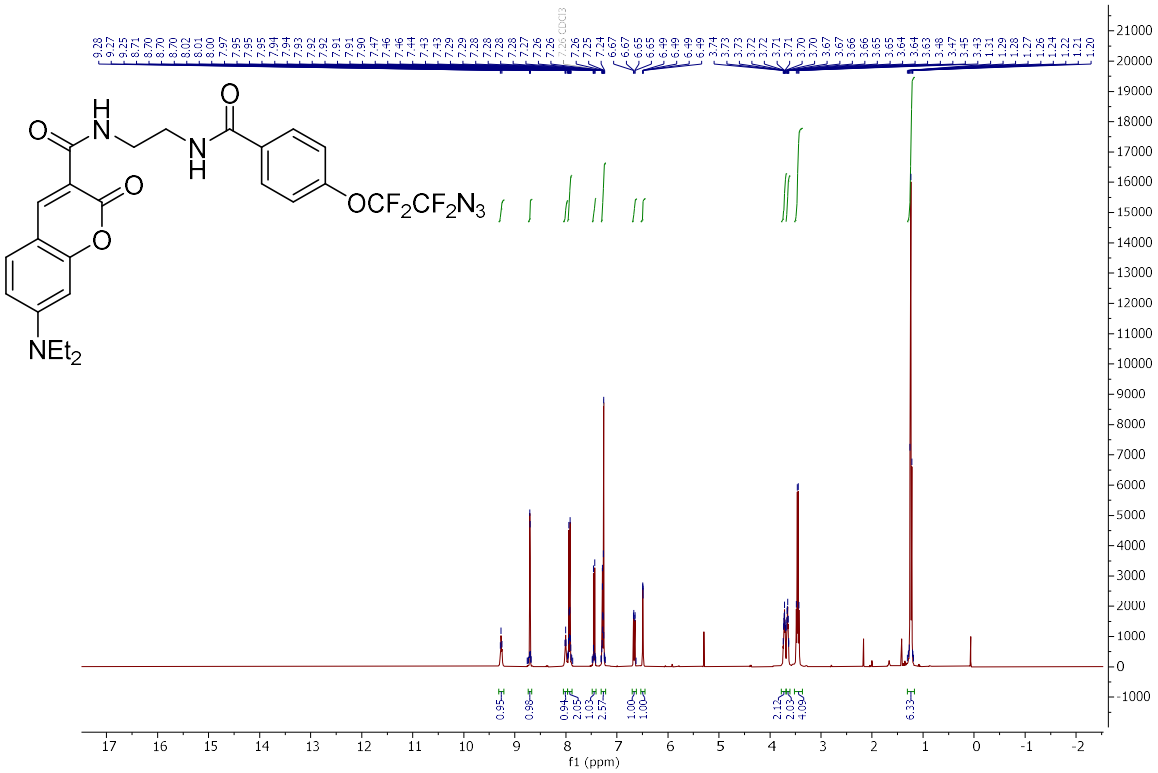


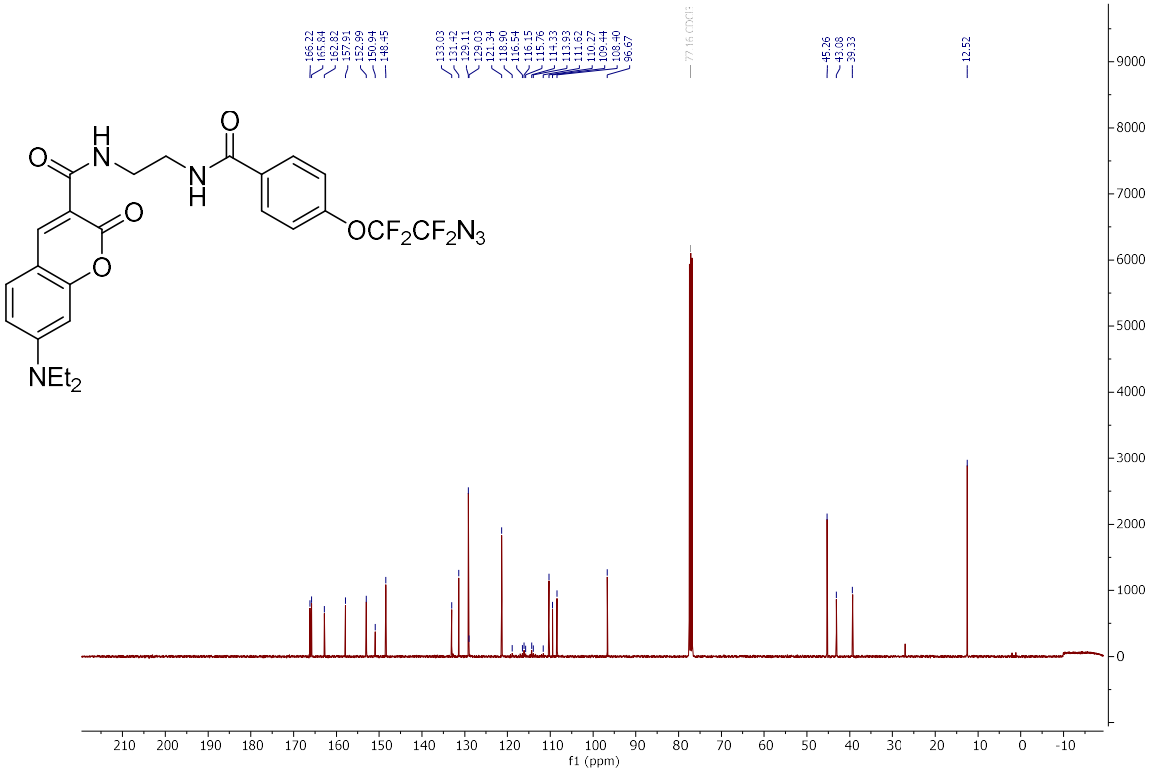
^{19}F NMR (376 MHz, $CDCl_3$) spectrum of **10**



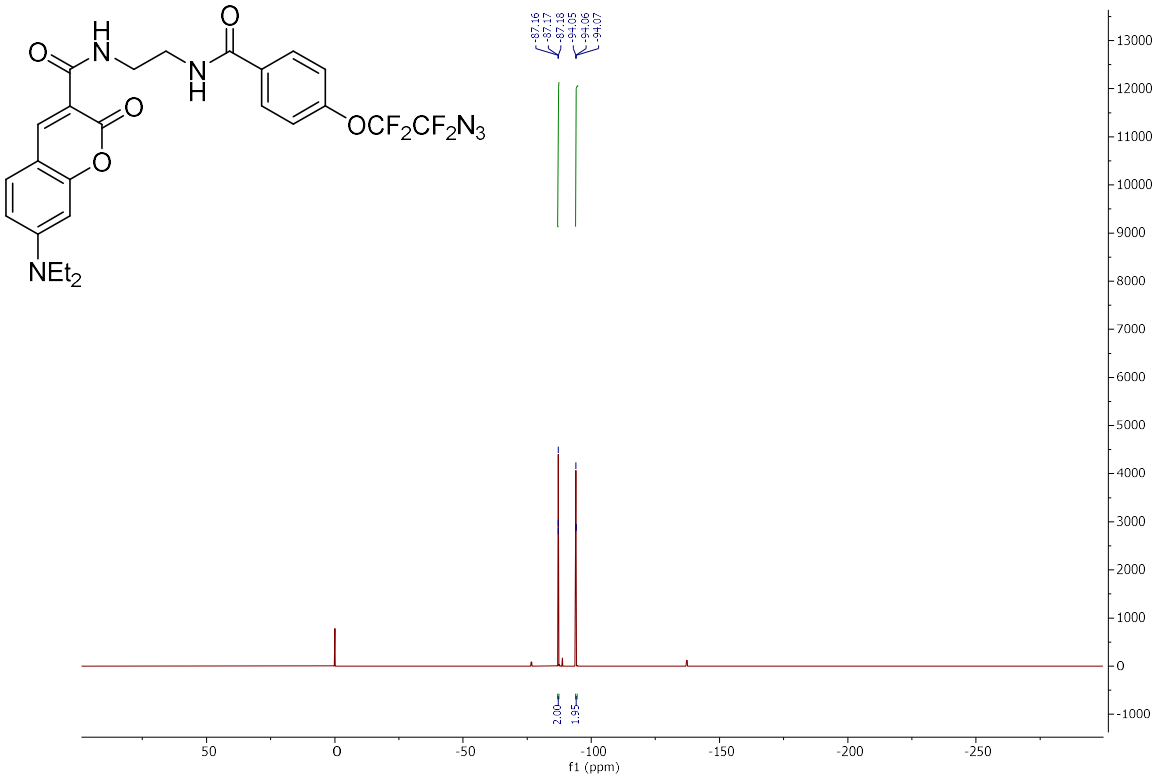
^{1}H NMR (401 MHz, $CDCl_3$) spectrum of **11**



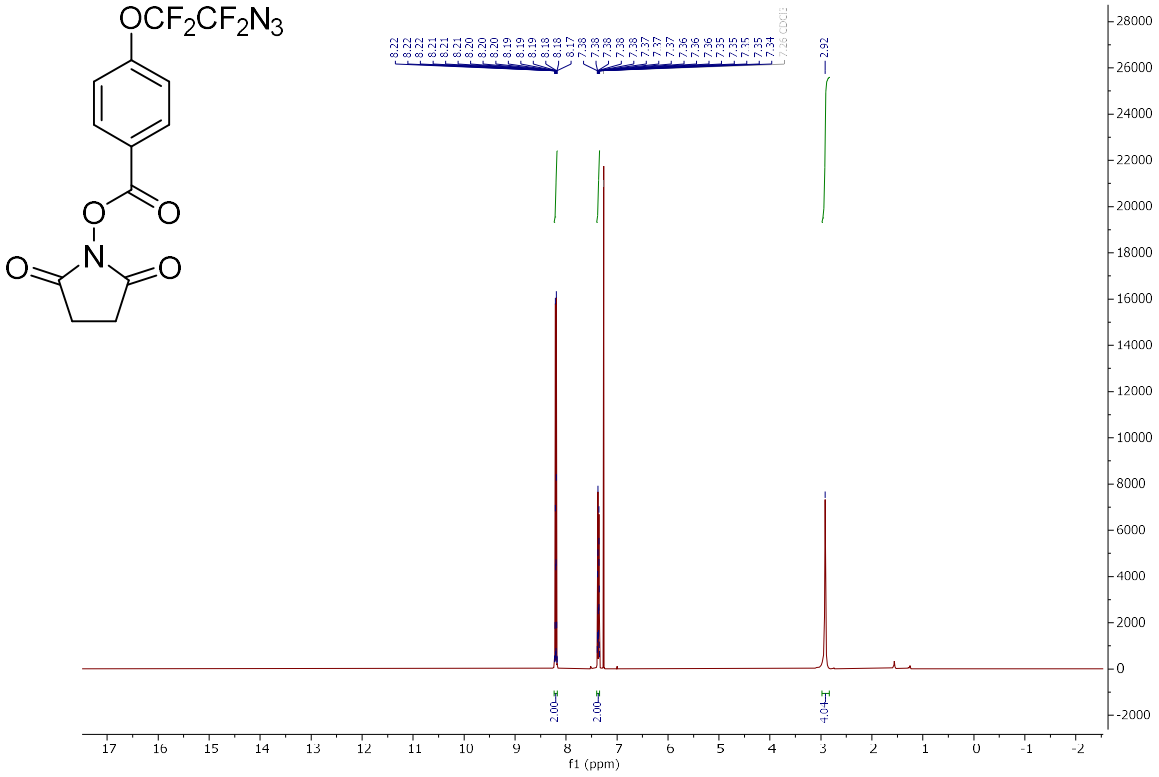
^{13}C {^{1}H} NMR (101 MHz, $CDCl_3$) spectrum of **11**



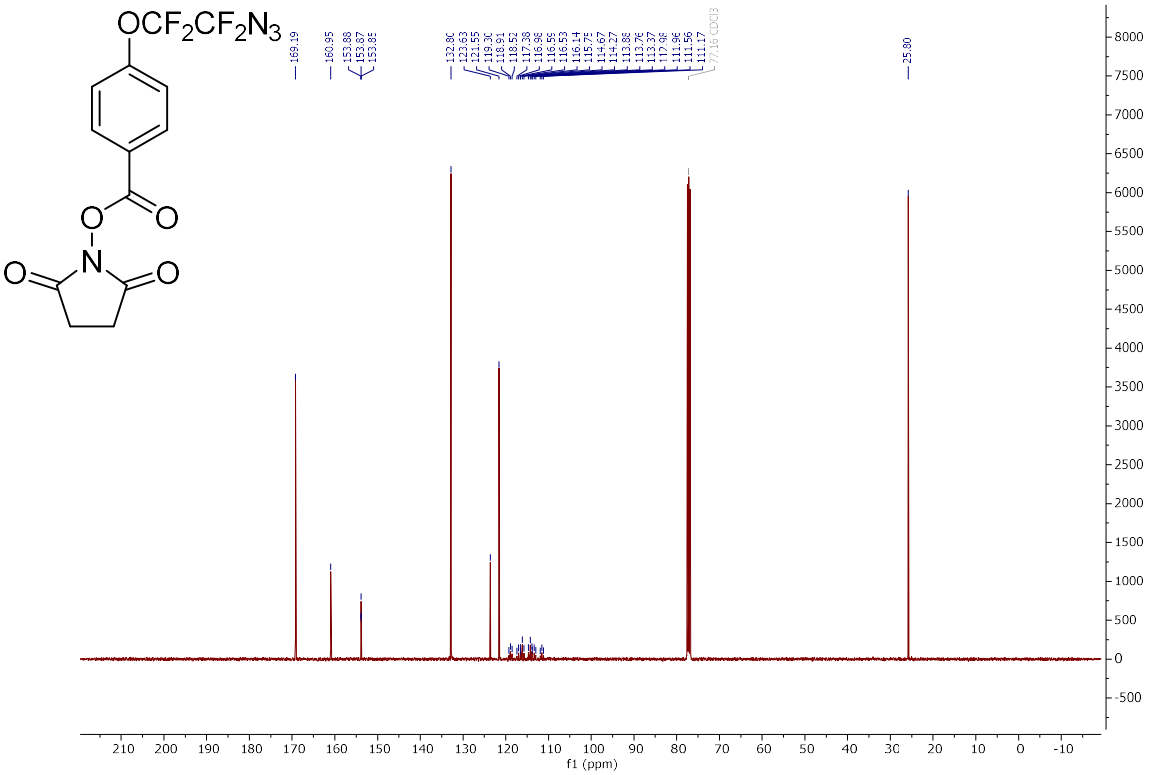
^{19}F NMR (376 MHz, $CDCl_3$) spectrum of **11**



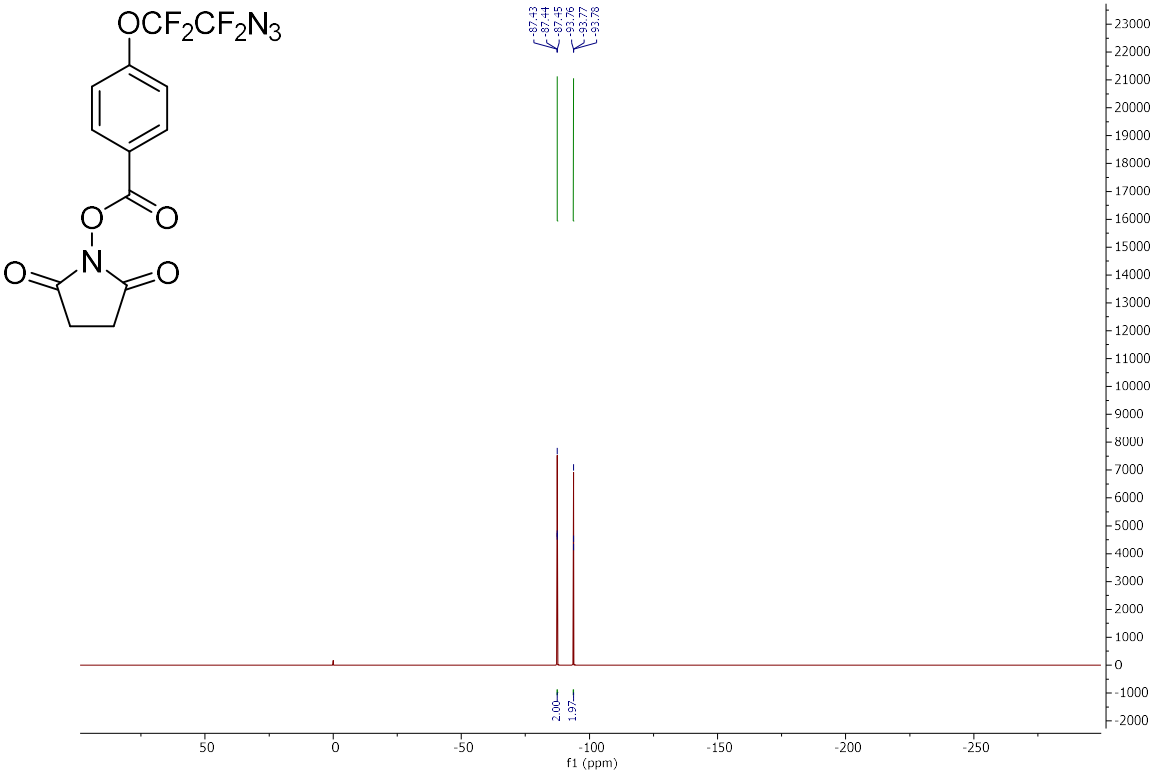
^{1}H NMR (401 MHz, $CDCl_3$) spectrum of **12**



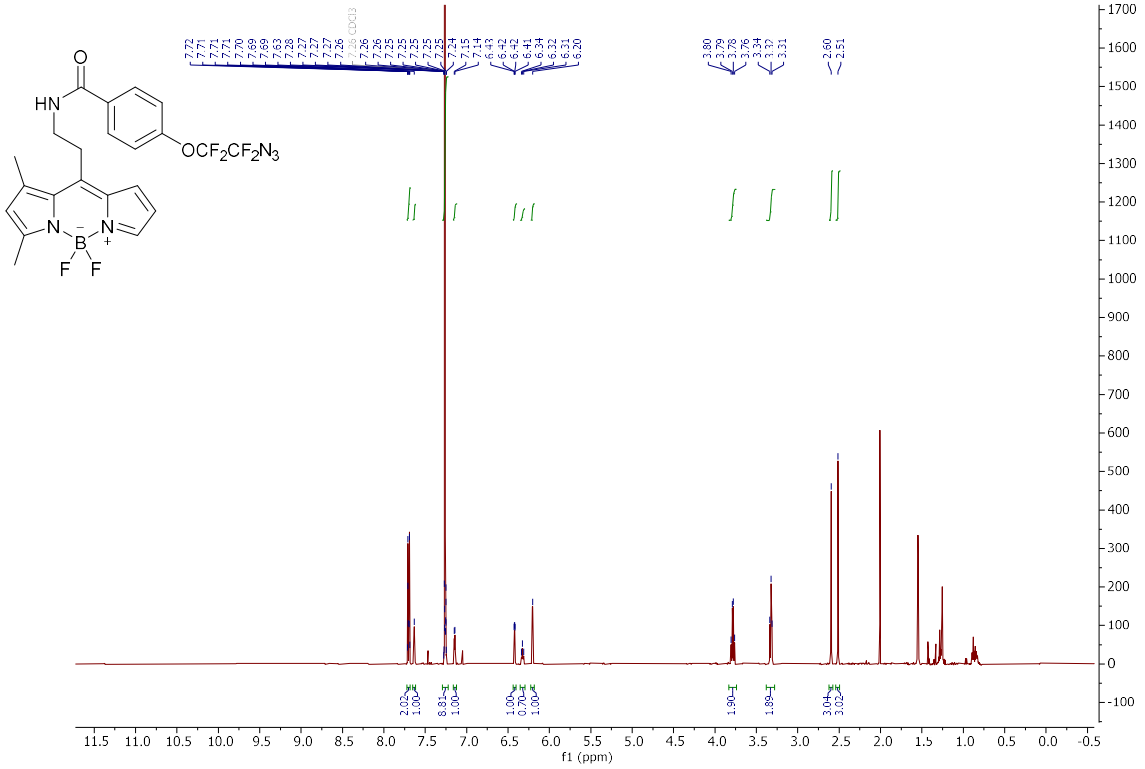
^{13}C {^{1}H} NMR (101 MHz, $CDCl_3$) spectrum of **12**



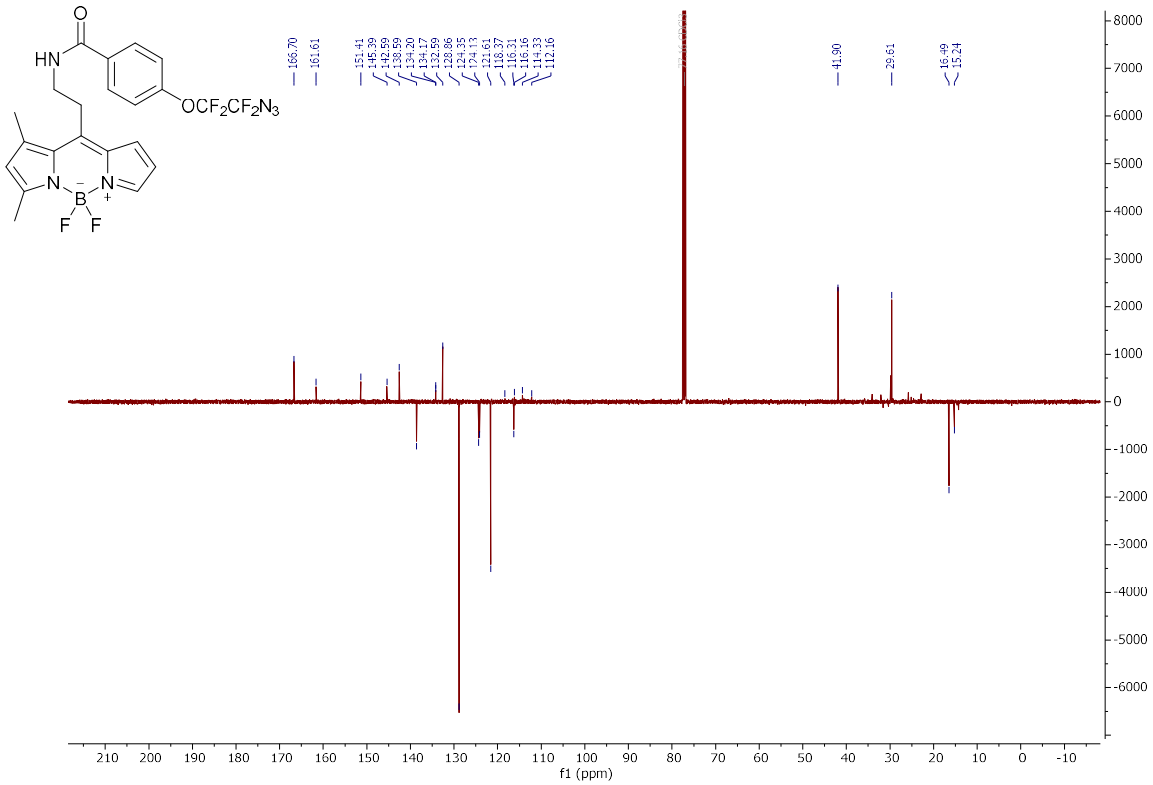
^{19}F NMR (376 MHz, $CDCl_3$) spectrum of **12**



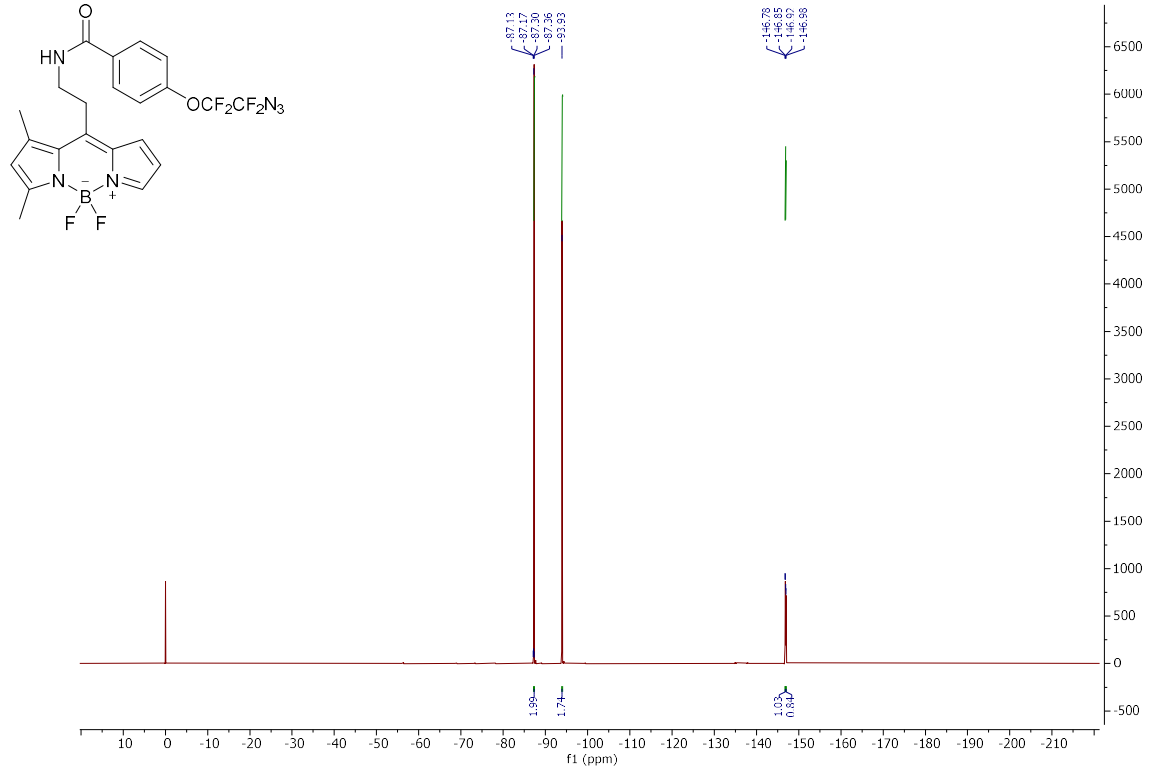
^{1}H NMR (401 MHz, $CDCl_3$) spectrum of **13**



^{13}C {^{1}H} NMR (101 MHz, $CDCl_3$) spectrum of **13**



^{19}F NMR (376 MHz, $CDCl_3$) spectrum of **13**



^{11}B NMR (160 MHz, $CDCl_3$) spectrum of **13**